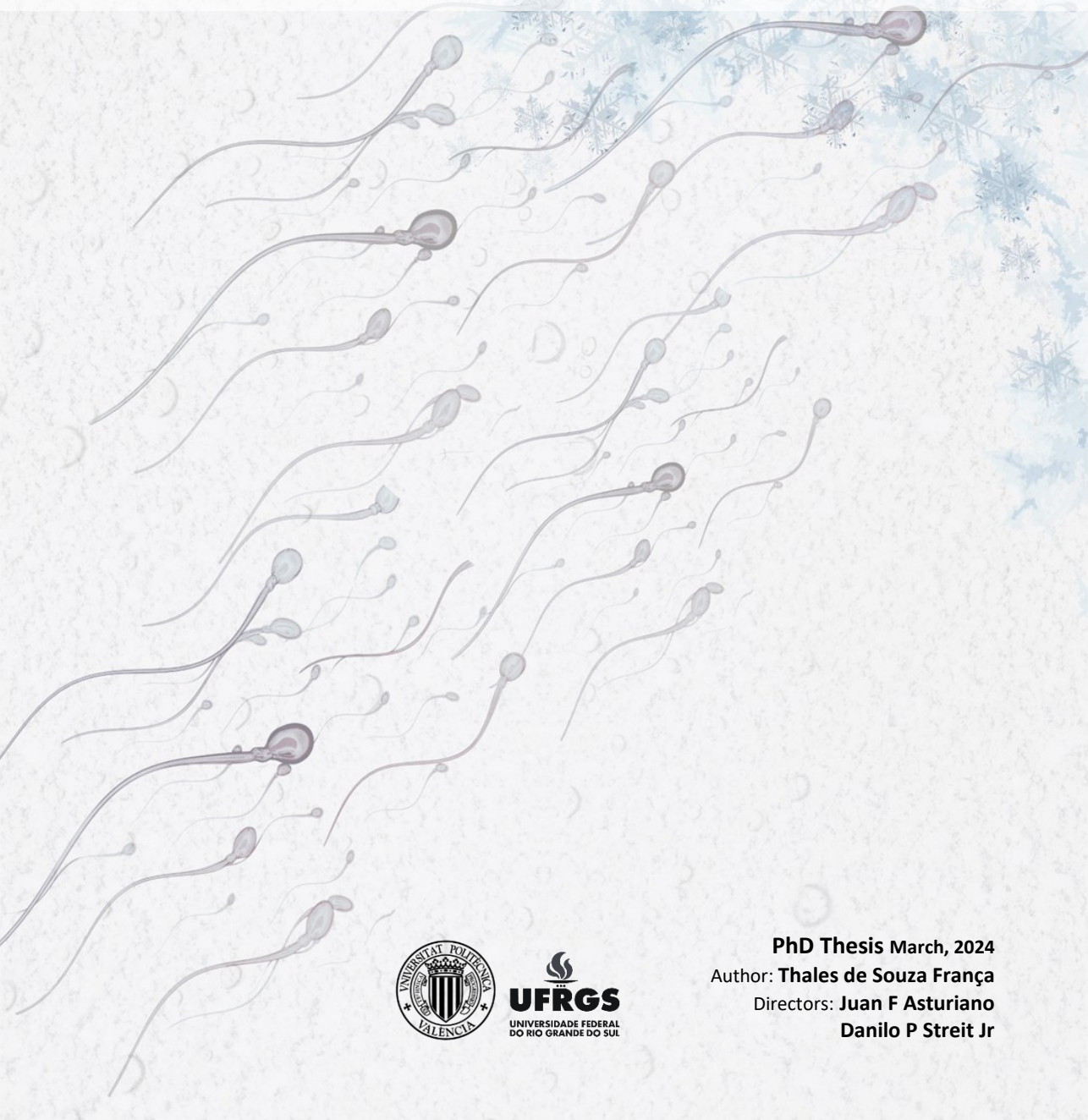
# DEVELOPMENT AND APPLICATION OF LOW-COST AND ENVIRONMENT-FRIENDLY TECHNIQUES FOR FISH SPERM CRYOPRESERVATION



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# DEVELOPMENT AND APPLICATION OF LOW-COST AND ENVIRONMENT-FRIENDLY TECHNIQUES FOR FISH SPERM CRYOPRESERVATION

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This Thesis has been submitted in accordance with the requirements for the degree of Doctor at the Universitat Politècnica de València and Federal University of Rio Grande do Sul.

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**Ao meu pai, Jorge Morais França (in memoriam)**

***"Ignorance is the cause of fear."***

— Lucius Annaeus Seneca

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## SUMMARY

Fish sperm cryopreservation is a technique that can increase the reproduction in captive efficiency of freshwater and marine fishes. The technique is applied in environmental conservation programs and aquaculture. Over the last few decades, protocols for sperm cryopreservation from many fishes have been established. However, the researchers' main focus was successfully freezing and thawing sperm, neglecting the period in which the gametes remain in contact with the cryoprotective solution until fertilization. Exposure of sperm to cryoprotectant solutions after thawing can be harmful to the quality of the gametes since they can be toxic.

The vast majority of the established protocols use ultra-resistant plastic containers to store sperm during the cryopreservation process. These containers usually are not reused, generating highly polluting waste for the environment. Furthermore, in some countries, the containers usually used are sold by a few industries, which makes acquisition difficult and increases the product's price. Thus, the main objective of the thesis was to create and test low-cost methodologies that enhance the use of fish post-thaw sperm and make the sperm cryopreservation process more environmentally friendly.

The experiments in the Chapters 1 and 2 were developed in Brazil. We used the South American silver catfish *Rhamdia quelen*, a species considered an experimental model for native South American fishes. *In vitro* fertilization and sperm cryopreservation protocols for the species were already established; thus, we based our work on these protocols to apply innovations.

In Chapter 1, we tested the use of post-thawing dilution to reduce the toxicity of the cryoprotectant solution. This technique is commonly used in mammalian sperm cryopreservation protocols but has never before been applied to post-thaw sperm of South American fishes. South American silver catfish post-thaw sperm samples were diluted in a saline extender (1.1% NaCl - 325 mOsm $kg^{-1}$; pH 7.6). The post-thaw sperm diluted samples showed higher velocities, straightness, progression, and flagellar beat frequency than

the cells of undiluted samples (control). The post-thawing dilution also provided higher fertilization and hatching rates than the control group. Thus, the post-thawing sperm dilution proved to be a simple, cheap, and efficient methodology that should be included in the silver catfish sperm cryopreservation protocol.

In Chapters 2 and 3, we developed, tested, and described the methodology for using biodegradable gelatin (collagen) and hypromellose (HPMC) capsules as an alternative container to plastic straws in the fish sperm cryopreservation. In the second chapter, we observed that the biodegradable capsules maintained the kinetic parameters and reproductive capacity of South American silver catfish sperm just as effectively as plastic straws. The results indicated that the capsules could be an alternative container to plastic straws in the silver catfish sperm cryopreservation.

The experimental procedures in Chapter 3 were carried out in Spain. We apply the methodology developed in Chapter 2 to the cryopreservation of sperm from European eel *Anguilla anguilla*, gilthead seabream *Sparus aurata*, and European sea bass *Dicentrarchus labrax*. In these three species, biodegradable capsules preserved the sperm kinetic parameters and membrane integrity just as effectively plastic straws. We observed that DNA damage in European eel and European sea bass sperm samples cryopreserved in capsules and straws did not differ. On the other hand, gilthead seabream sperm samples showed higher DNA damage than those cryopreserved in straws. However, the damage level observed in samples stored in capsules is considered low, thus, may not compromise embryonic development. We observed the results and concluded that biodegradable gelatin and HPMC capsules could be used as alternative containers to plastic straws for sperm cryopreservation from the three aquaculture Mediterranean fish species.

## RESUMEN

La criopreservación de semen de peces es una técnica que puede aumentar la eficiencia de la reproducción en cautiverio de especies de peces de agua dulce y marinas. La técnica se aplica en programas de conservación ambiental y en la acuicultura. A lo largo de las últimas décadas, se han establecido protocolos para criopreservación de semen de diversas especies de peces. Sin embargo, el foco principal de los investigadores ha sido en tener éxito en el congelamiento y descongelamiento de los espermatozoides, no llevando en cuenta el período en que los gametos se quedan expuestos a la solución crioprotectora en un momento previo a la fertilización. Esta exposición de los espermatozoides a las soluciones crioprotectoras después del descongelamiento puede ser perjudicial a la calidad de los gametos, ya que pueden ser tóxicos.

La gran mayoría de los protocolos establecidos utilizan recipientes de plástico ultrarresistentes para almacenar el semen durante el proceso de criopreservación. Estos recipientes normalmente no se reutilizan, generando residuos altamente contaminantes al medio ambiente. Además, en algunos países, los recipientes normalmente utilizados son comercializados por pocas industrias, o que dificultan la adquisición y elevan el precio del producto. Así, el objetivo principal de la tesis fue crear y probar métodos de bajo costo que potencialicen el uso de los espermatozoides descongelados de peces y torne el proceso de criopreservación de semen menos contaminante al medio ambiente.

Los experimentos de los capítulos 1 y 2 se llevaron a cabo en Brasil. Utilizamos el jundiá gris *Rhamdia quelen*, especie considerada modelo experimental para peces nativos de América del Sur. Los protocolos para la reproducción artificial y criopreservación de semen de esta especie ya se han establecidos; por lo tanto, nos basamos en estos protocolos para aplicar innovaciones.

En el capítulo 1, probamos el uso de la dilución de semen descongelado para disminuir la toxicidad de la solución crioprotectora. La técnica se utiliza comúnmente en protocolos de criopreservación de semen de mamíferos, pero nunca antes se había aplicado al semen descongelado de peces

suramericanos. Muestras de semen descongelado de *R. quelen* se diluyeron en un diluyente salino (NaCl al 1,1% - 325 mOsm $kg^{-1}$; pH 7,6). Después, observamos que los espermatozoides de muestras diluidas mostraron mayores velocidades, rectitud, progresión y frecuencia de batido flagelar que las muestras no diluidas. La dilución del semen descongelado también proporcionó mayores tasas de fertilización y eclosión que el grupo no diluido. De esta manera, la dilución de semen descongelado de *R. quelen* resultó ser una metodología sencilla, económica y eficiente que debe incluirse en el protocolo de criopreservación del semen de la especie.

En los capítulos 2 y 3 desarrollamos y probamos la metodología para el uso de cápsulas de gelatina biodegradables (colágeno) y cápsulas de hipromelosa biodegradables (HPMC) como recipiente alternativo al uso de pajuelas de plástico en la criopreservación de semen de peces. En el segundo capítulo observamos que las cápsulas biodegradables mantuvieron los parámetros cinéticos y la capacidad reproductiva del esperma de *R. quelen* así como las pajuelas de plástico. Indicando así que las cápsulas pueden ser utilizadas como un recipiente alternativo al uso de las pajuelas de plástico en la criopreservación de semen de esta especie.

Los procedimientos experimentales del capítulo 3 se llevaron a cabo en España. En este capítulo aplicamos la metodología desarrollada en el capítulo 2 para la criopreservación del semen de anguila europea *Anguilla anguilla*, dorada *Sparus aurata* y lubina *Dicentrarchus labrax*. En estas tres especies, las cápsulas biodegradables conservaron los parámetros cinéticos y la integridad de la membrana de los espermatozoides, así como las pajuelas de plástico. Además, observamos que el daño al ADN en muestras de semen de anguila europea y lubina europea criopreservadas en cápsulas y pajuelas no difirió. Sin embargo, las muestras de semen de dorada mostraron mayor daño en el ADN que las criopreservadas en pajuelas. Aunque, el nivel de daño que observamos en las muestras almacenadas en las cápsulas se considera bajo, por lo que pueden no comprometer el desarrollo embrionario. Evaluamos los resultados y concluimos que las cápsulas de gelatina biodegradables y las cápsulas de HPMC biodegradables pueden utilizarse como recipientes alternativos al uso de pajuelas de plástico para la criopreservación de semen de las tres especies mediterráneas.

## RESUM

La criopreservació de l'esperma de peixos és una tècnica que pot augmentar l'eficiència de la reproducció en captivitat d'espècies de peixos d'aigua dolça i marins. Aquesta tècnica s'aplica en programes de conservació ambiental i en l'aqüicultura. Al llarg de les dècades passades, s'han establert protocols per a la criopreservació de l'esperma de diverses espècies de peixos. No obstant això, el focus principal dels investigadors ha estat tenir èxit en la congelació i descongelació dels espermatozoides, sense tenir en compte el temps en què els gamets queden exposats a la solució crioprotectora abans de la fecundació. Aquesta exposició dels espermatozoides a les solucions crioprotectores després de la descongelació pot ser perjudicial per a la qualitat dels gàmetes, ja que poden ser tòxics.

La gran majoria dels protocols establerts utilitzen recipients de plàstic ultrarresistents per emmagatzemar l'esperma durant el procés de criopreservació. Aquests recipients normalment no es reutilitzen, generant residus altament contaminants per al medi ambient. A més, en alguns països, els recipients normalment utilitzats estan comercialitzats per poques indústries, la qual cosa dificulta l'adquisició i augmenta el preu del producte. Així, l'objectiu principal de la tesi va ser criar i provar mètodes de baix cost que potenciïn l'ús dels espermatozoides descongelats de peixos i facin que el procés de criopreservació de l'esperma sigui menys contaminant per al medi ambient.

Els experiments dels capítols 1 i 2 es van dur a terme a Brasil. Vam utilitzar el jundia gris *Rhamdia quelen*, una espècie considerada com a model experimental per a peixos natius d'Amèrica del Sud. Els protocols per a la reproducció artificial i criopreservació de l'esperma d'aquesta espècie ja estan establerts; per tant, ens vam basar en aquests protocols per aplicar innovacions.

En el capítol 1, vam provar l'ús de la dilució de l'esperma descongelat per reduir la toxicitat de la solució crioprotectora. Aquesta tècnica s'utilitza comúment en protocols de criopreservació de l'esperma de mamífers, però mai abans s'havia aplicat a l'esperma descongelat de peixos sud-americans. Mostres d'esperma descongelat de *R. quelen* es van diluir en un diluent salí

(NaCl al 1,1% - 325 mOsm kg-1; pH 7,6). Després, vam observar que els espermatozoides de mostres diluïdes mostraven majors velocitats, rectitud, progressió i freqüència de batuda flagel·lar que les mostres no diluïdes. La dilució de l'esperma descongelat també va proporcionar majors taxes de fecundació i eclosió que el grup no diluït. D'aquesta manera, la dilució de l'esperma descongelat de *R. quelen* va resultar ser una metodologia senzilla, econòmica i eficient que ha d'incloure's en el protocol de criopreservació de l'esperma de l'espècie.

En els capítols 2 i 3 vam desenvolupar i provar la metodologia per a l'ús de càpsules de gelatina biodegradables (col·lagen) i càpsules d'hipromelosa biodegradables (HPMC) com a recipient alternatiu a l'ús de canuts de plàstic en la criopreservació de l'esperma de peixos. En el segon capítol vam observar que les càpsules biodegradables mantenien els paràmetres cinètics i la capacitat reproductiva de l'esperma de *R. quelen* així com els canuts de plàstic. Indicant així que les càpsules es poden utilitzar com a recipient alternatiu a l'ús de canuts de plàstic en la criopreservació de l'esperma d'aquesta espècie.

Els procediments experimentals del capítol 3 es van dur a terme a Espanya. En aquest capítol vam aplicar la metodologia desenvolupada al capítol 2 per a la criopreservació de l'esperma d'anguila europea *Anguilla anguilla*, daurada *Sparus aurata* i llobarro *Dicentrarchus labrax*. En aquestes tres espècies, les càpsules biodegradables van conservar els paràmetres cinètics i la integritat de la membrana dels espermatozoides, així com els canuts de plàstic. A més, vam observar que el dany a l'ADN en mostres d'esperma d'anguila europea i llobarro europeu criopreservades en càpsules i canuts no es va diferir. No obstant això, les mostres d'esperma de daurada van mostrar més dany a l'ADN que les criopreservades en canuts. Tot i això, el nivell de dany que vam observar a les mostres emmagatzemades en càpsules es considera baix, pel que poden no comprometre el desenvolupament embrionari. Vam avaluar els resultats i vam concloure que les càpsules de gelatina biodegradables i les càpsules d'HPMC biodegradables es poden utilitzar com a recipients alternatius a l'ús de canuts de plàstic per a la criopreservació de l'esperma de les tres espècies mediterrànies.

## RESUMO

A criopreservação de sêmen de peixes é uma técnica que pode aumentar a eficiência da reprodução em cativeiro de espécies de peixes de água doce e marinhas. A técnica é aplicada em programas de conservação ambiental e na aquicultura. Ao longo das últimas décadas, foram estabelecidos protocolos para criopreservação do sêmen de diversas espécies peixes. Porém, o foco principal dos pesquisadores foi no sucesso do congelamento e descongelamento dos espermatozoides, negligenciando o periodo em que os gametas ficam em contato com a solução crioprotetora até a fertilização. A exposição dos espermazoides às soluções crioprotetoras após o descongelamento pode ser prejudicial à qualidade dos gametas, já que elas podem ser tóxicas.

A grande maioria dos protocolos estabelecidos utilizam recipientes plásticos ultra-resistentes para armazenar o sêmen durante o processo de criopreservação. Esses recipientes normalmente não são reutilizados, gerando resíduos altamente poluentes ao meio ambiente. Além disso, em alguns países, os recipientes normalmente utilizados são comercializados por poucas industrias, o que dificulta a aquisição e eleva o preço do produto. Assim, o principal objetivo da tese foi criar e testar metodologias de baixo custo que potencializem o uso de espermatozoides descongealdos de peixes e torne o processo de criopreservação de sêmen menos poluente ao meio ambiente.

Os experimentos do capítulo 1 e 2 foram realizados no Brasil. Utilizamos o jundiá cinza *Rhamdia quelen*, espécie considerada modelo experimental para os peixes nativos sulamericanos. Os protocolos de reprodução artificial e criopreservação de sêmen desta espécie já foram estabelecidos; assim, nos baseamos nesses protocolos para aplicar as inovações.

No capítulo 1, testamos o uso da diluição do sêmen descongelado para diminuir a toxicidade da solução crioprotetora. A técnica é comumente utilizada em protocolos de criopreservação de sêmen de mamíferos, porém nunca antes aplicada no sêmen de peixes sul-americanos. Amostras descongeladas de sêmen de jundiá cinza foram diluídas em um diluidor salino (1,1 % NaCl - 325 mOsm $kg^{-1}$; pH 7,6). Observamos que os espermatozoides

das amostras diluídas apresentaram maiores velocidades, retilinearidade, progressão e frequência de batimento flagelar que as amostras não diluídas. A diluição do sêmen descongelado também propocionou maiores taxas de fertilização e eclosão que o grupo controle. Desta forma, a diluição do sêmen descongelado de jundiá cinza se mostrou uma metodologia simples, barata e efficiente que deve ser incluída no protocolo de criopreservação do sêmen da espécie.

Nos capítulos 2 e 3 desenvolvemos e testamos a metodologia de uso das cápsulas biodegradáveis de gelatina (colágeno) e das cápsulas biodegradáveis de hipromelose (HPMC) como um recipiente alternativo ao uso das palhetas plásticas na criopreservação do sêmen de peixes. No segundo capítulo, observamos que as cápsulas biodegradáveis mantiveram os parâmetros cinéticos e a capacidade reprodutiva dos espermatozoides de jundiá cinza tão bem quanto as palhetas plástica. Indicando assim que as cápsulas podem ser utilizadas como um recipiente alternativo ao uso das palhetas plásticas na criopreservação de sêmen dessa espécie.

Os procedimentos experimentais do capítulo 3 foram realizados na Espanha. Neste capítulo, aplicamos a metodologia desenvolvida no capítulo 2 para a criopreservação do sêmen de enguia europeia *Anguilla anguilla*, dourada *Sparus aurata* e robalo europeu *Dicentrarchus labrax*. Nessas três espécies, as cápsulas biodegradáveis preservaram os parâmetros cinéticos e a integridade de membrana dos espermatozoides tão bem quanto as palhetas pláticas. Observamos que os danos de DNA das amostras de sêmen de enguia europeia e robalo europeu criopreservadas em cápsulas e palhetas não se diferiram. Por outro lado, as amostras de sêmen de dourada mostraram maior dano de DNA que as criopreservadas em palhetas. Porém, o nível de dano que observamos nas amostras armazenadas em cápsulas é considerado baixo, assim podem não comprometer o desenvolvimento embrionário. Observamos os resultados e concluímos que as cápsulas biodegradáveis de gelatina e as cápsulas biodegradáveis de HPMC podem ser utilizadas como recipientes alternativos ao uso das palhetas plásticas para a criopreservação do sêmen das três espécies de peixes mediterrâneas.

# GENERAL INTRODUCTION

# 1. Fish sperm cryopreservation

## *1.1 Cryopreservation*

Cryopreservation methodologies were developed based on knowledge of cryobiology, the science that studies life at low temperatures and has as its object of study any biological material at a temperature below its physiological range. Cryopreservation is defined as conserving the viability of cells, tissues, and organs at low temperatures in quiescence, promoting the cessation of biochemical reactions, and restoring metabolic activities after thawing (Mazur, 1970). Biological materials of interest are generally stored according to their shelf life. If the samples are used for a short time, they are stored at 4 °C; this technique is known as short-term storage. However, if the objective is to maintain the cells for a long time, the long-term technique is applied, where the samples are stored in an ultra-freezer (-80 °C), suspended in liquid nitrogen vapor cylinders (-170 °C), or immersed in liquid nitrogen (-196 °C). At such low temperatures, kinetic energy levels are too low to allow molecules to move, promoting the material's conservation (Mazur, 1984).

## *1.2 History*

According to Sztein et al. (2018), cryobiology has been studied for centuries. In 1776, Lazzaro Spallanzani described sperm survival from humans, dogs, bulls, horses, frogs, and salamanders in hot and cold environments (Spallanzani, 1776). In 1866, Paolo Mantegazza, a physicist, anthropologist, and visionary, observed sperm samples from humans and frogs frozen at -14 and -17 °C. Furthermore, he envisioned that maintaining cells at low temperatures would facilitate artificial reproduction practices in animals and enable the creation of sperm banks from soldiers fighting in wars. Over the years, scientists have discovered and perfected techniques for maintaining cells at low temperatures. In 1949, Christopher Polge, Audrey Smith, and Alan Parkes first described glycerol as a cryoprotectant (Polge et al., 1949). The discovery of glycerol as a cryoprotectant is the beginning of a new era of cryobiology because its use enabled the development of sperm cryopreservation protocols for various species (Sztein et al., 2018). The first

published work showing successful cryopreservation of fish sperm was carried out by Blaxter in 1953. After this study, over the last few decades, fish sperm cryopreservation methodologies have evolved, increasing the number of protocols and their applications (Horváth and Urbányi, 2020).

### *1.3 Recipients*

The first step to achieving success in a cryopreservation protocol is choosing the correct container to store the sperm at low temperatures. The composition, thickness, and mainly the volume of sperm that the container holds directly influence the freezing and thawing curves of the sample and the purpose of using the samples (Cabrita et al., 2022).

In the early days of cryopreservation, glass tubes were used to cryopreserve fish sperm. Over the years, plastic containers: straws (0.25,0.3, 0.5, 1.2, 2, and 5 mL) and cryovials (1, 1.8, 2, 3.5, 4.5, and 5 mL) replaced glass tubes (Figure. 1).

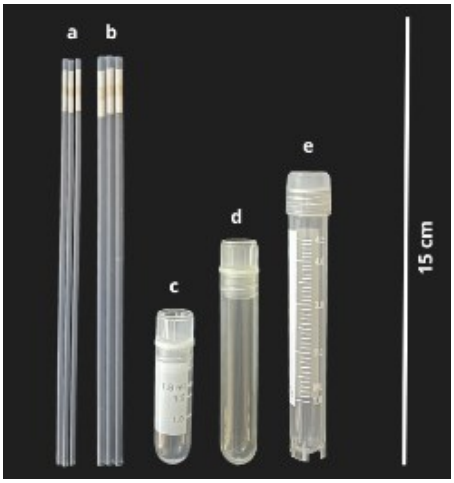


**Figure 1.** Plastic containers in fish sperm cryopreservation. Straws (a – 0.25; b – 0.5 mL) and cryovials (c – 1.8; d – 3.5; e – 4.5 mL).

All of these containers are manufactured using ultra-resistant plastic compounds, such as polypropylene and polyvinyl chloride (PVC). Most fish sperm cryopreservation protocols use low-volume straws (0.25 and 0.5 mL).

These containers are easy to handle and, due to their shape, are very efficient for storing sperm samples inside liquid nitrogen tanks. However, the low volume of the straws (0.25 and 0.5 mL) is a disadvantage when a large quantity of cells is needed, making the operation harder and slower.

Cryotubes are used to cryopreserve sperm in higher volumes than those supported by straws (Cabrita et al., 2001). These containers are also used in germ stem cells and reproductive tissues cryopreservation protocols (Cabrita et al., 2022). Cryotubes are resistant, easily labeled, and store a high volume of cells. On the other hand, they require a specific liquid nitrogen tank to store the samples in an orderly and safe manner. Furthermore, plastic cryotubes are manufactured by a few companies, which makes their accessibility in developing countries difficult.

Hard-gelatin and hard-hypromellose (hydroxypropyl methylcellulose – HPMC) capsules, widely used for administering medicines and other substances, appear as cryopreservation sperm containers (França et al. 2023). The main advantages of using capsules as recipients in cryopreservation include the physical resistance to ultra-low temperatures, safe labelling, biodegradability, different sizes (Figure. 2), low acquisition cost, and high accessibility on the market. However, the biodegradable capsules also have disadvantages, such as dissolution in contact with permeable cryoprotectants at room temperature (~25 °C), the use of an extender to thaw the sample, and the lack of specific equipment for storage in liquid nitrogen. Developing cell cryopreservation protocols using biodegradable capsules is still in the beginning. Thus, the disadvantages should be mitigated with protocol advances.

Low-volume containers are an excellent choice for forming germplasm and sperm banks for commercial larvae production. However, for large-scale commercial production of larvae, containers with larger volumes facilitate handling during animal reproduction (Cabrita et al., 2001; Herranz-Jusdado et al., 2019a; Maria et al., 2015).

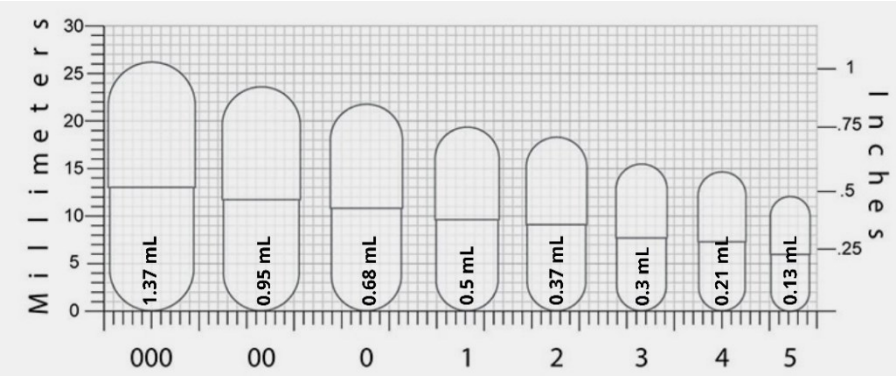


**Figure 2.** Hard-capsules sizes, length (mm and inches), and volume (mL). The wall of body capsules is 0.110 mm (size 000), 0.107 mm (size 00), 0.104 mm (size 0), 0.102 mm (size 1), 0.99 mm (size 2), 0.091 mm (size 3), 0.089 mm (size 4), and 0.086 mm (size 5). Figure adapted from https://www.icapsulepack.com.

### *1.4 Extenders*

The sperm of most fishes remain immotile inside the gonads, being in contact with the seminal plasma (Morisawa and Suzuki, 1980). Spermatozoa activation occurs when cells come into contact with a hypoosmotic solution in relation to seminal plasma in the case of freshwater species or a hyperosmotic solution in marine species. Thus, extenders with osmolality, pH, and ionic concentration adjusted according to the composition of the seminal plasma maintain the immotile spermatozoa, allowing the application of several techniques, including sperm cryopreservation (Alavi and Cosson, 2006).

Diluting fish sperm in an extender is essential for successful sperm cryopreservation. Using the extender in the cryoprotective solution aims to maintain the integrity of the sperm structure during the cryopreservation and thawing process. Its principal function is to maintain the osmotic pressure and pH of the sperm, in addition to providing nutrients and energy for the spermatozoa (Yongsheng et al., 2020). Due to the different compositions of the seminal fluid and the species' physiology, the composition of the seminal plasma is also different, thus making the use of the extender species-specific. The extender can be composed of just one chemical, as in the South American silver catfish (*Rhamdia quelen*) sperm

cryopreservation protocol that uses only fructose (Adames et al., 2015), or it can be composed of more than one, as in the P1 extender, composed of NaCl, $NaHCO_3$, $MgCl_2$, $CaCl_2$, and KCl), used in the European eel sperm cryopreservation protocol (Peñaranda et al., 2009).

Extenders can be composed of salts such as NaCl, KCl, $Na_2CO_3$, $MgSO_4$, $KHCO_3$, $CaCl_2$, $MgCl_2$, $KH_2PO_4$, and others. These chemical compounds aim to provide osmotic pressure and ionic presence in order to immobilize sperm and maintain intracellular pH during the cryopreservation process. They may also contain sugars such as glucose, sucrose, fructose, lactose, and trehalose. The sugars maintain osmotic pressure and act as an extracellular cryoprotectant, providing energy to sperm (Jawahar and Betsy, 2020).

### *1.5 Cryoprotectants*

As the extender, cryoprotectants are essential for the success of fish sperm cryopreservation. Extender and cryoprotectant form the cryoprotectant solution. Cryoprotectants are divided into two groups: permeable and non-permeable. Both groups have the same objective: to protect the cell against the formation of ice crystals. A cryoprotectant solution can contain only permeable or non-permeable cryoprotectants. However, the most common in fish sperm cryopreservation protocols is the presence of both.

Permeable cryoprotectants are substances with high solubility and low molecular weight capable of penetrating cells and performing their cryoprotective action throughout the cytoplasm and organelles (Fahy, 2010). These substances replace the existing water in the cellular environment and increase the permeability of the plasma membrane. These actions cause partial dehydration of the cell, reducing the freezing point, which consequently prevents the formation of ice crystals inside the cells. In fish sperm cryopreservation protocols, the choice and concentration of permeable cryoprotectants, the addition of cryoprotectants temperature, and the equilibrium time should be practiced assertively. Thus, the cryoprotectant solution will not be toxic to the cells and will promote

efficient protection during cryopreservation (Cabrita et al., 2022). Two of the most commonly used permeable cryoprotectants in fish sperm cryopreservation protocols are dimethyl sulfoxide ($Me_2SO$) and methanol (MeOH).

Non-permeable cryoprotectants are substances that have a high molecular weight. Therefore, they cannot penetrate inside the cell and promote protective action externally. As they are hydrophilic compounds, they bind to water molecules, increasing the viscosity of the solution and reducing the formation of ice crystals (Fahy, 2007). The non-permeable cryoprotectants used in fish sperm cryopreservation protocols are sugars, such as glucose, fructose, and trehalose, and sources rich in nutrients, such as egg yolk and milk powder.

### *1.6 Freezing and thawing*

Most cell damage happens during the freezing and thawing of cells. Therefore, these two moments are considered critical points in the cryopreservation process, and their execution should be well studied and planned (Mazur, 1984).

Fish sperm freezing is usually carried out using liquid nitrogen vapor. Sperm cooling can be carried out in two ways: an uncontrolled way and a controlled way. In the uncontrolled form, sperm is cryopreserved inside a Styrofoam box filled with liquid nitrogen. The samples are placed in a floating structure at a certain height from the surface of the liquid nitrogen, where they are in contact with the nitrogen vapor. Samples are exposed to liquid nitrogen vapor for an optimal time, typically a few minutes, and are then submerged in liquid nitrogen before being stored. Another way to carry out uncontrolled freezing is using a dry-shipper, a liquid nitrogen vapor tank to transport cryopreserved samples. This method is widely used in sperm cryopreservation protocols for South American fishes (Viveiros and Godinho, 2009) and is more efficient than the Styrofoam box for freezing a large number of samples (Horokhovatskyi et al., 2017).

Sperm freezing in a controlled way is carried out using an equipment called biofreezer. This equipment consists of a freezing chamber where the samples are placed, connected to a computer, and a liquid nitrogen tank. This equipment has variations, such as the electric freezer, which does not use liquid nitrogen vapor (Diogo et al., 2018). However, the operation of this equipment is of the same standard. The desired freezing curve is entered into software installed on the computer that controls the freezing chamber's internal temperature and performs the freezing curve accurately. Freezing fish sperm samples in biofreezers provides better standardization of protocols compared to uncontrolled freezing methods. The acquisition and maintenance costs and the difficulty of transportation mean that uncontrolled freezing methods are still the most used.

The samples should be thawed to restore sperm metabolism and evaluate the results of the cryopreservation process. Thawing fish sperm is customarily carried out in a water bath. The water temperature and submersion time vary according to the protocol for each species and the type of container used to store the samples. The premise is typically that the greater the frozen sperm volume, the greater the temperature and time of submersion of the samples. Thawing water temperatures can vary from 5 to 80 °C. The thawing time for straws (0.25-0.5 mL) can vary between 3 to 60 s, while for cryovials and macro tubes, it can be longer than 150 s (Cabrita et al., 2001; Herranz-Jusdado et al., 2019a; Riesco et al., 2017a).

### *1.7 Application*

Currently, the primary use of the fish sperm cryopreservation technique is in genetic material conservation programs through germplasm cryobanks, which are maintained mainly by public funding (Asturiano et al., 2017). Germplasm banks hold samples of freshwater and saltwater fishes and species used as experimental models in the laboratory. These samples can assist aquaculture, simplifying broodstock management on properties and serving as a genetic backup (Martínez-Páramo et al., 2017). Cryobanks are also a powerful tool for preserving genetic material from wild fishes and can assist environmental conservation programs in restructuring the natural stocks of species at a high extinction risk. Furthermore, germplasm banks

allow the conservation of strains, mutants or transgenic fish of species used as experimental models in biomedical research (Liu et al., 2019).

In aquaculture, sperm cryopreservation provides better use of breeders on properties that produce fry, allowing the reproduction period to be more flexible, conserving the genetics of animals selected by genetic improvement programs, and solving problems of desynchronization between males and females in the final maturation of the gametes (Asturiano et al., 2017). Furthermore, cryopreservation facilitates the production of inter-species hybrids and intra-species cross-breedings (Judycka et al., 2019). Fish sperm cryopreservation brings benefits to aquaculture; however, it presents challenges for its implementation. Among these challenges are the standardization of sperm cryopreservation methodologies for each species and the development of practical protocols that scale the freezing and thawing of samples to be used in oocyte fertilization (Judycka et al., 2019; Tiersch, 2011). Due to these challenges, sperm cryopreservation is still scarcely used in aquaculture compared to its use in bovine and horse reproduction. On the other hand, aquaculture is an activity that has shown constant evolution, not only productively but also technologically. With an eye on this market, large animal reproduction companies already offer product lines for fish sperm cryopreservation. Furthermore, the tendency is that new companies will emerge offering fish sperm cryopreservation services and invest in developing products aimed at this area (Asturiano et al., 2017; Martínez-Páramo et al., 2017).

## 2. South American silver catfish (*Rhamdia quelen*)

The South American silver catfish *Rhamdia quelen* (Figure 3) is a teleost fish belonging to the Siluriformes order and is part of the Pimelodidae family. This species has a Neotropical distribution from Argentina and Uruguay to south eastern Mexico. In Brazil, this fish is known as "jundiá cinza". Adult fish have an omnivorous eating habit and prefer crustaceans, insects, fish, plant remains, and organic debris (Silfvergrip, 1996).

**Figure 3.** South American silver catfish *Rhamdia quelen*. (Source: AQUAM group)

The growth rate of males is higher than that of females until the third or fourth year of life, when the situation is reversed. The calculated asymptotic growth of males is 52 cm and of females 66.5 cm (Gomes et al., 2000). South American silver catfish is a rheophilic and oviparous fish with external fertilization. According to Nakatani et al. (2001) sexual maturation is reached at approximately 16.5 cm in females and 13.4 cm in males. This species has two reproductive peaks per year, one in the spring and the other in the summer, with total spawning. There is no parental care. They reproduce in areas with clean and calm water and a stony bottom.

Over the years, South American silver catfish has been used as a model native species for the development of methodologies in several aquaculture areas, such as reproduction (Adames et al., 2015; Neumann et al., 2019), cryobiology (Da Costa et al., 2019, 2020); management (Becker et al., 2013; Corso et al., 2019) and health (Andrade et al., 2006). This was due to the species presenting desirable characteristics, such as the large quantity of gametes produced during the reproductive period and the calm temperament of the animals.

## 3. European eel (*Anguilla anguilla*)

The eel family constitutes a diverse group of Anguilliformes, encompassing over 800 known species of eels (Mapes and Mouillot, 2022). The genus *Anguilla* comprises 19 freshwater eel species (Watanabe, 2003). These species have an intricate catadromous life cycle involving oceanic spawning migrations from a few hundred to several thousand kilometers, depending on the species (Arai, 2014).

Among the species of the genus *Anguilla*, European eel *A. anguilla* is the one that carries out the most distant reproductive migration. This species travels 4000 to 6000 km between seas that bathe the European continent and the Sargasso Sea along the Atlantic Ocean (Arai, 2014). The European eel life history (Figure 4) begins with the departure of mature breeding animals from continental rivers and swimming to the Sargasso Sea, taking advantage of the Canary and North-equatorial currents. The animals arrive after 6-7 months, reproduce and die. Leptocephalus larvae return to the European coast helped by the Gulf Stream and North-Atlantic Drift for 8-9 months (Arai et al., 2000). Arriving at the coast, they undergo metamorphosis and transform into glass eels, then they enter the rivers where they grow until they reach puberty years later (more or less depending on the region's temperature) (Tesch, 2003).

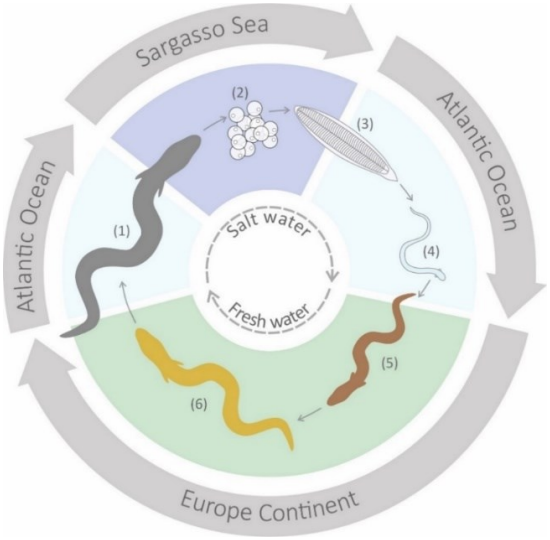


**Figure 4.** European eel *Anguilla anguilla* catadromous life cycle. Breeders carry (1) out reproductive migration from European continental rivers to the Sargasso Sea, crossing the Atlantic Ocean. Reproduction (2) occurs in the Sargasso Sea, and after hatching, the leptocephalus larvae (3) are carried to the European continent by the currents of the Atlantic Ocean. Arriving on the coast of the European continent, they undergo metamorphosis and transform into glass eels (4). They then enter continental rivers and continue growing, going through the elver (5) and yellow (6) phases until they reach the stage of reproductive migration (1). Adapted from (Williamson et al., 2023).

Over the years, human actions such as overfishing, pollution, and the construction of dams, among others, have reduced 90% of the species' natural stocks. Nowadays, the European eel (Figure 5) is classified as "Critically Endangered" in the Red List of the International Union for Conservation of Nature (IUCN) (Pike et al., 2020). The high consumer market demand and the risk of extinction of the species in the natural environment led to a significant mobilization of governments and the scientific community to seek to mitigate the problem. Thus, projects are funded for species conservation and developing and improving techniques that increase European eel production in aquaculture.

**Figure 5.** European eel *Anguilla anguilla* (Source: Carta Piscícola Española; https://www.cartapiscicola.es/#/home)

Sperm cryopreservation is a technique capable of assisting European eel's natural conservation and production in captivity. Therefore, short-term and long-term conservation protocols have been created and improved over the last two decades (reviewed by Herranz-Jusdado et al., 2019b). The established protocols are efficient, but new adjustments can be made to increase the economic and environmental sustainability of the cryopreservation process.

## 4. Gilthead seabream (*Sparus aurata*)

The gilthead seabream *Sparus aurata* (Figure 6) belongs to the superclass Actinopterygii, the Osteichthyes class, and the Perciformes order (Hanel and Costas, 2011). The species is classified as a subtropical fish, and its natural stocks are distributed in the region ranging from 15° to 62° N and from 7° W to 43° E. This region covers the Eastern Atlantic Ocean and the Mediterranean and Black Seas. In a natural environment, fish inhabit the surf zone at depths ranging from 30 to 150 m. The animals live in small shoals or solitary, have eurythermic and euryhaline characteristics, and are resistant to wide range of temperatures and salinities (Hanel nad Costas, 2011). The

gilthead seabream has an omnivorous feeding habit, adapting its diet according to the availability of food in its habitat. Their food preferences are gastropods and bivalves, but they also feed on echinoderms, polychaetes, and occasionally algae and bryozoans (Pita et al., 2002).

**Figure 6.** Gilthead seabream *Sparus aurata*. (Source: Scandinavian Fishing Year Book; https://fish-commercial-names.ec.europa.eu/fish-names/species/sparus-aurata_en.).

The sea bream is a species of fish that presents protandrous hermaphroditism. In the first two years, the fish grow and mature as males; when they reach 30 cm in length, they transition to females (Chaoui et al., 2006). During the male period, the testes are located in the ventral area and present asynchronous spermatogenesis, in the dorsal part, the gonad presents a non-functional ovarian area (Zohar et al., 1978). Considering the genetic similarity between males and females, morphological and behavioral disparities and sexual dimorphisms are attributed to a different expression of genes according to sex, resulting in a more or less pronounced transcription in one sex compared to the other (Pauletto et al., 2018).

The reproduction of the species can be carried out in captivity. Manipulation of water temperature and environmental photoperiod induces sexual maturation of fish (FAO, 2022a). Groups of animals of different ages are formed, following the ratio of 2 males for each female. The release of semen occurs naturally or through extraction. Spawning can occur spontaneous or be induced with the help of the hormone GnRHa in doses ranging from 5 to 20 mg/kg (FAO, 2022a).

The seabream is the world's fourth most-produced marine fish (FAO, 2022b). The exchange of knowledge between fish farmers and scientists has led to production growth over the years. Several areas of the species' production have been investigated over the last few decades (Mhalhel et al., 2023), one of them being the cryopreservation of the species' sperm (Cabrita et al., 2005; Fabbrocini et al., 2000; Gallego et al., 2012a). Adjustments in production management will always be welcome for the continued sustainable growth of aquaculture.

## 5. European sea bass (*Dicentrarchus labrax*)

The European sea bass *Dicentrarchus labrax* (Figure 7) belongs to the superclass Actinopterygii, Osteichthyes class, Perciformes order, and Moronidae family. The natural stocks of the species are distributed in the Eastern Atlantic Ocean, Azov, Black, North, Baltic, Mediterranean, and Adriatic Seas (11°- 72° N and 19° W - 42° E). The species is considered eurythermic, euryhaline, and resistant to temperature and salinity variations. Therefore, they are found in sheltered bays, coastal lagoons, estuaries, and offshore areas (López et al., 2015). European sea bass has a carnivorous eating habit and feeds on shrimp, molluscs, and other fishes (Abbate et al., 2012).

**Figure 7.** European sea bass *Dicentrarchus labrax*. (Source: Scandinavian Fishing Year Book; https://oceans-and-fisheries.ec.europa.eu/ocean/marine-biodiversity/seabass_en).

This species is a gonochoristic species lacking morphological secondary sex characters. However, it displays distinct sex-related growth patterns, with females growing faster and achieving larger sizes than males (Saillant et al., 2001). The age and size at which animals reach sexual maturity varies

depending on the environment (López et al., 2015). In the Mediterranean, males reproduce at two years old and 20-25 cm in length. Females spawn for the first time between 3 and 4 years old and are 29-34.5 cm long (López et al., 2015).

European sea bass is the world's sixth most-produced marine fish (FAO, 2022). Developing the production chain was essential to place this species in the most produced. Among the production areas is captive reproduction. According to Superio et al. (2021), *in vitro* fertilization through hormonal induction of breeders is the most efficient way to develop breeding programs and, consequently, the production of the species. Thus, one of the tools to facilitate the exchange of genetic material between fish farmers and create a genetic backup bank for breeding programs is sperm cryopreservation. Protocols for European sea bass sperm cryopreservation have been established and improved over the years (Fauvel et al., 1998; Martínez-Páramo et al., 2012, 2013). However, the search to make the technique more efficient from the point of view of environmental sustainability will always be welcome.

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# OBJECTIVES

The general objective of the thesis was to study, create, adapt, and test low-cost methodologies that enhance the use of fish sperm and make the sperm cryopreservation process more environment-friendly. The specific objectives include:

- ✓ To evaluate the quality and reproductive capacity of South American silver catfish cryopreserved sperm diluted after thawing in an extender.
- ✓ To develop and standardize cryopreservation protocols for South American silver catfish, European eel, gilthead seabream, and European sea bass using low-cost biodegradable gelatin and Hypromellose capsules (HPMC).
- ✓ To compare the sperm quality of South American silver catfish, European eel, gilthead seabream, and European sea bass sperm samples cryopreserved in straws and biodegradable capsules.

# CHAPTER 1

## Post-thaw dilution of *Rhamdia quelen* sperm improves the reproductive success

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## Abstract

The aim was to evaluate the effect of a post-thaw dilution of *Rhamdia quelen* sperm in 1.1 % NaCl (325 mOsm $kg^{-1}$; pH 7.6; 24 °C) solution on the quality and reproductive capacity. Sperm from eight males were cryopreserved in nitrogen vapor at − 170 °C for 18 h in 0.25 mL straws in a freezing medium containing 5% fructose, 5% Powdered milk, and 10% methanol. The samples were thawed and post-thaw diluted (1:20) in NaCl solution or not (control). The higher spermatozoa velocities were observed in the post-thaw diluted samples (curvilinear (VCL) - 69 ± 11 µm $s^{-1}$; average path (VAP) - 45 ± 8 µm $s^{-1}$; straight-line (VSL) - 43 ± 8 µm $s^{-1}$) compared to the control (VCL - 47 ± 10 µm $s^{-1}$; VAP - 31 ± 6 µm $s^{-1}$; VSL - 30 ± 6 µm $s^{-1}$). Greater straightness (STR), progression (PROG), and beat cross frequency (BCF) were observed in the post-thaw diluted samples (STR - 96 ± 7%; PROG - 666 ± 128 µm; BCF - 42 ± 2 Hz) than in control (STR - 95 ± 5%; PROG - 463 ± 92 µm; BCF - 40 ± 2 Hz). The strongly curled tail was the only morphology change that differ between the post-thaw diluted (5 ± 2 %) and control (2 ± 1 %). Membrane integrity, mitochondrial activity, and normal larvae rate were not different between treatments. Fertilization and hatching were higher in the post-thaw diluted sperm (93 ± 3%; 82 ± 9%) when compared to control samples (65 ± 13 %; 55 ± 17 %). Were used oocytes from one female, limiting these results. The post-thaw dilution improved the sperm kinetics and reproductive parameters. Thus, this methodology can be included in the sperm cryopreservation protocol for *R. quelen*.

# 1. Introduction

Fish sperm cryopreservation protocols have been developed in which several substances have been tested to preserve sperm viability during freezing and thawing (Martínez-Páramo et al., 2017). However, but the period after thawing when sperm are in contact with cryoprotective solutions has previously been neglected by researchers. During the freezing/thawing, cells suffer high stress and damage, so permeable cryoprotectants penetrate cells and inhibit this damage, thus these substances are the key to maintaining sperm viability after the cryopreservation process.

Permeable cryoprotectants are components present in cryoprotective solutions that prevent ice crystals' formation and reduce cryoinjuries. However, during the thawing process, the rising temperature increases the toxicity of the permeable cryoprotectants to cells (Best, 2015). In mammals, the common method of cryoprotectant removal involves stepwise dilution with a cryoprotectant-free medium, centrifugation, and resuspension of the sperm. These procedures can cause osmotic and physical damage associated with decreased fertilization rates (Alvarenga et al., 2012; Alvarez et al., 1993). Thus, to reduce the damage caused by cryoprotectants while avoiding centrifugation injury, we propose a new technique called post-thaw dilution, where the thawed sperm is diluted immediately after thawing and before fertilization.

NaCl has low commercial value, is accessible, and has previously been used as an extender in cryoprotective sperm solutions (López et al., 2015; Orfão et al., 2011) and activating medium (Viveiros and Godinho, 2009) for neotropical fishes. Seminal plasma is an essential factor in maintaining immobility and sperm quality in the reproductive tract (Figueroa et al., 2015). Neotropical fishes have seminal plasma with osmolalities between 216 and 313 mOsm $kg^{-1}$ (Di Chiacchio et al., 2017), pH between 6.5 and 8.5 (Tabares et al., 2005), and are rich in $Na^{+}$ ions (Egger et al., 2021; França et al., 2020). Therefore, NaCl solution at a concentration of 325 mOsm $kg^{-1}$ and a pH of 7.6 guarantees the immobility of sperm by promoting conditions similar to seminal plasma. In addition, the presence of NaCl at the time of activation potentiates the sperm kinetic parameters (Viveiros et al., 2019), increasing

the chances of fertilization.

The South American silver catfish (*Rhamdia quelen*) is one of the native American species with great potential for aquaculture (Valladão et al., 2018) and a commercial species that consumers appreciate (Gil Barcellos et al., 2004). Its culture is performed mainly in Uruguay, Argentina, and Brazil, where 36,017 fish farmers produce this catfish (Peixe-BR, 2020). This species has protocols for artificial reproduction in captivity (Sanches et al., 2011a), and its sperm cryopreservation protocol was defined by Adames et al. (2015), where the post-thaw sperm presented 49% of motility, 59% of fertilization rate, and 48% of hatching rate. Therefore, this study aimed to evaluate the effect of a post-thaw dilution, diluting South American silver catfish thawed sperm in 1.1% NaCl (325 mOsm $kg^{-1}$; pH 7.6; 24 °C) solution on kinetic parameters, morphology, mitochondrial activity, and membrane integrity of sperm cells, as well as on the rates of fertilization, hatching, and normal larvae production.

## 2. Materials and Methods

### *2.1 Fish handling and gametes collection*

The experiment was performed at the Federal University of Rio Grande do Sul Research Group AQUAM, Porto Alegre (30°04'24'' S; 51°08'11'' W), Rio Grande do Sul State, Brazil, during the spawning season (January 2020). Animal handling and experiments were performed according to with the Animal Use Ethics Committee of the Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS (N° 39527).

Two-year-old South American silver catfish (*R. quelen*) from artificial spawning and breeding were acquired from a commercial fish farm and transported to the laboratory, where they were maintained in concrete tanks (7 $m^3$) in a recirculating system for two months until the experiment. Fish were fed a commercial diet (32% crude protein, Acqua Fish, Supra®, Alisul, Brazil) twice a day (8:00 and 16:00) until apparent satiety. Animal health and behavior were monitored daily during feeding. Males (n = 8; 762 ± 168 g of body weight) with detectable running sperm under soft abdominal pressure, and females (n = 4; 437 ± 131 g) with bulging of the celomatic cavity and

reddish urogenital papilla were selected. The breeders were weighed, provided an identification chip, and transferred to two plastic tanks (500 L) in a recirculating system. The experimental parameters were as follows: water temperature, 24 ± 0.5 °C; pH, 7.0 ± 0.2; dissolved oxygen, 5.5 ± 0.5 mg $L^{-1}$; and natural photoperiod (14 h light:10 h dark).

The males received a single intraperitoneal dose of carp pituitary extract (CPE) at 3 mg $kg^{-1}$, 8 h before sperm collection. Females were induced with two hormonal doses of CPE, with the first dose of 0.5 mg $kg^{-1}$ at 22 h before collection and the second effective dose at 5 mg $kg^{-1}$ at 10 h (water 24 °C; 240 accumulated thermal units, ATU) before oocyte collection (Sanches et al., 2011a).

Before gametes collection, the urogenital papilla and proximities were cleaned and dried to avoid contamination by water, feces, urine, or blood. The sperm was collected in graduated plastic tubes through anteroposterior massage in the abdominal region of the fish. The volume of sperm was recorded, and the samples were kept in a Styrofoam box containing ice foam Super Cold® (15 ± 1 °C) (Sanches et al., 2011a) for approximately 30 min. Oocytes were collected in a dry plastic container, and after weighing, one gram of spawned eggs were used for the total estimated oocytes.

### *2.2 Experimental design*

The experimental design is summarized in Figure 1. The experiment was performed using a completely randomized block design with the sperm considered blocks and the male fish as the repetitions. Two treatments were compared: control post-thaw sperm (T1) and post-thaw diluted (T2) sperm. The samples were analyzed through tests of kinetic parameters, sperm morphology, mitochondrial activity, membrane integrity, fertilization, hatching, and normal larvae. All the analyses were performed using one straw for each male (n = 8) in triplicate.

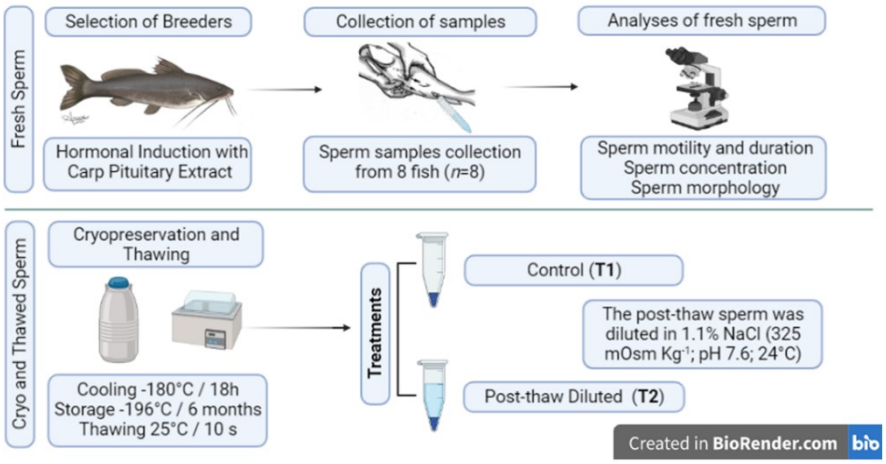


**Figure 1.** Experimental design to compare quality and reproductive capacity of control post-thaw *R. quelen* sperm (T1) and post-thaw diluted sperm in NaCl solution (T2). After the collect sperm samples from each male (n = 8) and before its cryopreservation, the quality of the samples was validated by means of sperm motility percentage, sperm motility duration, sperm concentration and sperm morphology (anomalies percentage). The straws from each male were individually thawed, immediately one part of sperm into the straw was used (T1) and un aliquot (50 µL) of the same sample was post-thaw diluted in 1 mL of NaCl solution (325 mOsm $kg^{-1}$; pH 7.6; 24 °C, diluted in distilled water) (T2). The same parameters were evaluated for each treatment, kinetics parameters, morphology, mitochondrial activity, membrane integrity, fertilization rate, hatching rate and normal larvae rate. For all analysis was performed using one straw per male in triplicate.

### *2.3 Evaluation of fresh sperm*

Immediately after collection, each sperm sample was subjectively evaluated under a light microscope (Nikon E200, Tokyo, Japan) at 400 × magnification to check for possible previous activation of the spermatozoa by contaminants or water, motility rate (0–100%), and duration of motility (s) (Carolsfeld et al., 2003). A sperm sample from each male (1 µL) was activated using distilled water (5000 µL) at a ratio of 1:5000 (sperm: distilled water) (Neumann et al., 2019).

The pH of the immobile sperm from each male was evaluated using a pH meter (Quimis Q400HM, Diadema, Brazil). An aliquot (1 mL) of a male sample was pipetted into plastic tubes (1.5 mL) and centrifuged (80–2B Analogic, Daiki, Sao Paulo, Brazil) at 1000 *g* (3341 rpm) for 30 min. The osmolality of

the seminal plasma (supernatant) was evaluated using an osmometer (Wescor Vapro 5520, Logan, USA). For sperm concentration and morphology, male samples were fixed in 10% buffered formaldehyde solution at a 1:999 dilution (1 µL sperm: 999 µL formaldehyde solution).

To evaluate sperm concentration, an aliquot (10 µL) of diluted sperm was pipetted into each counting field of a Neubauer hemocytometer chamber (Olen, Kasvi, Sao José dos Pinhais, Brazil) covered by a coverslip for 15 min to stabilize the cells. Using a microscope at 400 × magnification (Nikon E200, Tokyo, Japan) and a manual counter, the gametes were quantified by counting 10 squares. After cell counting, the sperm concentration was calculated using the equation described by (Sanches et al. 2011b).

To evaluate sperm morphology, the samples were diluted in Bengal Rose dye (4%) (Merck, Darmstadt, Germany) at a dilution of 1:10 in a plastic tube (1.5 mL). Smears of 20 µL of stained sperm were evaluated under an optical microscope at 1000 × magnification (Nikon E200, Tokyo, Japan) (Streit Jr. et al., 2004). Spermatozoa (n = 200) were evaluated from each sample (n = 8 males), and the number of normal and abnormal cells was expressed as a percentage. The following morphological changes were evaluated: broken tail, curled tail, strongly curled tail, distally curled tail, short tail, distal and proximal gout, loose head, degenerated head, microcephaly, and macrocephaly (Miliorini et al., 2011).

### *2.4 Sperm cryopreservation, thawing, and dilution*

Only samples with motility greater than 50% were cryopreserved. The sperm of each male was cryopreserved separately within 30 min of collection, following the methodology described by Adames et al. (2015). The freezing medium was composed of 5% D-fructose (Exodô Científca, Sumaré, Brazil), 5% powdered milk (Molico Skimmed®, Nestlé, Sao Paulo, Brazil), and 10% methanol (Merck, Darmstadt, Germany). All samples were individually diluted in the freezing medium at a ratio of 1:3 (62.5 µL sperm + 187.5 µL freezing medium). After dilution, the sperm was immediately drawn into 0.25 mL straws (Minitube, Tiefenbach/Landshut, Germany). Straws were conditioned in racks and frozen into a nitrogen vapor vessel (Dry-Shipper MVE SC4/2 V, Genex, Toronto, Canada) at − 180 °C for 18 h (Carolsfeld et al.,

2003). This procedure allows the samples to reach a temperature of – 180 °C at 10 min, a freezing rate of approximately - 11 °C $min^{-1}$. The cooling curve was measured using a digital thermometer (Gulterm 200, Gulton, São Paulo, Brazil) with a sensor PT-100. The samples were subsequently transferred to a cryogenic tank (MVE Millennium 2000, Genex, Toronto, Canada) at − 196 °C, and after six months, the straws were thawed in a water bath at 25 °C for 10 s (Adames et al., 2015).

Immediately after thawing, each straw was dried, placed in a plastic tube (1.5 mL), and a perforation was made at the top so that the contents flowed into the plastic tube. An aliquot (50 µL) was collected and post-thaw diluted in 1000 µL of the NaCl extender (325 mOsm $kg^{-1}$; pH 7.6; 24 °C; diluted in distilled water; Synth, Diadema, Brazil) (T2). The final ratio was 1:20 (sperm + freezing medium: extender). The sperm that remained from the straw was considered the control treatment (T1). Samples were used for analysis or fertilization approximately 2 min after thawing.

### *2.5 Post-thaw sperm analyses*

#### *2.5.1 Kinetics parameters (CASA)*

The movement of spermatozoa from the control post-thaw sperm (T1) and the post-thaw diluted sperm (T2) was evaluated using a 1 µL aliquot of each sample. Both were activated by distilled water (24 ± 0.5 °C) in a plastic tube (1.5 mL), with 600 µL for the control and 100 µL for the post-thaw diluted sperm. Immediately after motility was activated, 1 µL was placed in a Neubauer hemocytometer chamber, covered with a coverslip, and placed under a microscope (Bel Solaris, Milan, Italy) at 100 × magnification, connected to a digital video camera (Basler AC640–120uc, 658 × 492 pixels, 120 fps, Ahrensburg, Germany). Analysis of kinetic parameters was performed 10 s after the induction of sperm activation. Pylon Viewer 4 software (Version 4.1.0.3660 64-Bit; Basler, Ahrensburg, Germany) was used to video record the spermatozoa movement at a capture rate of 100 fps (Frames Per Second) (Adames et al., 2015). The videos from 10 s after activation were then separated into 50 images using the VirtualDub software (Version 1.10.04; Microsoft Virtual Studio, Redmond, USA), representing 0.5 s of video. The images were analyzed using the Computer-Assisted Sperm

Analyzer (CASA) free plugin software from ImageJ (Version 1.53e 64-Bit, National Institutes of Health, USA) (Wilson-Leedy and Ingermann, 2007). The parameters evaluated were motility rate (MOT, %), curvilinear velocity (VCL, μm $s^{-1}$), average path velocity (VAP, μm $s^{-1}$), straight-line velocity (VSL, μm $s^{-1}$), straightness (STR, %), wobble (WOB, %), progression (PROG, μm), and beat cross frequency (BCF, Hz). The following input variable values were used in the CASA plugin: a= 1; b= 40; c= 50; d= 10; e= 3; f= 10; g= 15; h= 5; i = 1; j = 15; k = 15; l= 25; m= 80; n = 80; o= 50; p = 60; q= 50; r = 561.7978; s = 0; and t = 0 (adapted from Neumann et al., 2019).

### *2.5.2 Morphology*

Control and post-thaw diluted sperm were fixed in 10% buffered formaldehyde solution at a 1:9 dilution (100 μL thawed sperm: 900 μL formaldehyde solution). Spermatozoa morphology was evaluated using the same methodology described for fresh sperm.

### *2.5.3 Mitochondrial activity*

Mitochondrial activity was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl) − 2,5-diphenyl tetrazolium bromide) (Invitrogen, Thermo Fisher Scientific, Waltham, USA). This technique is based on the reduction of MTT to formazan crystals by the mitochondrial succinate dehydrogenase enzyme, which is only active in living cells (Liu and Peterson, 1997). The MTT assay was performed according to the adapted method of Mosmann (1983). The samples were thawed, 50 μL of sperm was post-thaw diluted in 1000 μL NaCl extender (325 mOsm $kg^{-1}$; pH 7.6; 24 °C) into a plastic tube (1.5 mL) (T2), and the remaining sperm in the straw (control) was pipetted into another plastic tube (1.5 mL) without the extender solution (T1). Samples from each male were placed in plastic tubes and centrifuged (80–2B Analogic, Daiki, Sao Paulo, Brazil) at 1000 × g (3341 rpm) for 10 min. After centrifugation, the supernatant was removed, and 400 μL MTT (3-(4,5-dimethylthiazol-2-yl) − 2,5-diphenyl tetrazolium bromide) (Invitrogen, Thermo Fisher Scientific, Waltham, USA) stock solution (5 mg of MTT $mL^{-1}$ of PBS) was added. After adding MTT, 10 μL of each sample was collected and fixed in 10% buffered formaldehyde solution at a 1:99 dilution (10 μL sample: 990 μL formaldehyde solution) to determine the sperm concentration. The samples with MTT were incubated

at 28 °C for 2 h, centrifuged, and the supernatant was removed, followed by the addition of 400 µL of dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) and 100 µL of each sample was pipetted into a 96-well microplate (Kasvi, Sao José dos Pinhais, Brazil). Fifty-one wells were used, 48 for treatments (eight males × two treatments × three replicates) and three for the white control, which was subjected to the same treatments except that the sperm was not pipetted.

The optical density of the samples was measured using a spectrophotometer (SpectraMax M2®, San Jose, USA) at a wavelength of 570 nm. The MTT reduction rate (optical density) for each sample was calculated by determining the difference between the sample and control values. The results were normalized to 50 million cells, as adapted from (Aziz, 2006).

### *2.5.4 Membrane integrity*

The samples (n = 8) were thawed, and 50 µL of the control sperm (T1) was centrifuged (K14–1215, Kasvi, Sao José dos Pinhas, Brazil) at 1000 *x* g 10 min. After centrifugation, 25 µL was diluted in 75 µL of NaCl solution (325 mOsm $kg^{-1}$; pH 7.6; 25 °C, diluted in distilled water), and 50 µL was stained. For post-thaw diluted treatment (T2), 10 µL of sperm after thawed was immediately diluted in 190 µL of NaCl solution and centrifuged (K14–1215, Kasvi, Sao José dos Pinhas, Brazil) at 1000 × g 10 min. After the supernatant was discarded, the sample was resuspended in 200 µL of saline solution. Finally, 25 µL of the solution was diluted in 25 µL of saline solution and stained.

The membrane integrity was assessed by a staining procedure using SYBR-14 (Thermo Fisher Scientific, Waltham, USA), which penetrates through the membrane of live cells and stains them green, and propidium iodide (PI; Thermo Fisher Scientifc, Waltham, USA), which penetrates through the ruptured membrane of dead cells and stains them red. For staining, 50 µL of each sample was mixed with 0.25 µL SYBR-14 (0.02 mM) and incubated in the dark at room temperature (~25 °C) for 4 min. Then, 1 µL PI (1.19 mM) was added and incubated for 1 min in the dark at room temperature (~25 °C). This methodology was adapted from Hagedorn et al. (2009). Before analyzing the samples, was added 1950 µL of NaCl solution.

The evaluation was performed using an Accuri C6 flow cytometer (BD Biosciences, San Jose, USA) equipped with a 488 nm solid state laser and a 640 nm diode laser. Fluorescence signals of SYBR-14 were gathered via a 533/30 nm band-pass filter, and PI was collected using a 670 nm long-pass filter. Prior to the analyses, the flow cytometer was adjusted using a sperm sample that was heated to 100 °C per 40 s and then exposed to liquid nitrogen at − 196 °C causing death (100%) of cells (positive control). This sperm sample was stained only with PI, and the mortality of all cells was verified under a fluorescence microscope. The entire sperm population of South American silver catfish could be identified and gated. The results were plotted on a logarithmic scale (C6 Analysis Software, BD Biosciences, San Jose, USA). The sperm population was gated by referring to the expected forward- and side-scatter signals. Ten thousand events per sample were acquired.

### *2.5.5 Fertilization, hatching and normal larvae*

The oocytes were collected from each female (n = 4), and through visual evaluation, one spawning was chosen to be fertilized by the control sperm (T1), post-thaw diluted sperm (T2), and fresh sperm. A fresh sperm pool from three males was formed and used for quality control of oocytes. The motility and concentration of the control sperm (T1), post-thaw diluted sperm (T2), and sperm pool were analyzed following the same methodology as previously described. Firstly 0.2 mL of oocytes (223.1 ± 4.1 oocytes) was placed into 51 disposable plastic cups (50 mL) (eight males × two treatments × three replicates and three controls with fresh sperm). A volume of control sperm (T1), post-thaw diluted (T2), or fresh sperm was then pipetted into each cup to reach an ideal motile sperm: oocyte ratio of 70000 motile spermatozoa per oocyte (Neumann et al., 2019). The activation was promoted individually with 20 mL of distilled water (24 ± 1 °C). A subsequent mixing action was imposed on the gametes for 60 s, followed by the transfer of oocytes and spermatozoa to small circular sieves.

The eggs were incubated in small circular sieves with nylon nets in plastic tanks (500 L) in a recirculating system. The experimental parameters were as follows: water temperature, 24 ± 0.5 °C; pH, 7.5 ± 0.2; dissolved oxygen 5.5

± 0.5 mg $L^{-1}$.

The fertilization rates were evaluated after the embryonic blastopore closed, at approximately 12 h after fertilization (Pereira et al., 2006). Analysis was performed by counting the number of fertilized and non-fertilized oocytes, so all oocytes from each sieve were analyzed using a binocular stereomicroscope (Q7740SZ-T, Quimis, Diadema, Brazil) at 10 × and a manual counter. The result is given by the formula Fertilization (%) = (Number of fertilized/Total oocytes) x 100.

Hatching rates and normal larvae were observed 48 h after fertilization. The analyses were performed by counting all the larvae and classifying their morphology. Larvae without head, spine, and yolk sac abnormalities were considered normal (Jezierska et al., 2009). A binocular stereomicroscope was used at 10 × and a manual counter. The results are given by the formulas Hatching (%) = (Number of larvae/Total oocytes) x 100 and Normal Larvae (%) = (Number of normal larvae/Total larvae) x 100.

### *2.6 Statistical analyses*

Data are expressed as the mean ± standard deviation (SD). Data were tested for normal distribution using the Shapiro–Wilk test and homogeneity using the Bartlett test. After verifying compliance with the statistical assumptions, data were analyzed using a one-way ANOVA, followed by comparing the paired t-test averages. The level of significance for all statistical tests was set at 95% ($P < 0.05$). Statistical analysis and graph construction were performed using GraphPad Prism 7.04.

## 3. Results

### *3.1 Evaluation of fresh sperm*

The sperm characteristics were: volume 13 ± 4 mL, concentration 5.9 ± 2.1 × $10^{10}$ spermatozoa $mL^{-1}$, pH 8.1 ± 0.06, and osmolality 286 ± 7 mOsm $kg^{-1}$. Fresh sperm presented a subjective motility of 80 ± 27% and a motility duration of 46 ± 17 s. The analysis of sperm morphology showed 86.5 ± 5.7% of spermatozoa as normal or with abnormalities (Fig. 3).

### *3.2 Post-thaw sperm analyses*

#### *3.2.1 Kinetics parameters (CASA)*

The motility of the control sperm samples (51 ± 12%) did not differ (P > 0.05) from the post-thaw diluted samples (57 ± 14%) in NaCl solution (Fig. 2A).

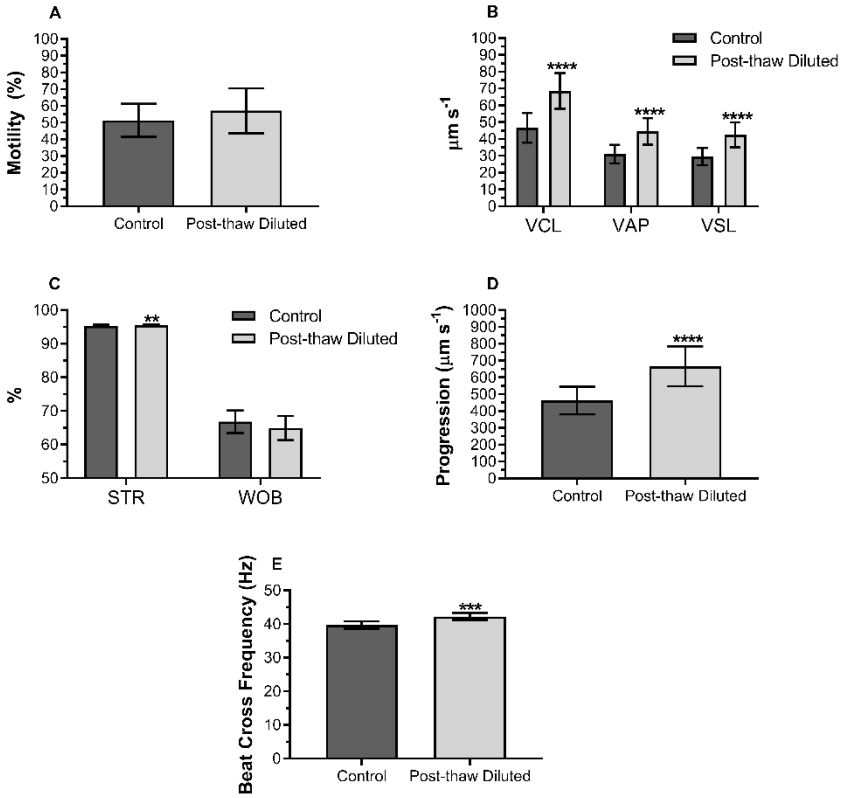


**Figure 2.** Motility rate (A), velocities (curvilinear, VCL; average path, VAP; straight-line, VSL) (B), straightness (STR) and wobble (WOB) (C), progression (D), and beat cross frequency (E) of the control thawed sperm (T1) (n = 8) and that post-thaw diluted in NaCl solution (T2) (n = 8) assessed by CASA. Bars indicate mean ± SD; Means followed by different numbers of asterisks differ ** p < 0.01; *** p < 0.001, **** p < 0.0001; paired t-test).

Higher velocities were observed when samples were post-thaw diluted in NaCl (VCL, 69 ± 11 μm $s^{-1}$; VAP, 45 ± 8 μm $s^{-1}$; VSL, 43 ± 8 μm $s^{-1}$) compared to the control samples (VCL, 47 ± 10 μm $s^{-1}$; VAP, 31 ± 6 μm $s^{-1}$; VSL, 30 ± 6 μm $s^{-1}$) (Fig. 2B). Higher straightness (STR), progression (PROG), and beat cross

frequency (BCF) were observed in the post-thaw diluted samples (STR, 96 ± 7%; PROG, 666 ± 128 µm; BCF, 42 ± 2 Hz) than in their control sperm counterparts (STR, 95 ± 5%; PROG, 463 ± 92 µm s, BCF, 40 ± 2 Hz) (Fig. 2C, D, and E). There was no difference in wobble (WOB) between control (67 ± 5%) and post-thaw diluted (65 ± 7%) sperm (Fig. 2C).

### *3.2.2 Morphology*

The number of normal spermatozoa in the control samples (17 ± 8%) and post-thaw diluted samples (24 ± 8%) did not differ ($P > 0.05$). The strongly curled tail was the only change that differed signifi*c*antly between the control (2 ± 1%) and post-thaw diluted (5 ± 2%) samples (Fig. 3).

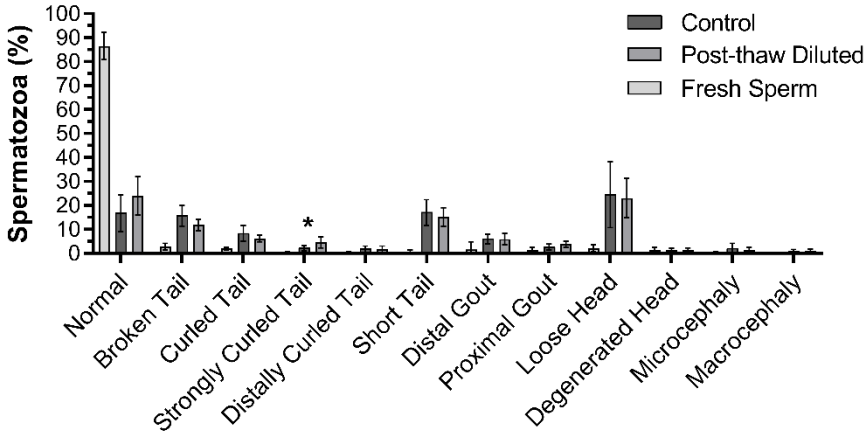


**Figure 3.** Morphology of fresh sperm (n = 8), control thawed sperm (n = 8), and post-thaw diluted in NaCl solution (n = 8) analyzed by Rose Bengal (4%) stain, observed and counted using an optical microscope (1000 × magnification) and manual counter, respectively. Bars indicate mean ± SD; *Means followed by an asterisk differ between control and post-thaw diluted ($p < 0.05$; paired t-test).

### *3.2.3 Mitochondrial activity*

The mitochondrial activity showed that the control (0.17 ± 0.05 Abs $s^{-1}$) and post-thaw diluted (0.2 ± 0.04 Abs $s^{-1}$) samples did not differ (Fig. 4A).

### *3.2.4 Membrane integrity*

There was no difference in the membrane integrity of control (59 ± 10%) and post-thaw diluted (64 ± 11%) sperm (Fig. 4B).

### *3.2.5 Fertilization, hatching and normal larvae*

The fertilization rate with a pool of fresh sperm (91%) showed that the spawning used was of good quality. A higher fertilization rate was observed when used post-thaw diluted sperm in NaCl solution (93 ± 3%) compared to control (65 ± 13%) (Fig. 4C). The same occurred for hatching rate, whereby control sperm (55 ± 17%) had a lower rate than post-thaw diluted samples (82 ± 9%) (Fig. 4C). For the normal larvae production rate, the control (89 ± 10%) and post-thaw diluted (89 ± 7%) samples did not differ (Fig. 4C). Oocytes from only one female were used, limiting these results' interpretation.

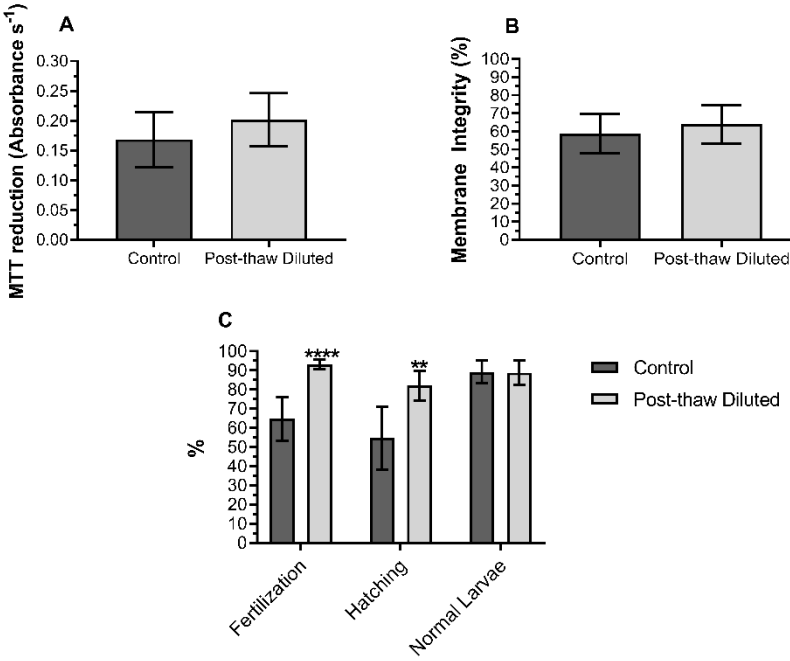


**Figure 4.** Mitochondrial activity (A) analyzed by MTT reduction rate (optical density) at 570 nm. The results were normalized to 50 million cells. Membrane integrity (B) analyzed by flow cytometry. The cells with intact membrane were stained green using SYBR-14 and the cells with rupture membrane were stained red using propidium iodide (PI). Fertilization, hatching, and normal larvae rate (C) were obtained after counting and evaluating all oocytes and larvae collected in sieves. All assessments were evaluated the sperm (n = 8) from control post-thaw sperm and post-thaw diluted in NaCl solution. Bars indicate mean ± SD; Means followed by different numbers of asterisks differ ** $p < 0.01$; **** $p < 0.0001$; paired t-test).

# 4. Discussion

Sperm cryopreservation is biotechnology that subjects cells to contact with toxic solutions and extremely low temperatures. These actions cause damage and decrease sperm quality after thawing, compromising fertilization and hatching rates. Thus, methodologies that mitigate these damages are always welcome. Post-thaw dilution in NaCl for thawed sperm is practical and inexpensive. Consequently, comparing sperm quality and reproductive capacity between control and post-thaw diluted sperm from the same straw is relevant. Using post-thaw dilution in a NaCl solution improved the kinetic parameters, maintained the membrane integrity and mitochondrial activity of sperm, and provided a higher fertilization and hatching rate.

The choice of ionic composition, osmolality, and temperature of solution for post-thaw sperm dilution was based on sperm characteristics to maintain sperm quality. Thus, NaCl was used since most of the ions in South American silver catfish seminal plasma are $Na^+$ and $Cl^-$ (Borges et al., 2005). The osmolality of the seminal plasma of fresh sperm was 287 ± 7 mOsm $kg^{-1}$; hence, to avoid sperm activation before fertilization, the NaCl solution osmolality was corrected to 325 mOsm $kg^{-1}$, and the temperature was maintained at 24 °C, which was the same temperature in which the animals were immersed until the moment of gamete collection. Thus, when thawed sperm samples were post-thaw diluted in NaCl, the ionic properties and temperature did not show substantial changes that could damage the gametes.

Methanol is a permeable cryoprotectant that enters cells through the pores of the cell membrane. Higher concentrations, medium temperatures, and exposure times can acidify the intracellular medium, which is toxic to cells (Best, 2015). Therefore, post-thaw dilution in NaCl solution decreased the methanol concentration in the medium, consequently decreasing its toxicity to gametes at room temperature (~25 °C) (Morris et al., 2012).

The analysis of sperm kinetic parameters is widely used to compare different experimental conditions (Gallego and Asturiano, 2018). For example, in a study with cats, sperm motility was greater in diluted samples (65.4%) than

in undiluted samples (59.2%). In addition, diluted sperm also had a higher progressive motility score (4.1) than undiluted sperm (3.6) (Chatdarong et al., 2010). Although our data showed no difference between treatments for motility rate, post-thaw dilution in NaCl provided better parameters for sperm velocity. The higher sperm velocities made a difference at the time of oocyte fertilization. In other studies, on *Rhamdia quelen* (Neumann et al., 2019), *Prochilodus lineatus* (Viveiros et al., 2010), and *Solea senegalensis* (Beirão et al., 2015), a high correlation between sperm velocities and fertilization rate has been reported. The progression observed in the post-thaw sperm diluted may have another factor that influenced the higher fertilization rate. The spermatozoa must move close to the oocyte for fertilization (Cosson et al., 2008). Therefore, the progression parameter, which indicates the total average distance travelled by spermatozoa, is a key factor for fertilization.

There is a classic definition by Morisawa et al. (1983) that sperm activation of carp in hypoosmotic saline solution strongly reduces sperm tail curling, which even led to the formulation of a diluent solution for the species by Saad and Billard (1987). Thus, we expected that the South American silver catfish post-thaw sperm would present a higher percentage of flagellum (tail) abnormalities and not when previously post-thaw diluted in a hypoosmotic solution of NaCl in relation to the cryopreservation solution in which it was submitted. This behavior did not occur with this species, but a significant percentage, albeit low, of the strongly curled tail abnormality. In the review of flagellum function in fish sperm by Boryshpolets et al. (2018), the authors related the study by Perchec et al. (1995) that determined osmotic damage as the main factor that causes alteration in sperm flagella. However, in fishes, sperm motility is usually activated by osmotic shock **(**Alavi and Cosson, 2006**)**.

In general, the highest percentage of abnormality is always associated with lower motility of post-thaw sperm, as their structure and physiological function have been damaged, resulting in a loss of potential function for kinetics parameters (Muchlisin and Azizah, 2009). So, in our study, a direct consequence of the increase in the strongly curled tail abnormality could be related to some sperm kinetics parameters or even to the productive parameters (fertilization, hatching, and normal larvae rates). However, in

none of these other evaluated parameters, there was a lower mean percentage in post-thaw sperm diluted in NaCl compared to control. In South American silver catfish, it has already been proven that the appearance of a strongly curled tail is associated with the physic-chemical actions to which sperm are exposed, mainly the cryopreservation process (Da Costa et al., 2019).

Membrane integrity is essential for sperm functions and is used as a sperm quality parameter (Cabrita et al., 2014). The sperm membrane of fish sperm is sensitive to small changes in the osmolality of the environment (Boryshpolets et al., 2020). It was expected that the post-thaw dilution could cause damage to the sperm membrane. However, comparing the integrity of the sperm membrane of the two treatments, we did not observe a difference. The same was presented when silver catfish post-thaw sperm was diluted in Ginsburg solution and centrifuged (Pérez-Atehortúa et al., 2022). Hence, the sperm membrane of the silver catfish is resistant to osmotic shock, as proven by post-thaw dilution.

In sperm, as in other cells, mitochondria are the organelles responsible for providing energy in the form of ATP (Boryshpolets et al., 2020). The success of fertilization and the duration of sperm motility are dependent on mitochondrial activity (Figueroa et al., 2019). Our results did not show a difference in mitochondrial activity between treatments. On the other hand, another study by our group (Pérez-Atehortúa et al., 2022) showed greater mitochondrial activity of post-thaw sperm diluted compared to undiluted. The difference between the results found in the studies may be due to the use of an extender containing other salts besides NaCl and a sperm pool. However, we know that mitochondrial activity was not the key to explaining the high fertilization using post-thaw diluted sperm in the present study.

The presence of permeable cryoprotectant at fertilization is toxic to oocytes and sperm, which can compromise sperm/oocyte interaction or embryonic development. At the same time, it can cause non-lethal damage to spermatozoa, which does not affect motility but reduces their ability to fertilize (Boryshpolets et al., 2020). Post-thaw dilution provided greater sperm velocity and decreased permeable cryoprotectant concentration at

the time of fertilization, resulting in a high fertilization rate (>80%) (Beirão et al., 2019). In another study carried out by our group (Pérez-Atehortúa et al., 2022), fertilizing oocytes from one female, post-thaw sperm of silver catfish diluted in the Ginsburg extender and centrifuged showed fertilization (77%) and hatching rates (55%) lower compared to the present study. This showed us that centrifugation of post-thaw diluted sperm is not necessary to achieve high fertilization and hatching rates. The fact that we used oocytes from only one female in fertilization limits the interpretation of our results, but the use of dilution in the present study showed the highest rates of fertilization (93%) and hatching (82%) ever reported for this species. We did not find differences in the normal larvae rate using the thawed sperm samples from the control and post-thaw diluted treatments. Other studies that used thawed sperm of the same species also observed an offspring of more than 90% of normal larvae (Neumann et al., 2019; Pérez-Atehortúa et al., 2022). Therefore, we believe that the emergence of South American silver catfish larvae with morphological problems is not related to the use of cryopreserved sperm.

## 5. Conclusions

The post-thaw dilution of South American silver catfish sperm in NaCl solution (325 mOsm $kg^{-1}$; pH 7.6; 24 °C) improved the sperm kinetic parameters, as well as fertilization and hatching rates. Our reproductive results are limited because we use oocytes from only one female even though this methodology can be included in the sperm cryopreservation protocols for this species and should be investigated for other species concerning their seminal properties, hence improving cryopreservation protocols for fish sperm.

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# CHAPTER 2

## Fish sperm cryopreservation using biodegradable containers: New low-cost and environment-friendly methodology

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## Abstract

Containers used to cryopreserve sperm are made with non-biodegradable plastic compounds, having a high monetary and environmental cost. Therefore, the development of biodegradable alternative containers for cell cryopreservation is necessary. Thus, this study aimed to evaluate the efficiency of hard-gelatin and hard-hydroxypropyl methylcellulose (HPMC) capsules as low-cost and biodegradable alternative containers for sperm cryopreservation. Sperm from 12 South American silver catfish *Rhamdia quelen* were individually cryopreserved in plastic straws 0.25 mL (as control), hard-gelatin, and hard-HPMC capsules. The quality of post-thaw sperm cryopreserved in the different containers was checked by measuring spermatozoa membrane integrity, kinetic parameters, mitochondrial activity, fertilization, hatching, and normal larvae rates. The samples cryopreserved in straws showed a higher percentage of membrane integrity (68%) than those frozen in hard-gelatin (40%) and hard-HPMC capsules (40%). However, we did not observe differences between the samples stored in straws and hard capsules for the rest of tested sperm parameters. Thus, based on the high sperm fertility capability, both capsules were efficient as cryopreservation containers for maintaining sperm functionality.

## 1. Introduction

Cryopreservation aims to suspend and preserve the development, functions, and growth of cells or tissues at low temperatures for later use with the least possible loss in efficacy. Over the years, scientists have reported essential discoveries in cryobiology (Sztein et al., 2018). Thus, they improved cells and tissue cryopreservation protocols (Nebot et al., 2020; Oldenhof et al., 2020; Paredes et al., 2020; Pomeroy et al., 2022) to become an important tool nowadays. Among the applications of this technique, we can cite the genetic preservation of plants and animals, the artificial fertilization in humans and animals, as well as the creation of medicine for cancer treatment, and the delivery of medicines and vaccines based on mRNA technology (Murray and Gibson, 2022).

The type of packaging container is important in cryopreservation because the volume, shape, and material are directly related to the cooling and heating of the samples (Yang and Huo, 2022). Ampoules and glass capillaries were used until the 1960s when polypropylene or polyvinyl chloride (PVC) straws began to be used (Pickett and Berndtson, 1974). Nowadays, the most used containers are straws (0.25, 0.3, 0.5, 1.2, and 5 mL) made of PVC, cryovials made of polypropylene, and freezing bags made of PVC (Yang and Huo, 2022). Polypropylene and PVC are recyclable, but after coming into contact with biological materials, they must be sterilized before recycling. Thus, they are generally not reused, making them waste with a high potential for polluting the environment. In addition, they are manufactured and supplied by a few manufacturing companies, which raises their prices and makes accessibility difficult depending on the country.

Hard capsules are widely used in pharmaceuticals to deliver drugs, minerals, vitamins, and other substances to target absorption sites (Zilhadia et al., 2022). They are made from biodegradable polymers. One of these polymers used in manufacturing hard capsules is gelatin. It consists of one of the l-amino acids linked to d-amino acids by peptide bonds, forming a linear polymer with a molecular weight between 15,000 and 25,000 Daltons (Price, 2006). As an alternative to the use of polymers of animal origin, Hypromellose (hydroxypropyl methylcellulose – HPMC) is a nongelatinous

polymer of vegetable origin that is also used for manufacturing hard capsules. Gelatin and HPMC hard capsules are soluble in water, and their degradation depends on the characteristics of the medium, such as pH, temperature, and exposure time (Al-Tabakha, 2010; Ling, 1978). Because they are made from natural, biocompatible, and water-soluble materials, both capsules are biodegradable. In addition, they have a low cost and are easily found in the market, regardless of the country. Nowadays, discarding plastic waste is a serious global environmental problem. Burning plastic materials, such as polyethylene, polypropylene, and PVC, produces high carbon dioxide emission that contributes to global warming growth (Luckachan and Pillai, 2011). Biodegradable polymers are an alternative to minimize environmental problems and can be used in several fields, including the biomedical area (Samir et al., 2022). Thus, this study aimed to evaluate the efficiency of hard-gelatin and hard-HPMC capsules as low-cost and biodegradable alternative containers for sperm cryopreservation.

## 2. Materials and Methods

### *2.1 Fish handling and gametes collection*

The experiment was performed at the São Paulo State University (UNESP), Registro, São Paulo, Brazil (24°32'04.7" S; 47°51'42" W), during the spawning season. Animal handling and experiments were performed according to the Animal Use Ethics Committee of the Federal University of Rio Grande do Sul (UFRGS) (N° 39527), and Animal Use Ethics Committee of the São Paulo State University (UNESP) (Project Number 01/2021).

We used 20 two-year-old *Rhamdia quelen* males (403 ± 139 g) and 5 females (484 ± 155 g) from UNESP/Registro aquaculture laboratory. The animals were maintained in 10,000 L geomembrane tanks in a recirculation system and fed once a day until the apparent satiety with a commercial diet (32% crude protein, 4-6 mm). Animals' health and behavior were monitored daily during feeding. The feeding was restricted 24 h before the selection of breeders to avoid gametes contamination by feces. The males able to reproduce were selected when they showed detectable running sperm under soft abdominal pressure, and females showed the bulging of the celomatic cavity and

reddish urogenital papilla. During the selection, breeders were individually weighed, and transferred to plastic tanks (500 L) in an indoor recirculation system equipped with heaters and aeration. The experimental parameters were as follows: water temperature, 24 ± 0.3 °C; pH, 7.1 ± 0.3; dissolved oxygen, 6 ± 0.5 mg $L^{-1}$; and photoperiod (14 h light:10 h dark).

Males were hormonally induced using a single intramuscular dose of carp pituitary extract (CPE) at 2.5 mg $kg^{-1}$ (Sanches et al., 2010). The oocytes were obtained just before the fertilization trials. To promote the final maturation and ovulation, the females received two hormonal doses of CPE, with the first dose of 0.5 mg $kg^{-1}$ and 8 hours later, the second dose at 5 mg $kg^{-1}$. Sperm and oocytes were collected after 10 hours (water 24 °C; 240 accumulated thermal units, ATU), counted from the single administration of hormone to males and the second dose of females (Adames et al., 2015).

Before the gametes collection, the urogenital area was cleaned and dried to avoid contamination by mucus or water. The first sperm-released samples were discarded to prevent contamination with urine, feces, or blood (Marques et al., 2021). The sperm samples of each animal were collected in dry graduated plastic tubes through anteroposterior massage in the ventral region of the fish. Immediately, the sperm kinetics parameters were evaluated with the Computer Assisted Sperm Analysis (CASA - open-source plug-in ImageJ version 1.53e 64-Bit, National Institutes of Health, USA) (Sanches et al., 2010) to detect the absence of contamination and to select the samples to be used in the experiment. Thus, sperm from 12 males (n=12) that presented a motility percentage of at least 60% were selected and kept in a Styrofoam box containing ice foam Super Cold® (15 ± 1 °C) for a maximum of 60 minutes until cryopreservation. Oocytes were stripped in a dry plastic container, and stored in the same conditions of sperm (15 ± 1 °C) (Sanches et al., 2011).

### *2.2 Experimental design*

The experimental design is summarized in Fig. 1. The experiment was performed using a completely randomized design, with each male considered a block. Three cryopreservation recipients were compared:

plastic straw manufactured by PVC with a 0.25 mL volume (Minitube, Tiefenbach, Germany), hard-gelatin capsule manufactured by collagen, size 0 – locked length 21.7 mm, and with a 0.68 mL volume (Capsule Connection, Prescott, USA), and hard-HPMC capsule manufactured by hydroxypropyl methylcellulose, size 0 – locked length 21.7 mm, and with a 0.68 mL volume (Capsule Connection, Prescott, USA). After thawing, samples were analyzed through tests of membrane integrity, kinetic parameters, mitochondrial activity for the sperm, beyond fertilization rate, hatching rate, and percentage of normal larvae. All the analyses were performed using one sample for each male (n=12) in triplicate.

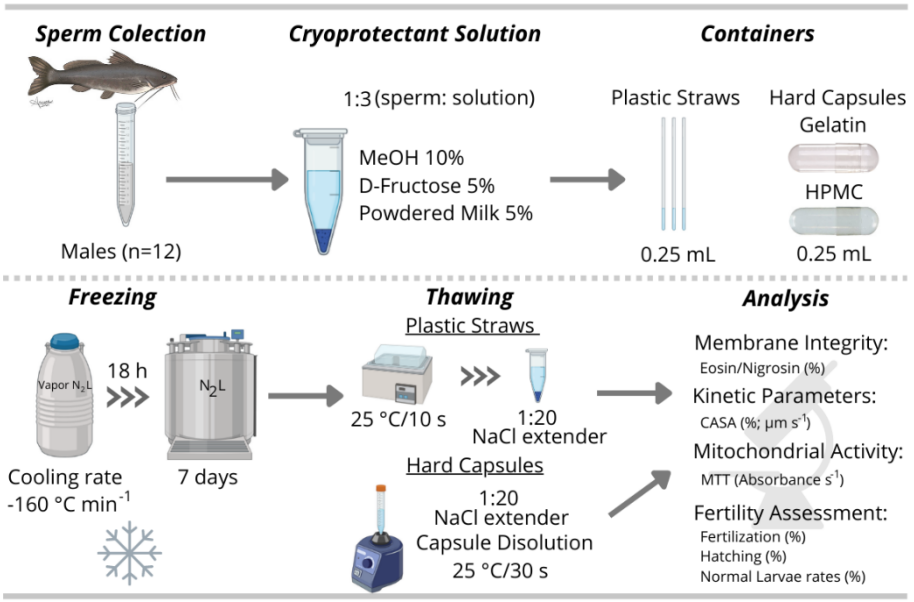


**Figure 1.** Experimental design to compare quality and fertility capability of post-thaw sperm after cryopreservation in plastic straws (0.25 mL), hard-gelatin capsules, and hard-HPMC capsules (Size 0 – locked length 21.7 mm, 0.68 mL). Sperm samples from 12 *R. quelen* (n=12) were collected and cryopreserved in the different containers, which were filled with 0.25 mL in all the cases. After sperm collection, membrane integrity and kinetic parameters of fresh samples were performed. Then, membrane integrity, kinetic parameters, mitochondrial activity, fertilization, hatching, and normal larvae rates were evaluated in the post-thaw sperm. All the analyses were performed in triplicate.

### *2.3 Cryopreservation and thawing*

The sperm was individually cryopreserved: 62.5 µL of sperm and 187.5 µL of cryoprotectant solution were used in each sample, assuming a ratio of 1:3 (sperm: cryoprotective solution). The cryoprotectant solution used consisted of 5% powdered milk (w/v) (Molico Skimmed®, Nestlé, São Paulo, Brazil), 5% D-fructose (w/v) (Êxodo Científica, Sumaré, Brazil), and 10% methanol (v/v) (Merck, Darmstadt, Germany) dissolved in distilled water (Adames et al., 2015). The sperm was diluted in the cryoprotectant solution and immediately containers were filled with 0.25 mL each: 10 plastic straws, 10 hard-gelatin capsules size 0, and 10 hard-HPMC capsules size 0 per male. The samples were placed in racks and frozen into a nitrogen vapor vessel (Dry-Shipper MVE SC4/2V, Genex, Toronto, Canada) at -180 °C for 18 h. This process allows the samples to reach a temperature of –180 °C at 10.5 minutes, a freezing rate of approximately -11 °C $min^{-1}$ (França et al., 2022). After this procedure, the samples were transferred to a liquid nitrogen tank (MVE Millennium 2000, Genex, Toronto, Canada) at -196 °C, where they remained for one week until thawing.

Sperm samples cryopreserved in straws were thawed in a water bath at 25 °C for 10 s (Adames et al., 2015), aliquot of thawed sperm was diluted in 1 mL of NaCl extender solution (325 mOsm $kg^{-1}$, pH 7.6, 25 °C; diluted in distilled water; Synth, Diadema, Brazil) reaching a ratio 1:20 (post-thaw sperm: extender) (França et al., 2022). The capsules were individually removed from the racks; the upper part was broken, and inserted into plastic tubes (15 mL) containing 5 mL of NaCl extender solution (325 mOsm $kg^{-1}$, pH 7.6, 25 °C; diluted in distilled water; Synth, Diadema, Brazil). Immediately, the tube containing the sample immersed in the extender was shaken in a vortex for 30 s to dissolve the capsule. After thawing, sperm analyses and fertilization trials were performed.

### *2.4 Membrane integrity*

The percentage of cells with intact membranes was evaluated by the method adapted from Blom (1950) using the dyes Eosin Y (3%; w:v) and Nigrosin (5%; w:v), both diluted in 3% (w:v) sodium citrate. A sperm aliquot of 20 µL was

dyed with 10 µL of each dye. Later, 10 µL were used to make the slide-through smear. After completing and air drying the slides, they were photographed using a smartphone (iPhone 8, Apple, Los Altos, USA) coupled to an optical microscope (Nikon E200, Tokyo, Japan) at 400X magnification. The photos were transferred to a computer and analyzed using a plug-in Cell Counter (Image J). The percentage of spermatozoa with intact membrane was quantified in 500 cells per male, considering the spermatozoa with the unstained head as intact.

### *2.5 Spermatozoa kinetic parameters*

An aliquot of 1 µL of fresh sperm was pipetted into a plastic tube (2 mL), then 600 µL of distilled water (0 mOsm $kg^{-1}$) at 25 °C were added, reaching a ratio of 1:600 (sperm: activator). For post-thaw sperm an aliquot of 20 µL from the straws or capsules was activated with 100 µL of distilled water (25 °C), reaching a final ratio of 1:5 (thawed sperm: activator). After activation, 5 µL of the mix was pipetted into a Neubauer chamber under an optical microscope (Bel Solaris, Milan, Italy) at 100x magnification with a camera (Basler AC640-120uc, 658 × 492 pixels, 120 fps, Ahrensburg, Germany) attached for video recording. Videos of spermatic movements were captured at 100 frames per second (FPS). This video was recorded by a computer connected to the camera using Pylon Viewer 4 software (Version 4.1.0.3660 64-Bit; Basler, Ahrensburg, Germany). Subsequently, the videos from 10 s after activation were edited into 50 images, representing 0.5 seconds of video, with the VirtualDub software (Version 1.10.04; Microsoft Virtual Studio, Redmond, USA).

The images were analyzed using the Computer-Assisted Sperm Analyzer (CASA) free plug-in software from ImageJ (Version 1.53e 64-Bit, National Institutes of Health, USA) (Wilson-Leedy and Ingermann, 2007). The parameters analyzed were: percentage of motility sperm in relation to static sperm (MOT - %); curvilinear velocity (VCL - µm $s^{-1}$), average path velocity (VAP - µm $s^{-1}$), straight line velocity (VSL - µm $s^{-1}$); sperm straightness (STR - %), wobble (WOB - %), progression (PROG - µm), and beat cross frequency (BCF - Hz) (Neumann et al., 2019; Sanches et al., 2010). The variables used to analyze the samples in the CASA plug-in were: a=1; b=40; c=50; d=10; e=3;

f=10; g=15; h=5; i=1; j=15; k=15; l=25; m=80; n=80; o=50; p=60; q=50; r=561.7978; s=0; t=0.

### *2.6 Mitochondrial activity*

Mitochondrial activity was analyzed using the MTT assay described for this species by França et al. (2022). An aliquot of 1 mL of post-thaw sperm from each male was placed in plastic tubes and centrifuged (80-2B Analogic, Daiki, São Paulo, Brazil) at 1,000 × g for 10 min. Then, the supernatant was removed, and 400 µL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Invitrogen, Thermo Fisher Scientific, Waltham, USA) stock solution (5 mg of MTT $mL^{-1}$ of PBS) was added. After adding MTT, 10 µL of each sample was collected and fixed in 10% buffered formaldehyde solution at a 1:99 dilution (10 µL sample: 990 µL formaldehyde solution) to determine the sperm concentration. The samples with MTT were incubated at 28 °C for 2 h, centrifuged as explained before, and the supernatant was removed, followed by the addition of 400 µL of dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany). Later, 100 µL of each sample was pipetted into each well of a 96-well microplate (Kasvi, São José dos Pinhais, Brazil).

The optical density of the samples was measured using a spectrophotometer (SpectraMax M2®, San Jose, USA) at a wavelength of 570 nm. The MTT reduction rate (optical density) for each sample was calculated by determining the difference between the sample and control values. The results were normalized to 50 million cells.

### *2.7 Fertility assessment*

The post-thaw sperm samples were used immediately after thawing and analyzed. In addition, a fresh sperm pool from three males (272 ± 46 g) was formed and used as control. Oocytes obtained after hormonal induction using CPE were used in equal quantities of 0.2 mL (211 ± 7 oocytes). They were placed into 111 disposable plastic cups (50 mL) (12 males × 3 types of containers × triplicate and 3 three controls with fresh sperm). A volume of post-thaw sperm from straws, hard-gelatin capsules, hard-HPMC capsules, and fresh sperm was pipetted into each cup in the fixed ratio of 70,000 motile spermatozoa per oocyte (Neumann et al., 2019). The activation of the sperm

and hydration of the oocytes was carried out with 20 mL of distilled water (24 ± 1 °C) and gentle mixing for one minute, followed by the transfer of eggs to incubation system. The incubation was performed in small circular sieves with nylon nets in plastic tanks (500 L), with constant water quality control, temperature, and oxygen supply.

The fertilization rate was evaluated 12 h after fertilization trial. The blastopore is closed in this embryonic development stage (Pereira et al., 2006). The result is given by the formula: Fertilization (%) = (Number of fertilized/Total oocytes) x 100. The hatching and normal larvae rates were evaluated 48 hours after fertilization. Larvae without head, spine or yolk sac abnormalities (Jezierska et al., 2009) were considered as normal. The results were obtained after applying the following formulas: Hatching (%) = (Number of larvae/Total oocytes) x 100; and Normal Larvae (%) = (Number of normal larvae/Total larvae) x 100. These analyses were performed using a binocular stereomicroscope (Q7740SZ-T, Quimis, Diadema, Brazil) at 10 × and a manual counter.

### *2.8 Statistical analyses*

Normality (Shapiro-Wilk test) and homogeneity (O'Neill & Mathews test) were verified. When necessary, data were transformed (LOG), and outliers exclude when present. After verifying compliance with the statistical assumptions, data were analyzed using a one-way ANOVA followed by Tukey's test.

In order to more comprehensively assess the set of data obtained and the behavior of the responses through the parameters evaluated in the post-thaw sperm cryopreserved using plastic straw, hard-gelatin and hard-HPMC capsules the principal component analysis (PCA) was performed. The PCA reduces the dimensionality of the data, performing a linear combination and rejecting a part of the components with the least variation. For the analysis, all data were standardized, only principal components with eigenvalues >2 were considered, and ellipses were plotted, separating the experimental groups. All analyses were performed with a 95% confidence level. The graphs and analyses were performed using R (RStudio version 2022.07.01) and GraphPad Prism (Version 9.3.0) software.

## 3. Results

Membrane integrity results of fresh and post-thaw sperm cryopreserved in different containers (straw, hard-gelatin, and hard-HPMC capsules) are shown in Fig. 2. Post-thaw samples showed a lower number of spermatozoa with intact membrane compared to fresh samples. The samples cryopreserved in straws showed a higher ($P$<0.05) number of cells without membrane damage than sperm stored in capsules.

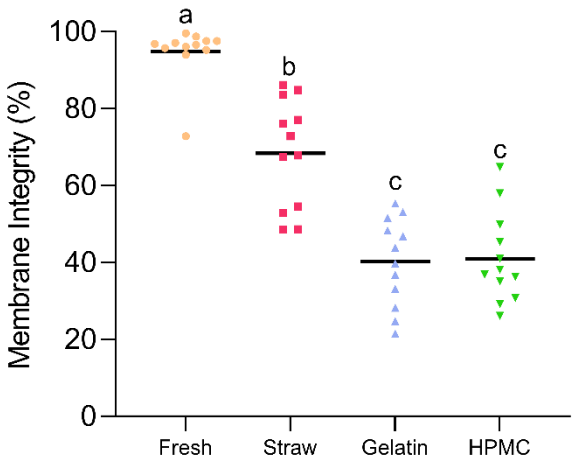


**Figure 2.** Membrane integrity of spermatozoa from fresh and post-thaw sperm samples (n = 12) cryopreserved in straws, hard-gelatin, and hard-HPMC capsules. All samples were evaluated using eosin/nigrosine staining and the number of spermatozoa was counted with plug-in Counter in Image J. Values are shown individually, and horizontal lines indicate the means; different letters indicate differences (P < 0.05; Tukey's test) between means.

After cryopreservation, all samples reduced the spermatozoa motility (Fig. 3A), velocities (Fig. 3B), and progression (Fig. 3D) independently on the used container. There was no difference among the post-thaw sperm motility, velocities, and progression of samples cryopreserved in different containers. However, the post-thaw sperm cryopreserved in straws showed higher straightness and beat cross frequency than fresh sperm (Fig. 3C, E).

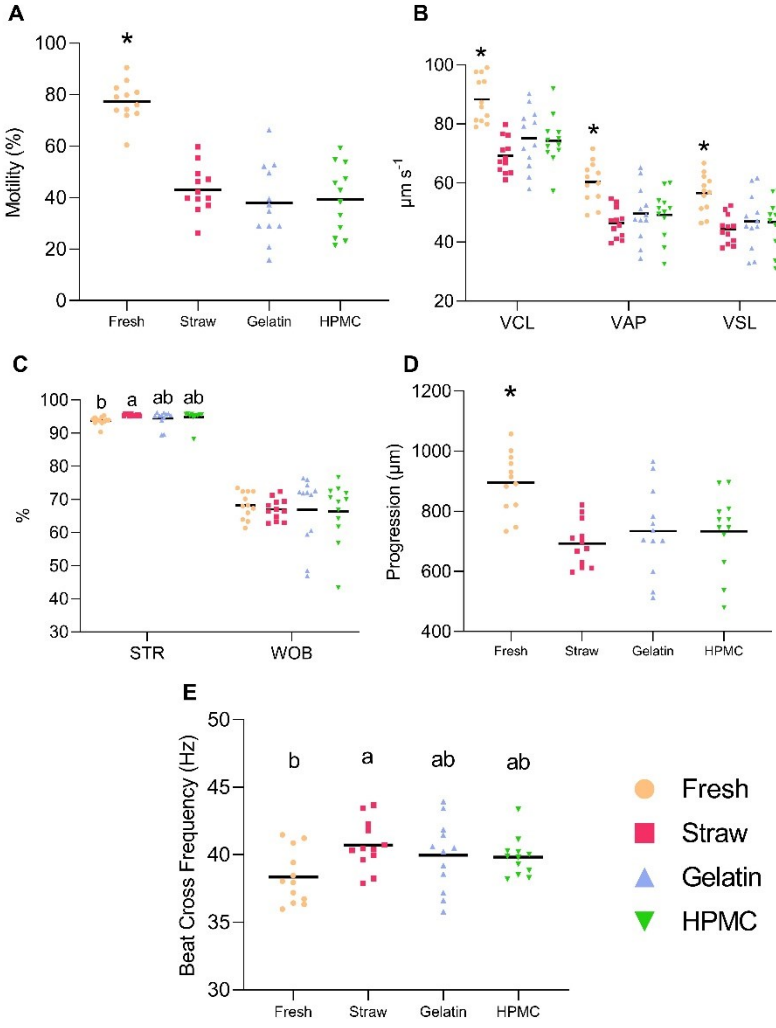


**Figure 3.** Sperm kinetic results of fresh and post-thaw sperm samples cryopreserved in straws, hard-gelatin, and hard-HPMC capsules. Graphs show motility (MOT – A), velocities (VCL, VAP, VSL – B), straightness (STR – C) and wobble (WOB – C), progression (PROG – D) and beat cross frequency (BCF – E). Values are shown individually, and horizontal lines indicate the means. Different letters and asterisks indicate differences ($P < 0.05$; Tukey's test) between means.

The comparison between containers for post-thaw sperm mitochondrial activity is shown in Figure 4A. Cryopreserved sperm in hard-gelatin capsules showed higher mitochondrial activity when compared to that in hard-HPMC

capsules, while sperm samples cryopreserved in straws showed intermediate values. The fertilization rate with a pool of fresh sperm (75%) showed that the oocytes used were good quality. When post-thaw sperm cryopreserved in straws, hard-gelatin capsules, or hard-HPMC capsules was used the fertilization rate did not differ (Fig. 4B). Differences were also not observed for hatching and normal larvae rates.

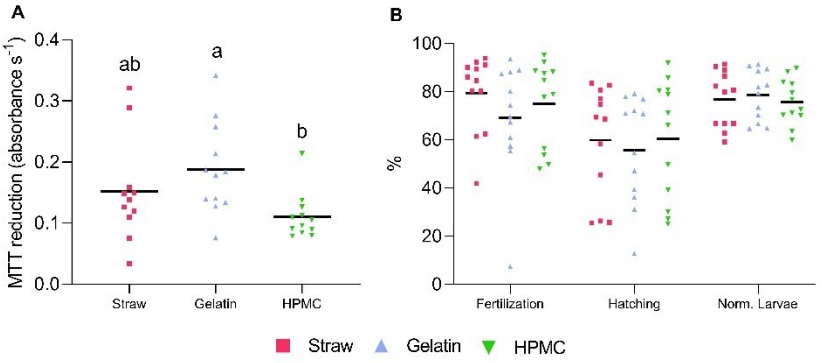


**Figure 4.** Mitochondrial activity (A) was analyzed by MTT reduction rate (optical density) at 570 nm. The results were normalized to 50 million cells. Fertilization, hatching, and normal larvae rates (B) were obtained after counting and evaluating all embryonic and larvae collected in sieves. All analyses evaluated the post-thaw sperm samples (n = 12) cryopreserved in straws, hard-gelatin capsules, or hard-HPMC capsules. Values are shown individually, and horizontal lines indicate the means. Different letters indicate differences ($P < 0.05$; Tukey's test) between means.

The scores of the PCA are shown in Fig. 5A. The first principal component (PC1) showed an eigenvalue of 4.96 and included 38.17% of the variation in the data set, while the second principal component (PC2) had an eigenvalue of 2.84, representing 21.89%. The ellipses designed for each treatment overlapped, showing no difference between the parameters evaluated for sperm cryopreserved in plastic straws, hard-gelatin, and hard-HPMC capsules. Figure 5B presents the projection of the loading vectors, showing a pattern of correlation between the variables analyzed. In PC1, the variables with higher contribution were VSL (−0.949), PROG (−0.943), VAP (−0.933), WOB (−0.885), and MOT (−0.799). While in PC2 hatching rate (0.873), normal larvae rate (0.825), fertilization rate (0.767), VCL (0.484), and membrane integrity (0.404) showed a higher contribution. Thus, in PC1, the kinetic

parameters promoted higher contributions, while in PC2, the fertility capability variables had higher contributions.

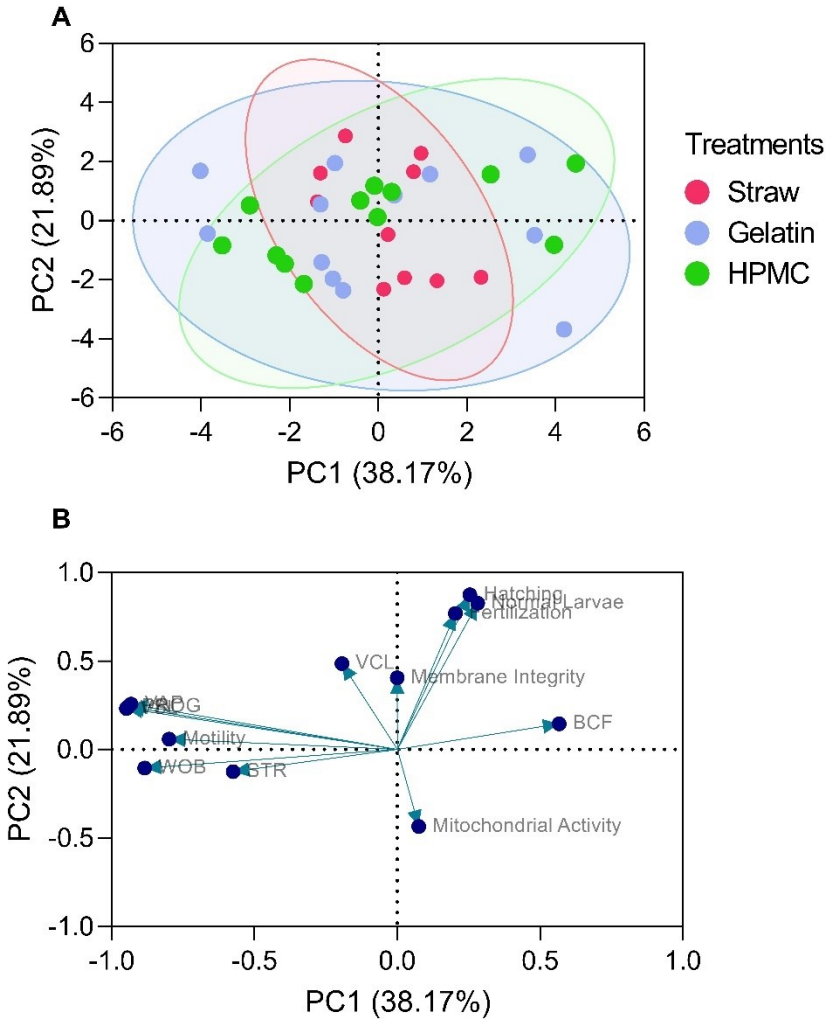


**Figure 5.** Principal Component Analysis (PCA). Score plot (A) of the first two principal components (PC1 and PC2) for the variables analyzed post-thaw sperm cryopreserved in plastic straw, hard-gelatin, and hard-HPMC capsules. The ellipses separating the groups with a 95% confidence level. Loading plot (B), where the arrows show the loading of variables presented in the PC1 and PC2 axes. All parameters analyzed in the post-thaw sperm stored in the three containers.

## 4. Discussion

Cryopreservation is a biotechnique that stores numerous animal and vegetable cells (Murray and Gibson, 2022). The usual is preserving these cells at low temperatures using plastic containers (Meneghel et al., 2020). After using these containers, they are not recycled, thus becoming a polluting agent for the environment. Therefore, biodegradable containers are welcome for cell cryopreservation. This is the first study using a hard capsule as a container to cryopreserve cells. Hard-gelatin and hard-HPMC capsules are environment-friendly, biocompatible, and low-cost. Sperm cells cryopreserved in these biodegradable containers showed kinetic parameters, mitochondrial activity, fertilization, hatching, and normal larvae rates similar to cells stored in plastic straws.

The cryopreservation process maintains live cells and tissues alive despite the damage, called cryoinjuries (Estudillo et al., 2021). Cryoinjuries promote a decrease in qualitative cellular parameters after the cryopreservation process. In previous studies, other authors reported post-thaw sperm quality decrease in humans (Yan et al., 2021), bovines (Zoca et al., 2021), equines (Oddi et al., 2021) and fish (Gallego and Asturiano, 2019). We also observed a sperm quality decrease after the cryopreservation process in plastic straws, hard-gelatin, and hard-HPMC capsules compared with fresh sperm quality. Cryoinjuries mainly occur when cells pass through the temperature zone between -5 and -60 °C, thus making cooling and thawing critical moments for the success of the technique (Mazur, 1970, 1984). The cryopreservation process can compromise fish cell membrane integrity (Ciereszko et al., 2020). Previously, studies reported that the sperm membrane integrity of fish sperm cryopreserved in 5 and 2 mL containers does not differ from samples stored in plastic straws (Beirão et al., 2021; Herranz-Jusdado et al., 2019a). On the other hand, in the present study, we observed a higher number of cells with damaged membranes when cryopreserved in biodegradable capsules. The damage may have been caused during the thawing samples; as they undergo agitation, the collision between the spermatozoa may have caused damage to the membranes. However, even with lower membrane integrity than sperm stored in straws, gametes cryopreserved in

biodegradable capsules were able to fertilize and produce viable larvae.

Analysis of sperm kinetic parameters using computer-assisted sperm analysis (CASA) are widely used to evaluate the quality of sperm from wild species (Van der Horst, 2021), stallion, bull, boar (Amann and Waberski, 2014), human (Diaz et al., 2022), and fishes (Gallego and Asturiano, 2018). Nowadays, sperm motility is the best quality biomarker of fish spermatozoa (Gallego and Asturiano, 2019). We observed post-thaw sperm motility range from 38 to 43%, and we did not observe a statistical difference between treatments. Other studies using plastic straws as a container for *R. quelen* sperm cryopreservation reported post-thaw motility of 42% (Pérez-Atehortúa et al., 2022) and 47% (Adames et al., 2015). Thus, we can see that in this experiment, the cryopreservation protocol was well executed. Furthermore, the results showed the capability of using biodegradable capsules to maintain post-thaw sperm motility. In fish, there is competition between spermatozoa for the fertilization of oocytes (Gage et al., 2004). So, when a fixed ratio of motile sperm per oocyte is used at fertilization, sperm velocities and progression are determining parameters for differentiating treatments (Gallego, et al., 2013a). Our group reported that *R. quelen* sperm with higher velocities and progression had the higher reproductive capability (França et al., 2022). We did not observe differences in these parameters on samples cryopreserved in the three tested containers in the present study. The sperm samples cryopreserved in capsules presented values of STR, WOB, and BCF similar to fresh sperm and sperm cryopreserved in straws. Increased STR, BCF, and decreased spermatic velocities after cryopreservation have also been reported in *R. quelen* post-thaw sperm (Neumann et al., 2019). Thus, it indicates that there was a decrease in sperm swimming efficiency. However, to achieve better accuracy in evaluating these parameters, analyses can be performed using multi flash stroboscopic illumination and high-speed video techniques (up to 1,000 FPS) to assess these parameters (Boryshpolets et al., 2018; Cosson, 2019).

Mitochondria are the organelles responsible for providing energy to fish sperm in the form of ATP (Kholodnyy et al., 2020). Therefore, the mitochondrial activity of gametes is essential for reproductive success (Figueroa et al., 2019). Furthermore, mitochondria are organelles sensitive

to low temperatures (Tsvetkov and Naydenova, 1987), so validating their functionality after the cell cryopreservation process is essential. The oxidoreductases, mainly succinate dehydrogenase enzyme in the mitochondrial membrane, reduce MTT to thiazolyl blue tetrazolium bromide, showing activity only in viable cells (Liu and Nair, 2010). Our study observed that mitochondrial activity in sperm cryopreserved in hard-gelatin and hard-HPMC capsules did not differ from samples stored in straws. This result corroborates the similarity of containers in the results obtained by the tests of sperm kinetic parameters and reproductive capability. Sperm quality is primarily defined as its ability to fertilize oocytes and consequently generate a normally developing embryo (Ciereszko et al., 2020). We observed that the samples cryopreserved in the three containers showed fertilization rates around 70%, which can be considered high (Beirão et al., 2019). Our previous study with the same species (Pérez-Atehortúa et al., 2022) showed a fertilization rate of using post-thaw sperm stored in straws of 77% and a hatching rate of 55%, values similar to those observed in the present study. The present study proves that high fertilization and normal hatch rates can be achieved using biodegradable capsules as containers. Finally, we found no differences between containers for the production of normal larvae, but our experiment resulted in lower values than those previously reported by other studies (França et al., 2022; Neumann et al., 2019; Pérez-Atehortúa et al., 2022). The lower result can be explained by the higher number of animals used in this study compared to the other studies. Thus, the oocytes used in the present study had a post-extrusion storage time greater than 45 minutes, which caused a decrease in the percentage of normal larvae (Sanches et al., 2011a).

Principal Component Analysis is an exploratory analysis that helps to understand an overview of the behavior of experimental variables. Here, we can see the post-thaw behavior of sperm cryopreserved in plastic straws, hard-gelatin capsules, and hard-HPMC capsules. In general, we observed that the interactions of loadings PC1 and PC2 explained 60% of the total variability of the collected data. The evaluation with ellipses did not show a difference between the containers. Furthermore, the loading analysis shows us that the kinetic parameter most related to reproductive capability is the VCL. Other

studies have shown a high correlation between this velocity with the fertilization rate of fishes; *Rhamdia quelen* (Neumann et al., 2019), *Prochilodus lineatus* (Viveiros et al., 2010), *Colossoma macropomum* (Gallego et al., 2017), and *Takifugu niphobles* (Gallego et al., 2013a). The BCF presented an antagonistic behavior to the other kinetic parameters, which indicates that the sperm showed high MOT, PROG, STR, WOB, and velocities but lower BCF. This observation corroborates the report that swimming speed is linearly proportional to the frequency of the flagellar beat (Cosson, 2019). A decrease in velocities and an increase in BCF were also observed in other studies with *Rhamdia quelen* (Neumann et al., 2019; Pérez-Atehortúa et al., 2022). However, as mentioned above, specific analyses should be performed to observe the flagellum movements reaching accurate results. Moreover, we observed that the mitochondrial activity in the present study promoted a negative contribution concerning reproductive capability variables. Conversely, the authors reported a positive correlation between sperm mitochondrial activity and successful oocyte fertilization (Figueroa et al., 2019). Our study used a fixed ratio of motile sperm per oocyte at the fertilization time, which may have masked the effect of the mitochondrial activity in the PCA analysis.

## 5. Conclusions

Our data show that hard-gelatin and hard-HPMC capsules maintained the functionality of sperm and the capability to produce viable larvae after cryopreservation. They offer a low acquisition cost and environmentally friendly alternative to plastic straws. Thus, the present study is a starting point for developing biodegradable containers for the cryopreservation of other cells, as well as specific equipment for storing cryopreserved samples in capsules.

### Declaration of interest

TSF, ICG, and DPSJ filed (September/2022) a patent (pending) application on the methodology described in the study. The remaining authors have no

conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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# CHAPTER 3

## Successful cryopreservation in biodegradable containers of sperm from aquaculture Mediterranean fishes

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## Abstract

We aimed to evaluate the efficiency of hard-gelatin and hard-hydroxypropyl methylcellulose (HPMC) capsules as biodegradable alternative containers to plastic straws in European eel (*Anguilla anguilla*), gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) sperm cryopreservation. Sperm samples from each European eel (n=12) were diluted 1:8:1 (sperm: extender P1+5% egg yolk: methanol). Gilthead seabream (n=12) samples were individually diluted in a cryoprotectant solution of 5% $Me_2SO$ + NaCl 1% plus BSA (10 mg $mL^{-1}$) at a ratio of 1:6 (sperm: cryoprotectant solution). European sea bass (n=10) sperm from each male was diluted in non-activating medium (NAM) at a ratio of 1:5.7 (sperm: NAM), and 5% of $Me_2SO$ was added. The diluted European eel and sea bass sperm aliquots (0.5 mL) were individually filled in plastic straws (0.5 mL), hard-gelatin, and HPMC capsules (0.68 mL). Gilthead seabream diluted sperm (0.25 mL) were filled in plastic straws (0.25 mL) and identical capsules described. All samples were frozen in liquid nitrogen vapor and stored in a liquid nitrogen tank. Sperm kinetic parameters were evaluated by CASA-Mot software. Sperm membrane integrity was performed using a Live and Dead KIT and an epifluorescence microscope. To quantify DNA damage, the alkaline comet assay was performed and TailDNA (TD-%) and Olive Tail Moment (OTM) were evaluated by CaspLab software. Sperm cryopreservation of the three Mediterranean species in straws, gelatin, or HPMC capsules reduced the kinetic parameters and cell membrane integrity. Generally, the post-thawing samples cryopreserved in straws and capsules did not differ for the kinetic parameters and cell membrane integrity, except for European sea bass sperm, where the samples stored in gelatin capsules showed higher velocities (VCL - 100; VSL - 76; VAP - 90 µm $s^{-1}$) than the sperm stored in HPMC capsules (VCL - 87; VSL - 59; VAP - 73 µm $s^{-1}$). The cryopreservation process did not damage the sperm DNA of European eel and European sea bass, regardless of the containers used. On the other hand, gilthead seabream sperm cryopreserved in gelatin (TD - 9.8%; OTM - 9.7) and HPMC (TD - 11.1%; OTM - 11.2) capsules showed higher DNA damage than fresh samples (TD - 3.6%; OTM - 2.7) and the sperm stored in straws (TD -

4.4%; OTM - 5.2). The hard-gelatin and HPMC biodegradable capsules can be used as an alternative to straws for European eel, gilthead seabream, and European sea bass sperm cryopreservation.

# 1. Introduction

In 2020, global aquaculture achieved a remarkable milestone, producing 122.6 million tons of aquatic organisms, representing a noteworthy 2.7% increase compared to the previous year's output (FAO, 2022). This notable growth in aquaculture production can be attributed to the ever-increasing demand for food and the economic development, spurred by the relentless growth of the world's population (Costello et al., 2020). As fish capture encounters stagnation or decline in certain regions, the spotlight has turned to aquaculture as a crucial solution to meet the surging food demands. Looking ahead to 2030, some regions and countries are targeting a substantial increase of 35 to 40% in the production of aquatic organisms through aquaculture. The surge in aquaculture production contributes to a proportional increase in the amount of waste released into the environment. Therefore, measures that make production more sustainable from an environmental point of view and reduce waste should be practiced (Campanati et al., 2022). One of them is the use of the circular economy, which aims to prevent resource depletion, close energy and materials loop uses, and facilitate sustainable development (Prieto-Sandoval et al., 2018). This entails optimizing resource utilization to enhance efficiency and minimize waste generation and emissions by aquaculture (Chary et al., 2023). This ambitious effort requires innovating and advancing sustainable techniques within the industry (FAO, 2022).

Cryopreservation is a biotechnique that maintains cells and tissues at extremely low temperatures, ensuring their functionality, development, and growth can be preserved even after thawing, with minimal loss of efficiency. Among its various applications, fish sperm cryopreservation is a valuable tool for enhancing aquaculture practices, particularly in optimizing broodstock management at hatcheries while safeguarding natural fish stocks (Asturiano et al., 2017; Cabrita et al., 2022). Nowadays, several protocols are established for the cryopreservation of fish sperm (Cabrita et al., 2022). Most protocols use plastic straws (0.25, 0.5, 1, 2 and 5 mL) or cryovials (1, 2, 3 and 5 mL) as sperm storage containers. These containers are made of Polyvinyl chloride (PVC) or polypropylene, both plastic and recyclable. However, it is a common

practice not to recycle these containers after use due to their contact with biological samples. Consequently, this leads to waste with significant pollution potential, given that these containers are made from durable plastics. Furthermore, the limited number of industries manufacturing plastic containers results in higher market prices and hinders accessibility in certain countries. To find a biodegradable alternative to plastic containers for fish sperm cryopreservation, a groundbreaking methodology has been reported by França et al. (2023). This innovative approach employs hard-gelatin capsules and hard-hydroxypropyl methylcellulose (HPMC) capsules as the storage containers for freshwater fish sperm. These capsules are crafted from biodegradable and biocompatible by products from animal and plant production, making them environmentally friendly and compatible with biological samples. Moreover, their widespread availability on the market and cost-effectiveness makes them a practical and accessible solution. Using these capsules represents a significant step towards reducing environmental harm compared to plastic containers.

The European eel *Anguilla anguilla* is a catadromous fish species that performs a long-spawning migration, reaching thousands of kilometers along the Atlantic Ocean (Arai, 2014). Since the 1980s, natural eel stocks have been decreasing due to anthropic actions. Today the species is on the Red List of the International Union for Conservation of Nature (IUCN), categorized as "Critically Endangered" (Pike et al., 2020) the highest classification level before extinction rating. Moreover, the European eel is a highly prized delicacy in European and Asian cuisine, boasting great economic value. This places a considerable strain on the wild eel population due to the high demand, emphasizing the urgency for implementing a captive breeding program. Such a program aims to alleviate the fishing pressure on natural eel populations, ensuring their sustainable preservation for the future (Asturiano et al., 2017). In last decades, protocols for cryopreserving the sperm of this species have undergone continuous development and enhancement (Herranz-Jusdado et al., 2019b). Our research group has established the latest and most efficient protocol in this regard (Herranz-Jusdado et al., 2019a).

Gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) hold prominent positions as two of the top ten most produced marine fishes globally (FAO, 2022b). Concentrated mainly in the Mediterranean Sea, their combined production in 2021 reached 619,000 tons (FAO, 2023a,b). As the aquaculture sector continues to expand in the Mediterranean Sea with a positive growth trajectory (Zoli et al., 2023), adopting new strategies to mitigate potential environmental impacts becomes crucial. In this context, sperm cryopreservation can be an important tool in producing these species, assisting in genetic improvement programs (genetic backup), and genetic editing for the development of infertile animals, since fish escape from cages are recurrent and cause serious environmental problems (Alvanou et al., 2023). Gilthead seabream and European seabass sperm cryopreservation protocols have been developed and improved for decades (Cabrita et al., 2005; Chambeyron and Zohar, 1990; Fauvel et al., 1998). Nowadays, protocols for sperm cryopreservation of gilthead seabream (Gallego et al., 2012a) and European sea bass (Martínez-Páramo et al., 2012) in plastic straws are already established. However, new protocol adjustments are always welcome to make it cheaper and environment-friendly.

In several areas, the use of biodegradable polymers is an alternative to minimize the pollution caused by plastic (Samir et al., 2022). By adopting these sustainable alternatives, the fish sperm cryopreservation process becomes more ecologically responsible, positively contributing to environmental conservation and sustainability efforts. Thus, we aimed to evaluate the efficiency of hard-gelatin and hard-hydroxypropyl methylcellulose (HPMC) capsules as biodegradable alternative containers in European eel, gilthead seabream and European sea bass sperm cryopreservation.

## 2. Materials and methods

Reagents were purchased from Sigma Aldrich (Madrid, Spain), unless otherwise stated.

## *2.1 Ethics statement*

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 (BOE, 2013). The protocol used with European eel was approved by the Experimental Animal Ethics Committee from the Universitat Politècnica de València (UPV). Protocols used with gilthead seabream (1295/2022) and European sea bass (1273/2022) were approved by the Institute of Aquaculture Torre de la Sal (IATS) Experimental Animal Ethics Committee and CSIC Ethics Committee. Final permissions (European eel: 2023-VSC-PEA-0039; gilthead seabream: 2022-VSC-PEA-0230; European sea bass: 2022-VSC-PEA-0213) were given by the local government (Generalitat Valenciana).

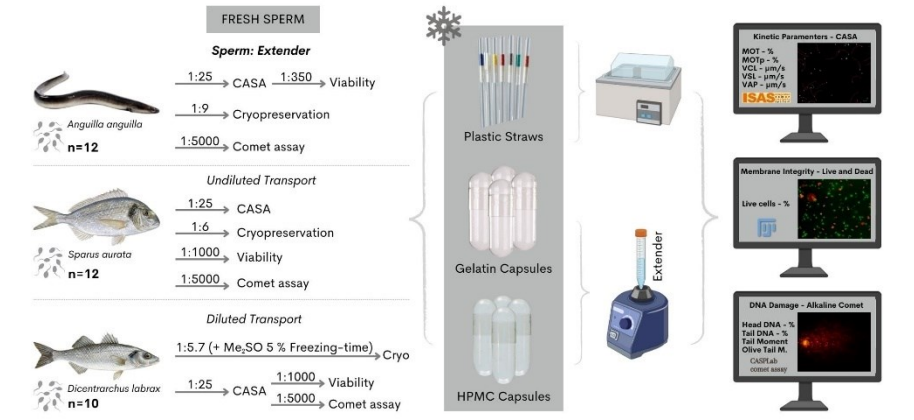


**Figure 1.** Fresh sperm dilutions and experimental design to compare European eel (*Anguilla anguilla*; n = 12), gilthead seabream (*Sparus aurata*; n = 12), and European sea bass (*Dicentrarchus labrax*; n = 10) post-thawing sperm quality after cryopreservation in plastic straws, hard-gelatin capsules, and hard-HPMC capsules. Plastic straws were thawed in water-bath and capsules in tubes with extender under vortex shake. The sperm kinetic parameters, membrane integrity, and DNA damage of fresh and post-thawing sperm were performed in triplicate.

## *2.2 Experimental design*

The experimental design is summarized in Fig. 1. The study was performed using European eel, gilthead seabream, and European sea bass sperm samples. The motility was compared in fresh and post-thawing sperm

samples after being cryopreserved in three containers: plastic straw (0.25 or 0.5 mL - IMV Technologies, l'Aigle, France), hard-gelatin capsule (Nadiprana SL, Els Pallaresos, Spain) manufactured with collagen, size 0 – locked length 21.7 mm, and with a 0.68 mL volume, and hard-HPMC capsule (Nadiprana SL, Els Pallaresos, Spain) manufactured with hydroxypropyl methylcellulose and having the same size. After thawing, samples were evaluated testing in triplicate their kinetic parameters, membrane integrity, and grade of DNA damage.

### *2.3 Fish handling and sperm collection*

#### *2.3.1 European eel*

All experimental procedures were performed at the Fish Reproduction Laboratory of the Universitat Politècnica de València (UPV, Spain). European eel males (n = 36; 127 ± 21 g) from commercial fish farm (Valenciana de Acuicultura S.A.; Puzol, Spain) were transferred to UPV. The animals were accommodated in three 96-L freshwater aquaria in the laboratory, 12 fish in each aquarium, and gradually acclimatized to seawater (salinity = 38 ± 0.5 g $L^{-1}$) during a week. Aquaria water was kept at 20 °C and covered to decrease the light, reducing the eels stress. After 10 days of acclimatization, the hormonal protocol to induce sexual maturation started. Eels were weekly anesthetized using a benzocaine solution (60 ppm – Thermo Fisher, Kandel, Germany) and received an intraperitoneally injection of 1.5 IU $g^{-1}$ fish of recombinant human chorionic gonadotropin (rhCG; Ovitrelle, Merck S. L., Madrid) (Gallego, Mazzeo, et al., 2012). From the seventh week of hormone treatment, the animals began to produce sperm. After 24 hours of hormone administration, the sperm samples were collected weekly by abdominal massage and collected in a plastic tube (15 mL) (Pérez et al., 2000). The samples were maintained at 4 °C until experimental procedures.

#### *2.3.2 Gilthead seabream and European sea bass*

The sperm collection was performed at the Institute of Aquaculture of Torre la Sal (IATS, Ribera de Cabanes, Castellón, Spain) during the species spawning season (gilthead seabream: Dec/2022; European sea bass Feb-Mar/2023). Gilthead seabream (n = 32; 997 ± 114 g) and European sea bass (n = 12; 3.4

± 0.5 kg) males were maintained in 5000 L tanks in an open seawater system with salinity (39 ± 0.5 g $L^{-1}$), natural temperature (10.2 ± 2 °C), and photoperiod (11 h light: 13 h dark). Animals of both species were fed by hand using commercial fish feed once a day to apparent satiation.

Before the sperm collection, the animals were not hormonally induced following the IATS reproduction protocol. Both breeder species produce a sperm volume of at least 2 mL, which does not justify the use of hormone. Each animal was anesthetized with Ethyl 3-aminobenzoate methanesulfonate (Tricaine methanesulfonate, MS-222 - 60 ppm). The genital area was cleaned with distilled water and dried to avoid contamination of samples by seawater, urine, and feces. Sperm from each male was individually collected by a gentle abdominal massage using a syringe and placed in a plastic tube (15 mL). In a previous test, it was observed that undiluted gilthead seabream fresh samples could be transported to the UPV without loss of quality. On the other hand, undiluted European sea bass sperm samples showed decreased quality after transport. Thus, individualized gilthead seabream fresh sperm samples were transported undiluted, while individualized European seabass samples were diluted in two tubes containing non-activation medium (NAM seabass - in mM: NaCl 59.83, KCL 1.47, $MgCl_2$ 12.91, $CaCl_2$ 3.51, $NaHCO_3$ 20, glucose 0.44; BSA 1% (w:v); 310 mOsm $kg^{-1}$ and pH adjusted to 7.7) described by (Fauvel et al., 1998). In the first plastic tube (15 mL), 1430 µL of fresh sperm were diluted in 8145 µL NAM seabass reaching a ratio of 1:5.7 (sperm: extender). In the second plastic tube (2 mL), 80 µL of fresh sperm were diluted in 1920 µL of NAM seabass, reaching a ratio of 1:24 (sperm:extender) (Fig. 1). All samples were kept in a cooled box at 4 °C and transported (approximately 50 min) to the Fish Reproduction Laboratory at UPV.

### *2.4 Sperm samples selection*

Once at the Fish Reproduction Laboratory at UPV, fresh sperm samples from the three species were selected for the experiments using the Computer-Assisted Sperm Analyzer (CASA ISASv1; Proiser R+D, S.L., Spain), following the method described by Gallego et al. (2013b). After CASA evaluation, 12 European eel sperm samples demonstrating a minimum of 65% MOT, 12

gilthead seabream samples exhibiting at least 55% MOT, and 10 European sea bass samples with a minimum of 55% MOT were selected for the experiment.

### *2.5 Cryopreservation and thawing*

The European eel sperm samples (n = 12) were individually diluted at a proportion of 1:8:1 (sperm: P1 extender plus 5% egg yolk (v:v): methanol) in plastic tubes (1.5 mL). The egg yolk (from commercial hen eggs) was diluted in P1 extender, then the methanol was added, followed by the sperm. Diluted samples were incubated for 1 h at 4 °C, permitting the cryoprotectant penetration into the cells (Herranz-Jusdado et al., 2019a). Further, the 8 plastic straws, 8 hard-gelatin, and 8 hard-HPMC capsules per male were filled with 0.5 mL of diluted sperm. Immediately, the samples were frozen (both in straws and capsules) for 3 min, 3 cm over the liquid nitrogen vapor, and then thrown into the liquid nitrogen (Herranz-Jusdado et al., 2019a). Afterward, all the samples were stored in a liquid nitrogen tank (Minitube, Tiefenbach, Germany) at -196 °C for one month. For thawing, the plastic straws were individually submerged in water at 40 °C for 13 s (Herranz-Jusdado et al., 2019a). The capsules were individually removed from the nitrogen tank. The upper part of each capsule was broken with a clamp pression and placed inside plastic tubes with a capacity of 15 mL. These tubes contained 5 mL of P1 extender previously warmed at 40 °C in a water bath. As soon as the capsule was placed in the extender, the tube was shaken in a vortex (VWR Test tube shaker – model 4441378, Leuven, Belgium) at 2000 rpm for 25 seconds to dissolve the capsule (França et al., 2023). The post-thawing samples were maintained at 4 °C until analyzed.

Gilthead seabream sperm samples (n = 12) were cryopreserved using a solution of 5% of the permeable cryoprotectant dimethyl sulfoxide ($Me_2SO$) in extender NaCl 1% plus BSA (10 mg $mL^{-1}$). The cells were diluted in the cryoprotectant solution at a ratio of 1:6 (sperm: cryoprotectant solution) (Cabrita et al., 2005; Fabbrocini et al., 2000). Immediately, 8 plastic straws, 8 hard-gelatin, and 8 hard-HPMC capsules per male were filled with 0.25 mL of diluted sperm. Without equilibrium time, the samples were frozen (both in straws and capsules) for 10 min, 1 cm over the liquid nitrogen vapor (Gallego

et al., 2012a). Later, the containers were stored in a nitrogen liquid tank at -196 °C for two weeks. Plastic straws were thawed in a water bath at 60 °C for 5 s (Gallego et al., 2012a), and capsules were thawed as described above, but using the extender NAM seabream -(in mM: NaCl 75, $NaHCO_3$ 20, $MgCl_2$ 12.9, $CaCl_2$ 2.65, KCl 1.5, glucose 4.4, bovine serum albumin (BSA) 0.015; 280 mOsm $kg^{-1}$ and pH adjusted to 7.7 described by (Castro-Arnau et al., 2022*)*) previously warmed at 40 °C in a water bath. All post-thawing samples were maintained at 4 °C until analyzed.

In the European sea bass sperm samples (n = 10) previously individually diluted in NAM sea bass (200 mOsm $kg^{-1}$; pH 7.7) were added 5% Me2SO (v:v), reaching a final concentration of 4.29% $Me_2SO$ and a final ratio of 1:6 (sperm: cryoprotectant solution). Then, 8 plastic straws, 8 hard-gelatin, and 8 hard-HPMC capsules per male were filled with 0.5 mL of diluted sperm. Immediately, the samples were frozen 6.5 cm over the liquid nitrogen vapor for 15 min (Martínez-Páramo et al., 2012). After, the containers were stored in a liquid nitrogen tank at -196 °C for one month. Plastic straws were thawed in a water bath at 35 °C for 15 s (Martínez-Páramo et al., 2012). Capsules were thawed using the previously described method, except they were placed in extender NAM sea bass pre-warmed at 35 °C in a water bath. The post-thawing samples were maintained at 4 °C until analyzed.

### *2.6 Sperm kinetic parameters*

The evaluation of European eel (n = 12), gilthead seabream (n = 12), and European sea bass (n = 10) fresh samples and post-thawing sperm samples cryopreserved in plastic straws and biodegradable capsules were carried out in triplicate using CASA-Mot system. Before the analysis, European eel samples were diluted in P1 medium (in mM: NaCl 125, $NaHCO_3$ 20, $MgCl_2$ 2.5, $CaCl_2$ 1, KCl 30; and pH adjusted to 8.5 (Peñaranda et al., 2010). Gilthead seabream sperm was diluted using as extender a non-activating medium NAM seabream. To evaluated European sea bass samples, fresh sperm previously diluted in a 1:25 ratio (sperm: NAM sea bass) was used (Fig. 1).

All samples were activated using artificial seawater (ASW - in mM: NaCl 354.7, $MgCl_2$ 52.4, $CaCl_2$ 9.9, $Na_2SO_4$ 28.2, KCl 9.4, in distilled water) with 2%

BSA (w:v), pH adjusted to 8.2 and osmolality of 1100 mOsm $kg^{-1}$. Briefly, a fresh sperm diluted aliquot of 0.5 µL was activated with 4.5 µL ASW. The post-thawing samples cryopreserved in plastic straws were evaluated by mixing 0.2 µL of sperm in 10 µL of ASW, and in the case of the samples stored in capsules, an aliquot of 1 µL was mixed with 5 µL of ASW. The samples activation was performed in a counting chamber ISAS Spermtrack 10 (Proiser R+D, S.L., Spain) under a microscope in negative phase with a 10× magnification (Nikon Eclipse 80i, Tokyo, Japan) with a camera (ISAS 782M, Proiser R+D, S.L., Spain) attached for video recording connected to a computer. Videos of spermatozoa displacements were captured at 60 frames per second for 1 s. For all samples, the analyses were performed in triplicate 10 s after activation using CASA software. The kinetic parameters analyzed were the percentage of total motile spermatozoa (MOT - %), the percentage of spermatozoa swimming forward (MOTp - %), curvilinear velocity (VCL - µm $s^{-1}$), straight line velocity (VSL - µm $s^{-1}$), and average path velocity (VAP - µm $s^{-1}$).

### *2.7 Sperm membrane integrity*

The percentage of viable spermatozoa was analyzed in every fresh and thawed sample from European eel (n = 12), gilthead seabream (n = 12), and European sea bass (n = 10). The evaluation was performed using a fluorescence kit (LIVE/DEAD Sperm Viability Kit, Thermo Fisher Scientific, MA, USA) composed of SYBR-14 at a final concentration of 2 µM and propidium iodide (PI) at 240 µM. Firstly, European eel fresh sperm previously diluted (1:25) was diluted again in P1 at a ratio of 1:350 (diluted sperm: extender). Post-thawing sperm frozen in plastic straws was diluted in P1 at a ratio of 1:15 (post-thawing sperm: extender), and those samples cryopreserved in biodegradable capsules were not diluted to perform this evaluation. In all the cases 50 µL aliquots were used, 1.5 µL of SYBR-14 were added and incubated for 10 min. Then 3 µL of PI were added and incubated 5 min more (Gallego et al., 2012a).

Gilthead seabream fresh sperm samples were diluted with NAM seabream at a ratio of 1:1000 (fresh sperm: extender). Post-thawing sperm from plastic straws was diluted in the same extender at a ratio of 1:20 (post-thawing

sperm: extender), and the samples cryopreserved in capsules were not diluted. Similarly, in all the cases 50 µL aliquots were used, 1.5 µL of SYBR-14 were added and incubated for 10 min, and 3 µL of PI were added and incubated 5 min more (Gallego et al., 2012a).

For European sea bass, the fresh sperm samples previously diluted (1:25) was diluted again in NAM sea bass at a ratio of 1:1000 (fresh sperm:extender). Post-thawing sperm from plastic straws was diluted in NAM sea bass at 1:20 (post-thawing sperm:extender), and the samples cryopreserved in capsules were not diluted. In this case, 2 µL of SYBR-14 were added to 50 µL of samples, and after 5 min, 0.2 µL of PI were added and incubated 5 min more (Gallego et al., 2012a). All the samples from the three species were incubated at 25 °C in the dark and immediately visualized.

For observation, two sperm-stained aliquots of 20 µL were pipetted onto each corner of the histological slide and covered with a coverslip under a fluorescence microscope (Nikon Eclipse 80i, Tokyo, Japan). With the aid of a camera (Moticam 1080, Xiamen, China) attached to the microscope and the Motic Image Plus software (Version 3.1.1, Motic, Xiamen, China), photos were taken in the same field using 450-490 nm filter to visualize viable gametes stained by SYBR-14, and 510–560 nm fluorescence filters to visualize non-viable spermatozoa stained by PI. The percentage of viable cells in relation to non-viable cells was evaluated using the FIJI software (Version 1.53t, Image J, National Institutes of Health, Bethesda, USA) with the cell counter plug-in, where the images were superimposed and at least 400 cells on each slide were counted.

### *2.8 DNA fragmentation quantification*

The alkaline comet assay was performed to determine the DNA fragmentation of fresh and post-thawing sperm samples using the protocol described by (Cabrita et al., 2005) with adaptations. Before the analyses, the histological slides were prepared with normal melting point agarose (0.5% diluted in PBS). After removing the excess of agarose, the slides were stored in the dark at 4 °C.

To prepare the samples, 1 µL of fresh sperm, 10 µL of sperm cryopreserved in straws, or 200 µL of sperm cryopreserved in capsules were diluted in 5 mL of extender (European eel - P1; gilthead seabream - NAM seabream; European sea bass - NAM sea bass). A positive control, aimed to cause severe damage to DNA sperm using hydrogen peroxide ($H_2O_2$ 30%), also was prepared to certify the accuracy of the analysis. A work solution was prepared, diluting $H_2O_2$ in PBS at a ratio of 1:10. In each fresh and post-thawing sample of European eel was added 2 µL, gilthead seabream 250 µL, and European sea bass 350 µL. Later, the positive control samples were incubated for 15 min at 4 °C. Then, all the samples were centrifuged at 4000 x g for 5 min at 4 °C and resuspended in 200 µL of the extender used for sperm dilution in each species. After this procedure, 10 µL of the samples were pipetted into a plastic tube (1.5 mL). In these same tubes, 180 µL of low melting point agarose (0.5% diluted in PBS) were added. The slides received two 75 µL aliquots (semen + agarose), one from each end of the slide, which were covered with coverslips. The slides were stored for 30 min at 4 °C for the agarose to let the agarose solidify, and then the coverslips were gently removed. The European eel samples slides were exposed to a lysis solution ($Na_2$ EDTA 100 mM; NaCl 2.5 M; Tris pH 10 10.0 mM; 1% lauryl sarcosine; Triton X-100 1% - diluted in distilled water and adjusted to pH 10) for 30 min at 4 °C. Gilthead seabream and European sea bass samples slides were exposed to a lysis solution for 60 min at 4 °C. Then, they were exposed to denaturizing solution (lysis solution containing dithiothreitol 10 mM) for 30 min at 4 °C, and finally, a denaturizing solution with lithium diiodosalicylate 4 mM for 90 min at room temperature. Once the cell lysis phase was concluded, the slides were subjected to electrophoresis (Biorad PowerPac, Basic Sub-Cell GT Horizontal Electrophoresis System, Hercules, USA) at 15 v, 300 mA for 10 min submerged in an electrophoresis solution ($Na_2$ EDTA 1 mM; NaOH 0.3 M – diluted in distilled water and adjusted to pH 13). Once finished the electrophoresis step, the slides were removed from the cube and washed three times with the neutralization solution (Tris HCl 0.4 M - diluted in distilled water and adjusted to pH 7.5). After this step, the slides were fixed using methanol for 3 min and stored in the dark at 4 °C.

The cells were stained with PI (0.5 mM) to evaluate the comets. Aliquots of PI (10 µL) were pipetted on each side of the slide and covered by coverslips. The samples were observed under an epifluorescence microscope (Nikon Eclipse 80i, Tokyo, Japan) at 400 x magnification and using an excitation filter of 450-490 nm. One slide per male and treatment was observed, evaluating at least 50 cells per slide. The images were acquired with a camera (Moticam 1080, Xiamen, China) attached to the microscope and connected to a computer with Motic Image Plus software (Version 3.1.1, Motic, Xiamen, China). The images of comets were individually analyzed using the CASP Lab software (version 1.2.3). From the several parameters analyzed by the software, the percentage of tail DNA (TailDNA - %) and Olive tail moment (OTM) were used to characterize DNA damage of sperm samples (fresh and cryopreserved at different containers) from the three species. The TailDNA % refers to the proportion of DNA migrated from the cell nucleus. Moreover, the OTM is the product of the TailDNA % and the median migration distance that occurs due to the distance between the comet's center head and tail gravity center (Fig. 2). Greater values of these parameters indicate higher cellular DNA fragmentation. Fresh and post-thawing samples of European eel (n = 8), gilthead seabream (n = 10), and European sea bass (n = 7) cryopreserved in plastic straw and hard biodegradable capsules were evaluated in triplicate.

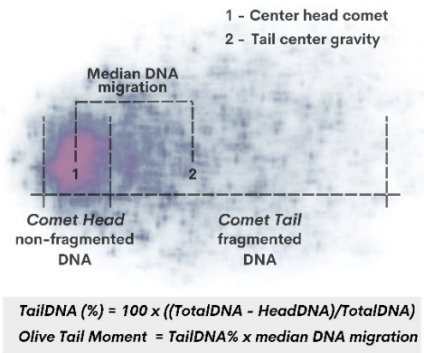


**Figure 2.** Schematic image demonstrating TailDNA (%) and Olive Tail Moment formulas used by CaspLab software after the alkaline comet assay. Both parameters show DNA fragmentation that indicates cellular DNA integrity. The median DNA migration is the distance between the center head comet and tail center gravity; numbers indicate both points.

### *2.9 Statistical analyses*

The data are presented as means ± SD. Normality (Shapiro-Wilk test) and homogeneity (O'Neill & Mathews test) were verified. When necessary, data were transformed using LOG. After verifying compliance with the statistical assumptions, data were analyzed using a one-way ANOVA followed by Tukey's test. All analyses were performed with a 95% confidence level. The graphs and analyses were performed using R (RStudio version 2022.07.01) and GraphPad Prism software (Version 9.3.0).

## 3. Results

European eel, gilthead seabream, and European sea bass sperm kinetic parameters results of fresh and post-thawing sperm cryopreserved in plastic straw, hard gelatin, and hard HPMC capsules are shown in the Figure 3. The cryopreservation process, independently of the used container, decreased the post-thawing sperm MOT and MOTp of the three species. In addition, no differences were observed for these parameters between the samples cryopreserved in plastic straws and hard biodegradable capsules (Fig. 3A).

European eel fresh samples showed higher VCL values (179 ± 14 μm $s^{-1}$) than those found in samples cryopreserved in capsules (gelatin – 139 ± 9 μm $s^{-1}$; HPMC – 143 ± 18 μm $s^{-1}$), and higher VAP values (133 ± 18 μm $s^{-1}$) than sperm stored in hard HPMC capsules (109 ± 11 μm $s^{-1}$). In gilthead seabream samples, the cryopreservation process caused a reduction of the post-thawing sperm velocities independently of the used container. A similar result was observed in European sea bass samples, but the sperm cryopreserved in hard gelatin capsules showed higher velocities (VCL 101 ± 15 μm $s^{-1}$; VSL 76 ± 18 μm $s^{-1}$; VAP 90 ± 18 μm $s^{-1}$) than those stored in hard HPMC capsules (VCL 87 ± 7 μm $s^{-1}$; VSL 59 ± 11 μm $s^{-1}$; VAP 73 ± 10 μm $s^{-1}$) (Fig. 3B).

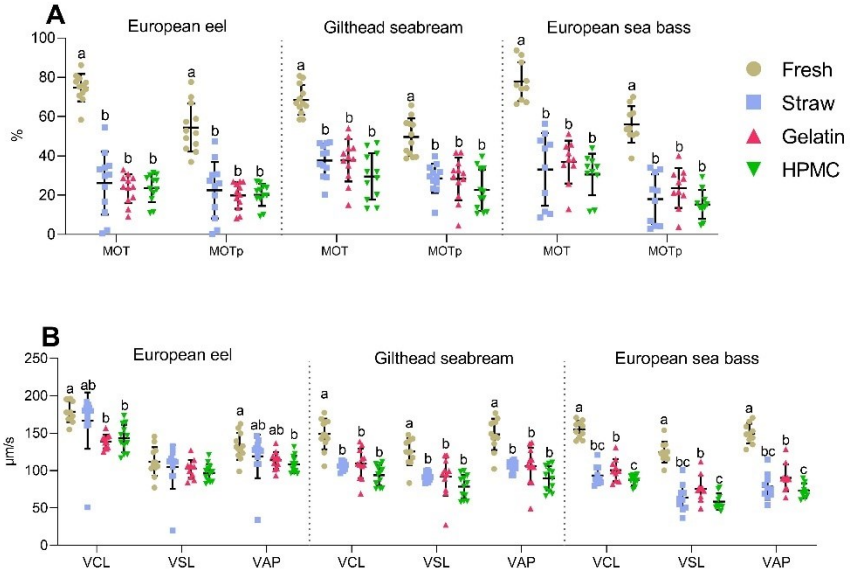


**Figure 3.** Sperm kinetic results of European eel (n = 12), gilthead seabream (n = 12), and European sea bass (n = 10) fresh and post-thawing sperm. The samples were cryopreserved in plastic straws, hard-gelatin, and hard-HPMC capsules. Graphs show motility (MOT – A), progressive motility (MOTp - A), and velocities (VCL, VSL, VAP – B) evaluated by CASA-Mot. Values are shown individually, and horizontal lines indicate the means ± SD. Different letters indicate differences ($P < 0.05$; Tukey's test) between means.

The fresh and post-thawing sperm membrane integrity percentage of the three studied species is shown in the Figure 4. European eel sperm membrane integrity of samples cryopreserved in hard HPMC capsules (65 ± 12%) did not differ from the value observed in fresh sperm (76 ± 11%). However, samples cryopreserved in plastic straws (61 ± 12%) and hard gelatin capsules (62 ± 14%) showed a significantly smaller percentage of live spermatozoa. In the gilthead seabream and European sea bass samples, a decrease of viable cells was observed after the cryopreservation process, independently on the used container.

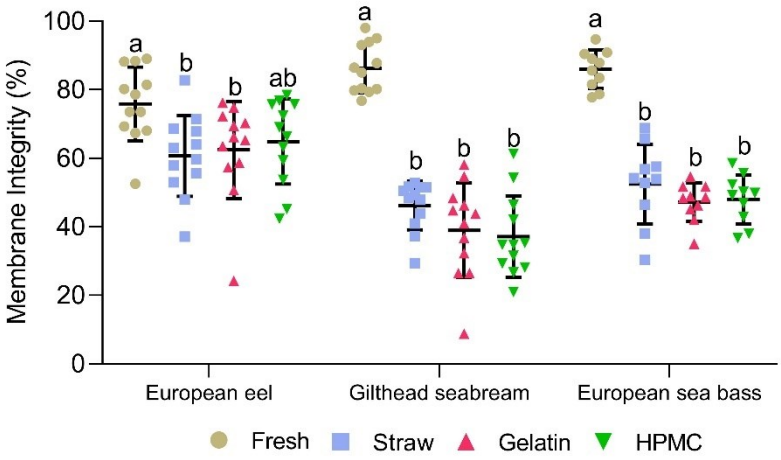


**Figure 4.** Sperm membrane integrity of European eel (n = 12), gilthead seabream (n = 12), and European sea bass (n = 10) fresh and post-thawing sperm. The samples were cryopreserved in plastic straws, hard-gelatin, and hard-HPMC capsules. Graphs show viable cells percentage observed by epifluorescence microscope using SYBR-14 (2 µM) and propidium iodide (PI - 2.4 mM) stains. Photos were taken, and the results were calculated after counting cells using the FIJI software. Values are shown individually, and horizontal lines indicate the means ± SD. Different letters indicate differences ($P < 0.05$; Tukey's test) between means.

The comet assay showed that European eel and European sea bass sperm samples did not show differences of DNA fragmentation after the cryopreservation in different containers (Fig. 5). However, in gilthead seabream, the sperm samples cryopreserved in capsules showed higher Tail DNA (gelatin – 10 ± 2%; HPMC – 11 ± 6%) and OTM (gelatin – 10 ± 3; HPMC – 11 ± 6) than fresh (Tail DNA – 4 ± 3%; OTM – 3 ± 2) and cryopreserved in plastic straws samples (Tail DNA – 4 ± 1%; OTM – 5 ± 2), evidencing a higher DNA damage through the freezing-thawing process.

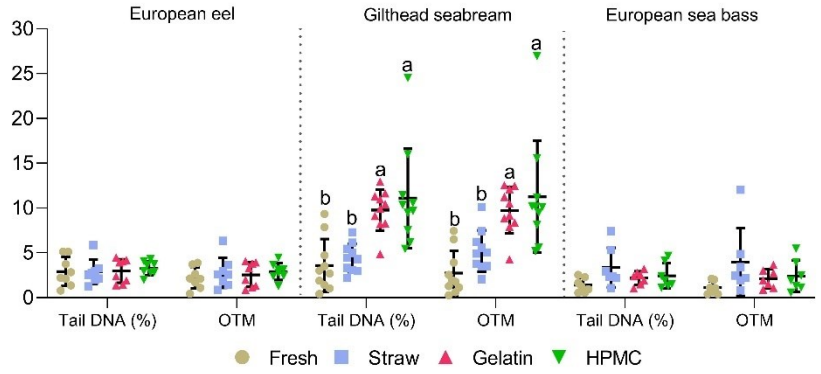


**Figure 5.** Sperm DNA fragmentation of European eel (n = 8), gilthead seabream (n = 10), and European sea bass (n = 7) fresh and post-thawing sperm. The samples were cryopreserved in plastic straws, hard-gelatin, and hard-HPMC capsules. Graphs show DNA in the comet tail percentage and the Olive tail moment index calculated observed by epifluorescence microscope using PI stain and evaluated by CaspLab software. Values are shown individually, and horizontal lines indicate the means ± SD. Different letters indicate differences ($P < 0.05$; Tukey's test) between means.

## 4. Discussion

The present study is the first one testing biodegradable capsules as an alternative to plastic straws in the cryopreservation of sperm from three marine fish species with high environmental and commercial interest. We observed that biodegradable capsules were as efficient as plastic straws in preserving sperm kinetic parameters, membrane integrity, and DNA integrity after European eel, gilthead seabream, and European sea bass sperm cryopreservation.

During cryopreservation, cells are exposed to high thermal and osmotic stress, mainly during freezing and thawing, causing damage to the cells (Mazur et al., 1972). This damage caused during cryopreservation is called cryodamage, which can fully or partially compromise cellular functionality. Thus, cryopreserved sperm generally showed lower kinetic parameters than fresh sperm due to the damage caused during the cryopreservation process (Horváth and Urbányi, 2020). Sperm motility is the main biomarker of sperm quality (Gallego and Asturiano, 2019). In the present study, we observed in

the three species that samples cryopreserved in plastic straws, hard-gelatin, or hard-HPMC capsules showed lower MOT and MOTp than fresh sperm samples. The same was observed in other studies by our group in European eel (Herranz-Jusdado et al., 2019a,c; Peñaranda et al., 2009) and by other authors in gilthead seabream (Beirão et al., 2012) and European sea bass (Martínez-Páramo et al., 2012, 2013). On the other hand, the kinetic parameters of sperm samples cryopreserved in straws and capsules did not differ. These results support the efficiency of the sperm cryopreservation protocols for the three species in biodegradable capsules developed in this study. Sperm velocities are kinetic parameters only observed in motile spermatozoon and are related to the potential fertilization capacity (Gallego et al., 2013a, 2017). In the present study, we observed that the sperm velocities of European eel and gilthead seabream samples cryopreserved in the three containers did not differ. European sea bass thawed sperm cryopreserved in HPMC capsules showed a difference of VCL -13%, VSL -23%, and VAP -19% for sperm cryopreserved in gelatin capsules. We observed that after the thawing procedure, the HPMC capsules did not dissolve as well as the gelatin capsules. Small fragments left in the middle may have made it difficult for the sperm to swim, reducing the velocities. However, cryopreserved sperm in plastic straws showed similar velocities to samples stored in HPMC capsules. This may have happened due to the presence of the extender at the time and after defrosting because sperm dilution after thawing can increase sperm velocities (França et al., 2022). The present study results show that the capsules maintained sperm kinetic parameters after the cryopreservation process, as well as straws of three crucial species of environmental and commercial fish from the Mediterranean.

The evaluation of cell viability by checking the membrane integrity and functionality is a widely employed method in research to assess the effectiveness of a sperm cryopreservation procedure (Cabrita et al., 2010). The plasma membrane consists of a dual layer of lipids with distinct hydrophobic and hydrophilic regions. This membrane shields and isolates the cell from the external environment while also regulates the movement of substances into and out of the cell, primarily through transmembrane proteins. Maintaining the integrity and functionality of the plasma

membrane is crucial for the survival of cells, including cryopreserved spermatozoa (Cabrita et al., 2022). As expected, we observed that cryopreservation of European eel, gilthead seabream, and European sea bass sperm induced a decrease of the number of viable spermatozoa. A similar decrease of membrane integrity was observed by (Herranz-Jusdado et al., 2019a) when they tested the cryopreservation of European eel sperm in 2 and 5 mL containers, and the use of different cryoprotectant solutions. In addition, decreases in sperm membrane integrity in samples from both gilthead seabream (Beirão et al., 2012) and European sea bass (Martínez-Páramo et al., 2012) has also been reported. However, when sperm samples from the three species cryopreserved in different containers were compared, we did not observe any difference between them in terms of post-thawing sperm membrane integrity. These results support the idea that the use of biodegradable capsules as containers could be successfully applied to preserve sperm membrane integrity, and it can be used in aquaculture and conservation projects of Mediterranean fish species.

The ultimate objective of spermatozoa is to transmit the male genetic information to the offspring, making imperative to prioritize the preservation of spermatozoa's genomic information during the design of a cryopreservation protocol (Cabrita et al., 2022). Over the years, several studies have reported DNA damage in post-thawing fish sperm evaluated by alkaline comet assay (Cabrita et al., 2011; Martínez-Páramo et al., 2013; Riesco et al., 2017b; Rodrigues et al., 2020, 2021). Our study is the first to report results about DNA fragmentation in European eel fresh and post-thaw sperm using comet assay analysis.

We did not observe an increase in sperm DNA damage in European eel and sea bass after freezing and thawing. In addition, we did not find any difference in the TailDNA and OTM between sperm samples of both species cryopreserved in plastic straws and biodegradable capsules. We also observed no difference in DNA damage parameters between gilthead seabream fresh and cryopreserved sperm in straws, coinciding with the reported by (Cabrita et al., 2005). On the other hand, our results showed greater DNA damage in post-thawing sperm cryopreserved in biodegradable capsules compared to fresh and cryopreserved sperm in straws. The

permeable cryoprotectant $Me_2SO$ is toxic to the cells, and the toxicity is accentuated by the increase in temperature during thawing and the time of exposure to the cryoprotectant solution post-thawing (Best, 2015). When we were fixing our protocol, we first thawed gilthead seabream sperm samples frozen in capsules at 60 °C. However, this resulted in the absence of sperm motility. Thus, we decided to reduce the thawing temperature to 40 °C, and observed that the sperm kinetic parameters were similar to those of sperm samples cryopreserved in straws. In this way, decreasing the thawing temperature of capsules could minimize DNA damage to spermatozoa cryopreserved in these containers. Anyhow, we observed a Tail DNA of 10 and 11% in sperm cryopreserved in gelatin and HPMC capsules, respectively. In our opinion, and considering previous studies (Cabrita et al., 2005; Herráez et al., 2017) these results indicate low sperm DNA damage and it is probable that the oocyte could repair this level of damage during early embryogenesis, without jeopardizing a right embryonic development and, consequently, a regular larvae production. Our results showed that the use of biodegradable capsules as containers for the cryopreservation of sperm from Mediterranean fishes of commercial interest preserves the sperm DNA integrity at a level than can be considered good enough to maintain normal embryonic development.

In this study, our data showed that it is possible to cryopreserve European eel, gilthead seabream, and European sea bass sperm using hard-gelatin and hard-HPMC capsules. The sperm quality parameters evaluated in the present study are widely used in fish reproduction studies (Cabrita et al., 2022; Ciereszko et al., 2020). In addition, they have a high correlation with reproductive success for several fish species (Beirão et al., 2019; Gallego et al., 2013a, 2017). Even though we did not evaluate the fertilization capacity of the cryopreserved sperm samples, we observed that they presented similar sperm quality to those stored in plastic straws. Thus, the cryopreservation methodology of the three Mediterranean species sperm in gelatin or HPMC capsules can be apparently used without losing sperm reproductive potential. Consequently, using capsules as a container for sperm cryopreservation from marine fish species of high commercial interest

can make larvae production more sustainable from an environmental and economic point of view.

## 5. Conclusion

We observed that biodegradable hard-gelatin and hard-HPMC capsules could maintain the sperm quality of European eel (*Anguilla anguilla*), gilthead seabream (*Sparus aurata*), and European sea bass (*Dicentrarchus labrax*) after cryopreservation. Thus, the capsules can be considered as an alternative container to plastic straws for storing sperm of marine fish species at ultra-low temperatures. Our study describes the methodology for using these containers in three species, which could be adapted to other ones. In addition, it paves the way for developing this research area intending to reduce the cost and the amount of plastic waste generated in the sperm cryopreservation process.

### Declaration of interest

TSF and DPSJ filed (September/2022) a patent (pending) application on the methodology described in the study. The remaining authors have no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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# CONCLUSIONS

Throughout the chapters presented in this thesis, the objective was to develop low-cost and eco-friendly methodologies to optimize the use of sperm and make the fish sperm cryopreservation process more environmentally sustainable. The findings of this thesis can facilitate and increase larvae production in captivity using post-thaw sperm using cryopreservation protocols that generate fewer pollutants in the environment. Among the most relevant achievements of this thesis, the following stand out:

i. Post-thaw dilution of South American silver catfish sperm was successful. With the technique used, post-thaw diluted samples showed a greater difference of 32% in curvilinear speed, 30% in sperm progression, 29% in fertilization rate, and 28% in hatching rate compared to undiluted samples.

ii. The cryopreservation protocols were developed for cryopreservation of sperm from four fish species using biodegradable gelatin capsules and biodegradable HPMC capsules as containers. A patent (BR 10 2022 017817) was filled for the developed methodology.

iii. We observed that biodegradable gelatin capsules and HPMC capsules were as efficient as plastic straws in storing sperm from four fish species during cryopreservation. Thus, the observed results prove that the capsules are an alternative to this commonly used container.

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