

Carlota Francisca Cerqueira Fernandes Martins Gonçalves

**Standardization of a protocol for purple sea urchin
(*Paracentrotus lividus*, Lamark 1816) sperm cryopreservation**



Universidade do Algarve

Faculdade de Ciências e Tecnologia

2022/2023

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(*Paracentrotus lividus*, Lamark 1816) sperm cryopreservation**

Master's degree thesis in Aquaculture and Fisheries

Supervisors:

Elsa Cabrita (CCMAR-FCT, University of Algarve)

Florbela Soares (IPMA, EPPO, Olhão)



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1816) sperm cryopreservation**

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Abstract

Aquaculture, as a result of growing seafood consumption and overexploitation of wild populations, has the potential to provide a sustainable alternative to meet market demand. This study developed reproductive strategies for the production and conservation of the purple sea urchin, *Paracentrotus lividus*, as this species is highly exploited for their rich content gonads, sea urchin “roe”. In that aim, three experiments were conducted to standardize a protocol for sea urchin sperm cryopreservation: the first experiment tested the optimal freezing rate using two different freezing rates, $-5^{\circ}\text{C}/\text{min}$ and $-10^{\circ}\text{C}/\text{min}$, and which storage equipment was more adequate to pack sea urchin sperm, 500 μL straws and 1.2 mL cryovials. Our results showed that while analyzing sperm motility parameters and cell survival, treatments with $-10^{\circ}\text{C}/\text{min}$ freezing rates and straws as storage equipment produced significantly higher results, thus they were chosen for the following experiments. As cryoprotectants are one of the most important aspects when selecting a cryopreservation protocol, the second experiment tested three permeable cryoprotectants, DMSO, ethylene glycol, and methanol at different concentrations (5%, 10%, and 20%), and used sperm motility parameters, cell viability, and DNA fragmentation as tools to evaluate post-thaw sperm quality. We discovered through these tests that 20% DMSO had greater results than all the other treatments. Finally, the third experiment involved examining quality tests such as sperm motility, cell viability, DNA fragmentation, and fertilization rates after the addition of a non-permeable cryoprotectant (trehalose or sucrose). The results were clear and adding a nonpermeable cryoprotectant to 20% DMSO had no influence. Further research should be conducted to explore higher cooling rates, more sperm quality testing, and alternative combinations of permeable and nonpermeable cryoprotectants.

Keywords: *Paracentrotus lividus*, conservation, sperm, cryopreservation, cryoprotectants, quality assays

Resumo

À medida que a população mundial cresce e o consumo de alimentos e produtos aquáticos aumenta, há uma necessidade de encontrar alternativas sustentáveis para satisfazer a crescente oferta. O *Paracentrotus lividus* é uma espécie de ouriço-do-mar distribuído essencialmente no Mar Mediterrâneo e no leste do Oceano Atlântico, e é uma espécie fundamental dos ecossistemas marinhos bentônicos, especialmente no Mediterrâneo, onde ajuda a manter a estabilidade ecológica. Como muitos outros seres marinhos, os ouriços-do-mar enfrentam ameaças devido a mudanças ambientais, como a alteração do meio físico e químico, competição interespecífica por recursos, poluição, destruição de habitat, e atividades humanas, como pesca excessiva para consumo humano, ou para descoberta de novos medicamentos. Um dos impactos mais preocupantes é a exploração comercial de suas gônadas (ovas de ouriço-do-mar), consideradas uma iguaria na América do Norte e do Sul, na Ásia e na Europa, especialmente no Norte. Neste contexto, a aquacultura tornou-se um excelente aliado em situações críticas onde determinadas espécies, como os ouriços-do-mar, são alvo.

Consequentemente, este estudo desenvolveu estratégias reprodutivas *ex situ* para produção e preservação de *P. lividus*, padronizando um protocolo de criopreservação de esperma. No entanto este tipo de procedimentos por norma são bastante interespecíficos podendo causar danos celulares se não for eficiente, e como tal é necessário implementar testes de qualidade. Para alcançar um protocolo adequado, foi necessário realizar três experiências com diferentes objetivos. A primeira experiência, testou duas taxas de congelação diferentes, $-5^{\circ}\text{C}/\text{min}$ e $-10^{\circ}\text{C}/\text{min}$, e ainda avaliou dois tipos de armazenamento, palhinhas de 0,50 ml e crioviais de 1.2 ml. Os resultados obtidos foram avaliados através de testes de mobilidade dos espermatozoides e viabilidade celular, permitindo mostrar diferenças significativas ($P\text{-value} = 0.008$) nos tratamentos com taxa de $-10^{\circ}\text{C}/\text{min}$ criopreservados em palhinhas para a mobilidade progressiva bem como para a velocidade em linha reta ($P\text{-value} = 0.008$). Para a velocidade circular ($P\text{-value} = 0.009$) as diferenças significativas foram direcionadas para os tratamentos de DMSO a 10% para as duas taxas de congelação em palhinhas e ainda no coeficiente de linearidade também de observaram diferenças ($P\text{-value} = 0.030$) para os tratamentos armazenados em palhinhas com concentração de DMSO 5% e o DMSO 10% congelados na taxa $-10^{\circ}\text{C}/\text{min}$ e $-5^{\circ}\text{C}/\text{min}$ respetivamente. Contudo na mobilidade total ($P\text{-value} = 0.353$) e viabilidade celular ($P\text{-value} = 0.407$) não foram encontradas diferenças estatísticas, todavia podemos afirmar que as maiores percentagens atingidas foram nos tratamentos de palhinhas congelados na taxa de $-10^{\circ}\text{C}/\text{min}$ ($31.79 \pm 14.21\%$ e $29.93 \pm 4.56\%$). No geral as diferenças foram encontradas essencialmente nos tratamentos em que era utilizada a taxa de congelamento de $-10^{\circ}\text{C}/\text{min}$ e as palhinhas como forma de armazenamento, tendo então sido posteriormente escolhidas como condições para a seguinte experiência.

Para a segunda experiência foi analisado a influencia de três crioprotetores permeáveis, DMSO, etileno de glicol, e metanol, a três concentrações diferentes, 5, 10, e 20%, sendo que os seus efeitos foram avaliados por três testes de qualidade diferentes, mobilidade dos espermatozoides, viabilidade celular, e percentagem de ADN fragmentado. A escolha de um crioprotetor e concentração ideal são parâmetros essenciais no desenvolvimento de protocolos, já que este tem a capacidade de penetrar na célula e agir como mecanismo de defesa contra os danos causados pelo congelamento, que outrora poderiam ser letais para a esta. Os resultados mostraram que o DMSO 20% e o DMSO 10% obtiveram diferenças significativas na mobilidade total tendo $1.58 \pm 0.21\%$ e $1.29 \pm 0.25\%$ de

espermatozoides móveis respetivamente (P -value = 0.002), por outro lado para a mobilidade progressiva não se observaram diferenças entre tratamentos (P -value = 0.074). Quando analisadas as velocidades, mais uma vez o DMSO 20% e o DMSO 10% obtiveram diferenças em relação aos outros tratamentos na velocidade em linha reta, contudo para a velocidade curvilínea não apresentaram diferenças (P -value = 0.030) entre crioprotetores e suas respetivas concentrações, assim como o coeficiente de linearidade também não apresentou (P -value = 0.471). Relativamente á viabilidade celular, o DMSO 20% e o DMSO 10% obtiveram diferenças significativas (P -value = 0.001), com o DMSO 20% a destacar-se com $53.81 \pm 9.04\%$ células viáveis, contudo na fragmentação de DNA não foram encontradas diferenças (P -value = 0.289). Dado as evidências, os tratamentos DMSO 20% e DMSO 10% foram os tratamentos que demonstraram ser mais eficazes na proteção celular, no entanto o DMSO 20% foi escolhido como crioprotetor permeável para a otimização deste protocolo. Segundo vários autores a suplementação com crioprotetores não permeáveis é benéfica para muitos seres vivos, e no caso dos invertebrados marinhos os açúcares em concreto são os mais utilizados, especialmente a trealose e a sacarose. Dada esta informação a terceira experiência tem como objetivo testar os efeitos da suplementação da trealose e a sacarose, com duas concentrações, 5% e 15%. Para tal, avaliou-se a mobilidade dos espermatozoides, a viabilidade celular, a percentagem de ADN fragmentado, e ainda a percentagem de ovos fertilizados. Através dos testes de qualidade, observaram-se diferenças significativas na mobilidade total (P -value = 0.006), sendo o DMSO 20% ($1.22 \pm 0\%$), o melhor tratamento, contudo os restantes parâmetros de mobilidade, de viabilidade, de fragmentação de ADN e de taxas de fertilização não apresentaram diferenças estatísticas. Ainda assim DMSO 20% suplementado com 5% de Sacarose apresentaram maiores percentagem de células viáveis ($70.39 \pm 11.37\%$), e maior taxa de fertilização ($37.77 \pm 9.17\%$).

De forma sucinta, é necessário realizar mais investigações que permitam abordar as falhas deste estudo e executar em conjunto outros testes de qualidade. Estudos complementares deverão utilizar outras taxas de congelação já que diferentes autores utilizam taxas mais altas, realizar testes de toxicidade e stress oxidativo para a infabilidade dos resultados e utilizar mais pools para mitigar os erros. É essencial também testar outros crioprotetores permeáveis e não permeáveis e diferentes concentrações.

Tendo em consideração os resultados aqui obtidos, concluímos que o DMSO é de facto o melhor crioprotetor, no entanto testes de toxicidade deverão ser realizados em estudos futuros para entender os seus efeitos nas taxas de fertilização. Para além disso a suplementação com açúcares mostrou ser irrelevante, criando a oportunidade de testar outros crioprotetores não permeáveis. É também importante realçar que o protocolo de quantificação de fragmentação de DNA obteve incoerências e como tal futuros estudos devem insistir no melhoramento do protocolo.

Palavras-chave: *Paracentrotus lividus*, conservação, esperma, criopreservação, crioprotetores, testes de qualidade

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Abbreviations

ASW- artificial sea water

BSA- bovine serum albumin

CASA- computer assisted sperm analysis

CPA- cryoprotectant agent

DMSO- dimethyl sulphoxide

DNA- deoxyribonucleic acid

DNA_t - tail DNA

EG- ethylene glycol

FAO- food and agriculture organization

F₁- first generation

Kg- kilogram

KCl- potassium chloride

LIN- linearity coefficient

L- liter

MetOH- methanol

mA- milliampere

mL- milliliters

mM- millimolar

Min- minute

M- molar

NaCl- sodium Chloride

Na₂-EDTA- ethylenediaminetetracetic acid disodium

n- number

PBS- phosphate buffered solution

PI- propidium iodide

PM- progressive motility

ROS- reactive oxygen species

SCD- sperm chromatin dispersion

SCSA- sperm chromatin structure assay

SE - standard error

TM- total motility

Tris-HCl- tris(hydroxymethyl)aminomethane hydrochloride

TUNEL- terminal deoxynucleotidyl transferase-mediated dUTP-biotin end-labelling

V- volt

VCL- curvilinear velocity

VSL- straight-line velocity

°C- degrees Celsius

μL- microliter

1. Introduction

1.1 The role of Aquaculture in the production of aquatic foods

Aquatic food products have been an essential component of the human diet since mankind began fishing in the waters and producing food through aquaculture (Tacon and Metian, 2013). This animal source food is nutrient dense and high in bioavailable micronutrients such as vitamins D and B, minerals (calcium, phosphorus, iodine, zinc, iron, and selenium), and long-chain n-3 polyunsaturated fatty acids, making aquatic food consumption crucial for prenatal brain development and adult cardiovascular health (Garlock et al., 2022; Hibbeln et al., 2019; Mohan et al., 2021; Rimm et al., 2018). As the world's population grows and food consumption rises, aquaculture appears to be a possible viable option for meeting the rising supply need while addressing nutritional deficiencies (Golden et al. 2021; Filipski and Belton, 2018; Smith et al. 2010). Global apparent consumption of aquatic foods increased at a 3% annual rate from 1961 to 2019, roughly twice the rate of annual global population growth (1.6%) (Fig. 1.1), while aquatic food consumption per capita increased from 9.0 kg in 1961 to a record high of 20.5 kg in 2019, before slightly declining to 20.2 kg in 2020. Rising salaries, as well as urbanization, advancements in post-harvest methods, and dietary patterns, are all expected to increase aquatic food consumption, resulting in an estimated 21.4 kg per capita in 2030 (FAO, 2022).

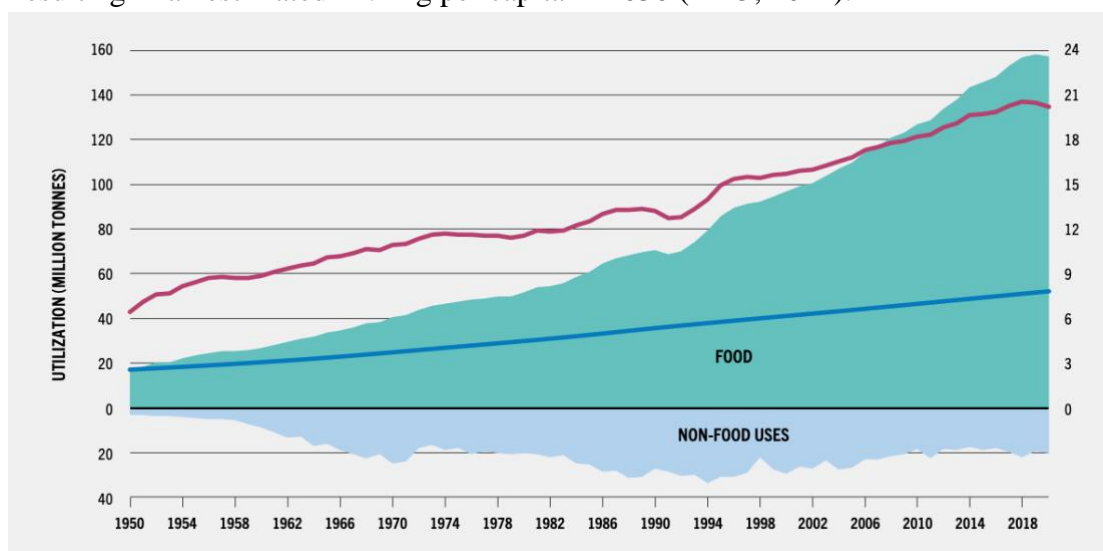


Figure 1.1- World fisheries and aquaculture production: utilization and apparent consumption (Data excludes aquatic mammals, crocodiles, alligators, caimans, and algae. Data is expressed in live weight equivalent. Source: FAO, 2022)

In 2020, aquaculture contributed 49% of total aquatic food production (178 million tonnes), with 157 million tonnes (89%) for human consumption only (Fig. 1.1). Of that total, marine waters produced 63% (112 million tonnes), with aquaculture accounting for 30%, while inland areas contributed 37% (66 million tonnes), with aquaculture providing for 83% (Fig.1.2) (FAO, 2022).

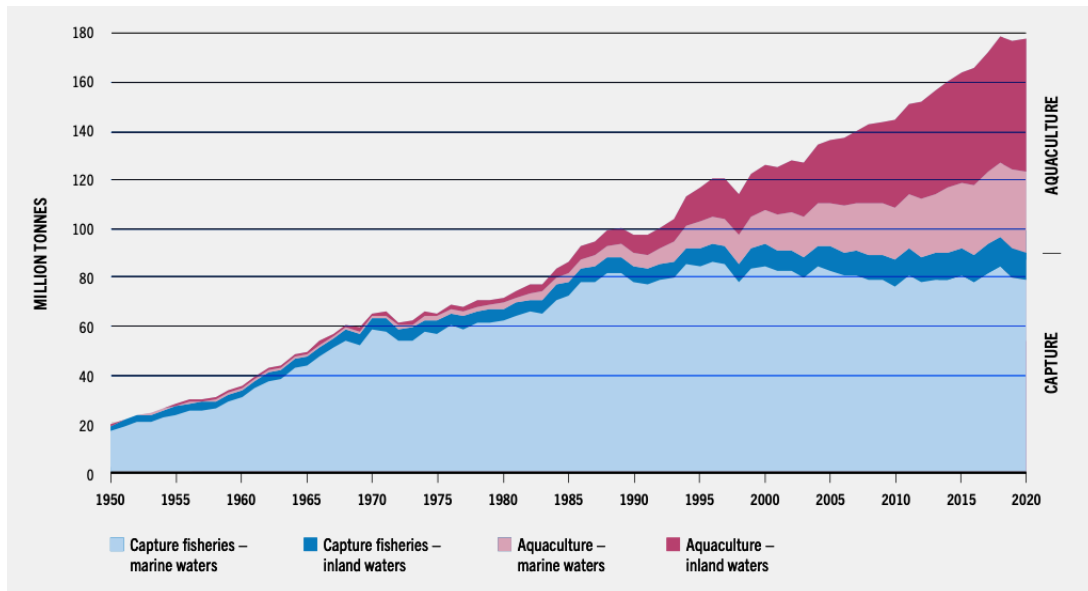


Figure 1.2- World capture fisheries and aquaculture production (Data excludes aquatic mammals, crocodiles, alligators, caimans, and algae. Data is expressed in live weight equivalent. Source: FAO, 2022)

Aquatic foods supply at least 20% of the average per capita animal protein intake for 3,3 billion people, particularly in low-income countries in South Africa, and some small island developing States, where aquatic foods contribute half or more of total animal protein intake, averting severe declines in food and nutrition security (Béné et al., 2016; Belton et al., 2018; FAO, 2022). Driven by increased seafood consumption and overexploitation of wild populations, aquaculture is a potential answer for providing a sustainable alternative to meet market demand. (Castilla-Gavilán et al., 2018; Grosso et al., 2022). Sea urchins are a great example of how aquaculture is becoming a critical answer for certain species, for a variety of reasons, such as the pressure to secure high-quality roe or to cope with the environmental challenge of sea urchin barrens, or in light of global food industry changes or drug discovery pressure. Although there are still very few companies producing some sea urchin species, a lot

of open innovation and collaborative work is being done to create profitable and sustainable sea urchin aquaculture production (Rubilar and Cardozo, 2021).

1.2 Sea urchin harvesting, production, and consumption

The sea urchin *Paracentrotus lividus* (Lamarck, 1816), commonly referred to as the Purple Sea Urchin, is a marine echinoderm species belonging to the phylum Echinodermata. These members of the Echinoidea class are mostly found in the Mediterranean Sea and the eastern Atlantic Ocean, ranging from Portugal to Norway, south to Morocco, the Canary Islands, the Azores, and Madeira (Boudouresque and Verlaque, 2007, 2013; Domínguez et al., 2015; Furesi et al., 2016). They prefer shallow rocky waters with depths ranging from a few meters to roughly 30 meters, though they have been observed in deeper areas (Boudouresque and Verlaque, 2020; Casal et al., 2020). The *Paracentrotus lividus*, like many other sea urchins, is a keystone species of benthic marine ecosystems especially in the Mediterranean (Hereu et al., 2005; Prado et al., 2012), that helps maintain ecological stability. They are herbivores that predominantly feed on algae, kelp, and other small marine plant material, and their grazing activity helps regulate the density and composition of macrophyte populations. They are also the primary prey for various fish species, starfish, and mollusks (Boudouresque and Verlaque, 2020).

However, like many marine species, they face threats due to environmental changes like alteration of the physical environment, inter-specific competition for resources, and human activities such as overfishing, pollution, and habitat destruction (Machado et al., 2019; Rilov, 2016; Yeruham et al., 2015). One of the most concerning implications has been the commercial exploration of their gonads (sea urchin roe), which are regarded as a delicacy in North and South America, Asia, and Europe, particularly in the northern Mediterranean countries (Bertocci et al., 2014; Boudouresque and Verlaque, 2007; Stefánsson et al., 2017), due to their rich content gonads with bright yellow or orange coloration (Bertocci et al., 2014; Boudouresque and Verlaque, 2020; Mol et al., 2020; Zupo et al., 2019). According to the most recent official statistics, Chile is the world's largest exporter of sea urchins, exporting 2103,12 tonnes in 2019, 2305,01 tonnes in 2020, and 1614,31 tonnes in 2021, followed by Canada, the United States of America, and Spain (FAO, 2023a). Japan

imports the most sea urchin products, and like practically every other country, it is on a downward trend, with 11339,14 tonnes imported in 2019, 11275,28 tonnes in 2020, and 10708,12 tonnes in 2021. Saudi Arabia is estimated to be the second largest importer, followed by the United States of America and Italy (FAO, 2023a). The global capture production of *Paracentrotus lividus* increased from 149 to 958 tonnes between 2018 and 2023, with the only data available in Portugal ranging from 45 to 126 tonnes between 2016 and 2018 (FAO, 2023b). Overharvesting for this species has led to early indications of local sea urchin populations disruption (Carreras et al., 2020; Farina et al., 2020; Hur et al., 2002; Pais et al., 2007; Parrondo et al., 2022) and can possibly raise serious concerns about the conservation of littoral populations, therefore conservation efforts and sustainable harvesting practices are crucial to maintaining the ecological balance of marine ecosystems where these sea urchins reside.

1.3 Conservation of endangered species

There are two basic conservation strategies that can be used to preserve genetic diversity, both of which aim to protect all existing gene diversity while also protecting species integrity, adaptability, disease resistance, and reproductive fitness (Comizzoli, 2017). *In situ* conservation and *ex situ* conservation (Floris et al., 2020; Hanson and Ellis, 2020).

In situ conservation refers to the preservation of ecosystems and natural habitats, as well as the maintenance and recovery of viable populations of species in their natural environments and, in the case of domesticated or cultivated species, in the environments where they have developed their distinctive features (Nawaz et al., 2020; Singh et al., 2018; Visconti, 2019; Irfan and Alatawi, 2019). *Ex situ* conservation is the preservation of biological diversity components outside of their natural habitats (Maestro et al., 2019; Hanson and Ellis, 2020), such as gene banks, zoological infrastructures, or samples of sperm, ovules, embryos, tissue explants, or DNA kept under special artificial conditions (Maxted, 2013). Biobanks have long been recognized as a valuable resource for research and commercial development, particularly in the human health sector, accompanied by the livestock, crop production, and health sectors (Groeneveld et al., 2016; Mascher et al., 2019). However, in the last decade, long-term biobanking has expanded beyond long-

established biobanks in museums and botanical gardens. The growing awareness of the biodiversity crisis and the increasing risk of extinction of wild and captive populations has led to the creation of new biobanking initiatives aimed at contributing to wildlife conservation. (Breithoff and Harrison, 2020; Comizzoli, 2017). As demonstrated by Bell et al., (2008), using aquaculture technologies to produce and release juveniles reared in hatcheries can be a useful tool for restocking commercial species that are locally extinct or threatened due to overexploitation from fishing pressure, such as *Paracentrotus lividus*.

Other initiatives, such as selective breeding, can help to ensure genetic variability, and cryopreservation can be an excellent way to get a significant volume of genetically diverse material from biobanks. Biobanks, on the other hand, can be used to broaden and deepen knowledge by making marine biological resources available to scientists for research (Paredes, 2016, 2019, 2022).

1.4 Cryopreservation

Gamete preservation and cryopreservation are two common practices in artificial reproduction and gene bank management (Contreras et al., 2019). Among many different applications, gamete preservation in aquatic animals is a significant breakthrough for artificial reproduction since it allows fertilization to be synchronized based on gamete accessibility (Mylonas et al., 2017), it facilitates transportation if it needs to be moved between different locations, improves gamete economy by allowing the use of total volume, which is especially important when sperm volume is limited (Cabrita et al., 2010; Robles et al., 2009), and it also enables the cryobanking of genetic resources from an growing number of endangered species (Asturiano et al., 2017; Martínez-Páramo et al., 2017).

Based on storage conditions, gamete can be preserved in two ways: refrigerated storage, also known as short-term storage, which ranges from -1°C to -35 °C or long-term storage, also known as cryopreservation, which extends from -80°C to -196 °C (Cabrita et al., 2010; Ma et al., 2021). Long-term storage causes cell damage in several aspects, including damage to the plasma membrane, organelles, and chromatin, the mechanical forces caused by ice crystal formation, both inside and outside the cells, and osmotic stress (Murray and Gibson, 2022). The viability of the

cryopreservation protocol will determine the conservation of each cell structure as well as its functionality (Robles et al., 2009), as many factors influence cryopreservation success, including interactions between cryoprotectant, type of extender used, cooling rate, thawing rate, and storage type, as well as individual animal variation (Whaley et al., 2021; Yang and Tiersch, 2020; Martínez-Páramo et al., 2017).

1.4.1 Cryoprotectants

When water constitutes more than 50% of tissue mass, freezing water, both intra and extracellular, exerts a strong influence on the harmful biochemical and structural changes that occur in unprotected freezing damage. As a result, when cells are exposed to temperatures below zero, they end up dying (Wolkers and Oldenhof, 2021) making it necessary to protect intracellular structures and biomolecules with cryoprotectants that can pass through the cellular membrane (Sieme et al., 2016). Cryoprotectants (CPAs) can divert water from the intracellular to the extracellular environment, lowering the freezing point of the solution and, as a result, limiting water crystallizations and therefore cellular damage after thawing/warming (Bhattacharya, 2018).

CPAs are classified into two types based on their capacity to cross the cell membrane: permeable cryoprotectants, which are low molecular weight chemicals and can penetrate the cell membrane (dimethyl sulfoxide (DMSO), glycerol, propylene glycol, and ethylene glycol), and non-permeating cryoprotectants that have a high molecular weight and therefore cannot enter cells (2-methyl-2,4-pentanediol, polymers such as polyvinyl pyrrolidone, hydroxyethyl starch, and sugars like glucose, fructose, sorbitol, mannitol, sucrose, trehalose, and raffinose) (Bhattacharya, 2018; Fahy and Wowk, 2015; Tsai et al., 2018; Whaley et al., 2021).

When selecting cryoprotectant agents, it is important to consider whether they are biologically acceptable, capable of penetrating cells, and have low toxicity (Pegg, 2007), and in order to achieve the best cell and tissue survival rate, the sample volume, cooling rate, warming rate, and CPA concentrations should be optimized based on the different cells and type of tissues (Yavin and Arav, 2007). The effect of cryoprotectants will also depend on the type of biological material and on the freezing-thawing rates applied during cryopreservation and material recovery.

1.4.2 Freezing and Thawing rates

1.4.2.1 Freezing Rate

Ice first forms in the extracellular spaces of tissues at normal cooling rates, and it excludes solutes, indicating that the extracellular solute concentration increases as water is removed from the extracellular fluid to form ice crystals. The resulting osmotic gradient across cell membranes works by osmosis to drive water out of cells, dehydrating cells and increasing intracellular solute concentration, this increased solute concentration lowers the intracellular freezing point, preventing the formation of intracellular ice (Bojic et al., 2021). This process can harm tissues in two ways, firstly, solutes like salts, which are normally kept at safe concentrations, become more concentrated to cause osmotic stress (Lovelock, 1953), and secondly, extracellular ice directly harms tissues by puncturing or crushing cells membranes or disrupting extracellular structures (Wowk, 2007; Takamatsu and Zawlodzka, 2006). Intracellular ice formation can occur when a sample is cooled too quickly for water to exit a cell. Intracellular ice crystals are far more dangerous than their extracellular counterparts because they can disrupt the cell's internal structures, such as lysosomes (Persidsky, 1971), which can be fatal (Wesley-Smith et al., 2015; Yu et al., 2017). The size of the ice crystals, their intracellular location, their mechanism of growth, their shape, and the rate of warming are all factors that affect lethality (Fuller et al., 2004; Seki and Mazur, 2008; Grout et al., 1990).

1.4.2.2 Thawing Rate

During warming, ice crystals can form around an initial nucleus. The process of ice formation is most rapid just below the freezing point and slows as the temperature drops, being that the peak of nucleation occurs well below the freezing point. In other words, when the temperature for crystallization is no longer optimal, the cooled sample first passes through an ice growth zone (with few nuclei available to seed the growth), then enters the nucleation zone. As a result, rewarming the sample increases the risk of recrystallization and devitrification once it reaches freezing temperatures (Bojic et al., 2021).

1.5 Techniques and Parameters to assess sperm quality

Aquatic animal's reproductive success is determined by a variety of factors, in both natural and artificial spawning, sperm quantity and quality may influence fertilization capacity and reproductive success in males, adding to that, cryopreservation can be a useful tool, but it can also cause damage, as previously stated. Whereas an extensive quality analysis of samples is required to ensure the benefits of cryopreservation (Cabrita et al., 2014).

1.5.1 Sperm motility

There are numerous consequences of oxidative damage during sperm cryopreservation. Aside from sperm membrane damage, oxidative stress disrupts mitochondrial activity, promotes intracellular enzyme efflux, and impairs several axonemal proteins, resulting in sperm motility loss (Peris-Frau et al., 2020), making it critical to assess post-thawed semen quality to achieve the best protocol efficiency results. Sperm motility is therefore regarded as the best sperm quality biomarker for aquatic animals (Beirão et al., 2019; Mylonas et al., 2017). In fact, some aquatic species have shown strong correlations between sperm motility, fertilization, and hatching rates (Gallego et al., 2018; Esposito et al., 2020).

The proportion of motile sperm has been routinely assessed using a bright field microscope for visual estimation, but manual assessment has proven to be slow, approximate, and basic (only percentage of total and progressive motility), furthermore, it is highly sensitive to subjective errors and requires technicians to be trained in order to increase the consistency of the estimation (Broekhuijse et al., 2011). Since the early 1980s (Amann and Katz, 2004), quantitative computer-assisted methods (CASA) (Rurangwa et al., 2004) have made significant advances in sperm evaluation by analyzing more than 500 sperm cells per sample and tracking the movement pattern of each sperm cell (Amann and Waberski, 2014). They permit analysis of parameters that are proven to be important for a successful fertilization such as total motility (TM %), progressive motility (PM%), curvilinear velocity (VCL ($\mu\text{m/s}$)), defined as the time/average velocity of a sperm head along its actual curvilinear trajectory, linear velocity (VSL ($\mu\text{m/s}$)) defined as the time/average velocity of a sperm head along the straight line between its first detected position and

its last position, and linearity coefficient (LIN %) defined as the linearity of the curvilinear trajectory. Other parameters like average path velocity, wobble and straightness coefficient, lateral head displacement, and beat cross frequency have been also described by other authors (Alqu  zar-Baeta et al., 2019; Gallego et al., 2013).

1.5.2 Sperm viability

The true viability of spermatozoa is defined by their ability to move and fertilize an egg, but fish spermatozoa viability kits have also been used, being their purpose investigate the viability or integrity of their membranes. These tests are based on dual-staining protocols with fluorescent dyes, flow cytometry protocols, and confocal microscope observations, with the most commonly used stains being a membrane-permeate nucleic acid stain (SYBR 14 dye) and a conventional dead cell stain (propidium iodide) or rhodamine 123 (Segovia et al., 2000; Grzyb et al., 2003). When these two stains are applied to the milt, live sperm cells with intact membranes fluoresce green, while cells with damaged cell membranes fluoresce red. SYBR can pass through the sperm head's cell membrane and stain the nucleic acids of viable cells, whereas propidium cannot pass through the membrane of living cells but can penetrate and stain the nuclear DNA of degenerated or dead sperm, and Rhodamine 123 can stain viable cells' functional mitochondria (Segovia et al., 2000). Sperm can also be stained with trypan blue and counted under a microscope to determine the percentage of spermatozoa that are intact (viable, unstained) and damaged (dead, red-stained) (Rurangwa et al., 2002).

Dual DNA staining with SYBR-14 and propidium iodide (PI) is one of the most commonly used stains for assessing sperm viability, particularly in marine animals (Gallo et al., 2018). This technique has been used successfully to assess sperm viability in fishes (Bouwmeester et al., 2022; Diogo et al., 2018; Herranz-Jusdado et al., 2019; Sandoval-Vargas et al., 2021), invertebrates such as oysters (Riesco et al., 2017; Boulais et al., 2017), some ascidians (Gallo et al., 2019), abalone (Gwo and Lei, 2022), mussel (Galo et al., 2020), and sea urchins (Gallo et al., 2018; Rodrigues, 2021).

1.5.3 DNA Fragmentation

Even though antioxidants protect biological systems (Kruk et al., 2019; Pisoschi et al., 2021), the amount of antioxidants in fish sperm and spermatozoa during cryopreservation is insufficient, due to sperm dilution in the cryopreservation extender (Cabrita et al., 2011; Martínez-Páramo et al., 2012). The high content of unsaturated fatty acids in sperm cells, as well as the low antioxidant capacity of diluted sperm, are critical aspects in sperm cell susceptibility to ROS attacks, being oxidative stress one of the primary causes of fish spermatozoa integrity and functionality impairment during cryopreservation (Sandoval-Vargas et al., 2021).

There are several methods for determining sperm DNA damage, including Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling or TUNEL assay in which single- and double-stranded DNA breaks are both labeled with fluorescein-thiocyanate (FITC)-dUTP in a single-step staining method before the sample undergoes fluorescence microscopic or flow cytometric analysis; the sperm chromatin dispersion or SCD method that assesses the dispersion of DNA fragments after denaturation with optical and fluorescence microscopy, SCSA assays that measure the susceptibility of sperm DNA to denaturation using flow cytometry (Dutta et al., 2020), as well as determination of 8-hydroxy-2-deoxyguanosine (8-OHdG) where the presence of 8 OHdG can be quantified by flow cytometry using assays incorporating labeled avidin, which binds to 8OHdG with great affinity (Aitken et al., 2012). Nevertheless, the comet assay is the most used method for determining the percentage of fragmented chromatin in aquatic animals such as fish sperm (Cabrita et al., 2005; Martinez-Páramo et al., 2009; Shaliutina et al., 2013; Žegura and Filipič, 2019; Zilli et al., 2003), invertebrates (Gajski et al., 2021) like mollusks such as clams (Châtel et al., 2017), abalone (Vosloo and Vosloo, 2017), oysters (Anjos et al., 2021; Akcha et al., 2012; Barranger et al., 2014; Riesco et al., 2019), and echinoderms like sea urchins (Pruski et al., 2009; Oliviero et al., 2019). The comet assay, also known as single-cell gel electrophoresis, employs fundamental principles of permeability and electrophoretic movement of fragmented DNA principles. The test is named after the distinctive “comet” appearance caused by stained unwound DNA fragments that form a tail after electrophoretic movement in the agarose gel, while unfragmented DNA remains in the comet's head. As a result,

DNA damage is quantified by measuring the distance between the nuclear genetic material (comet head) and the migrated unwound DNA tail (Simon et al., 2016).

2. Material and Methods

2.1 Broodstock conditioning

The *Paracentrotus lividus* broodstock first generation (F1) sea urchins used in this study were maintained at IPMA facilities in Olhão (EPPO), in a 5500 L tank inside baskets. The tanks had an open water system with a flow rate of 6L/min, with 36‰ salinity, and a temperature range of 21-22°C, during the months that the sampling occurred. The individuals were subjected to a natural photoperiod (14L:10D during the breeding season) and were fed with sea lettuce (*Ulva lactuca*).

For all trials, it was used 100 sea urchin males and 10 females.



Figure 2.1 -Baskets inside of the 5500 L fiberglass tank containing F1 sea urchin (*Paracentrotus lividus*) generation at EPPO, Estação Piloto de Piscicultura de Olhão (Instituto Português do Mar e da Atmosfera in Olhão, Portugal)

2.2 Gamete collection

For each trial, sea urchins were injected into the coelomic cavity with 1mL of 0.5M of potassium chloride (KCl) (Figure 2 (b)) and placed upside down on top of a beaker to collect gametes using the dry method (Paredes *et al.*, 2019). After collecting the gametes, they were placed on a styrofoam rack within a styrofoam box with ice and stored in 5mL Eppendorf tubes, with the exception of female gametes, which were given artificial seawater (ASW) to keep them hydrated. Any sample that appeared contaminated (feces, an unusual color, or an excessive amount of liquid) was discarded.

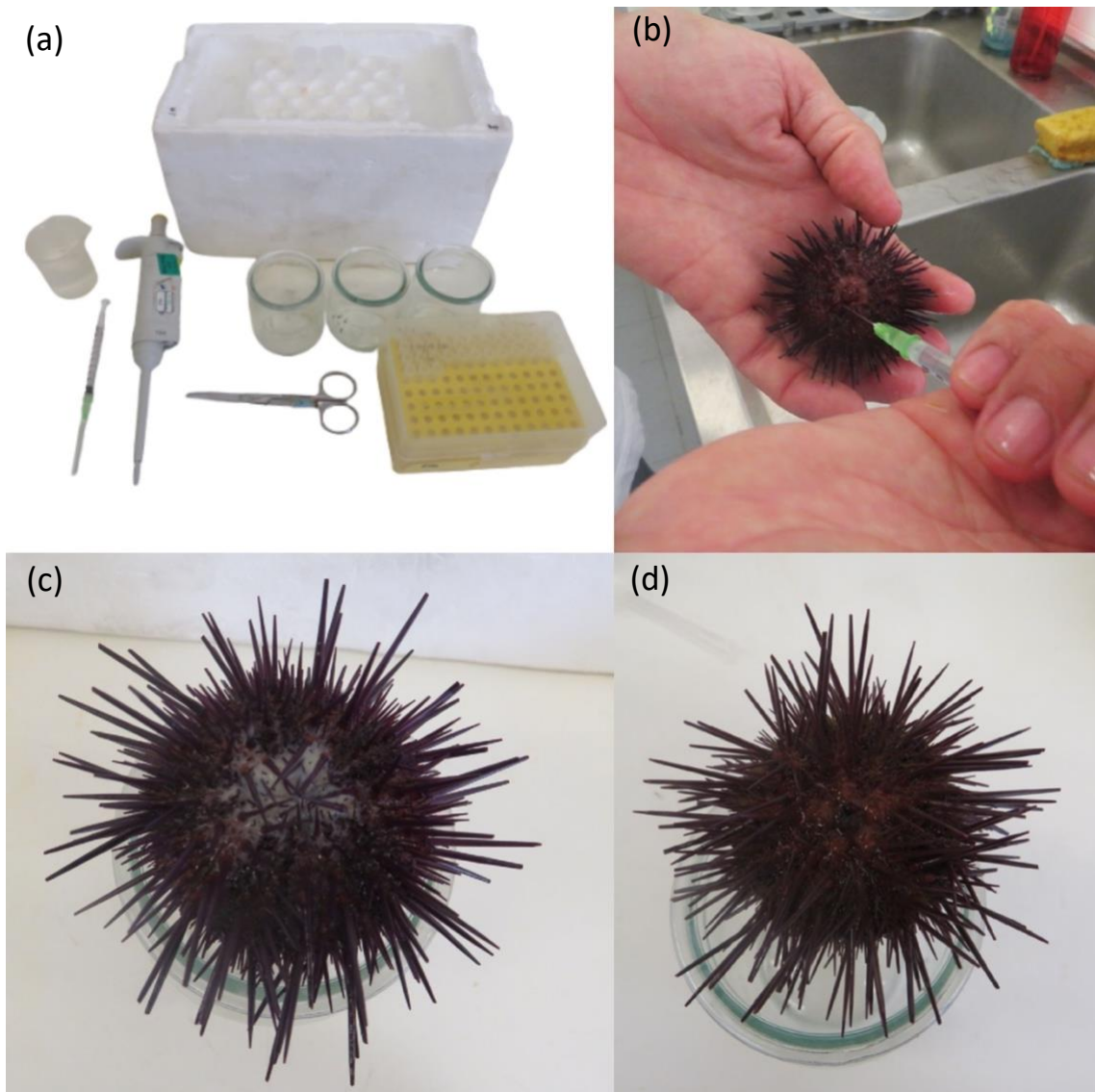


Figure 2.2 - Induction spawning technique. (a) Material used to perform artificial spawning; (b) Injection of potassium chloride in *Paracentrotus lividus* individual; (c) Male releasing gametes; (d) Female releasing gametes.

2.3 Experiment 1: Evaluating which storage equipment is the most efficient, and what is the most effective freezing rate

The sperm of 30 individuals were collected as previously described to form pools (n=3) in order to determine which storage equipment and freezing rate are the most efficient.

The extender solution was composed of an ASW medium with two distinct permeable cryoprotectants, DMSO (Dimethyl sulfoxide) and EG (Ethylene Glycol), at final concentrations of 5% and 10%. After diluting the sperm in the extender at a rate of 1:10 (v/v), the mixtures were loaded into two different types of containers, 0.50 mL straws and 1.2 mL cryovials, and cooled in a programmable biofreezer (Asymptote Grant EF600, UK) at a fast and slow controlled rate (5 °C/min and 10 °C/min) before being plunged directly into liquid nitrogen and kept in a cryogenic tank. Straws were thawed in a 30°C water bath for 15 seconds and cryovials in a 38°C water bath for 5 minutes before sperm quality was determined using two distinct methods: sperm motility and membrane integrity.

2.4 Experiment 2: Testing different permeable cryoprotectants at different concentrations

For the second experiment, the sperm of 40 individuals were collected and divided into pools (n=4) in order to examine the competency of different permeable cryoprotectants and determine the optimal concentration for those. Following sperm collection, the samples were processed similarly to the first experiment, but with three different permeable cryoprotectants, DMSO, EG, and MetOH (Methanol), at final concentrations of 5%, 10%, and 20%. The mixtures were then dispensed into 0.50 mL straws after dilution with the extender and cooled at a controlled rate of 10 °C/min in a programmed biofreezer (Asymptote Grant EF600, UK). Sperm quality was determined by measuring sperm motility, membrane integrity, and DNA fragmentation.

2.5 Experiment 3: Testing the benefit of adding a nonpermeable cryoprotectant and the optimal concentration

The sperm of 30 individuals was collected as previously described in order to create three pools (n=3). Following sperm collection, the samples were diluted in the extender at a rate of 1:10 with DMSO at a final concentration of 20%, and trehalose and sucrose at final concentrations of 5% and 15%. After diluting the sperm in the extender, the solutions went through the same procedure as in experiments one and two: they were loaded into 0.50 mL straws and cooled at a controlled rate of 10 °C/min in a programmed biofreezer (Asymptote Grant EF600, UK). Sperm quality has been determined by measuring sperm motility, membrane integrity, DNA fragmentation, and fertilization rates.

2.6 Evaluation of sperm quality

2.6.1 Sperm motility

Spermatozoa motility was assessed on both fresh and frozen samples. Fresh samples were diluted/activated at 1:100 with artificial seawater with 0.05% (final concentration) of BSA as described by Fabbrocini and D'Adamo, and cryopreserved samples were diluted at 1:10. After dilution, 20 µL sperm solution were placed in the Mackler chamber. A computer-assisted sperm analysis (CASA) system (ISAS Integrated System for Semen Analysis, Proiser, Valencia, Spain) was utilized in conjunction with a phase contrast microscope (Nikon E-200, Nikon, Tokyo, Japan) equipped with an x10 negative phase contrast objective. The images were taken with a Basler A312f camera (Basler AfC, Germany) and analyzed using CASA software to determine total motility (TM%), progressive motility (PM%), curvilinear velocity (VCL (m/s)), straight-line velocity (VSL (m/s)), and linearity (LIN%). Except for the fresh samples, motility parameters were only measured 5 minutes after activation.

2.6.2 Membrane integrity

The viability of spermatozoa was performed as described by Rodrigues, (2021) and was determined both in fresh and post-thawed samples. Aside from the post-thawed samples, the sperm samples were diluted in PBS to a ratio of 1:100 (1 μ L of sperm to 99 μ L PBS). For 100 μ L of diluted sperm, 0.5 μ L of SYBR green (1 000x concentrated in DMSO) (SYBR 14 - Sigma, Portugal) and 1 μ L of 2.4mM propidium iodide (PI - Sigma, Portugal) were added. After 5 minutes, 5 μ L of the sample was placed in a slide with a coverslip and examined under a fluorescent microscope (Nikon Eclipse E200). The samples were analyzed, and images were captured with a digital camera (VisionCam5, WWR) and the imaging software pylon Camera Software Suite 7.3.0 (Basler AfC, Germany). Three photos with at least 100 cells were taken so that the number of live cells (stained with green dye SYBR-14) and dead cells (cells permeable to the PI dye stained with red) could be counted.

2.6.3 DNA Fragmentation

For the DNA fragmentation test, the protocol described by Cabrita et al., (2005) was used, with some adaptations for sea urchin sperm. After splattering the slides with 40 μ L of agarose (normal agarose 0.5% in PBS), the sperm was diluted 1:1000 with 1% NaCl solution and 20 μ L of the diluted sperm was added to 150 μ L of low melting point agarose (0.5% in PBS). Afterward, 40 μ L of the previous solution was pipetted twice and covered with two coverslips before being placed horizontally at 4°C to solidify for each slide. After 15 minutes, the coverslips were quickly and gently removed. The samples then were exposed to a lysis solution (2.5M NaCl, 100mM Na₂-EDTA, 10mM Tris (base) pH 10, 1% Triton X-100, 1% lauryl sarcosine) for 1 hour at 4°C, and after were placed horizontally in an electrophoresis cube (BioRad, Spain) filled with electrophoresis solution (0.3M NaOH, 1mM Na₂-EDTA, pH 13). After 20 minutes of unwinding in the electrophoresis solution, 10 minutes of alkaline electrophoresis at 25V, 300mA, and 4°C were performed. The slides were therefore removed and neutralized (0.4M Tris-HCl, pH 7.5) with two 5 minute washes before being fixed for 3 minutes in 98% ethanol.

To visualize the comets, 10 μ L of 0.24M propidium iodide was pipetted into the sample and covered with a coverslip. After 5 minutes, the slides were placed in the

fluorescent microscope and photographs of at least 100 comets were taken for each slide (the equipment and software used is the same as used with viability). The comet assessment was carried out using the imaging system Komet software v6.0 (Andor Technology, Ireland), and DNA fragmentation was measured as a percentage of DNA in the tail (% DNAt).

2.6.4 Fertilizations

Frozen sperm samples and oocytes from F1 females (n=12) were utilized to analyze sperm capacity to fertilize an egg. Both gametes were collected using the technique previously described. The number of oocytes was estimated in an optical microscope (Nikon Eclipse Si RS) using a Sedgewick-Rafter cell-counting chamber, and the concentration per milliliter was determined. The oocytes were then mixed in a pool to ensure the same oocyte quality in all fertilizations and dispersed by the 36 fertilization 50 mL Falcons (3 male pools x 4 treatments x 2 replicates), with a target of 10,000 oocytes per Falcon and a sperm to oocyte ratio of 20 000 spermatozoa/oocyte (Rodrigues, 2021). Each Falcon had 40 mL of ASW. CASA software was used to calculate sperm concentration as previously mentioned. After assessing sperm concentration, dilutions were determined for each male to achieve 10×10^8 spermatozoa/mL. For each fertilization, the required amount of active sperm from each male was added to the Falcon's carrying eggs. Because of the high precipitation of oocytes, the falcons were gently stirred every 30 minutes for 1.5 seconds. After fertilization (120 minutes), 3 aliquots of each fertilization were counted with a counting chamber (Sedgwick-Rafter) in an optical microscope to determine the number of eggs in cell division, respect to the total number (Fig 2.3).

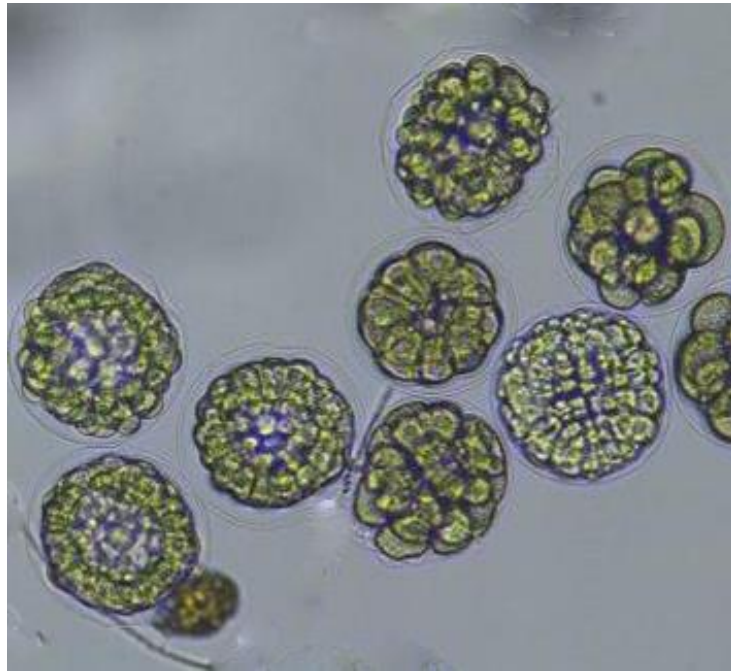


Figure 2.3-Fertilized *Paracentrotus lividus* eggs in cell division.

Source: João Araújo

2.7 Statistics

The statistical analysis was carried out with the help of SPSS Statistics 29.0 software (IBM Co., Hong Kong). All results are represented as means \pm standard error (mean \pm S.E.M). Shapiro-Wilk and Levene's tests were used to determine the normality and homogeneity of variances, and data transformation ($\arcsin(\sqrt[2]{x})$) was performed when the data was not normally distributed.

Given that the data in each experiment had different distributions, variance homogeneity, and responses to data transformation, a parametric (ANOVA) and a non-parametric (Kruskal-Wallis) test were employed according to the violation of their assumptions. If there was statistical evidence that the experimental groups differed, post-hoc testing for both tests was performed, using a Tukey HSD and Dunn's test to identify which groups were significantly higher or lower. The level of significance was considered with a *P-value* <0.05 .

3. Results

3.1 Experiment 1: Evaluating which storage equipment is the most efficient, and what is the most effective frizzing rate

To standardize a cryopreservation protocol for sea urchin sperm, two important aspects were evaluated: first, two different storage equipment were chosen to assess which one was the best to contain the biological material, cryovials, and straws, and then two controlled freezing rates, $-5^{\circ}\text{C}/\text{min}$ and $-10^{\circ}\text{C}/\text{min}$, were set to evaluate which one preserves better the cells. Also, two different cryoprotectants were chosen, although their effectiveness was not assessed in this experiment.

The sperm motility parameters (Fig. 3.1) revealed that there were no significant differences between treatments in terms of total motility (Fig. 3.1A) (P -value = 0.353), however, in terms of percentages the best results were obtained with straws rather than cryovials. The significantly different treatments in progressive motility were obtained (Fig. 3.1B) with 5% DMSO and 10% DMSO cryopreserved in straws. For the velocities results, sperm cryopreserved with 10% DMSO at $-5^{\circ}\text{C}/\text{min}$ ($9.64 \pm 1.04 \mu\text{m}/\text{s}$) freezing rate and 5% DMSO at a $-10^{\circ}\text{C}/\text{min}$ freezing rate ($8.89 \pm 0.47 \mu\text{m}/\text{s}$), both in straws, were the significantly different treatments (P -value = 0.008), while for VCL (Fig. 3.1D), 10% DMSO frozen at a $-5^{\circ}\text{C}/\text{min}$ ($25.68 \pm 3.49 \mu\text{m}/\text{s}$) and $-10^{\circ}\text{C}/\text{min}$ ($25.88 \pm 0.80 \mu\text{m}/\text{s}$) rates in straws were the significantly different treatments. The linearity coefficient (Fig. 3.1E), showed that 10% DMSO frozen at a $-5^{\circ}\text{C}/\text{min}$ ($38.65 \pm 5.23\%$) and 5% DMSO frozen at a $-10^{\circ}\text{C}/\text{min}$ rate ($37.33 \pm 2.73\%$), were the significantly different treatments (P -value = 0.030).

Another test that was performed in order to evaluate the experiment's objectives was cell viability. This test indicated that there were no significant differences across treatments (P -value = 0.407). However, straws frozen at $-10^{\circ}\text{C}/\text{min}$ outperformed other treatments with both 5% DMSO ($31.79 \pm 14.21\%$) and 10% DMSO ($29.93 \pm 4.56\%$).

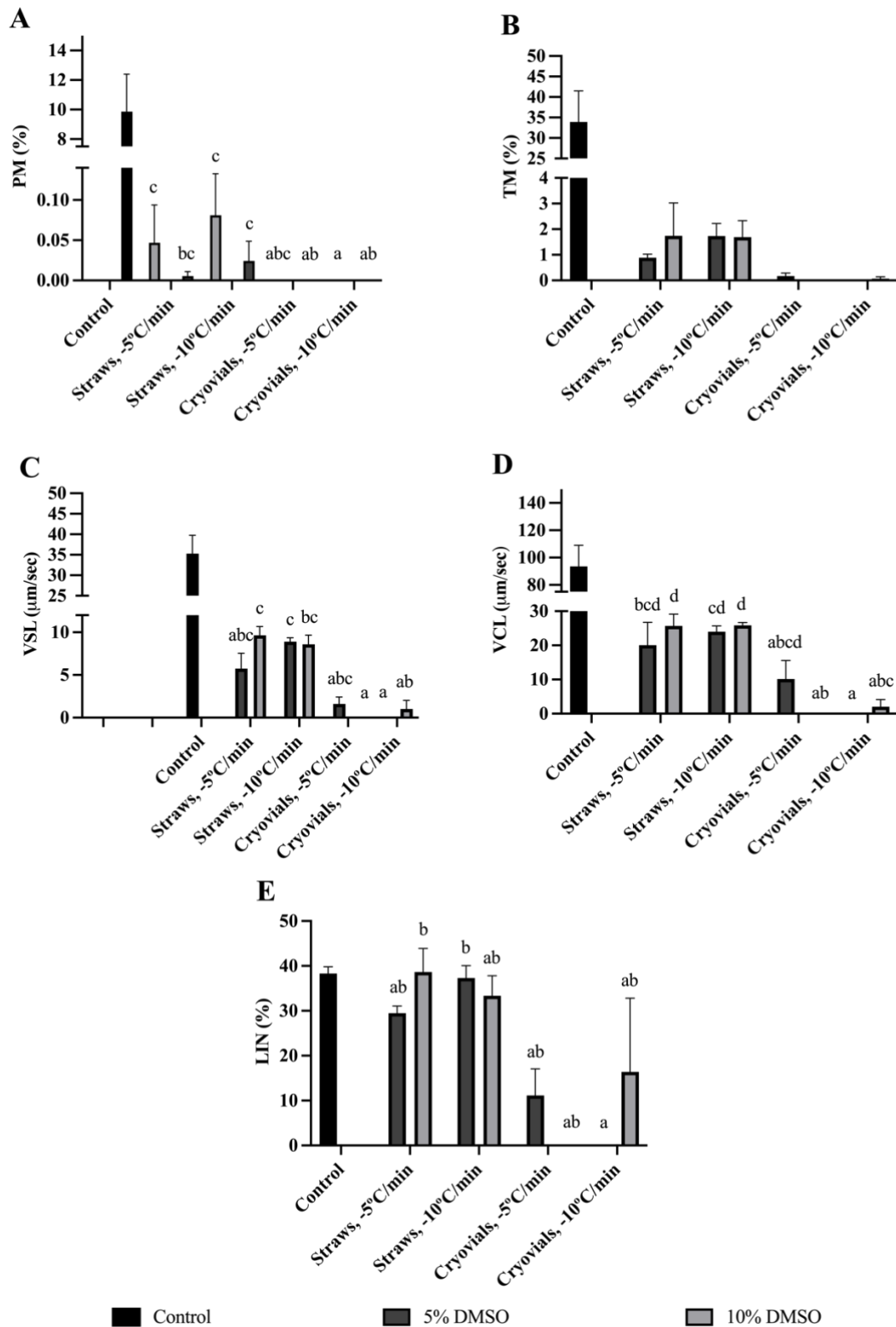


Figure 3.1- Motility parameters for *P. lividus* fresh and post-thawed sperm. (A) TM (Total motile sperm (%)); (B) PM (Progressive motile sperm (%)); (C) VSL (Straight-line velocity (μm/s)); (D) VCL (Curvilinear velocity (μm/s)); (E) LIN (Linearity Coefficient (%)). Data is shown in mean ± S.E.M of 3 pools for each experimental group (fresh and cryopreserved). Different letters indicate significant differences among the post-thawed groups (Kruskal-Wallis followed by a Dunn's post- hoc; P < 0.05).

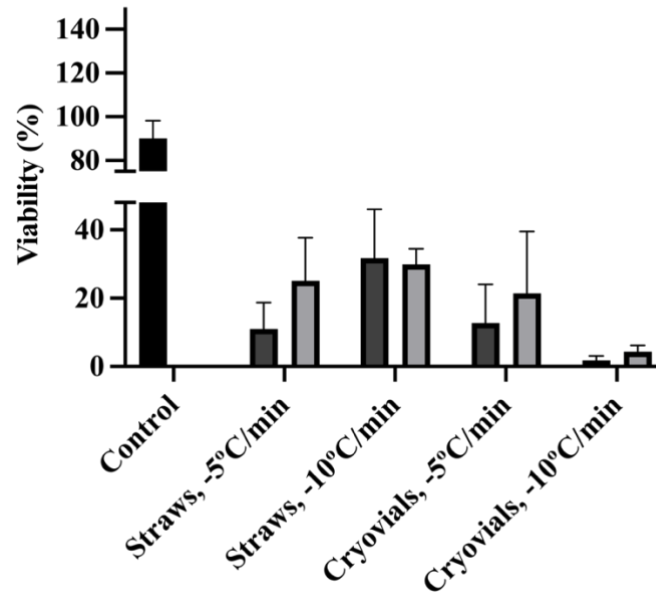


Figure 3.2-Percentage of viable cells in *P. lividus* fresh and post-thawed sperm. Data is shown in mean \pm S.E.M of 3 pools for each experimental group (fresh and cryopreserved). No letters indicates no differences among the post-thawed groups (One-Way ANOVA; $P < 0,05$).

3.2 Experiment 2: Testing different permeable cryoprotectants at different concentrations

The sperm motility parameters recorded after thawing are shown in Fig. 3.3. Considering all the evaluated parameters the best results were obtained with the 20% DMSO based extender. In terms of total motility (Fig. 3.3A) there were significant differences (P -value = 0.002), with 20% DMSO being the significantly higher treatment with $1.58 \pm 0.21\%$ motile sperm, followed by 10% DMSO $1.29 \pm 0.25\%$ motile sperm, as for the other progressive motility there were no significant treatments (P -value = 0.074) (Fig. 3.3B). In regard to velocity results, 20% EG was the significantly higher treatment in VSL with $11.99 \pm 1.27 \mu\text{m/s}$, but there were no statistical differences between treatments for the VCL (P -value = 0.081) and LIN (P -value = 0.471). Methanol at all concentrations and ethylene glycol at 5% were consistently the treatments with the lowest results in parameters such as total motility and VSL, with occasional exceptions.

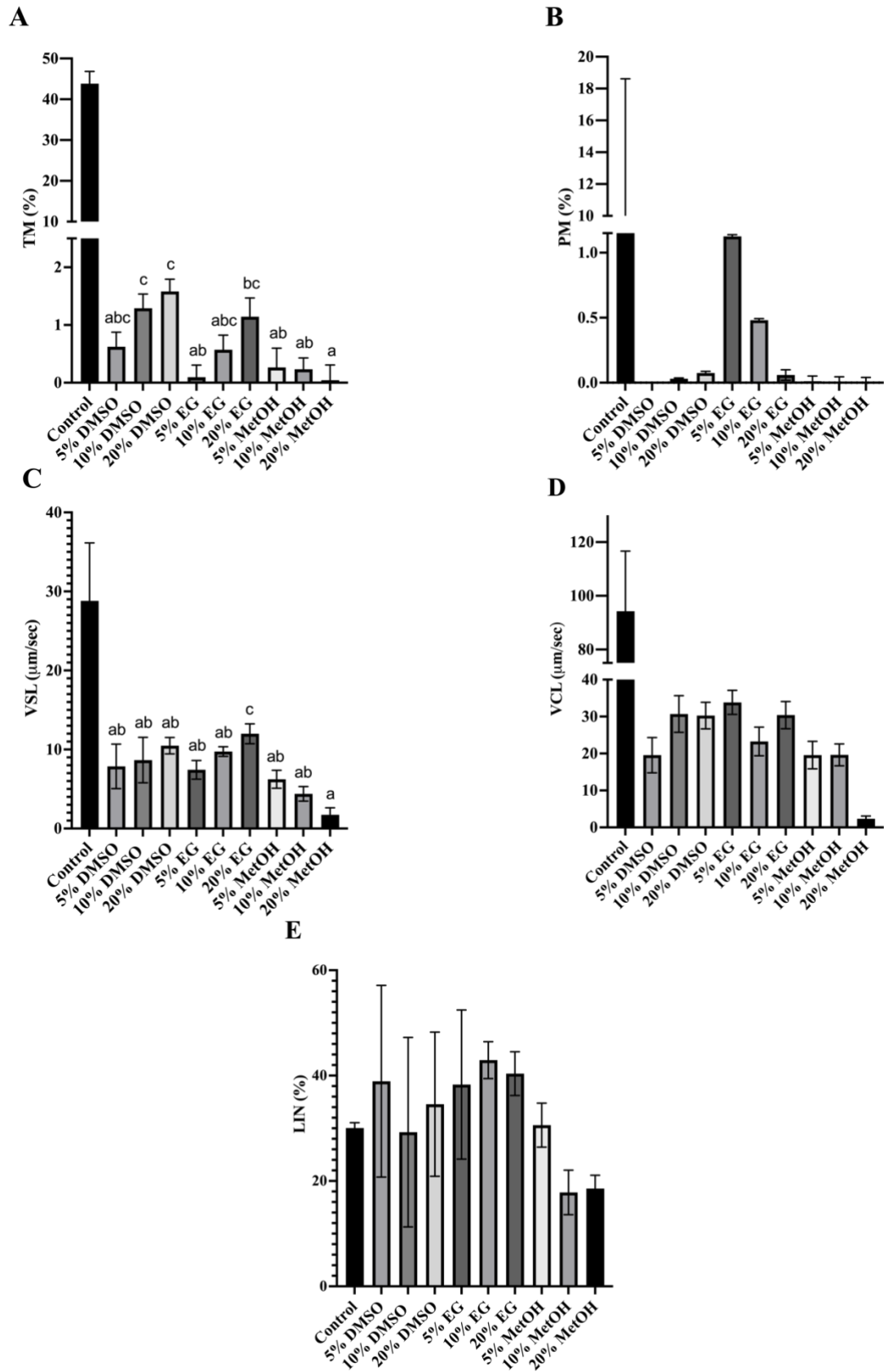


Figure 3.3- Motility parameters for *P. lividus* fresh and post-thawed sperm. (A) TM (Total motile sperm (%)); (B) PM (Progressive motile sperm (%)); (C) VSL (Straight-line velocity ($\mu\text{m}/\text{s}$)); (D) VCL (Curvilinear velocity ($\mu\text{m}/\text{s}$)); (E) LIN (Linearity Coefficient (%)). Data is shown in mean \pm S.E.M of 3 pools for each experimental group (fresh and cryopreserved). Different letters indicate significant differences among the post-thawed groups (Kruskal-Wallis followed by a Dunn's post- hoc; $P < 0.05$).

In regard to cell viability (Fig. 3.5), DMSO at 10% and 20% concentrations yielded $50.96 \pm 12.05\%$ and $53.81 \pm 9.04\%$ viable sperm cells, respectively, very similar to the control ($60.19 \pm 8.14\%$), making these significant different from the other treatments for this approach. In terms of the treatments with the worst results, methanol at 5, 10, and 20% and ethylene glycol at 5% were the considerably worst treatments among the experience. Regarding DNA fragmentation (Fig. 3.6) there were no significant differences between treatments (P -value = 0.289). As a result of these findings, DMSO at a concentration of 20% was chosen as the best treatment and as the permeable cryoprotectant to be tested alongside a non-permeable one in the third experiment.

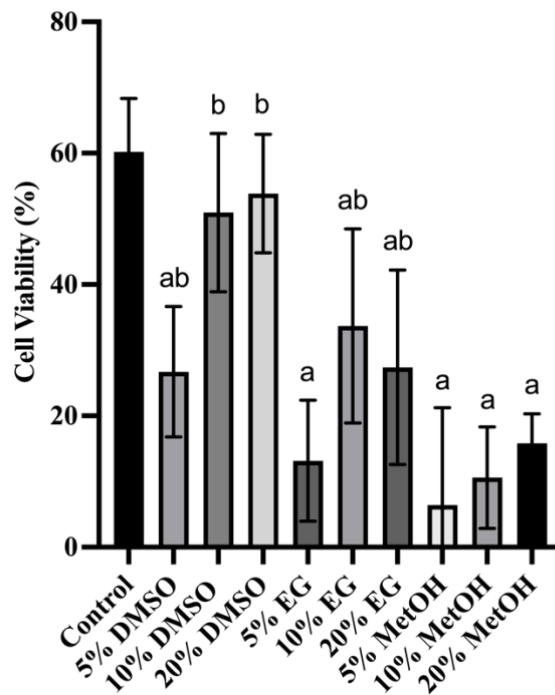


Figure 3.4 - Percentage of viable cells in *P. lividus* fresh and post-thawed sperm. Data is shown in mean \pm S.E.M of 4 pools for each experimental group (fresh and cryopreserved). Different letters indicate differences among the post-thawed groups (One-Way ANOVA followed by a Tukey's HSD post- hoc; $P < 0.05$).

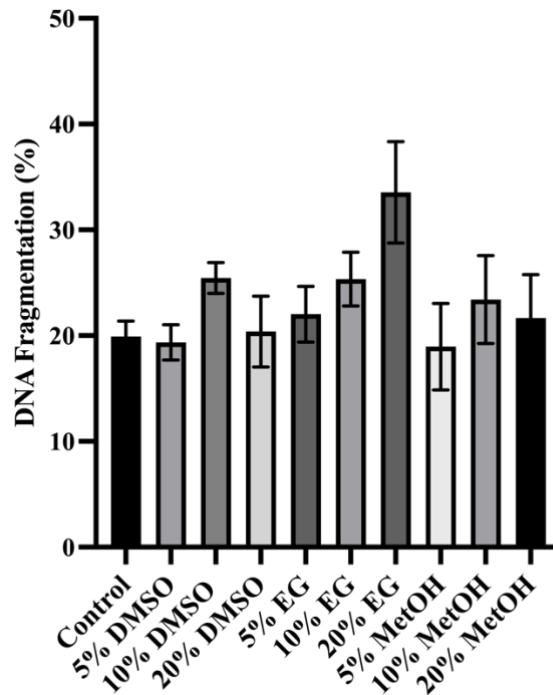


Figure 3.5-Percentage of DNA fragmentation in *P.lividus* fresh and post-thawed spermatogonia. Data is shown in mean \pm S.E.M of 4 pools for each experimental group (fresh and cryopreserved). Different letters indicate significant differences among the post-thawed groups (One- Way ANOVA; $P < 0,05$).

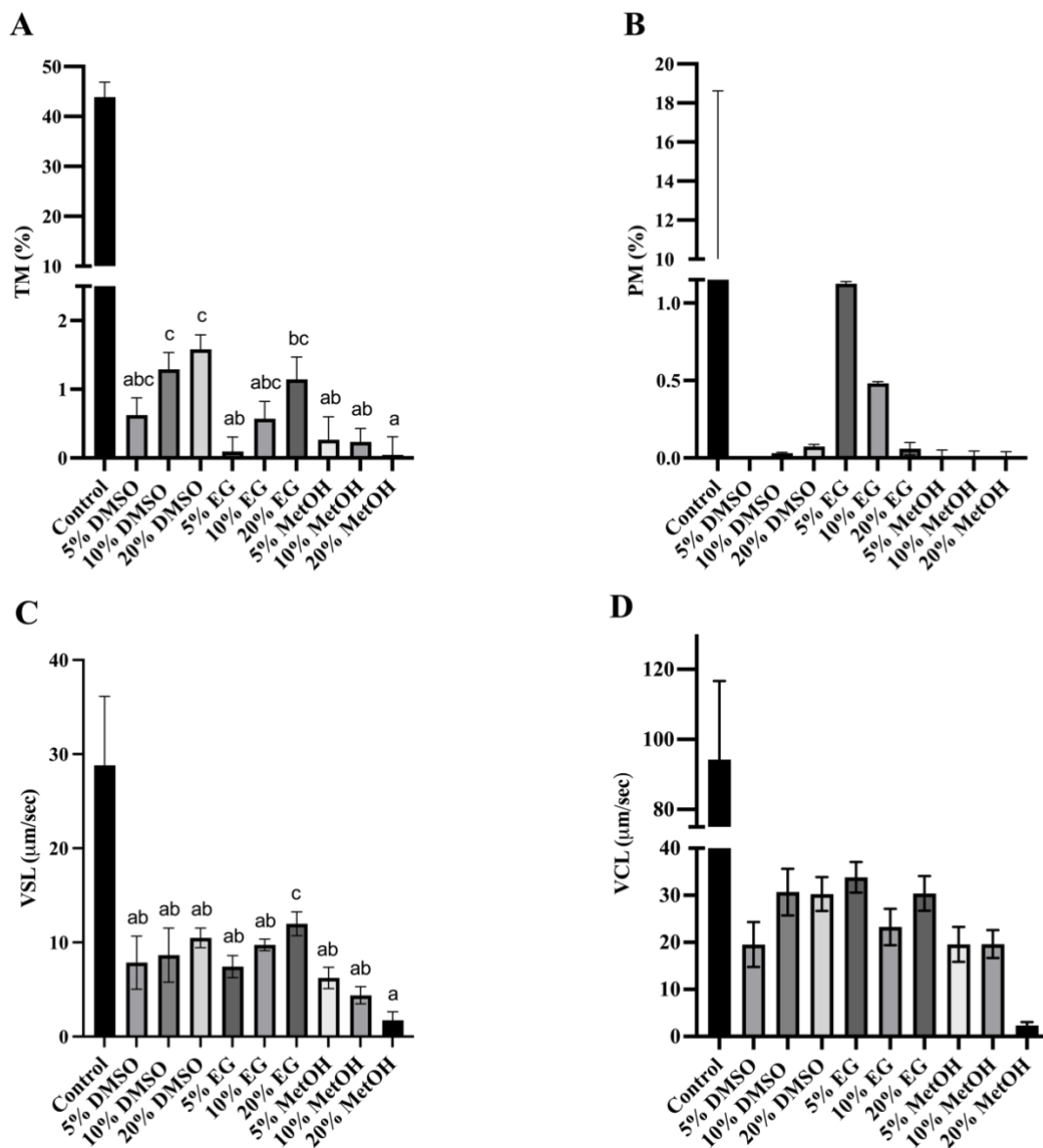
3.3 Experiment 3: Testing the benefit of adding a non-permeable cryoprotectant and the optimal concentration

The results were extremely consistent when testing the addition of a non-permeable cryoprotectant (Figs.3.6-3.9) with no significant differences between the treatments. Sperm frozen with 20% DMSO without the addition of a non-permeable cryoprotectant was considered the best treatment. In terms of motility parameters, 20% DMSO was the significantly different treatment for total motile sperm ($1.22 \pm 0\%$), but there was no statistical evidence of a significantly different treatment for the other parameters such as progressive motility (P -value = 0.342), VSL (P -value = 0.406), VCL (P -value = 0.320), and LIN (P -value = 0.237).

Although there were also no significant differences between treatments for sperm viability (Fig. 3.8) in this experiment, 20% DMSO supplemented with 5% Sucrose showed the best cell viability results with a mean of $70.40 \pm 11.37\%$ viable cells, almost equally good as the control with a mean of $79.40 \pm 11.37\%$ viable cells.

There were unexpected results in terms of DNA fragmentation, with all treatments having less DNA fragmentation than the control ($50.58 \pm 6.03\%$). The lowest percentage was 20% DMSO supplemented with 5% trehalose ($22.70 \pm 1.00\%$), while the highest was 20% DMSO supplemented with 5% sucrose ($26.34 \pm 3.48\%$). Regardless, there was no statistical evidence of a significant treatment.

In this experience, unlike the previous two, a different test allowed, to evaluate the quality of cryopreserved sperm supplemented with the various treatments studied, which was the capacity of sperm cells to fertilize an egg. The results (Fig. 3.10) revealed that there were no significant differences between treatments for this test.



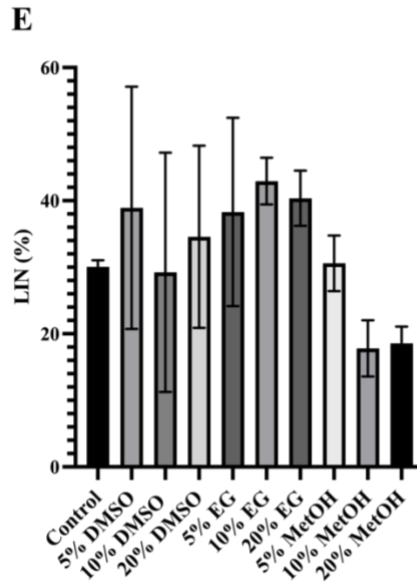


Figure 3.6 - Motility parameters for *P. lividus* fresh and post-thawed sperm. (A) TM (Total motile sperm (%)); (B) PM (Progressive motile sperm (%)); (C) VSL (Straight-line velocity ($\mu\text{m/s}$)); (D) VCL (Curvilinear velocity ($\mu\text{m/s}$)); (E) LIN (Linearity Coefficient (%)). Data is shown in mean \pm S.E.M of 3 pools for each experimental group (fresh and cryopreserved). Different letters indicate significant differences among the post-thawed groups (One-Way ANOVA followed by a Tukey's HSD post- hoc, and Kruskal-Wallis followed by Dunn's post hoc; $P < 0.05$).

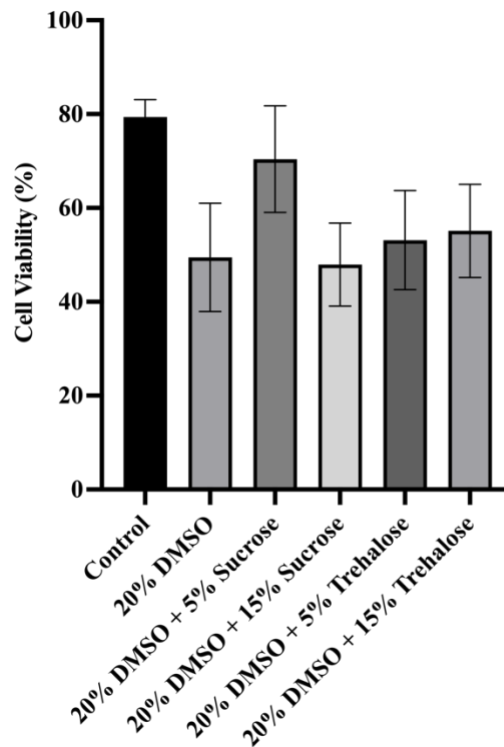


Figure 3.7 - Percentage of viable cells in *P. lividus* fresh and post-thawed sperm. Data is shown in mean \pm S.E.M of 4 pools for each experimental group (fresh and cryopreserved). No letters indicate no differences among the post-thawed groups (One-Way ANOVA; $P < 0.05$).

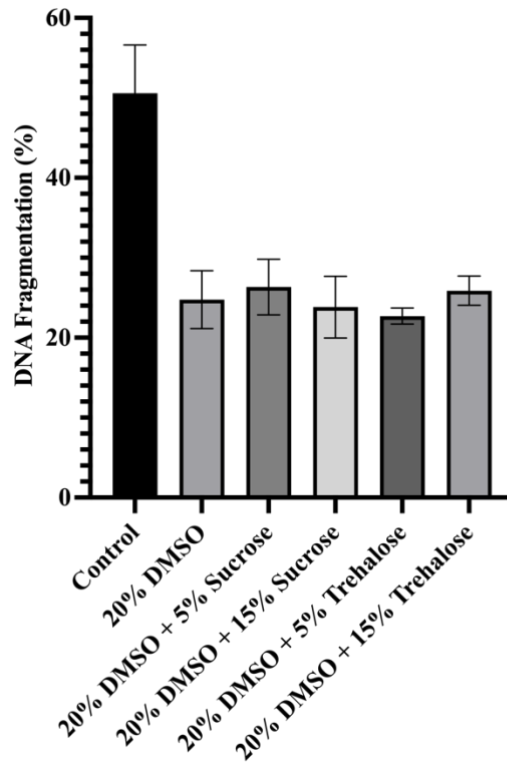


Figure 3.8 - Percentage of DNA fragmentation in *P. lividus* fresh and post-thawed sperm. Data is shown in mean \pm S.E.M of 3 pools for each experimental group (fresh and cryopreserved). No letters indicate no differences among the post-thawed groups (One-Way ANOVA; $P < 0.05$).

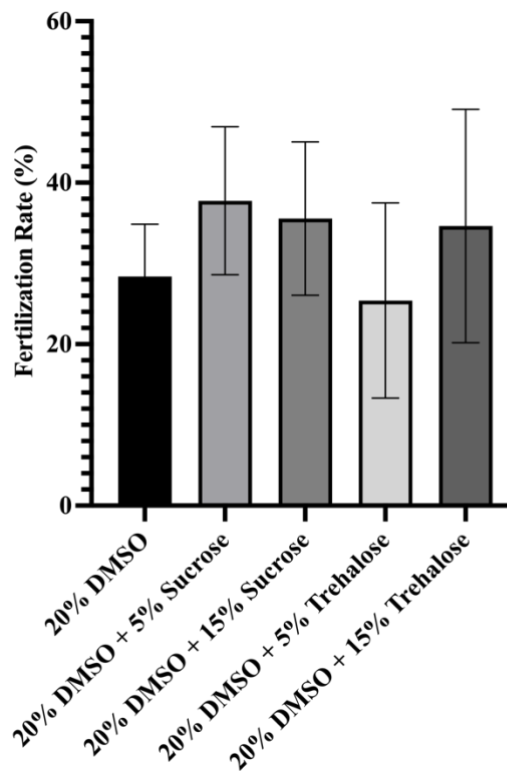


Figure 3.9 - Percentage of *P. lividus* fertilized eggs fresh and post-thawed sperm. Data is shown in mean \pm S.E.M of 3 pools for each experimental group (fresh and cryopreserved). No letters indicate no differences among the experimental groups (One-Way ANOVA; $P < 0.05$).

4. Discussion

4.1 Experiment 1: Evaluating which storage equipment is the most efficient, and what is the most effective freezing rate

Cryopreservation is a fundamental and crucial technique for storing organs, tissues, cells, and other biological materials at extremely low temperatures for long periods of time. It has made several improvements since its inception and is now investing heavily in protocol optimization research (Whaley et al., 2021). One of the main limitations of this technique is that without the proper cryopreservation protocol, cells may be unable to endure the physical and chemical stressors generated by cooling and thawing and hence cannot fertilize an egg (Barbas and Mascarenhas, 2009; Cabrita et al., 2010; Purdy, 2006). One of the major challenges for cryopreservation is the optimization of freezing or cooling because if we cool the cells slowly, their extracellular fluids form ice crystals, compressing the cells and eliminating all intracellular components. Rapid cooling, on the other hand, can cause severe damage to all the critical intracellular structures and cellular components, resulting in intracellular ice formation, and the vital components of the cells may come out, resulting in cell death due to shrinkage (Bhattacharya, 2018). Another important consideration is the container for frozen sperm. There are a number of methods for packaging spermatozoa for freezing in different species, including glass ampoules or vials, straws, flat aluminum packets, pellets, and macrotubes that come in different volumes, nonetheless, cryovials and straws are among the most commonly used for fish and invertebrates. Both methods have their advantages as well as disadvantages (Thilak Pon Jawahar and Betsy, 2020).

In that aim, we first evaluated the use of two different cooling rates ($-5^{\circ}\text{C}/\text{min}$ and $-10^{\circ}\text{C}/\text{min}$) using programmable freezing, which is thought to provide more precise and accurate results than non-programmable freezing (Xu et al., 2022), and then we evaluated which storage equipment (straws and cryovials) was most suitable for freezing sea urchin sperm. Our findings allowed us to choose $-10^{\circ}\text{C}/\text{min}$ to be the best freezing rate for *Paracentrotus lividus* sperm, but other studies suggest other freezing temperatures, for example, Mladenov et al., (2008) tested rates of $-5^{\circ}\text{C}/\text{min}$ and $-50^{\circ}\text{C}/\text{min}$, and conclude that $-5^{\circ}\text{C}/\text{min}$ was the best temperature; Kang et al., (2004) tested three different rates ($-50^{\circ}\text{C}/\text{min}$; $-30^{\circ}\text{C}/\text{min}$, $-10^{\circ}\text{C}/\text{min}$), and came to the

conclusion that $-30^{\circ}\text{C}/\text{min}$ was the best, and finally, Fabbrocini et al., (2014) tested $-90^{\circ}\text{C}/\text{min}$ and $-20^{\circ}\text{C}/\text{min}$, and stated that $-20^{\circ}\text{C}/\text{min}$ was the best rate. As a result, we cannot claim that our findings were inconsistent with the literature once Mladenov et al., (2008) and Kang et al., (2004) tested in different sea urchin species, *Evechinus chloroticus* and *Urechis unicinctus* respectively, and freezing rates can be species specific. As for Fabbrocini et al., (2014), they used non-programmable freezing and different types of containers to freeze sperm which can achieve different results than ours. Relatively to the type of containers, we ended up selecting the straws, once we had significant differences in progressive motility (P-value = 0.008), VSL (P-value = 0.008), VCL (P-value = 0.009), LIN (P-value = 0.030), constantly on the treatments using straws. Again, we cannot say that these findings are inconsistent with the literature because there is little information about studying the use of straws and cryovials for sea urchin sperm. However, Mladenov et al., (2008) and Kang et al., (2004) used straws in their studies, while Fabbrocini et al., (2014) obtained better results with cryovials, but their conditions were different than those evaluated here and in other studies.

4.2 Experiment 2: Testing different permeable cryoprotectants at different concentrations

Another significant challenge in cryopreservation is preventing the formation of ice crystals within cells and tissues during freezing, because ice crystals can damage essential biological components and structures, resulting in cell death, as previously mentioned. Permeable or penetrating cryoprotectants have the ability to penetrate cells and lower the freezing point of intracellular fluids. This prevents the formation of large, damaging ice crystals, which is essential for cell survival. It is crucial to highlight, however, that the cryoprotectant and its concentration must be carefully calibrated for each type of biological material, as some CPAs may be harmful at high doses.

Amongst the different types of permeable cryoprotectants available, DMSO is the most used permeable CPA for sperm cryopreservation, with concentrations ranging from 5% to 20% (Elliott et al., 2017; Gwo, 2000; Magnotti et al., 2016; Paredes, 2015, 2016). Once sperm have distinct ideal CPA and concentration ranges, their supplementation range is highly varied (Elliot et al., 2017; Guo and Weng, 2020). For invertebrates such as sea urchins, oysters, and sea cucumbers, DMSO is still the most

used CPA, but others such as EG, PG, and MetOH are also used (Dong et al., 2007; Xu et al., 2020). In this study, we explored different permeable cryoprotectants such as DMSO, ethylene glycol, and methanol at different concentrations of 5, 10, and 20%, and we measured motility parameters, cell viability, and DNA fragmentation as these are proven to be good quality sperm parameters. With those tests, we found that 20% DMSO was the most suitable permeable cryoprotectant, but there is little information about how cryoprotectant concentration affects motility kinetics on sea urchin sperm, even though Kang et al., (2004), stated that for the sea urchin, *Anthocidaris crassisapina*, 15% DMSO was the best concentration, and Mladenov et al., (2008) found that contrary to fertilization ability, high concentrations of DMSO were better for mitochondrial function and membrane integrity in post-thaw samples. We can confirm that relative to total motility there were statistical differences (P-value = 0.002) for 10 and 20% DMSO, and regarding cell viability, there were also statistical differences for the high concentrations of DMSO, which is therefore consistent with Mladenov et al., (2008).

4.3 Experiment 3: Testing the benefit of adding a non-permeable cryoprotectant and the optimal concentration

Non-penetrating cryoprotectants are substances or compounds that, unlike penetrating CPAs, can not enter cell membranes and instead establish a protective barrier surrounding cells or tissues to prevent ice crystal formation and protect biological material from freezing damage. One example of those substances are sugars, that in addition to being an energy source, sugars have been widely used as a CPA or CPA supplementation in diverse research for marine invertebrate, different cells and types of tissues (Paredes, 2015). In the range of existing sugars, trehalose and sucrose proved to be the most efficient, as both disaccharides disrupt water structure to reduce the repulsive hydrating force, which, when intensified, leads to the effect of stress on the membrane, causing freeze-induced membrane damage, however their effectiveness is dependent on their concentration in the medium (Tsai et al., 2018; Yoon et al., 1998). With that being said, in our third experiment we tested the benefit of adding a non-permeable cryoprotectant and its optimal concentration and for that we used two sugars, trehalose and sucrose, and two concentrations, 5% and 15%. Our results showed that there was no benefit of supplement 20% DMSO with trehalose or

sucrose, because when taking into consideration motility parameters the only differences obtained were with 20% DMSO in total motility (P-value = 0.006), and as for progressive motility, VSL, VCL and LIN there were no significant differences. Other tests assessed in this experiment like the percentage of viable cells, DNA fragmentation, and percentage of fertilized eggs also showed no differences between treatments. It is worth highlighting though, that when assessing DNA fragmentation all treatments had less DNA fragmentation than the fresh (control), which can be due to the failure of the protocol used or to a change in the quality of fresh samples.

These results can not be compared with other studies, as supplementation with trehalose and sucrose is mostly used in sea urchin embryos and larvae (Bellas and Paredes, 2011; Odintsova et al., 2009; Paredes and Bellas, 2014), despite that Fabbrocini et al., (2014) used trehalose as supplementation but there were no data if using DMSO only produce the same results. However other species of invertebrate sperm have been supplemented with trehalose and sucrose such as *Crassostrea angulata*, for which Anjos et al., (2021) found that 20% DMSO supplemented with 0.45M (approximately 15%) trehalose produced better results than sperm with 20% DMSO or sperm with DMSO supplemented with 0.45M sucrose.

5. Conclusion

We can conclude from this study that more testing with higher cooling rates is needed to evaluate whether $-10^{\circ}\text{C}/\text{min}$ is, in fact, the best cooling rate for *Paracentrotus lividus*. Although 20% DMSO produced good results in post-thaw sperm quality, more research into the influence of cryopreservation on sperm is needed. The addition of a non-permeable cryoprotectant may interfere with the results due to the small number of pools, but other substances at different concentrations should also be investigated. Another issue that may be investigated further is the development of the fertilized eggs to see if they can develop into larvae and grow without constraints.

6. References

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