**CATARINA MIRANDA CASTILHO DOS ANJOS**

# Establishment of a genetic resource bank for restocking management in Portuguese oyster (*Crassostrea angulata*) and striped venus clam (*Chamelea gallina*)

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**Faculdade de Ciências e Tecnologia**

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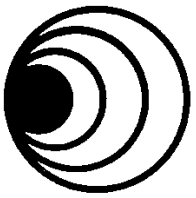
**Doutoramento em Ciências do Mar, da Terra e do Ambiente,**
**Ramo: Pescas e Aquacultura**

**Trabalho efetuado sob a orientação de:**

Professora Doutora Elsa Cabrita
Doutora Domitília Matias



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**Declaração de autoria de trabalho**

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

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*In the middle of difficulty lies opportunity.*

*Albert Einstein*

# ACKNOWLEDGMENTS

I want to express my deepest gratitude to everyone who has been with me throughout this journey, both on a scientific and personal level.

A heartfelt thank you to all the members of the AQUAGROUP, EEMT - IPMA Tavira, and Necton teams, with a special mention to Ana Luísa Santos, Daniel Duarte, Filipa Pinheiro, Patrícia Diogo, Elvira Fatsini, Catarina Oliveira, Ana Rato, Victória del Pino, and Ana Coelho.

Maurícia Vinhas, thank you so much for the support you provided during the long hours of work on the cytometer, optimizing protocols, and running samples.

My sincere appreciation goes to my advisors, Elsa Cabrita and Domitília Matias, for their exceptional support and guidance throughout these years.

Lastly, to my family, including Sofia Carril and Marietta Drumond, and especially to my parents, your unwavering support means the world to me.

# FUNDING

Catarina Anjos was the recipient of doctoral grants SFRH/BD/130910/2017 and COVID/BD/151914/2021 from the FCT. This work was supported by the projects 0139_VENUS_5_E (Interreg POCTEP), ASSEMBLE + JRA2-H2020-INFRAIA-2016-2017 (No 730984), EBB-EAPA_501/2016 (Interreg Atlantic Area) and received Portuguese national funds from FCT - Foundation for Science and Technology through projects UIDB/04326/2020 (DOI:10.54499/UIDB /04326/2020), UIDP/04326/2020 (DOI:10.54499/UIDP/04326/2020) and LA/P/0101/2020 (DOI:10.54499/LA/P/0101/2020) and from the operational programs CRESC Algarve 2020 and COMPETE 2020 through project EMBRC, PT ALG-01-0145-FEDER-022121.

# ABSTRACT

Bivalves are essential for fisheries, aquaculture, and ecosystems, serving as nutrient-rich resources for human consumption. Despite their significance, many bivalve resources, including *Crassostrea angulata* (Portuguese oyster) and *Chamelea gallina* (striped venus clam) in Europe, evidence signs of depletion due to environmental change, anthropogenic impact, and overexploitation, requiring rehabilitation measures. One possible strategy involves establishing a genetic resource bank via cryopreservation. However, cryopreservation presents challenges, requiring optimization of freezing and thawing conditions, particularly the cryoprotectant solution, and understanding cryodamage. The present thesis aims to explore and establish conditions to store and preserve the genetic resources of *C. angulata* and *C. gallina* populations. **Chapter 1** provides contextual background, on the current situation of bivalve production and the importance of these resources, with special attention on the endangered and valuable species for aquaculture/fisheries, *C. angulata* and *C. gallina*. The chapter addresses the fundamental principles of cryobiology and current knowledge on bivalve cryopreservation methodologies for sperm and larvae. This chapter discusses the value of cryodamage assessment tools, emphasizing "omics" molecular tools for high-potential analysis. **Chapters 2** and **3** aim to optimize and develop new cryopreservation protocols for the target species. **Chapter 2** investigates the effect of the cryoprotectant supplementation with sugars (trehalose and sucrose) on the post-thaw sperm quality of *C. angulata*. Several methodologies not commonly used in bivalve cryopreservation works were employed, including the determination of reactive oxygen species levels, acrosome integrity and the activity of antioxidant enzymes (superoxide dismutase - SOD, glutathione reductase - GR and glutathione peroxidase - GPX). Sugars supplementation, especially trehalose reduced lipid peroxidation and ROS levels having a positive effect in plasma membrane and acrosome integrity. **Chapter 3** evaluates the larval quality of *C. angulata* and *C. gallina* exposed and cryopreserved with cryoprotectant solutions that differ in the permeant agent (dimethyl sulfoxide - DMSO and ethylene glycol - EG). The work aimed to understand the effects of cryoprotectant exposure and, cryopreservation on malformations, movement, and enzymatic activity compared with non-exposed

larvae. The methodologies for cryopreserving D-larvae of both species were established for the first time. **Chapter 4** investigates *C. angulata* D-larvae cryodamage during cryoprotectant exposure and cryopreservation, using RNA sequencing. This molecular approach was essential for providing evidence that the freezing process was the critical step rather exposure. Furthermore, identified 11 genes as relevant biomarkers of freezability for D-larvae quality assessment. This thesis presents strategies for cryopreserving the genetic material of *C. angulata* and *C. gallina* and for cryodamage evaluation.

**Keywords:** Cryopreservation, Portuguese oyster (*Crassostrea angulata*), striped venus clam (*Chamelea gallina)*, sperm, larvae and cryodamage assessment.

# RESUMO

Os bivalves são importantes recursos para os sectores das pescas e da aquacultura, vista à sua relevância na dieta humana devido ao seu valor nutricional em proteínas, vitaminas e ácidos gordos essenciais. Além disso, como organismos filtradores tem um papel preponderante na regulação do ciclo de nutrientes em ecossistemas, removendo partículas orgânicas e inorgânicas suspensas na água. Contudo, mundialmente, a maioria destes recursos apresentam sinais de declínio, devido às alterações climáticas, impactos antropogénicos e sobre-exploração. Esta situação preocupante não afeta apenas os bancos naturais de bivalves, mas também a atividade pesqueira e produção aquícola destes recursos. Na Europa, a *Crassostrea angulata* (ostra Portuguesa) e *Chamelea gallina* (pé-de-burrinho), são exemplos de recursos essenciais para os setores da pesca e aquacultura em risco de desaparecer. Surge, assim, a necessidade de implementar medidas que visem mitigar a perda de recursos genéticos e recuperar essas populações. Uma abordagem possível para a recuperação dos bancos naturais e a preservação da diversidade genética destas espécies passa pela implementação de bancos de recursos genéticos usando a tecnologia de criopreservação. A criopreservação é um processo que suspende as atividade celular através de temperaturas negativas (≈ -196°C) e permite a preservação do material biológico por longos períodos. No entanto, para um processo de criopreservação bem-sucedido, é necessário submeter o material biológico a um conjunto de etapas de preparação, para evitar os danos inerentes ao processo de congelação e descongelação. A seleção da solução crioprotetora, é uma das etapas mais importantes para o processo. Além das estratégias para proteger, é imperativo aplicar metodologias de avaliação de danos, visando uma compreensão mais abrangente sobre o que está a acontecer e implementar medidas para reduzir ou evitar os danos. O propósito principal desta tese foi explorar e estabelecer condições para armazenar e preservar os recursos genéticos das populações de *C. angulata* e *C. gallina*, para no futuro estabelecer um programa para reconstruir as populações. O **Capítulo 1** proporciona um contexto abrangente sobre os conceitos abordados ao longo da tese. Neste segmento é abordado a situação atual da produção de bivalves a nível mundial e em Portugal. Destaca-se a importância destes recursos, com especial atenção a duas espécies de bivalves europeias, *C. angulata* e

*C. gallina*, abordando as causas de declínio destes recursos. Os princípios fundamentais da criobiologia são apresentados neste capítulo, com foco ao tipo de crioprotetores e estratégias para otimizar as suas propriedades protetoras, condições de congelação, formas de armazenamento e condições de descongelação. Também são apresentados o estado da arte da criopreservação de bivalves para espermatozoides e larvas, além das várias metodologias para avaliar os danos inerentes à criopreservação em ambos os matérias biológicos. Por conseguinte, o valor das "ómicas" como ferramenta para detetar danos moleculares à criopreservação é explorado. Os **Capítulos 2 e 3** tiveram como objetivo otimizar e desenvolver protocolos de criopreservação para espermatozoides e larvas-D de *C. angulata* e *C. gallina*. No **Capítulo 2** foi explorado o efeito de suplementar o crioprotetor permeável com açúcares (trealose e sacarose) na qualidade dos espermatozoides descongelados de *C. angulata*. Os açúcares são crioprotetores não-permeáveis, que ao serem combinados com crioprotetores permeáveis apresentam-se como uma estratégia bem-sucedida para reduzir os danos inerentes à criopreservação em espermatozoides de diversas espécies, permitindo estabilizar os constituintes da membrana plasmática. Neste estudo, foi pretendido aplicar um conjunto de técnicas para avaliar a qualidade do sémen descongelado, que não são comumente utilizadas em trabalhos de criopreservação de bivalves. As técnicas incluíam a determinação dos níveis de espécies reativas de oxigénio (ROS), a integridade do acrossoma e atividade das enzimas antioxidantes (superóxido dismutase – SOD; glutationa redutase – GR; glutationa peroxidase – GPX). A incorporação de açúcares na solução crioprotetora, em particular a trealose, melhorou a integridade da membrana plasmática e reduziu os níveis de ROS, acrossomas danificados (apenas trealose) e a degradação de lípidos. Assim, evidenciamos que a suplementação com açúcares parece ter um papel importante na proteção dos espermatozoides de *C. angulata* em relação ao stress oxidativo, promovendo a qualidade destes após descongelação, sendo esta compreensão dos resultados possível pela exaustiva avaliação de danos. O objetivo do **Capítulo 3** foi desenvolver metodologias para a criopreservação de larvas-D de *C. angulata* e *C. gallina*, para as quais não existiam protocolos previamente instituídos. Sendo assim, as larvas-D das espécies-alvo, foram expostas e criopreservadas com duas soluções crioprotetoras que diferiam no composto permeável (dimetilsulfóxido – DMSO e

etilenoglicol - EG). Os crioprotetores permeáveis são essências para o sucesso da criopreservação, uma vez, que são capazes de passar pela membrana plasmática substituindo a água intracelular e evitando a formação de cristais de gelo, e, consequentemente, a rutura celular após descongelamento. Contudo, este tipo de compostos apresenta um certo nível de toxicidade para a célula, devendo ser adaptado de acordo com o tipo de material genético e a espécie. A avaliação da qualidade das larvas-D antes e depois da exposição às soluções crioprotetoras e após descongelação, foi analisada considerando alterações morfológicas, parâmetros de movimento e atividade das enzimas antioxidantes (SOD, GR e GPX). Os resultados evidenciaram que nos testes de exposição aos crioprotetores, o DMSO promove alterações morfológicas e aumenta a atividade da GPX nas larvas-D de *C. angulata* em relação às frescas e ao EG. Quanto ao EG, este diminui significativamente a velocidade das larvas-D de ambas as espécies, sugerindo uma resposta de stress durante a exposição aos crioprotetores. Após a criopreservação verificou-se uma redução significativa na morfologia e nos parâmetros de movimento em relação às larvas-D frescas de ambas as espécies, salvo na morfologia da *C. gallina*. Foi estabelecido pela primeira vez as metodologias de criopreservação para ambas as espécies. No **Capítulo 4**, foi aplicado o RNA-seq para avaliar os danos moleculares induzidos pela criopreservação nas larvas-D de *C. angulata*, com foco nos dois passos críticos de exposição às soluções crioprotetoras e após a descongelação. Este trabalho concluío que o passo crítico foi o processo de congelar e descongelar, mostrando alterações moleculares relacionadas à formação da concha e a malformações, permitindo assim validar os resultados do **Capítulo 3**. Este estudo identificou 11 genes como biomarcadores moleculares para estudos futuros de danos inerentes à criopreservação. Nesta tese, é providenciado um referencial para as metodologias de criopreservação de espermatozoides e larvas-D, visando estabelecer um banco de recursos genéticos de *C. angulata* e *C. gallina* para suportar futuros programas de aquacultura e repovoamento.

**Palavras-chave:** Criopreservação, ostra Portuguesa (*Crassostrea angulata*), pé-de-burrinho (*Chamelea gallina*), espermatozoides, larvas-D e identificação de criodanos.

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# LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| % | Percentage |
| (v/v) | Volume/Volume |
| (w/v) | Weight/Volume |
| °C | Degree Celsius |
| µg | Microgram |
| µl | Microliter |
| µm | Micrometre |
| 7-AAD | 7-aminoactinomycin D |
| AFPI | Antifreeze proteins |
| AFPII | Antifreeze proteins |
| AFPs | Antifreeze proteins |
| ANOVA | Analysis of variance |
| ASMA | Automated sperm morphology analysis system |
| ATP | Adenosine triphosphate |
| BP | Biological processes |
| bp | Base pair |
| BSA | Bovine Serum Albumin |
| CASA | Computer-assisted sperm analysis |
| CAT | Catalase |
| CC | Cellular components |
| CPAs | Cryoprotectant solutions |
| DAVID | Database for Annotation, Visualization and Integrated Discovery |
| DEGs | Differentially expressed genes |
| DHA | Docosahexaenoic acid |
| DHE | Dihydroethidium |
| D-larvae | D stage of bivalve larvae |
| DMSO | Dimethyl sulfoxide |
| DMSO + Sucrose | 10% (v/v) DMSO supplemented with 0.45 M (w/v) sucrose |
| DMSO + Trehalose | 10% (v/v) DMSO supplemented with 0.45 M (w/v) trehalose |
| DNA | Deoxyribonucleic Acid |
| e.g. | exempli gratia |
| EG | Ethylene glycol |
| EGFR | Epidermal growth factor receptors |
| EPA | Eicosapentaenoic acid |
| FC | Fold Change |
| FCGR1A | Fc gamma receptor Ia |
| FDR | False Discovery Rate |
| FITC-PSA | fluorescein isothiocyanate-labeled *Pisum sativum* agglutinin |
| FSC-H | Forward scattered |
| FSW | Filtered and UV- sterilized seawater |

| | |
|---|---|
| g | Force of gravity |
| g | Gram |
| GO | Gene Ontology |
| GPX | glutathione peroxidase |
| GR | glutathione reductase |
| GRB | Genetic resource bank |
| GSEA | Gene Set Enrichment Analysis |
| h | Hour |
| hpf | Hours post fertilization |
| ISAS | Integrated System for Semen Analysis |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| L | Liter |
| LIN | Linearity |
| LN | Liquid nitrogen |
| M | Molar |
| MDA | Malondialdehyde |
| MF | Molecular functions |
| mg | Milligram |
| Min | Minute |
| ml | Millilitre |
| mM | Millimolar |
| Mm | Millimeter |
| NaCl | Sodium Chloride |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NGS | Next-generation sequencing |
| nM | Nanomolar |
| nm | Nanometer |
| p | p-value |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PG | Propylene glycol |
| PI | Propidium iodide |
| PM | Progressive motility |
| PUFA | Polyunsaturated fatty acids |
| PVP | Polyvinylpyrrolidone |
| PVP-40 | Polyvinylpyrrolidone with an average molecular weight of 40,000 |
| RefSeq | Reference Sequence |
| RIN value | RNA integrity number |
| RNA | Ribonucleic acid |
| RNA-seq | RNA sequencing |
| ROS | Reactive oxygen species |
| RT-Qpcr | Quantitative RT-PCR |
| s | Second |
| SCSA | Sperm Chromatin Structure Assay |

| | |
|---|---|
| SD | Standard deviation |
| SNK | Student–Newman–Keuls |
| SOD | superoxide dismutase |
| Spz | Spermatozoa |
| SSC-H | Side scatter |
| SYTOX | SYTOX® Green Nucleic Acid Stain |
| TM | Total motility |
| U/g | Units per gram |
| VAP | Average path velocity |
| VCL | Curvilinear velocity |
| VSL | Straight-line velocity |

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# PREAMBLE

This thesis comprises six main chapters, with chapters 2, 3 and 4 organized according to the format of scientific articles. To enhance the reader's comprehension throughout this dissertation, each chapter begins with an introductory passage outlining the main objectives of its respective content. **Chapter 1** is a general introduction to the Portuguese oyster (*Crassostrea angulata* currently *Magallana angulata*) and the striped venus clam (*Chamelea gallina*) species characterization and importance, bivalve sperm, and larvae cryopreservation and cryodamage assessment methodologies. **Chapters 2 and 3** focus on the development of cryopreservation methodologies for the target species. **Chapter 2** focuses on the optimization of a cryopreservation protocol for *C. angulata* sperm through the supplementation of cryoprotectant solution with sugars (trehalose and sucrose). **Chapter 3** addresses the effect of two freezing solutions that differ in the permeant cryoprotectant (dimethyl sulfoxide and ethylene glycol) in D-larvae prior and post-cryopreservation. In **Chapter 4** a transcriptomic tool was used to understand if the cryodamage in D-larvae is related to exposure to cryoprotectant solution or cryopreservation. Finally, in **Chapters 5 and 6** a general discussion of the results and conclusions of this dissertation is presented.

The trials, methodological approaches, and all the manuscripts included in this thesis were developed, designed, and executed by the author, with the collaboration of students and colleagues.

# CHAPTER 1. GENERAL INTRODUCTION

## 1.1. Bivalve production and the importance of preserve natural populations

Bivalves are an essential fisheries and aquaculture resource in coastal areas with a high contribution to the social and economic activities in those areas (Cardoso et al., 2013). Furthermore, bivalves perform crucial functions in ecosystems since they are filter feeders that generally improve water quality by the removal of inorganic and organic particles, thereby influencing nutrient cycling (Kreeger et al., 2018; Vaughn and Hoellein, 2018). Additionally, they perform a valuable ecosystem service by the fixation of environmental carbon by the shell because have the capacity to synthesise calcium carbonate (Vaughn and Hoellein, 2018). Apart from their ecological value, bivalves are low trophic species and due to their high protein content, vitamins A and D, minerals, and fatty acids (such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)), they represent an excellent source of nutrients for human nutrition and consumption (Orban et al., 2006; Tan et al., 2020a, 2020b).

The European Commission defined the guidelines of sustainable aquaculture production until 2030, focusing on the production of low trophic level species, such as bivalves, in order to improve production, nutrition, environment, and life (FAO, 2022). Altogether, these factors contributed to the continuous increase of bivalve production and the values registered nowadays. In 2020, global mollusc production reached 17.7 million tonnes, with cupped oysters representing 30.7% of global mollusc production (FAO, 2022). In European countries such as France, Spain and Italy, shellfish aquaculture represents between 61.6-75.4% of aquaculture production of aquatic animals (FAO, 2022). Mollusc aquaculture production in Portugal, in 2021, reached 9,120 tonnes, representing 50.9% of the total aquaculture production, with oysters (2,293 tonnes) and clams (3,585 tonnes) being the predominant produced organisms (DGRM, 2022). Simultaneously, molluscs fisheries in Portugal, in 2022, reached 17,895 tonnes, with 1,690 tonnes of clams and 1,449 tonnes of cockles harvested (DGRM, 2022). However, bivalve exploitation is an ancient activity, specifically in Portugal. This activity depends either on harvesting, where clams and cockles from wild populations emerge as the main captures, or on production, with the sector being dominated by clams and oysters

(Oliveira et al., 2013). Due to edaphic-climatic and geographic conditions, the culture of bivalves has great potential in Portugal (Matias, 2013). Nonetheless, most of the bivalve resources reveal signs of depletion due to climate change pressure, anthropogenic impact, and overexploitation. In last years, climate change significantly affected bivalves natural populations and consequently the activities such as fisheries and bivalve production (Oyarzún et al., 2018; Rato et al., 2022). Bivalves are mostly reared in coastal lagoons, which represent their natural environment where the conjugation of factors, such as changes in current dynamics, shifts in salinity and temperature, and oxygen depletion, mainly occur. This led to the mismatch of spawning events between males and females and resulted in a reduction of natural recruitment in some species (Joaquim et al., 2016). The impact of climate change is therefore leading to a pressing necessity to develop mitigation measures, such as to preserve and manage natural populations' genetic resources.

In Europe, there are two bivalve species with high socio-economic potential: the Portuguese oyster (*Crassostrea angulata* currently *Magallana angulata*) and the striped venus clam (*Chamelea gallina*). The banks of natural populations reveal signs of depletion due to some factors mentioned previously such as climate change, pollution and overexploitation, but also due to the fact that some of these species suffer hybridization with introduced species (*C. angulata* x *C. gigas*) (Chiesa et al., 2021; Joaquim et al., 2016). Due to the worrying situation of both species and since these are important commercial species, it is necessary to develop approaches to overcome this loss of resources. Therefore, it is crucial to improve aquaculture production technologies and fisheries management strategies to ensure biodiversity preservation (Anjos et al., 2017; Joaquim et al., 2014). With the aim to preserve these resources, arises the need to create genetic resource banks (GRB), thus providing the chance to preserve representative samples and further reconstruct the original population (Martínez-Páramo et al., 2017). Additionally, there is also the need to develop new technologies for the continued production of larvae in hatcheries.

### 1.1.1. Portuguese oyster – *Crassostrea angulata* (Lamarck, 1819)

The Portuguese oyster, *C. angulata* currently *Magallana angulata*, has a wide distribution, being found in Southern China, Taiwan, Vietnam, Japan, Spain, Morocco and Portugal (Chiesa et al., 2021; Fabioux et al., 2002; Grade et al., 2016; In et al., 2017; Sekino et al., 2016; Vaschenko et al., 2013; Wang et al., 2010). In China, this species is also known as the Fujian oyster and shows a high cultural and economic importance accounting for about 41.2-50% of the total oyster farmed (Di et al., 2020; Wang et al., 2023).

Until the 1970s, the Portuguese oyster, *Crassostrea angulata*, was a key species for the European shellfish industry, however, due to a viral disease, it suffered major mortalities, and its exploitation collapsed. The collapse of natural populations was promoted not only by the pathologies onset, but also by poor remediation and management measures (Batista et al., 2005). To overcome this situation, the Pacific oyster (*Magallana gigas,* formerly *Crassostrea gigas*) was initially introduced in France to support local farming production (Batista et al., 2005; Boudry et al., 1998; Grizel and Heral, 1991). Nevertheless, both species *C. angulata* and *C. gigas* can hybridize, creating a risk to pure populations. Currently, in Europe, only three pure populations of Portuguese oyster exist, namely in the Sado River estuary (Chiesa et al., 2021), Mira River estuary (Fabioux et al., 2002) and Guadalquivir River (Michinina and Rebordinos, 1997). Therefore, it is important to find tools for ensuring the conservation and recovery of these Portuguese oyster populations. There is already knowledge about the biology of *C. angulata*, with several studies in artificial hybridization (Jiang et al., 2021; Soletchnik et al., 2002), biology and physiology (Anjos et al., 2017; Sousa and Oliveira, 1994), pathologies (Batista et al., 2015, 2005) and sperm cryopreservation (Kuo and Gwo, 2022; Riesco et al., 2019, 2017a) areas. However, much work remains to be done to introduce *in vitro* and *in vivo* management measures.

### 1.1.2. Striped venus clam – *Chamelea gallina* (Linnaeus, 1758)

The striped venus clam, *Chamelea gallina,* can be found on the Adriatic coast of Italy (Orban et al., 2006), in the Mediterranean and the Black Sea (Kosyan and Divinsky, 2019; Özden et al., 2009) and on the southern coast of the Iberian Peninsula (Joaquim et al., 2014). Fisheries of striped venus *C. gallina* have high economic importance in Europe, but the natural populations of this species reveal signs of depletion, causing a decrease in the catches (Joaquim et al., 2014). This negative trend can be associated with fishing pressure that, combined with a short lifespan, creates inter-annual fluctuations in stock abundance and periodic recruitment scarcity, in the last decades (Delgado et al., 2013; Joaquim et al., 2016). Therefore, it is necessary to develop measures to preserve this genetic resource. Despite its economic importance, there are few studies concerning this species. These focused mainly on reproductive biology (Bargione et al., 2021; Delgado et al., 2013; Joaquim et al., 2016), population ecology (Dağtekin and Özyurt, 2023), artificial production methodologies in the hatchery (Joaquim et al., 2016) and genetic diversity (Carducci et al., 2020; Öztürk and Altınok, 2021). In this way, additional investigation is required to establish reproductive management methodologies in captivity, aiming to establish production protocols that can support future conservation and rebuild programs for these resources. Other technologies, in particular cryopreservation, can be instrumental in managing reproduction and contributing to the preservation of biodiversity.

## 1.2. Cryopreservation

Cryopreservation can be a helpful tool in reproductive management and for the preservation of biodiversity. This technique allows the storage of valuable genetic lines as endangered species or selective lines (Adams et al., 2008). In aquaculture production, there are several advantages, especially for hatcheries once it can simplify broodstock management (Martínez-Páramo et al., 2017) and allows for extending the running period due to the availability of material after the reproductive season (Cabrita et al., 2010; Hassan et al., 2015). In several species of invertebrates, it is possible to cryopreserve gametes (sperm and oocytes), embryos and larvae (Martínez-Páramo et al., 2017; Paredes, 2015). As previously mentioned,

cryopreservation is a method that can guarantee the genetic profile of native species, but it is necessary to be complemented with the zootecnhological production development of aquatic species. In fact, aquaculture and cryobanking should support management and conservation activities during the restocking and stock enhancement programs (Joaquim et al., 2016; Yang and Huo, 2022).

The fundamental principles of cryopreservation – freezing and thawing – are essential to ensure the success of cryopreservation. The success of the protocol may be compromised because of cellular damage caused by ice crystallization and the toxicity of cryoprotectants used to control ice formation. Therefore, it is necessary to protect the cell, and this can be done by adapting the steps of the cryopreservation protocol according to the type of biological material (gametes, embryos or larvae), being this process species-specific (Martínez-Páramo et al., 2017).

### 1.2.1. Cryoprotectants

#### *1.2.1.1. Permeant and non-permeant*

As in any type of biological material, it is necessary to protect the cells against cool damage during cryopreservation and, for that, cryoprotectants are essential compounds that interact with the inner and outer biological environment. Cryoprotectants counteract ice-induced effects due to damages in water membrane permeability, facilitating continued cellular dehydration at low subzero temperatures and reducing intracellular freezing (Wolkers and Oldenhof, 2021). Moreover, they adjust the cell environment and prevent ice formation by lowering the freezing point of the solution (Ozimic et al., 2023). While these agents protect the cell against ice crystal formation and dehydration, they have an associated level of toxicity (Elliott et al., 2017; Ozimic et al., 2023; Sieme et al., 2016), as mentioned before. For this reason, it is crucial to assess their protective effect versus their toxicity.

There are two types of cryoprotectants, permeating and non-permeating. The first one penetrates the cells and acts inside to prevent intracellular ice formation (Elliott et al., 2017). Examples of these agents include glycerol, dimethyl

sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG) and methanol. The second one remain outside the cells interacting with the plasma membrane, and, due to its high viscosity, interfere with the physical and chemical properties of the extender solution (Fuller, 2004; Tsai et al., 2018). Additionally, they can greatly improve outcomes when used as an additive (Raju et al., 2021). Sugars (e.g. trehalose, sucrose and glucose), proteins (e.g. albumin) and synthetic polymers (e.g. Polyvinylpyrrolidone or PVP, Ficoll) are some examples of these second compounds. Also, both categories of cryoprotectants promote a protective vitrification state during freezing, preserving biomolecular and cellular activities (Sieme et al., 2016).

In nature, certain organisms have naturally evolved specific physiological and biochemical adaptations to inhabit cold environments, entering a reversible state of suspended animation (Elliott et al., 2017). These adaptations include the presence of naturally-derived cryoprotectants (e.g. trehalose and glucose) or antifreeze proteins, among others. For instance, Rozsypal et al. (2013) reported that during the winter, the insect *Cydia pomonella* maintains high and relatively constant concentrations of trehalose and proline, explaining its ability to survive at low temperatures. Additionally, Antarctic fishes possess antifreeze proteins (AFPs) in the blood that enable them to survive in subzero temperatures (Devries, 1971). These lessons from nature have been adopted and applied with success in several works, where their capacity as cryoprotectants or cryoprotectant additives was explored (Hassan et al., 2017a; Labbé et al., 2018; Zilli et al., 2014). Sugars have been shown as effective cryoprotective agents, mitigating cryoinjuries due to their dual action of 1) interacting with the plasma membrane and 2) creating a protective environment through their high viscosity. These agents are large molecules that typically interact with the lipid bilayer during the cooling phase to maintain plasma membrane integrity when cells undergo dehydration (Tsai et al., 2018). Additionally, sugars can interfere with the colligative properties of the cryoprotectant solution, creating a highly viscous environment, where the surrounding of the cells is stabilized, protecting the plasma membrane from cold damage (Nicolajsen and Hvidt, 1994; Woelders et al., 1997).

According to Hassan et al. (2015), the combination of permeant and non-permeant cryoprotectants can improve sperm viability by acting as a membrane

fluidity regulator and by increasing the membrane hydrophobicity. Furthermore, when sugars are combined with permeant cryoprotectants, they can promote cellular dehydration by replacing the water in the membrane. This supplementation with sugars also reduces the exposure time, as well as the concentration of the permeating agent, which tends to be more harmful (Tsai et al., 2018). The freezing solution can be composed of one or several cryoprotectant agents, preferably combining physiological salts, nutrients, buffering components, permeating and non-permeating cryoprotectants, antioxidants, and scavengers (Wolkers and Oldenhof, 2021).

Cryoprotectants play a fundamental role during cryopreservation, but they can also induce certain levels of toxicity. For this reason, it is important to evaluate biological material previous to freezing through cryoprotectant exposure experiments.

#### *1.2.1.2. Cryoprotectant exposure experiments*

Cryoprotectant exposure trials, commonly referred to as toxicity tests in the field of cryopreservation, are experimental procedures conducted to assess the impact of cryoprotectant solutions on the normal functions of biological material, such as cells or complex organisms. As mentioned before, cryoprotective agents are substances utilized to safeguard cells and tissues during cryopreservation but can also have toxic effects on the biological material (Hassan et al., 2015; Simon and Yang, 2018). during pre-freezing and post-thawing (Chao and Liao, 2001), especially when used in high concentrations. In this way, it is crucial to understand the level of toxicity associated with the cryoprotectant in use, as this agent has the function of protecting the cells without compromising its functionality (Elliott et al., 2017). Therefore, the toxicity tests are a crucial preliminary step to ensure that the functionality and structural integrity of biological material is not compromised by the cryoprotectant toxicity prior to the freezing and thawing steps.

The adjustment of the cryoprotectant type and concentration is imperative according to the species and/or biological material type. Understanding how potential cryoprotectant agents interact with the biological material is key to optimizing cryopreservation protocols (Raju et al., 2021). Generally, toxicity tests involve exposing biological samples to various types and concentrations of

cryoprotectant agents and assessing their impact. This pre-trial plays a crucial role in the selection of the cryoprotective agents and their respective concentrations, as well as determining the duration for which biological materials should be exposed to these agents without causing irreversible harm (e.g. Heres et al., 2019; Riesco et al., 2017; Simon & Yang, 2018). A longer exposure to the cryoprotectant solution might be required in cell with low permeability to ensure adequate penetration (Raju et al., 2021). Furthermore, these tests allow to save time and effort for the following steps of freezing/thawing (Yang and Huo, 2022).

There are some strategies to mitigate or neutralize the toxic effects of cryoprotectants, such as 1) supplementation of cryoprotectant solution by combining different permeant and/or non-permeant agents to reduce the overall toxicity; 2) replacement of the agents of cryoprotectant solution by less toxic compounds; or 3) stepwise addition or gradually increasing cryoprotectant concentration; 4) removal by washing out the cryoprotectant solution after thawing (Awan et al., 2020; Best, 2015).

In summary, the selection of an appropriate cryopreservation solution relies on a delicate balance between toxicity tolerance and cryoprotection (Hassan et al., 2015; Simon and Yang, 2018). Ideally, toxicity tests are conducted first to identify and eliminate the most harmful cryoprotective agents. Once have been qualified in the toxicity test, the selected cryoprotective agents must also demonstrate their ability to suppress ice crystal formation without promoting osmotic damage, ensuring their overall effectiveness in the cryopreservation process. Herein, temperature exposure plays also an important role since the toxicity of some cryoprotectants increases at high temperatures (approximately 22°C) compared with low temperatures (4°C or even less) (Bojic et al., 2021).

### 1.2.2. Freezing and thawing conditions

To establish a cryopreservation protocol with successful outcomes, it is important not only to select the appropriate type and concentration of cryoprotectants but also to consider the freezing and thawing conditions. Freezing is the process that transforms liquid water into ice crystals, yielding different types

of injuries that could be lethal to the biological material (Wolkers and Oldenhof, 2021). The formation of extracellular ice can cause mechanical damage in the cell membrane and lead to cell dehydration above the tolerance limit, while intracellular ice can injure intracellular structures (Bryant et al., 2023). To avoid the previously mentioned constraints during the cooling process, it is necessary to remove the water or prevent ice formation in the biological material. To achieve this, two approaches are commonly employed: slow freezing and vitrification.

Slow freezing cryopreservation is a standard method that promotes cells to lose intracellular water through the gradual reduction of the temperature while exposed to low concentrations of cryoprotectant solution, to prevent intracellular ice formation (Wolkers and Oldenhof, 2021; Yang and Huo, 2022). The extent and rate of cellular dehydration during freezing are influenced by the cooling rate which can assume a range of values from 0.1 to 50 °C/min (Wolkers and Oldenhof, 2021) and can be composed of single or multiple steps of cooling (Yang and Huo, 2022). The cooling rate has an important role in cell survival according to the "Two Factor Hypothesis" suggested by Mazur et al. (1972). In cells undergoing slow cooling the death can be attributed to the toxic effects of the solute, whereas those cooled too fast die due to intracellular ice formation. Taking these factors into account, a successful cooling rate needs to be slow enough to allow intracellular water movement across the plasma membrane, reducing intracellular ice formation, but fast enough to minimize cryoprotectant exposure time, which generally has an inherent level of toxicity (Tiersch et al., 2007). The optimal cooling rate differs according to cell size and permeability (Mazur, 1977, 1963), so the challenge is to find the cooling velocity that maximizes the cryosurvival (Mazur, 1984), particularly when we are working with whole organisms composed of multiple cell types and tissues, such as embryos and larvae. For example, cryopreservation protocols for molluscan sperm rely on faster cooling rates composed of a single rate (Hassan et al., 2015), whereas methodologies for embryos and larvae are stepwise protocols which can include different cooling velocities that are slower rather than those used for sperm (Yang and Huo, 2022). Other factors that can impact the selection of optimal cooling rate are the type and concentration of cryoprotectant solution and packaging container characteristics (volume, shape and material) (Mazur, 1977).

Vitrification or ice-free cryopreservation directly passes the samples to a stable, glass-like state, suspending chemical reactions (Bojic et al., 2021; Bryant et al., 2023). Throughout the process, samples solidify without ice crystal formation due to extremely high cooling velocities and elevated cryoprotectant concentrations (Wolkers and Oldenhof, 2021). The challenge is to ensure similar mass and heat transfer while reducing cryoprotectant harmfulness (Fahy and Wowk, 2015). Vitrification has proven to be a suitable method in embryos and many types of mammalian cells (Rienzi et al., 2017), but no successful results have been reported for molluscan larval cryopreservation (Acosta-Salmón et al., 2007; Adams et al., 2004; Chao et al., 1997). However, it is also not an approach with practical potential for bivalve aquaculture because it is usually performed in a microliter level volume to achieve an ultra-fast cooling rate (Yang and Huo, 2022), which becomes time-consuming when dealing with for example 50 million oyster offspring to freeze and store.

To achieve the desired temperature, which should be around -196°C, for long-term storage different freezing technologies or devices can be used. These can be categorized as non-programmable and programmable systems (Liu et al., 2015). Non-programmable methods include passive coolers, placement on dry ice, suspension in liquid nitrogen vapour and electric ultrafreezers. Passive cooling systems represented by Mr. Frosty® and CoolCell® are freezing containers used in combination with a -80°C freezer. These systems are widely used for the cryopreservation of cell lines, being a practical option when intended to freeze small sample numbers, with low cost and low footprint (Kilbride and Meneghel, 2021). Cryopreservation employing dry ice and liquid nitrogen is inexpensive, does not require expensive equipment, and is practical for field use (Martínez-Páramo et al., 2017). Freezing in liquid nitrogen vapour involves placing samples in a tray floating over the liquid nitrogen surface inside of a styrofoam box, where the distance of the samples to the liquid nitrogen surface determines the cooling rate (Liu et al., 2015). This method is commonly used due to its simplicity and effectiveness, low equipment cost, and the ability to process a larger number of samples (Hassan et al., 2015; Liu et al., 2015). Nevertheless, to be repeatable it is necessary to report centimetres over the liquid nitrogen surface, the time of suspension and the exact volume of the samples (Paredes, 2015). Electric ultrafreezers have been raised as an

alternative method to conventional freezing in liquid nitrogen once are more cost-efficient (Diogo et al., 2018) when compared with programmable equipment.

Programmable methods involve special equipment to control cooling rates, with or without the resource of liquid nitrogen (Kilbride and Meneghel, 2021; Liu et al., 2015). These devices, commonly referred to as biofreezers, controlled-rate freezers or programmable freezers, provide controlled, accurate and reproducible cooling (Kilbride and Meneghel, 2021; Martínez-Páramo et al., 2017). Also, it allows the application of very complex freezing rates including two or multiple-step freezing rates and holding periods (Herráez et al., 2012). Although, this involves high costs to acquire and maintain the equipment especially when they consume liquid nitrogen, the availability of infrastructure and technicians can be an issue.

Overall, the freezing method needs to be straightforward, reliable and reproducible, following available infrastructures and resources and always taking into account the best post-thawing outcomes.

In bivalves the available protocols to freeze gametes, embryos and larvae generally rely on the use of a slow cooling approach (Demoy-Schneider et al., 2020; Hassan et al., 2015; Paredes, 2015; Yang and Huo, 2022), applying both programmable and non-programmable systems for sperm (e.g. Demoy-Schneider et al., 2018; Hassan, Li, & Qin, 2017; Hassan, Li, Liu, et al., 2017; Riesco et al., 2017)and programmable for embryos and (e.g. Heres et al., 2021; Labbé et al., 2018; Suquet et al., 2014).

As mentioned before, cryopreservation relies on the use of deep low temperatures to suspend the vital functions of the biological material and recover it later (Wolkers and Oldenhof, 2021). Therefore, after exposure to the cryoprotectant solution and freezing, storage and thawing becomes necessary. Storage can be for short or extended periods in liquid nitrogen tanks or ultrafreezers to prevent time-sensitive cellular degradation (Kilbride and Meneghel, 2021). After thawing, which is a critical step, the vital functions of the cryopreserved biological material need to be recovered. Rapid thawing is necessary to prevent recrystallization (Suquet et al., 2000), being the warming duration and temperature inversely correlated (Hassan et al., 2015). Thawing is usually performed either on air or in a water bath (Paredes,

2015). These protocols can adopt some procedures to washout the cryoprotectant solution to avoid their toxicity and prevent osmotic shock. Other ultra-fast techniques, such as laser warming (Daly et al., 2018; Jin et al., 2014), nanowarming (Manuchehrabadi et al., 2017), and electromagnetic heating (Luo et al., 2006), have arrived for the thawing of vitrified samples, but still representing a technological challenge when several samples need to be processed at the same time, especially under production conditions.

The way cells and biological material are frozen and thawed affects how well they make it through the deep freeze and come back to life. Freezing and thawing conditions must be optimized according to the type and complexity of the biological material, in order to avoid any damage by crystallization. The seamless integration of the freezing and thawing conditions with the right cryoprotectant solution is essential for establishing effective cryopreservation protocols.

### 1.2.3. Bivalve cryopreservation

The cryopreservation knowledge for marine invertebrates has increased in the last decades (Paredes, 2015), being reported several methodologies for gametes and larvae of different species, namely corals (Daly et al., 2018), bivalves (Heres et al., 2023; Riesco et al., 2019) and sea urchins (Lago and Paredes, 2023). Until now, more than 80 scientific reports have been published on sperm and larvae from several bivalve species (Martínez-Páramo et al., 2017; Paredes, 2015; Yang and Huo, 2022). The majority of the works published in this area, focused on the cryopreservation of gametes and larvae of *C. gigas* (Labbé et al., 2018; Paredes, 2015), being sperm the most widely studied parameter (Hassan et al., 2015). Nevertheless, the cryopreservation of bivalve larvae seems to have a huge potential due to the availability of diploid organisms immediately after thawing (Labbé et al., 2018; Suquet et al., 2014). Moreover, larval cryopreservation allows the storage of progeny genetic material, that is particularly advantageous when cryopreservation protocols are not fully developed for gametes of both sexes (Paredes et al., 2021), which is the case of most bivalve species. However, it can also be especially difficult

since bivalve embryos and larvae of bivalves have complex internal structures and present different stages of development and metamorphosis (Helm et al., 2004).

So far, knowledge about cryopreservation methodologies for *Crassostrea angulata* and *Chamalea gallina* is scarce. There are few studies about *C. angulata* sperm cryopreservation, where authors established the first protocols (Kuo and Gwo, 2022; Riesco et al., 2017a) and identified cellular and molecular tools to assess the sperm cryodamage (Riesco et al., 2019). Regarding larvae, there are no reports on both species, thus it is crucial to deepen investigate methods and tools, to successfully preserve both species and identify the principal damage.

Cryopreservation requires the selection of specific parameters according to the species and type of biological material (Martínez-Páramo et al., 2017). The specific parameters included extenders, cryoprotectant types and concentrations, development stage, concentrations of biological material in straws, freezing rate, and thawing conditions (Labbé et al., 2018). The correctness of procedures will allow the establishment of successful protocols to store *in vitro* the genetic material using cryobanks to further rebuild the natural banks of these two species.

### 1.2.4. Tools to evaluate cryodamage in sperm and larvae

One of the major challenges of cryopreservation is to prevent cryodamage to biological structures (plasma membrane, mitochondria and chromatin) and cell functions from a molecular and biochemical point of view (Cabrita et al., 2010; Sieme et al., 2016). Osmotic and thermal stress, intracellular ice crystallization, high levels of reactive oxygen species (ROS) and an imbalance in the antioxidant defence system are some examples of consequences that may compromise the cryopreserved material quality (Amidi et al., 2016; Yánez-Ortiz et al., 2022). Pini et al. (2018) define sublethal damage as any non-fatal biochemical or physiological alteration induced by cryopreservation, however, it is this type of damage that underlies significant fertility challenges by spermatozoa. This brings the necessity to evaluate sublethal cryodamage by developing quality assessment tools. Nevertheless, to date, there are no universal quality biomarkers for sperm and larval quality evaluation. Therefore, it is advisable to use a combination of different

analytical methods to evaluate the post-thaw quality (Cabrita et al., 2010; Yang and Huo, 2022).

The analysis of post-thaw sperm quality in bivalves has been focused mainly on sperm motility, plasma membrane integrity, and fertilization success (e.g. Hassan, Li, & Qin, 2017; Hassan, Li, Liu, et al., 2017; Horváth et al., 2012; Riesco et al., 2017; Suquet et al., 2016; Vitiello et al., 2011). However, few studies investigate the specific sperm structure and functions that are being damaged, through lipid peroxidation (Riesco et al., 2019), DNA fragmentation (Gwo et al., 2003; Kuo and Gwo, 2022; Riesco et al., 2019), acrosome integrity (Kuo and Gwo, 2022; Liu et al., 2016), mitochondrial activity (Demoy-Schneider et al., 2018; Kuo and Gwo, 2022; Paniagua-Chávez et al., 2006), ATP content (Demoy-Schneider et al., 2018; Suquet et al., 2016), morphology (Demoy-Schneider et al., 2018; Kuo and Gwo, 2022; Suquet et al., 2016), oxidative stress (Kuo and Gwo, 2022) and gene expression (Riesco et al., 2019), marking an emerging trend in recent research for bivalves.

Motility is the most used quality assessment method (Boulais et al., 2019; Cabrita et al., 2010), since it is essential for the sperm to reach the oocyte and for fertilization to occur (Boulais et al., 2019). Spermatozoa motility, as in other species, is commonly affected by cryopreservation, producing a reduction of all motility kinetic parameters post-thawing (Hassan et al., 2015; Riesco et al., 2017a), which is mostly likely a consequence of cryodamage. Overall, these methodologies promote a relevant reduction of sperm motility (10 - 50%) in bivalves (Demoy-Schneider et al., 2018; Kuo and Gwo, 2022; Riesco et al., 2017a; Yang et al., 2021). Therefore, the optimization of the cryoprotectant solutions and the methodology of cryopreservation are essential to improve sperm motility and overall quality. The evaluation of sperm motility can be performed through observation, based on qualitative measurements, or through automated systems. The use of computed assisted sperm analysis (CASA) systems allows the precise quantification of sperm motility according to several parameters namely total motility (TM, %), progressive motility (PM, %), straight-line velocity (VSL μm/s), curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s) and linearity (LIN, %) and leads to more reproducible results across studies (Rurangwa et al., 2004).

In many bivalve cryopreservation studies, sperm motility is typically assessed manually through visual observation on a microscope slide using a phase-contrast objective coupled with a microscope, involving the distinction between motile and non-motile spermatozoa to estimate its proportion (Horváth et al., 2012; Yang et al., 2021). Alternatively, some researchers applied a classification-based approach to categorize sperm motility (Gwo et al., 2003; Vitiello et al., 2011). Both visual methods are subjective, and obtaining feasible results requires operators with extensive training and clear criteria (Hassan et al., 2015). However, for a more precise and reliable assessment, the CASA system is preferred over the other methods for evaluating movement (Demoy-Schneider et al., 2018; Riesco et al., 2017a).

To assess the functional integrity of bivalve spermatozoa, other methods can be employed to quantify or qualify damage associated with specific structures such as the plasma membrane, acrosome, mitochondria and chromatin (Xin et al., 2020). Plasma membrane integrity or viability is a conventional technique to assess if the sperm plasma membrane is intact or compromised. The sperm plasma membrane serves as a primary barrier to protect against external injuries and respond to physiological fluctuations (Hossain et al., 2011). Since this semipermeable barrier is rich in polyunsaturated fatty acids (PUFA), it is more susceptible to osmotic and temperature stress factors, making it a crucial factor to be evaluated (Cabrita et al., 2010; Sieme et al., 2016). Several studies in bivalves employ cell viability as a quality indicator of the post-thaw sperm, reporting an increase in membrane permeability following the freezing/thawing process (Hui et al., 2011; Liu et al., 2016; Smith et al., 2012b). The assessment of plasma membrane integrity in sperm is accomplished by applying fluorescent probes, using either a single staining (e.g. Propidium Iodide - PI) (Riesco et al., 2019)or a double staining (e.g. PI and Syber-green) (Liu et al., 2016; Paniagua-Chávez et al., 2006; Smith et al., 2012b; Yang et al., 2021). Overall, the percentage of viable cells can be estimated by counting the dead/live cells in a fluorescence microscope (Hassan et al., 2017a, 2017b; Liu et al., 2016; Riesco et al., 2017a), which is time-consuming. Alternatively, high throughput equipment such as the flow cytometer can be used, having the ability to analyze thousands of cells per second and provide objective results (Paniagua-Chávez et al., 2006; Riesco et al., 2019; Yang et al., 2021). Flow cytometry can also be employed to analyze the post-

thaw sperm components related to acrosome integrity, mitochondrial activity and DNA fragmentation, through the application of specific fluorescent probes (Hassan et al., 2015; Hossain et al., 2011).

The acrosome is a membranous organelle derived from the Golgi apparatus that covers the anterior portion of the sperm head. The reaction induced by this organelle is crucial to the fertilization process since the release of acrosomal enzymes allows the penetration of spermatozoa into the egg. Like other invertebrate species, *C. angulata* and *C. gallina* also possess an acrosome (Erkan and Sousa, 2002; Sousa and Oliveira, 1994) and its damage could be one of the causes behind low fertilization rates. In bivalves, two studies evaluated the status of this organelle in fresh and post-thaw sperm of *C. angulata* and *Mytilus galloprovincialis* and reported that the acrosome integrity was affected by the cryopreservation, as well as the fertilization performance of *C. angulata* (Kuo and Gwo, 2022; Liu et al., 2016).

Mitochondria is one of the important organelles that spermatozoa possess that can be affected by cryopreservation procedures (Kuo and Gwo, 2022). The activity of mitochondria can be assayed using direct methods such as mitochondrial membrane potential, mitochondrial respiration or indirect ones by quantifying the energy content (adenosine triphosphate - ATP), necessary for motility activation (Boulais et al., 2015; Demoy-Schneider et al., 2018). In bivalves, Paniagua-Chávez et al. (2006) evaluated the post-thaw mitochondrial membrane potential through flow cytometry and found a significant correlation between fertilizing ability with cryoprotectant solutions and mitochondrial membrane potential. In turn, Demoy-Schneider et al. (2018) assessed the mitochondrial respiration and the ATP content in *Pinctada margaritifera* post-thaw sperm and reported a significant reduction in oxygen levels and ATP concentration.

The single-cell gel electrophoresis or comet assay is a classic method to measure deoxyribonucleic acid (DNA) strand breaks that are usually employed in sperm cryopreservation studies across different organisms (Cartón-García et al., 2013; Erraud et al., 2021; Riesco et al., 2017c). Ensuring DNA integrity is a crucial aspect of cryopreservation protocol development, to safeguard that spermatozoa fulfil their primary purpose of securely transmitting paternal genetic information to the next generation, thus preventing embryo abortion or malformations (Cabrita et

al., 2010; Figueroa et al., 2020). Several methods can be used to detect DNA damage. Riesco et al. (2019) used the method of the comet assay, whereas (Kuo and Gwo, 2022) used Sperm Chromatin Structure Assay (SCSA) to assess the DNA integrity between fresh and cryopreserved sperm from *C. angulata*, and no significant alterations were reported. Conversely, Smith et al. (2012) reported significant DNA damage in sperm as a result of cryopreservation for *Perna canaliculus*, through the use of the SCSA method.

Other quality parameters that have demonstrated their significance as tools to evaluate damage in cryopreservation studies include reactive oxygen species (ROS) determination and apoptosis detection, lipid peroxidation, and antioxidant enzymes activity (Martínez-Páramo et al., 2012; Riesco et al., 2017b; Sandoval-Vargas et al., 2021a). These parameters play an important role since they can help to detect damage that compromises the spermatozoa structures and functions previously mentioned (e.g. viability and acrosome), and consequently sperm quality of the cryopreserved material. This type of damage involves osmotic and thermal stress, elevated levels of ROS, and an imbalanced antioxidant defence system (Martínez-Páramo et al., 2012; Riesco et al., 2017b; Sandoval-Vargas et al., 2021a). ROS are molecules produced to protect the cell and regulate signal pathways (Ighodaro and Akinloye, 2018) and exist naturally in sperm and larvae of bivalves. However, when an imbalance of ROS occurs, in this case provoked by cryopreservation, several cell structures containing lipids, proteins or enzymes, and chromatin may be affected by oxidative stress (Sandoval-Vargas et al., 2021b), which could result in lipid peroxidation, protein denaturation and DNA fragmentation. To counteract this possible unbalance, sperm has several mechanisms to control ROS production usually by using molecule scavengers. The enzymes superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPX) constitute a group of antioxidant enzymes, present in sperm and larvae that enable the maintenance of homeostasis and prevent oxidative damage (Genard et al., 2011; Ragg et al., 2019; Sandoval-Vargas et al., 2021b). Thus, high levels of superoxide ions can be metabolized by SOD enzyme, which catalyzes the dismutation of these molecules to form oxygen and hydrogen peroxide, while CAT and GPX enzymes convert the hydrogen peroxide into water and oxygen (Amidi et al., 2016). Therefore, glutathione reductase (GR) regenerates

reduced glutathione from its oxidized form, which is required for GPX operation. Any changes in the activity of the antioxidant enzymes, such as freezing/thawing events, make the biological material more susceptible to oxidative stress or less prepared to counteract any oxidative process.

The interconnection of ROS levels, lipid peroxidation, and antioxidant enzymes activity points out the significance of a comprehensive evaluation of these factors. For example, Zhu et al. (2017) reported a reduction in levels of ROS and MDA, along with an increase in the enzymatic activity, in rabbit sperm when sugars were incorporated in freezing media. This example emphasizes the importance of considering multiple parameters in cryopreservation studies, as adjustments in freezing conditions can affect the delicate balance between oxidative stress and antioxidant defences. In bivalves' sperm cryopreservation, few studies explore these quality parameters (Kuo and Gwo, 2022; Liu et al., 2022a, 2022b; Riesco et al., 2019). For instance, in *C. angulata* sperm, Kuo & Gwo (2022) reported substantially higher oxidation levels in the frozen-thawed sperm. Riesco et al. (2019) evaluated the level of lipid peroxidation by determining the malondialdehyde (MDA) content in *C. angulata* and *C. gigas* sperm before and after cryopreservation. The authors reported no differences in sperm before and after cryopreservation in *C. angulata*; however, in *C. gigas*, there was a significant increase in the levels of thawed sperm.

In the case of bivalve larvae, the assessment is more challenging than sperm because of their complex internal structure, making it more difficult to identify which organelles or cells are affected by cryopreservation. Therefore, quality analysis is mostly focused on a global evaluation of the whole organism. For example, the most applied parameters to evaluate post-thaw quality are swimming activity, survival and development status over time (Suquet et al., 2012; Yang and Huo, 2022). Few studies investigated how larvae structures are affected during cryopreservation, throughout organogenesis and neurogenesis (Rusk et al., 2020; Suneja et al., 2014) and explore the cellular and molecular changes induced by the freezing and thawing process (Liu et al., 2022a, 2022b).

Oyster larvae also present movement that can be assessed using the same criteria as sperm motility, particularly in trochophore and D-larvae stages (Suquet et al., 2012; Yang and Huo, 2022). The prevailing method for characterizing post-

thawing larvae motility or swimming activity is the estimation of motile larvae by counting them over the total observed larvae (Heres et al., 2020; Labbé et al., 2018). However, Suquet et al. (2012) used average path velocity (VAP) determined by CASA software to establish an early and reliable estimation method. As reported for sperm, cryopreservation of larvae also induces a reduction of larval motility due to cryodamage (Heres et al., 2020; Labbé et al., 2018; Suquet et al., 2012).

There are methods to evaluate both individual cells and early-stage organisms such as larval stages. Morphological studies that describe or compare the characteristics of fresh and post-thawed sperm and larvae offer invaluable insights into the impact of cryopreservation on their structural integrity (Kuo and Gwo, 2022; Suneja et al., 2014). Cryodamage, in turn, can compromise the biological material functionality, including aspects such as sperm motility, fertilization capacity, and larval developmental potential due to structural damage inflicted, namely in the tail, acrosome and velum, respectively. The morphology of sperm and larvae has been observed using light microscopy (Espinoza et al., 2010; Rusk et al., 2020) scanning electron microscopy (Espinoza et al., 2010; Rusk et al., 2020) and transmission electron microscopy (Demoy-Schneider et al., 2018; Kuo and Gwo, 2022; Suquet et al., 2016), sometimes complemented with other tools such as fluorescent probes to label specific components of interest(Rusk et al., 2020) or morphology analysis systems for measurements (Demoy-Schneider et al., 2018; Suquet et al., 2012). The parameters most affected in sperm after thawing are typically the head, acrosome, and tail (Demoy-Schneider et al., 2018; Espinoza et al., 2010; Kuo and Gwo, 2022; Kurokura et al., 1990). When the size of these structures is measured, they are generally smaller than when they are fresh (Demoy-Schneider et al., 2018). In the case of the larvae, an Automated Sperm Morphology Analysis system (ASMA) is available to measure the morphological parameters (Suquet et al., 2012). Indeed, other techniques are being applied such as size and appearance of the shell, or organogenesis assessment to identify abnormalities in shell morphology and swimming and feeding structures (Rusk et al., 2020; Suneja et al., 2014).

Fertilization and hatching rate are widely applied to evaluate the performance of post-thaw sperm (Hassan et al., 2015), since the ultimate criterion

hinges on the sperm's competence to fertilize eggs and ensure the survival of larvae (Hassan et al., 2017b). In general, thawed sperm has a lower fertilization capacity than fresh sperm. Although fertilization and hatching rates reflect the sperm's ability to fulfil its objective, they need to be complemented with additional evaluation parameters to have a comprehensive understanding of its overall performance and the factors affecting its success during cryopreservation. Concerning embryos and larvae, the ultimate goal is long-term survival (Yang and Huo, 2022). It is generally reported as the development performance and survival over time.

## 1.3. Omics-based approaches for assessing cryodamage

The emerging methodologies of “omics” have been employed to predict sperm quality through the identification of potential molecular and protein indicators. In the cryobiology field, transcriptomics emerges as a helpful tool in the identification of different freezability biomarkers and in understanding the molecular consequences associated with freezing and thawing steps. In transcriptomics, the use of RNA sequencing (RNA-seq) has been widespread to investigate the underlying cryopreservation mechanism in spermatozoa, blastocyst, and larvae from mammals (e.g. Fraser et al., 2020; Larman et al., 2011) and fish species (H. Wang et al., 2022; Yang et al., 2019; Zhang et al., 2022), to elucidate about the molecular changes, in a global point of view, that occur during the cryopreservation process. For example, Wang et al. (2022) evaluated the impact of cryopreservation on the fish sperm *Ictalurus punctatus* by integrating sperm quality parameters with transcriptomic profiling. The authors reported increased oxidative stress and DNA fragmentation in cryopreserved sperm, impacting motility kinematic parameters. Transcriptome analysis revealed increased oxidative phosphorylation and compensatory gene changes to control ROS production and correct or remove affected proteins in the post-thaw fish sperm. Their approach provided a comprehensive understanding of the molecular mechanisms underlying cryoinjury and sperm response. Zhang et al. (2022), suggested that in cryopreserved

*Epinephelus moara* larvae, the impairment of the development of the larvae's central nervous system is due to the suppression of genes related to eye development, cranial nerve development, light sensory stimulation and neurotransmitter transport.

Likewise, in the field of cryobiology, proteomic and metabolomic analyses have both showcased their potential. Rusco et al. (2022), compared the entire proteome of fresh and cryopreserved rabbit semen, revealing that numerous proteins were cryodamaged, making the cell more prone to stressors during cryopreservation. Other authors used a metabolomic approach to evaluate the effect of trehalose on buck semen cryopreservation (Xu et al., 2022). The authors reported that trehalose improves plasma membrane integrity and motion in thawed sperm, protecting by altering amino acid synthesis and glycerol metabolism. An integrative approach using transcriptomics and proteomics was also used to evaluate the regulatory network of RNA and protein in response to cryopreservation stress in sheep semen (Bai et al., 2023). The findings suggested that the downregulation of Fc gamma receptor Ia (FCGR1A), could lead to decreased motility and viability, potentially impacting the fertility of frozen sheep sperm. All mentioned studies suggest that "omics" reveal molecular changes in cryopreservation. Studies across species, types of biological material and cryopreservation protocols contribute to a deeper understanding of cryoinjury mechanisms, guiding improved cryoprotective strategies.

For bivalves, to the best of current knowledge, there are only four studies in which cryodamage has been evaluated from a molecular point of view (Liu et al., 2023, 2022b, 2022a; Riesco et al., 2019). However, only the expression of selected genes has been measured in cryopreserved larvae of *Mytilus edulis* and *C. gigas* (Liu et al., 2023, 2022b, 2022a), as well as for *C. angulata* sperm (Riesco et al., 2019) to assess post-thaw quality. None of these studies used a transcriptomic approach. The application of an RNA-seq tool to assess post-thaw quality in bivalves would allow a deeper understanding of the changes in molecular networks and biological processes induced by cryopreservation.

In conclusion, assessing sperm and larval structures and metabolic pathways through a combination of different techniques can enable a more specific

understanding of the components and functions of sperm and larvae affected by cryopreservation. Understanding and improving cryodamage outcomes in diverse organisms remains a crucial aspect of achieving successful cryopreservation protocols.

## 1.4. Objectives

The present study intends to explore and establish conditions to store and preserve the genetic resources of *C. angulata* and *C. gallina* populations, to develop a future restocking program. The specific objectives of these main goals are:

❖ Optimize the cryopreservation protocol previously established for *C. angulata* sperm and explore assessment techniques to detect damage in the structure and functions of post-thaw sperm to enhance the quality of material stored in the cryobank.

❖ Design specific protocols for the cryopreservation and damage analysis of larvae in *C. angulata* and *C. gallina* with the purpose of creating a cryobank for these species.

❖ Develop a molecular tool to evaluate the larvae susceptibilities to cryopreservation procedures.

❖ Identification and validation of freezability biomarkers.

The accomplishment of these objectives will help answer the following questions: 1) Can we successfully store genetic material to recover the populations? What type of genetic material is possible to store for each species? 2) Is it possible to improve the post-thaw quality of *C. angulata* sperm by supplementating dimethyl sulfoxide with sugars? 3) Is it possible to establish freezing methodologies for the larvae of *C. angulata* and *C. gallina*? and 4) Can cryopreservation susceptibility be predicted using molecular tools? What types of freezability biomarkers are affected by the cryopreservation process?

# CHAPTER 2. SPERM CRYOPRESERVATION PROTOCOL

# PREAMBLE

This thesis targets the development of cryopreservation technologies for threatened species with high potential for aquaculture such as the Portuguese oyster. One of the most important factors during the development of cryopreservation protocols is the design of the cryoprotectant solutions. The combination of permeant and non-permeant cryoprotectants is a successful strategy in cryopreservation to improve post-thaw performance. During the cryopreservation process, structural and functional damage to the sperm may occur, emphasizing the importance of their evaluation. The objective of this chapter was to optimize the cryopreservation methodologies already established for Portuguese oyster sperm by supplementing the permeant cryoprotectant solution with sugars (trehalose and sucrose), while simultaneously developing different methodologies for the assessment of cryodamage.

This chapter allowed the improvement of the current cryopreservation protocols available for Portuguese oyster sperm. Also, allowed an exhaustive evaluation of post-thaw sperm through the application of a wide range of sperm quality analyses. Conventional techniques such as motility and viability, along with other specific tools, namely deoxyribonucleic acid fragmentation, reactive oxygen species levels, apoptosis, acrosome integrity, lipid peroxidation and antioxidant enzymes activities were employed to detect cryodamage related to the components and functions of the sperm. Sugars, especially trehalose, revealed to have a positive effect on the plasma membrane and acrosome integrity and reduce lipid peroxidation and superoxide ions levels.

This chapter's content represents an article published in Frontiers in Physiology, with Catarina Anjos as the first author.

# EFFECT OF TREHALOSE AND SUCROSE IN POST-THAW QUALITY OF *Crassostrea angulata* SPERM

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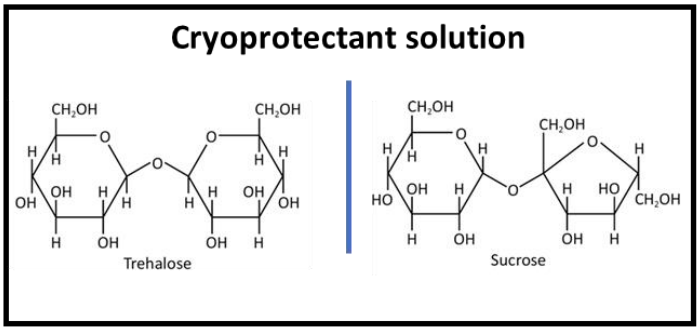


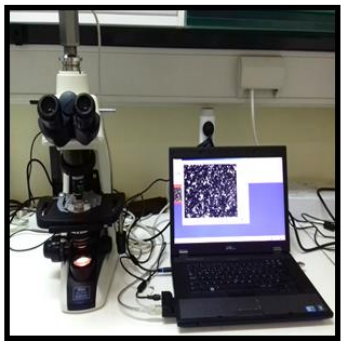

**[Frontiers in Physiology 2021, 12:749735]**

https://doi.org/10.3389/fphys.2021.749735

## 2.1. Abstract

Sperm cryopreservation can be a helpful tool in reproductive management and preservation of biodiversity. However, the freezing methodologies lead to some damage in structure and function of cells that may compromise post-thaw sperm activity. Cryoprotectant supplementation with sugars proved to be a successful strategy to reduce cryodamage in sperm of several species, once allowing to stabilize the plasma membrane constituents. Therefore, this study intends to understand the effects of sugars in the plasma membrane, DNA integrity, and oxidative response during Portuguese oyster sperm cryopreservation. Three cryoprotectants solutions with an initial concentration of 20% dimethyl sulfoxide (DMSO) and 20% DMSO complemented with 0.9 M trehalose or sucrose in artificial seawater were employed. Sperm samples of mature males were individually collected and diluted 1:10 (v/v) in artificial seawater followed by addition of cryoprotectants [1:1 (v/v)]. Thereafter, sperm was loaded into 0.5 ml straws, maintained at 4°C for 10 min, frozen in a programmable biofreezer at -6°C/min from 0 to -70°C, and stored in liquid nitrogen. Samples were thawed in a 37°C bath for 10 s. Several techniques were performed to evaluate post-thaw quality. Sperm motility and DNA integrity were analyzed by using computer-assisted sperm analysis (CASA) software and comet assay. Flow cytometry was employed to determine membrane and acrosome integrity and to detect intracellular reactive oxygen species (ROS) and apoptosis activity. Lipid peroxidation was determined by malondialdehyde (MDA) detection by using spectrophotometry. Sperm antioxidant capacity was evaluated through glutathione peroxidase, glutathione reductase, and superoxide dismutase. Motility was not affected by the extenders containing sugars; these compounds did not reduce the DNA damage. However, both the trehalose and sucrose protected plasma membrane of cells by increasing cell viability and significantly reducing MDA content. The same finding was observed for the ROS, where live cells registered significantly lower levels of ROS in samples cryopreserved with sugars. The activity of antioxidant enzymes was higher in treatments supplemented with sugars, although not significant. In conclusion, the addition of sugars seems to play an important role in protecting the *Crassostrea angulata* sperm membrane during cryopreservation, showing potential to improve the post-thaw sperm quality and protect the cells from cryoinjuries.

## 2.2. Introduction

Bivalves represent an important aquaculture supply, which has led to a great demand for this resource and consequently an increase in its production (Wijsman et al., 2019). Therefore, aquaculture needs to diversify the species produced for a greater offer. Portuguese oyster [*Crassostrea angulata* (*C. angulata*)] is a species with high potential for aquaculture production and market acceptance. It was a key species for the European shellfish industry in the 1970s; however, due to a disease and poor remediation and management measures, the natural populations decreased (Boudry et al., 1998). Nowadays, there are only three places with pure populations of Portuguese oyster, namely, in the Sado and Mira estuary (Fabioux et al., 2002) and the Guadalquivir River (Michinina and Rebordinos, 1997). Therefore, it is important to develop techniques to ensure the conservation and recovery of native populations of the Portuguese oyster, but also to enhance and support their aquaculture production (Anjos et al., 2017; Pogoda et al., 2019; Riesco et al., 2017a).

Cryopreservation methodologies are advantageous tools that may enhance conservation strategies and reproductive technologies. The applicability of sperm cryopreservation involves the maintenance of improved genetic lines or endangered species (Martínez-Páramo et al., 2017; Riesco et al., 2017a). In addition, it allows to extend the breeding season and synchronize spawning between males and females (Hassan et al., 2015). This procedure stores reproductive cells at low temperatures. Thus, one of the major challenges of cryopreservation is to prevent the associated cryodamage involving biological structures (plasma membrane, mitochondria, and chromatin) and functions of the cell in a molecular and biochemical point of view (Cabrita et al., 2010; Sieme et al., 2016). Spermatozoa characteristics make this type of cells very prone to suffer cryodamage, mostly due to the high content of polyunsaturated fatty acids of their membranes (Cabrita et al., 2014). Osmotic and thermal stress, intracellular ice crystallization, high level of reactive oxygen species (ROS), and imbalance in antioxidant defense system are some examples of consequences that may compromise the quality of the cryopreserved material (Amidi et al., 2016). These bring up the necessity to evaluate the lethal and sublethal cryodamage. The analysis of sperm quality in invertebrates has been performed by using several techniques such as motility, membrane

integrity, morphology, DNA integrity, lipid peroxidation, detection of intracellular ROS, mitochondrial membrane potential, and acrosome integrity (Akcha et al., 2012; Gallo et al., 2020, 2018; Le Goïc et al., 2013; Vignier et al., 2017). However, in cryopreservation studies, most research in bivalves applies only motility, plasma membrane integrity, and fertilization as tools to assess post-thaw sperm quality. Only a few works apply specific techniques such as malondialdehyde (MDA) content determination, DNA fragmentation, and acrosome integrity (Liu et al., 2016; Riesco et al., 2019; Smith et al., 2012b). Therefore, it is crucial to establish quality assessment methodologies that are already widely applied to mammals (Gangwar et al., 2020; Öztürk et al., 2020) and marine vertebrates (Riesco et al., 2017b; Sandoval-Vargas et al., 2021a). This will allow to understand more specifically which organelles and sperm functions are affected by cryodamage. By identifying the type of damage, it is possible to outline the strategy to prevent it. One common and successful strategy to prevent cryodamage is the supplementation of freezing media with compounds that protect cells against freezing injuries (Elliott et al., 2017). Antioxidants, proteins, and sugars are some examples of non-permeant cryoprotectants used in supplementation (Diogo et al., 2019; Martínez-Páramo et al., 2012; Riesco et al., 2017c). Sugars are large molecules that act outside of the cells, interacting with the plasma membrane. Due to their high viscosity, they interfere with the physical and chemical properties of the extender solution (Fuller, 2004). Furthermore, these are natural strategies that organisms and plants adopt to survive to adverse conditions (desiccation and freezing) (Gertrudes et al., 2017; Lencioni et al., 2015). Trehalose and sucrose are two disaccharides that have a positive effect during sperm cryopreservation when used as single or combined cryoprotectants in several species such as ram (Öztürk et al., 2020), stallions (Pérez-Marín et al., 2018), boar (Pezo et al., 2020), stone flounder (Lee et al., 2021), Greenshell™ mussel (Smith et al., 2012a), and Australian flat oyster (Hassan et al., 2017a, 2017b). A previous work developed by our group established several steps for cryopreservation protocol of *C. angulata* sperm (type of extender, type and concentration of permeant cryoprotectant, type and sperm concentration in package and freezing rate) (Riesco et al., 2017a). Nevertheless, supplementation of permeant cryoprotectant has not been yet evaluated for *C. angulata*.

This study aims to explore the effect of trehalose and sucrose, when combined with one permeant cryoprotectant as dimethyl sulfoxide (DMSO), on the structure and functions of cryopreserved spermatozoa. Simultaneously, the different cryodamage levels that compromise the quality of the spermatozoa during cryopreservation were evaluated through motility, DNA and plasma membrane integrity, content of ROS, apoptosis activity, acrosome integrity, lipid peroxidation, and antioxidant activity.

## 2.3. Material and methods

### *2.3.1. Reagents*

All the reagents used were acquired from Sigma-Aldrich (Saint Louis, MO, United States), unless otherwise indicated.

### *2.3.2. Sperm collection*

Breeders of *C. angulata* originated from one of the few pure banks of this species were acquired from the bivalve farm Viveiros Rio Mira, Lda (37°37′32.1′′N, 8°41′31.6′′W). Sperm was individually collected from each oyster through gonadal incisions as described by Riesco et al. (2017a) (Figure 2.1A). Sperm was then diluted in artificial seawater in a proportion of 1:10 and filtered with two mesh sizes (20 and 100 mM). Motility and concentration of fresh sperm were evaluated by using a computer-assisted sperm analysis (CASA) system to discard unsuitable samples. Only males with more than 40% of total motility and final concentration values between 1 and 2 × $10^9$ spermatozoa/ml were selected for freezing and thawing steps. A total of 10 sperm samples (n = 10) were collected and used in the following procedures.

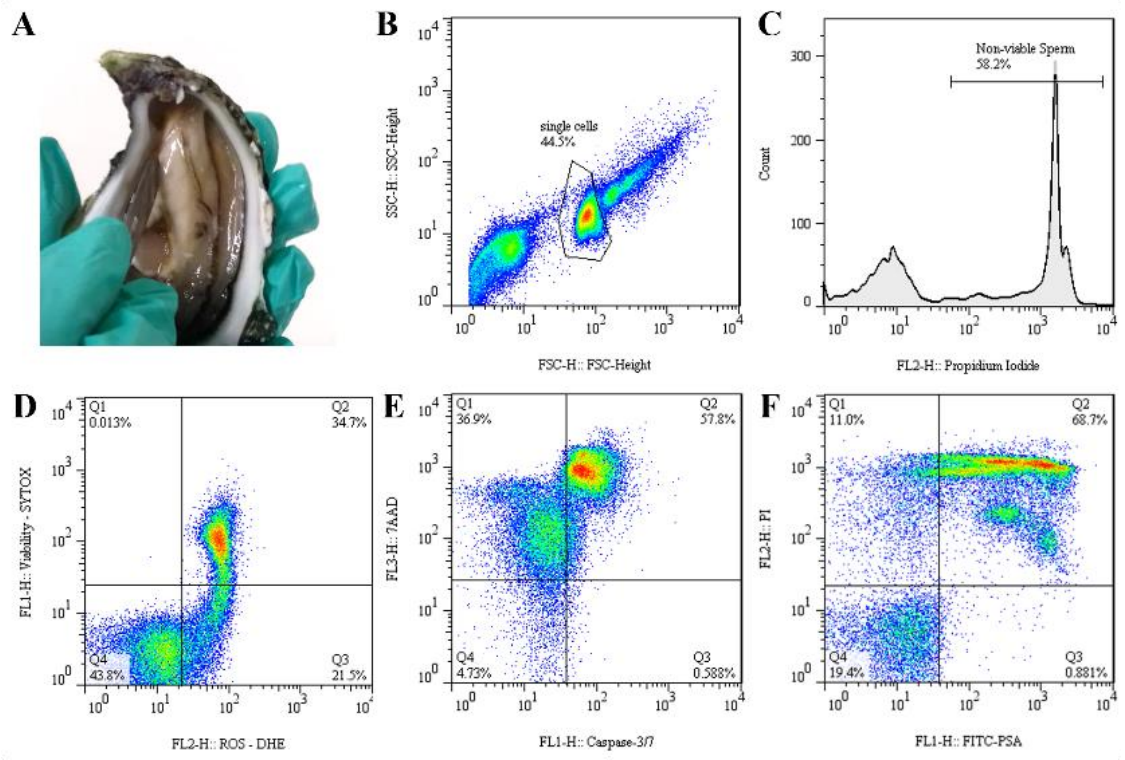


Figure 2.1 Gamete collection and flow cytometry data representation of *Crassostrea angulata* (*C. angulata*) thawed sperm: (A) Gonadal incisions to collect sperm. (B) Forward scatter (FSC-H) and side scatter (SSC-H) dot plot used to identify sperm population. (C) Histogram displaying propidium iodide (PI) fluorescence signal for identifying population with injured membrane. (D–F) Dot plots representing techniques of dual staining to detect (D) reactive oxygen species (ROS) by using the fluorescent probes dihydroethidium (DHE) and SYTOX® Green Nucleic Acid Stain (SYTOX) [dead cells with undetectable levels of ROS (Q1); dead cells with detectable levels of ROS (Q2); live cells with detectable levels of ROS (Q3); live cells with undetectable levels of ROS (Q4)], (E) caspase activity applying caspase-3/7 reagent with 7-aminoactinomycin D (7-AAD) [necrotic (Q1), late apoptotic (Q2), early apoptotic (Q3) and live (Q4) cells] and (F) acrosome integrity by fluorescein isothiocyanate-labeled Pisum sativum agglutinin (FITC-PSA) and PI labeling [dead cells, acrosome intact (Q1); dead cells, acrosome reacted (Q2); live cells, acrosome reacted (Q3); live cells, acrosome intact (Q4)].

### *2.3.3. Cryopreservation procedures*

Sperm samples were cryopreserved with three treatments that differ in the composition of the cryopreservation solutions. Thus, cryoprotectant solutions were composed by 20% (v/v) DMSO that was defined as control or 20% (v/v) DMSO supplemented with 0.9 M (w/v) trehalose or sucrose (DMSO + Trehalose or DMSO + Sucrose, respectively). The concentration of sugars was established according to Adams et al. (2004) and Hassan et al. (2017a) for sperm cryopreservation of *Crassostrea gigas* and *Ostrea angasi*, respectively. All the solutions were prepared freshly in artificial seawater and maintained at 4 °C. Each sample was mixed by using

one part of prediluted sperm with one part of each cryoprotectant solution (1:1 with a final concentration of 10% DMSO and 0.45 M trehalose or sucrose) followed by equilibration of 10 min. During this time, 0.5 ml French straws were filled with diluted sperm. The straws were subjected to a freezing rate of -6°C/min from 0 to -70°C in a programmable biofreezer (Asymptote EF600, Grant Instruments Ltd., Cambridge, United Kingdom), being then immersed into liquid nitrogen to be stored in a cryobank until further analyses. Sperm thawing was performed in a bath at 37°C for 10 s, immediately before the sperm quality assessment.

### *2.3.4. Post-thaw sperm quality assessment*

Sperm function or status was determined by conventional techniques such as motility and viability. However, more specific techniques (DNA fragmentation, detection of ROS and apoptosis, acrosome integrity, lipid peroxidation, and antioxidative defense) were determined to understand the extent of cryodamage and how cryoprotectant solutions applied in this trial (DMSO and DMSO supplemented with trehalose and sucrose) protect the sperm during freezing/thawing steps.

#### *2.3.4.1. Motility*

Computer-assisted sperm analysis system composed by ISAS software (ISAS, Proiser R + D S.L., Paterna, Valencia, Spain) was used to evaluate total sperm motility after thawing. For this, 10 ml of sample were loaded in a Makler chamber and movement was recorded with a video camera (ISAS 782C, Proiser R + D, S.L., Paterna, Valencia, Spain) connected to a phase-contrast microscope (Nikon Eclipse 200) with a 10X negative contrast objective. Software settings applied in this study were previously established by Riesco et al. (2017a) for *C. angulata* sperm, but connectivity was adjusted to 14. Motility determination was performed three times for each sample, being evaluated 10 sperm samples for each treatment (n = 10).

#### *2.3.4.2. Deoxyribonucleic acid fragmentation*

Comet assay was applied to quantify sperm DNA damage after thawing. This technique was performed by applying the method described for *C. angulata* sperm by Riesco et al. (2019). Spermatozoa were embedded in low melting point agarose (0.5% w/v) on agarose precoated slides. Slides were immersed in lysis solution (2.5

M sodium chloride (NaCl), 100m Methylenediaminetetraacetic acid disodium salt dihydrate, 10 mM Tris, 1% Triton X-100, 1% lauryl sarcosine, pH 10, 1 h at 4°C). Then, slides were immersed twice in neutralizing solution (0.4 M Tris, pH 7.5, 5 min at 4°C) and fixed in 100% ethanol (3 min). Slides were stained with propidium iodide (PI) (0.1 mg/ml) and photographed with a digital camera (F-view, Olympus Corporation, Tokyo, Japan) coupled to a fluorescent microscope (excitation filter 450–480 nm; Olympus IX 81, Olympus Corporation, Tokyo, Japan). Komet 6.0 software (Andor Technology Ltd., Belfast, United Kingdom) was used to quantify the percentage of DNA in tail of 100 cells per slide. Two slides were performed per sample (n = 5).

#### *2.3.4.3. Flow cytometry approach: membrane integrity, intracellular reactive oxygen species levels, caspase detection, and acrosome integrity*

Flow cytometry was used to characterize the quality of post-thawed sperm. Sperm quality was evaluated through plasma membrane integrity, ROS levels, apoptosis, and acrosome integrity. Samples were acquired in the FACS Calibur Flow Cytometer (Becton Dickinson Biosciences, San Jose, CA, United States) equipped with two laser excitation sources (488 and 633 nm), two scatter detectors [forward scatter (FSC) and side scatter (SSC)], four emission detectors (FL1—530/30 nm; FL2—585/42 nm; FL3—670LP nm; FL4—661/16 nm) and controlled by CellQuest Pro version 8.7 software. For each sample, 10,000–30,000 events were registered and all the data were displayed in logarithmic mode. FSC and SSC plot were used to gate sperm population and exclude debris and aggregates from analyses (Figure 2.1B). Negative (unstained) and positive (single-stained) controls were used when appropriate to set gates and regions of interest and to determine compensations in the double stain protocols. FlowJo version 7.6.1 software (FlowJo, Ashland, OR, United States) was used to analyze and exhibit the flow cytometry data. Several fluorescent probes and labelling techniques were applied to evaluate different aspects of sperm physiology related to their functions or organelles.

Plasma membrane integrity of post-thaw sperm was stained by using PI. This fluorescent probe has a high affinity for DNA and is membrane impermeant, only staining sperm with the compromised plasma membrane (non-viable). Sperm with

injured membrane emitted red fluorescence, which was detected at FL2 channel. The PI stock solution contained 1 mg/ml. For staining, 2.5 ml of PI was added to 10 ml of sperm and 500 ml of 1% (w/v) NaCl and incubated for 5 min in the dark. Sperm viability was quantified as the percentage of PI negative cells (membrane intact) for each sample of each treatment (n = 10; Figure 2.1C).

Reactive oxygen species levels in cryopreserved sperm were detected by using double staining with dihydroethidium (DHE, Thermo Fisher Scientific, Oregon, United States) and SYTOX® Green Nucleic Acid Stain (SYTOX, Thermo Fisher Scientific, Oregon, United States). DHE is oxidized in the presence of superoxide ions, intercalates with DNA of cells, and emits an orange fluorescence, which was captured in FL2 channel. SYTOX is a cell-impermeant DNA-binding green dye used to detect dead cells through FL1 channel. The DHE and SYTOX working solutions contained 500 and 1 mM, respectively. For staining, 5 ml of each sperm sample was diluted in 500 ml 1% NaCl and incubated in the dark with 1 ml DHE and 0.5 ml SYTOX for 10 and 5 min, respectively. The combination of dyes allowed to identify for each sample the percentage of four sperm subpopulations: dead cells with undetectable levels of ROS (Q1: DHE negative and SYTOX positive); dead cells with detectable levels of ROS (Q2: DHE and SYTOX positive); live cells with detectable levels of ROS (Q3: DHE positive and SYTOX negative); and live cells with undetectable levels of ROS (Q4: DHE and SYTOX negative) (Figure 2.1D). Only live sperm subpopulations were represented for each sample (n = 5).

Programmed cell death or apoptosis was characterized with the commercial Muser Caspase-3/7 Kit (Luminex Corporation, Austin, TX, United States). The caspase-3/7 probe is a green dye that binds to the DNA of cells in the presence of active effector caspases, producing fluorescence captured by FL1 channel. 7-aminoactinomycin D (7-AAD) fluorogenic probe also supplied in the kit, provided information that allowed differentiating between early- and late-stage apoptotic cells, by staining in red late-stage cells that were detected in the FL3 channel. The sperm labeling was carried out according to the instructions of the manufacturer. The caspase assay allowed the definition of four subpopulations of sperm: necrotic cells (Q1: caspase negative and 7-AAD positive); late apoptotic cells (Q2: caspase and 7-AAD positive); early apoptotic cells (Q3: caspase positive and 7-AAD

negative); and live cells (Q4: caspase and 7-AAD negative) (Figure 2.1E). Data of each treatment (n = 8–9) were expressed as percentages for each subpopulation of sperm.

Acrosome integrity in thawed sperm was detected by combining fluorescein isothiocyanate-labeled *Pisum sativum* agglutinin (FITC-PSA) with PI. FITC-PSA fluorochrome interacts with the carbohydrate moieties and links specifically to the inner acrosomal membrane, only staining damaged acrosome. As previously stated, PI stains spermatozoa with damaged membranes. The fluorescence was measurable on FL1 and FL2 detectors for FITC-PSA and PI probes, respectively. The FITC-PSA and PI working solution contained 5 μg/ml and 1 mg/ml, respectively. For staining, 5 μl of each sperm sample was diluted in 500 μl 1% NaCl and incubated in the dark with 3 μl FITC-PSA and 2 μl PI for 10 and 5 min, respectively. Four sperm subpopulations can be identified: dead cells, acrosome intact (Q1: FITC-PSA negative and PI positive); dead cells, acrosome reacted (Q2: FITC-PSA and PI positive); live cells, acrosome reacted (Q3: FITC-PSA positive and PI negative); and live cells, acrosome intact (Q4: FITC-PSA and PI negative) (Figure 2.1F). Data of each treatment (n = 8–9) were expressed as percentages for each live subpopulation of sperm.

#### *2.3.4.4. Lipid peroxidation and antioxidant enzymes activity*

Spectrophotometric methods were performed for assaying lipid peroxidation and antioxidant activity in post-thaw sperm. Lipid peroxidation was determined by MDA detection by using the Bioxytech MDA-586 Kit (Oxis Research, Portland, OR, United States). Sperm samples ($350 \times 10^6$ cells/ml) were used to obtain cell suspensions, following the protocol developed, for fish sperm by Martínez-Páramo et al. (2012) and adapted by Riesco et al. (2019) for Portuguese oyster. According to the instructions of the manufacturer, reagents provided by the kit were added to 100 μl of each supernatant. This was read in a microplate reader at 586 nm (Synergy 4, BioTek, Vermont, United States) by using a MDA standard, provided in the kit. Each sample (n = 8 for each treatment) was performed in triplicate and MDA levels were expressed as nmoles of MDA per million of spermatozoa (nmol/$10^6$ spz). Sperm antioxidant capacity was evaluated through superoxide dismutase (SOD), glutathione reductase (GR), and glutathione

peroxidase (GPX). Selected methodologies were previously adopted by Martínez-Páramo et al. (2012) for seabass sperm. Oyster sperm samples ($400 \times 10^6$ cells/ml) of each treatment were centrifuged (5,000 g, 5 min, 4°C) to obtain the pellets. Phosphate-buffered saline (PBS) (0.01 M) with 0.1% (v/v) Triton X-100 was added to sperm pellets and submerged in liquid nitrogen (20 s) to lyse the cells, then resuspend in PBS, and centrifuge. The supernatant was split in four subsamples to determine the enzymatic activity and protein content of each sample in triplicate. The enzymatic activities of SOD, GR, and GPX were evaluated with the SOD (Ransod), GR, and GPX (Ransel) assay kits (Randox Laboratories Ltd., Crumlin, United Kingdom) according to the protocols of the manufacturer. Xanthine oxidase was the method employed by the kit to determine SOD activity, while GR activity was determined through the oxidation of NADPH and GPX activity was evaluated by NADPH oxidation in the presence of cumene hydroperoxide. Protein quantification was assessed with the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, United States) following producer instructions. Absorbance was determined in a microplate reader (Synergy 4, BioTek, Vermont, United States) at 505 nm for SOD, 340 nm for GR and GPX, and 750 nm for proteins. Enzymatic activity was expressed as units of enzyme per g of protein (U/g protein) for each sperm sample of each treatment (n = 9–10).

### *2.3.5. Statistical analysis*

Percentage data were arcsine transformed to obtain homogenous variances (Zar, 2009). The total motility, DNA fragmentation, plasma membrane integrity, ROS levels, lipid peroxidation, and antioxidant enzyme activity parameters were analyzed through the one-way ANOVA followed by the Student–Newman–Keuls (SNK) test used to identify significant differences between cryoprotectant solutions (DMSO, DMSO + Trehalose, and DMSO + Sucrose). The results were assumed as significant at 5% level ($p < 0.05$) and reported as mean ± SD. Statistical analysis was undertaken by using the software program IBM software program IBM SPSS Statistics version 25 (IBM, New York, NY, United States).

## 2.4. Results

### *2.4.1. Motility*

Total motility of *C. angulata* spermatozoa after thawing was very similar for all the cryoprotectant solutions (Figure 2.2), showing no significant differences between treatments (DMSO: 1.73 ± 0.95%; DMSO + Trehalose: 0.87 ± 0.58%; DMSO + Sucrose: 0.90 ± 0.83%).

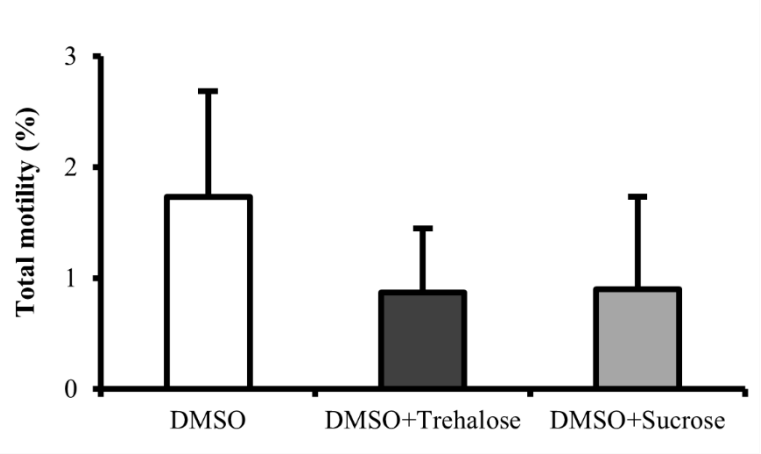


Figure 2.2 Motility of C. angulata sperm frozen with 10% dimethyl sulfoxide (DMSO) (white bar) and 10% DMSO supplemented with 0.45 M trehalose (black bar) or sucrose (gray bar). Results were expressed in mean percentages ± SD (n = 10). No significant differences between cryoprotectant solutions [one-way ANOVA followed by Student–Newman–Keuls (SNK) as post hoc test; $p < 0.05$].

### *2.4.2. Deoxyribonucleic acid fragmentation*

Deoxyribonucleic acid fragmentation results are shown in Figure 2.3. DMSO supplemented with sucrose had significantly higher values (20.64 ± 2.18%) of sperm with DNA damage when compared with the other two treatments. On the other hand, no significant differences were detected between DMSO supplemented with trehalose (14.95 ± 1.65%) and DMSO treatment (15.93 ± 1.47%). DNA fragmentation in thawed sperm did not seem to be reduced by sugars supplementation.

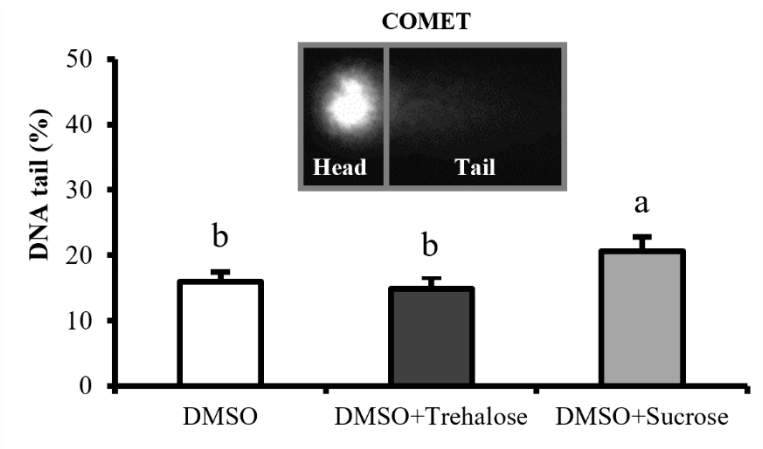


Figure 23 DNA fragmentation of *C. angulata* sperm frozen with 10% DMSO (white bar) and 10% DMSO supplemented with 0.45 M trehalose (black bar) or sucrose (gray bar). Results were expressed in mean percentages of DNA in the tail ± SD (n = 5). Different letters show differences between cryoprotectant solutions (one-way ANOVA followed by SNK as post hoc test; $p < 0.05$).

### *2.4.3. Flow cytometry approach: membrane integrity, intracellular reactive oxygen species levels, caspase detection, and acrosome integrity*

The percentage of viable cells was significantly lower in DMSO treatment (25.44 ± 9.89%) than in DMSO supplemented with sugars (Figure 2.4). However, no significant differences were found between trehalose (36.32 ± 11.33%) and sucrose (34.80 ± 8.04%). Therefore, the addition of both the sugars to DMSO seemed to improve post-thaw cell plasma membrane integrity, regardless of the sugar type.

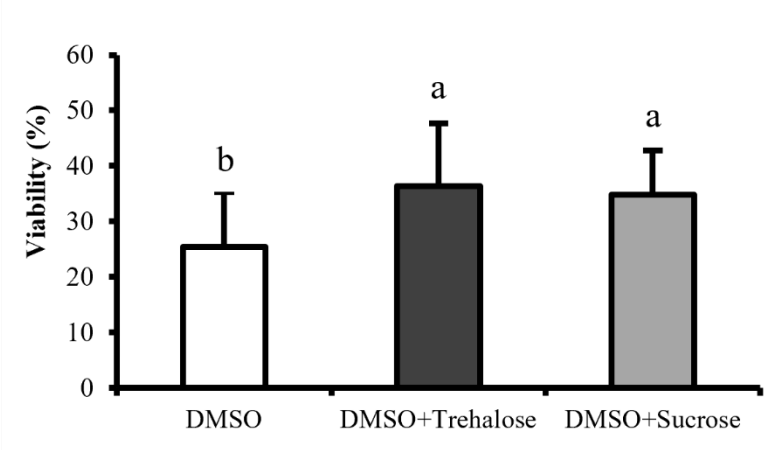


Figure 2.4 Plasma membrane integrity of *C. angulata* sperm frozen with 10% DMSO (white bar) and 10% DMSO supplemented with 0.45 M trehalose (black bar) or sucrose (gray bar) evaluated by flow cytometry. Results were expressed in mean percentages ± SD (n = 10). Different letters show differences between cryoprotectant solutions (one-way ANOVA followed by SNK as post hoc test; $p < 0.05$).

The levels of ROS in thawed sperm of *C. angulata* were detected with DHE and SYTOX fluorochromes being only represented the most relevant subpopulations: live sperm with detectable and undetectable levels of superoxide ions (Figure 2.5A). The cryoprotectants complemented with sugars showed a significantly higher percentage of living cells with undetectable levels of ROS (DMSO + Trehalose: 38.38 ± 6.21%; DMSO + Sucrose: 40.62 ± 2.73%) in comparison with DMSO (26.22 ± 5.00%). Therefore, the percentage of living cells with detectable levels of ROS was significantly higher in DMSO treatment (38.96 ± 4.18%) when compared to solutions containing sugars (DMSO + Trehalose: 30.50 ± 3.92%; DMSO + Sucrose: 27.56 ± 3.55%).

Programmed death cell (Figure 2.5B) allowed to identify four subpopulations: necrotic, late apoptotic, early apoptotic, and live sperm for each cryoprotectant solution. Early apoptotic and live cells were very similar between cryoprotectant solutions. DMSO showed a high percentage of necrotic cells (DMSO: 51.38 ± 25.24%; DMSO + Trehalose: 48.21 ± 28.04%; DMSO + Sucrose: 41.69 ± 22.25%), while sugar supplementation, especially sucrose, had high values of late apoptotic cells (DMSO: 44.96 ± 24.38%; DMSO + Trehalose: 49.10 ± 27.66%; DMSO + Sucrose: 55.13 ± 22.28%). However, no significant differences were found for each sperm subpopulation between treatments.

Figure 2.5C displays the live cells subpopulations identified in the acrosome integrity analyses. Sperm supplemented with sugars had higher values of live cells with acrosome intact (DMSO + Trehalose: 21.19 ± 3.60%; DMSO + Sucrose: 19.28 ± 5.78%) than DMSO treatment (15.85 ± 6.93%), however, with no significant differences. On the other hand, DMSO had a significantly higher number of cells with reacted acrosome (9.85 ± 3.71%) when compared to trehalose treatment (4.18 ± 3.32%).

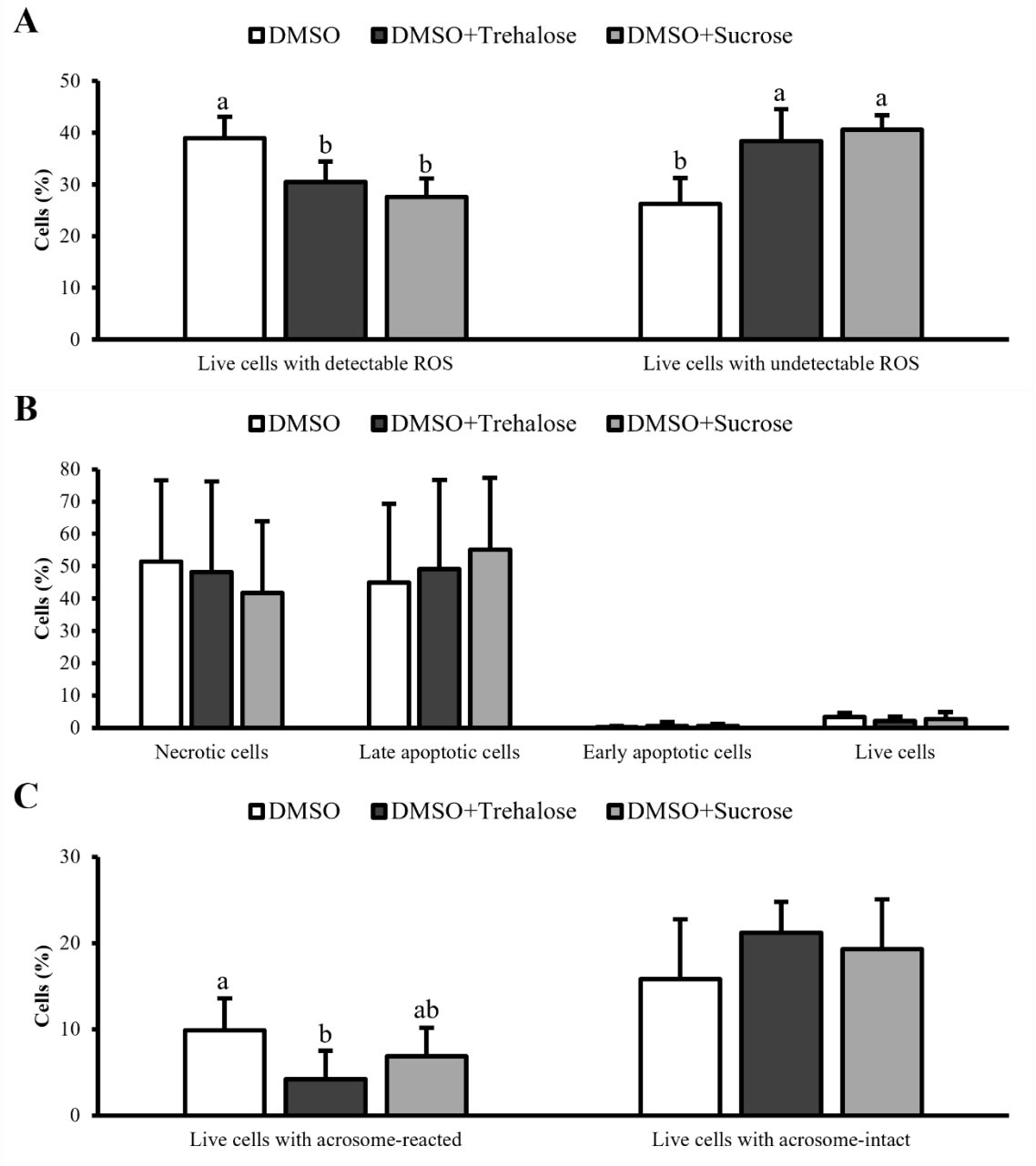


Figure 2.5 Flow cytometry assays of *C. angulata* sperm frozen with 10% DMSO (white bar) or 10% DMSO supplemented with 0.45 M trehalose (black bar) or sucrose (gray bar). (A) ROS levels through double staining with DHE and SYTOX. (B) Programmed cell death by caspase-3/7 detection. (C) Acrosome integrity by FITC-PSA combined with PI. Results were expressed in mean percentages ± SD (ROS: n = 5; caspase and acrosome: n = 8 for DMSO and n = 9 for DMSO with trehalose or sucrose). Different letters show differences between cryoprotectant solutions for each sperm subpopulation (one-way ANOVA followed by SNK as post hoc test; $p < 0.05$).

### *2.4.4. Lipid peroxidation and antioxidant enzymes activity*

Lipid peroxidation and antioxidant enzymes are shown in Figure 2.6. Lipid peroxidation assay revealed that sperm frozen with DMSO exhibited significantly higher MDA contents (53.49 ± 27.41 nmol/$10^6$ spz) when compared with sugar treatments (DMSO + Trehalose: 28.13 ± 12.35 nmol/$10^6$ spz; DMSO + Sucrose: 29.93 ± 10.95 nmol/$10^6$ spz) (Figure 2.6A). With respect to the activity of antioxidant enzymes, although treatments supplemented with sugars showed a trend of high enzymatic activity (Figures 2.6B–D), there were no significant differences between treatments.

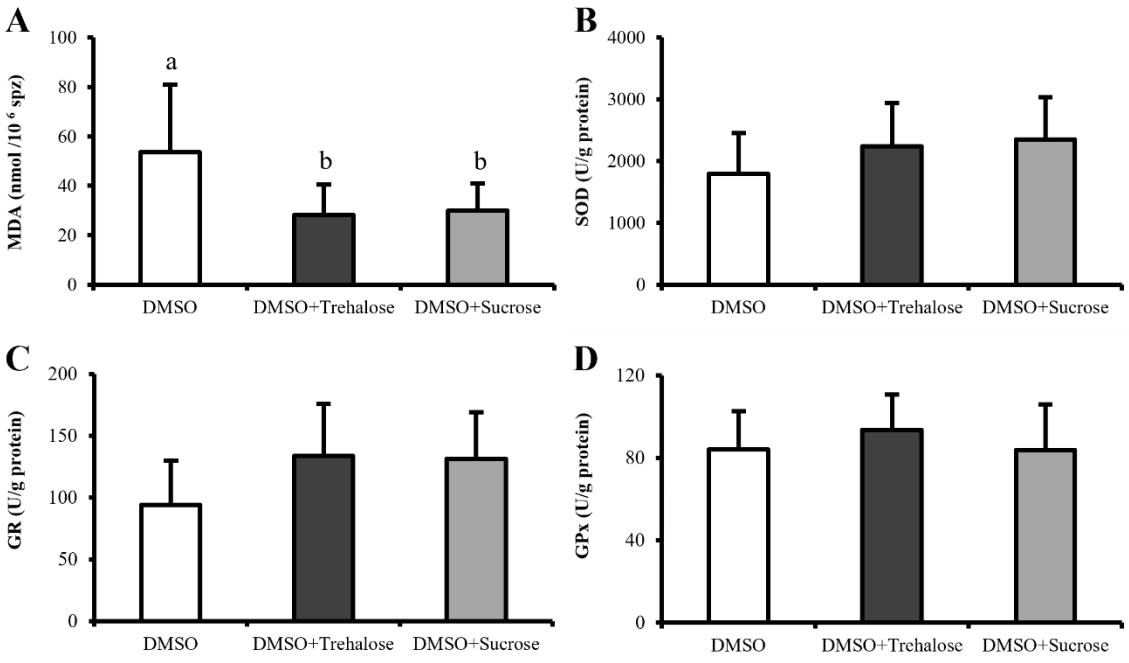


Figure 2.6 Spectrophotometric assays of *C. angulata* sperm frozen with 10% DMSO (white bar) and 10% DMSO supplemented with 0.45 M trehalose (black bar) or sucrose (gray bar). (A) Lipid peroxidation through malondialdehyde (MDA) levels (n = 8). (B–D) Enzyme activity of (B) superoxide dismutase (SOD) (n = 10), (C) glutathione reductase (GR) (n = 9) and (D) glutathione peroxidase (GPX) (n = 10). Results were expressed in mean ± SD. Different letters show differences between cryoprotectant solutions (one-way ANOVA followed by SNK as post hoc test; $p < 0.05$).

## 2.5. Discussion

Oysters are low trophic filter feeders that constitute an important resource for fisheries and aquaculture. However, due to bad management of seed production and anthropogenic impacts, some species such as the *C. angulata*, which shows potential for aquaculture diversification, are at risk of disappearing. Cryopreservation is a helpful method for genetic resources preservation. This method is a useful tool that may support the management of *C. angulata* pure natural banks and, at the same time, aquaculture production activities. However, the cryopreservation process produces cellular stress that generates damage in cells (Xin et al., 2020). Due to the complex dynamic structure of spermatozoa, it is necessary to establish different protocols for each species, toward the reduction of cryodamage. The supplementation with non-permeant cryoprotectants is a successful strategy to reduce cryodamage in cells through the use of high viscosity, high-molecular weight, and non-toxic compounds (Elliott et al., 2017). These strategies have been used in sea bass (*Dicentrarchus labrax*), dusky grouper (*Epinephelus marginatus*) and sea bream (*Sparus aurata*) improving either motility or cell viability or enhancing the antioxidant system by the addition of antioxidants (ascorbic acid, a-tocopherol), amino acids (taurine, hypotaurine), antifreeze proteins (AFPI, AFPIII), or sugars (glucose, sucrose) (Martínez-Páramo et al., 2012; Riesco et al., 2017c; Zilli et al., 2014).

In this study, sugars incorporated into the freezing media improved sperm plasma membrane integrity and reduced ROS levels, acrosome damage (only trehalose), and lipid peroxidation. This was probably due to high-molecular weight of sugars, which promoted cellular dehydration by replacing the water in the membrane that improved permeant cryoprotectant incorporation (De Leeuw et al., 1993). At the same time, due to a high viscous environment, the surrounding of the cells is stabilized, protecting the plasma membrane from cold damage (Nicolajsen and Hvidt, 1994; Woelders et al., 1997). This protective effect of sugars in post-thaw sperm was also reported for several species such as boar, Salmonidae fish, and Australian flat oyster (Gómez-Fernández et al., 2012; Hassan et al., 2017a; Nynca et al., 2016). Trehalose and sucrose have shown an important role as supplement of the freezing medium during cryopreservation of bivalves, being mainly applied in

larvae (Choi et al., 2008; Labbé et al., 2018) and less in sperm (Demoy-Schneider et al., 2018; Hassan et al., 2017a). Moreover, studies in bivalve sperm were performed with a low number of quality analyses both in the fresh and post-thaw samples, being these analyses focused mainly on sperm motility, plasma membrane integrity, and fertilization success(Hassan et al., 2017a, 2017b; Horváth et al., 2012; Suquet et al., 2016; Vitiello et al., 2011), thus lacking important information on sublethal damage. The use of a wide range of quality analyses in the establishment of a cryopreservation protocol is useful to investigate the specific structures and functions of spermatozoa that are being damaged (Cabrita et al., 2014). This information will support the establishment of strategies to mitigate cryodamage (Diogo et al., 2019; Hossen et al., 2021; Kim et al., 2020; Riesco et al., 2019). Therefore, in this study, the analysis performed on post-thaw sperm revealed the effects that trehalose and sucrose supplementation had on motility, DNA and plasma membrane integrity, ROS and apoptosis (caspases pathway) detection, acrosome integrity, lipid peroxidation, and antioxidant enzymes activities (SOD, GPX, and GR). This allowed an exhaustive evaluation of sperm quality and cell cryodamage. Sperm motility is a widely used quality indicator, since its activation is necessary for sperm to reach oocytes for successful fertilization (Boulais et al., 2019; Cabrita et al., 2008). In this study, post-thaw sperm motility did not show significant differences between cryoprotectant solutions; therefore, sugars supplementation did not have an effect on this parameter. In opposition, Hassan et al. (2017a) reported an improvement of post-thaw sperm motility of Australian flat oyster (*Ostrea angasi*) when using 10% DMSO supplemented with 0.45 M trehalose. In previous studies, our group showed that sperm motility did not seem so relevant when compared with other parameters that can jeopardize cells, due to the fact, that even with few motile cells, the long sperm motility duration of this species would allow oocytes to be fertilized (Riesco et al., 2017a). This fact is also supported in *Crassostrea virginica* by Yang et al. (2012), who showed that even low sperm motility after thawing did not compromise the ability of sperm to fertilize the oocytes and further develop into D-larvae. According to Figueroa et al. (2016), one structural damage that can compromise post-thaw sperm motility of *Salmo salar* was the damage inflicted to the mitochondria. Mitochondria is an organelle also present in bivalve sperm that participates in the synthesis of ATP, producing the majority of the energy necessary

for motility (Boulais et al., 2015). For this reason, some changes or damage in this structure or metabolic pathways can compromise sperm performance. In this way, in further studies, the evaluation of mitochondrial functionality such as plasma membrane potential and ATP content should be applied, since it could help to explain the motility results.

Deoxyribonucleic acid integrity evaluation is essential, since gametes need to provide secured genetic contribution to the offspring to avoid embryo abortion (Cabrita et al., 2010). Sperm freezing with sucrose addition showed significant higher DNA damage when compared to trehalose treatment and control. In equine sperm, DNA was not affected by cryopreservation by using different trehalose and sucrose concentrations (Pérez-Marín et al., 2018). Also, El-Sheshtawy et al. (2015) tested the effect of different concentrations of trehalose and sucrose (0.05, 0.1, and 0.2 M) on post-thaw quality of bull sperm and showed that freezing media supplemented with 0.05 M trehalose and 0.05 and 0.1 M sucrose produced low levels of sperm DNA fragmentation, while higher concentrations of these sugars make the sperm more prone to DNA damage. In this study, trehalose and sucrose were only tested at 0.45 M, taking into consideration the literature in similar species (Adams et al., 2004; Hassan et al., 2017a), being necessary in further studies to evaluate the effect of other concentrations. This will allow inferring about their possible effect on DNA protection and may justify our results with sucrose treatment, as demonstrated by El-Sheshtawy et al. (2015).

The membrane stabilization promoted by trehalose and sucrose allowed an improvement of sperm plasma membrane integrity. Apart from that, the high viscosity of sugars and the reduction in ice crystal formation may have contributed to the results obtained, as demonstrated by Nicolajsen and Hvidt (1994) and Woelders et al. (1997). Hassan et al. (2017a) showed that post-thaw sperm viability of Australian flat oyster (*Ostrea angasi*) was improved when a solution with 10% DMSO complemented with 0.45 M trehalose was applied. The membrane protection through sugars supplementation also reduced lipid peroxidation. Zhu et al. (2017) showed that trehalose supplementation improved plasma membrane integrity and reduced lipid peroxidation in rabbit post-thawed sperm. Sperm plasma membrane possesses high levels of lipids, which are prone to oxidation during stress events

such as cold exposure (Cabrita et al., 2014). Additionally, sugars can create bonds with the polar head of phospholipids in the plasma membrane (Anchordoguy et al., 1987), thus improving membrane protection and preventing lipid peroxidation. Altogether, these factors allowed a higher maintenance of the sperm plasma membrane integrity and stabilization during cryopreservation. Although this effect was not consistent for all the tested sugars, acrosome-reacted cells were lower in trehalose treatment when compared with control. This stabilization could be the responsible factor for acrosome protection, which is an essential structure for fertilization. It has enzymes engaged in lysing the oocyte membrane and their release allow the penetration of spermatozoa into the egg (Boulais et al., 2019). This study allowed understanding the effects of cryopreservation in different sperm structures essential to achieve successful fertilization. However, future studies are necessary to investigate fertilization success and offspring quality generated from *C. angulata* cryopreserved sperm.

Reactive oxygen species are molecules produced naturally to protect the cell and regulate signal pathways (Ighodaro and Akinloye, 2018). However, when an imbalance of ROS occurs, several cell structures containing lipids, proteins or enzymes, and chromatin may be affected by oxidative stress (Sandoval-Vargas et al., 2021b). In this study, there was a reduction in the formation of ROS in post-thaw sperm supplemented with sugars when compared to DMSO treatment. Concomitantly, low levels of lipid peroxidation were also detected in sperm treated with sugars, improving in these treatments the stabilization of the plasma membrane. Low levels of ROS and MDA associated with an improvement in rabbit sperm membrane integrity were reported by Zhu et al. (2017) when sperm was supplemented with trehalose. Trehalose and sucrose also showed a high protective effect against oxidative stress in boar post-thawed sperm by reducing the ROS levels and improving the cell viability when compared with lactose (Pezo et al., 2020). The relationship between ROS levels and lipid peroxidation was also reported by (Sandoval-Vargas et al., 2021a) for coho salmon (*Oncorhynchus kisutch*) sperm, which demonstrated that post-thawed samples with high levels of superoxide anions showed high MDA content and low levels of viable sperm. In this study, the sugars seemed to reduce the damage associated with oxidative stress by protecting

the lipids present in oyster sperm plasma membrane, thus avoiding their peroxidation by maintaining a balance of ROS.

Antioxidant enzymes are the first line of defense system of the cell, that keep ROS levels under control (Ighodaro and Akinloye, 2018). High levels of superoxide ions can be metabolized by SOD enzyme, which catalyzes the dismutation of these molecules to form oxygen and hydrogen peroxide, while catalase and GPX enzymes convert the hydrogen peroxide into water and oxygen (Amidi et al., 2016). Therefore, this protective mechanism acts to suppress or prevent the production of harmful molecules. Any changes in the activity of the antioxidant enzymes, such as freezing/thawing events, make the sperm more susceptible to oxidative stress. The detection of the activity of antioxidant enzymes in this study did not show any significant differences between treatments and control, although some authors have shown higher enzymatic activity in rabbit post-thawed sperm when sugars were incorporated in the freezing media (Zhu et al., 2017). This fact may suggest that sugars are not able to protect intracellular enzymes during cryopreservation and, probably, some enzyme denaturation or enzyme cell leakage may have occurred, but their effect is similar to the control.

Caspase-3/7 triggers the beginning of cell death from which the apoptosis process cannot be reversed, leading to cell alterations such as DNA and protein degradation (Elmore, 2007). Several studies have identified apoptosis activation in cryopreserved spermatozoa leading, at latter times, to a decrease in cell viability (Diogo et al., 2018; Riesco et al., 2017c). In this study, there were no significant differences in cellular apoptosis mechanisms identified through caspase-3/7 activities between control and sugar supplemented sperm. These results suggested that supplementation of sugars in the extender did not change cell death type or pathway (early apoptosis, late apoptosis, or necrosis by other mechanisms), according to the subpopulations detected by flow cytometry. The roles of cellular apoptosis and antioxidant enzymes in this study remain unclear. In future studies, these issues should be investigated, as well as, the effect of different freezing and warming rates on antioxidant enzymes activities in *C. angulata* post-thaw sperm.

This study contributed to the optimization of *C. angulata* sperm cryopreservation. Through the obtained results, it was possible to confirm selected

sugars as successful freezing media, additives to mitigate cryodamage, particularly in reducing ROS production, lipid peroxidation, and improving plasma membrane integrity. In particular, trehalose may have shown a higher protective effect during cryopreservation because its molecular structure creates more connections to phospholipid polar heads than the other tested sugar (sucrose) (Tsai et al., 2018). This effect was evident in reducing acrosome-reacted cells and in the registered levels of DNA fragmentation. This study corroborates the previous findings in several species (Hassan et al., 2017b; Öztürk et al., 2020; Zhu et al., 2017) that freezing media supplementation with trehalose is a good strategy to improve post-thaw sperm quality in *C. angulata*.

## 2.6. Conclusion

In *C. angulata* sperm cryopreservation, the supplementation with trehalose and sucrose of the freezing media containing DMSO improved plasma membrane integrity and reduced the oxidative stress and lipid peroxidation caused by ROS.

Sucrose supplementation did not protect chromatin and acrosome structures of *C. angulata* post-thaw sperm, revealing damage in DNA and acrosome structures.

Sperm cryopreservation with 10% DMSO and 0.45 M trehalose showed a high protective effect in *C. angulata* post-thaw sperm quality, once improved plasma membrane and acrosome integrity, and reduced lipid peroxidation and superoxide ions levels.

The establishment of a cryopreservation protocol that evaluates several post-thaw sperm quality parameters allowed to establish a strategy to mitigate cryodamage.

# CHAPTER 3. LARVAL CRYOPRESERVATION PROTOCOLS

# PREAMBLE

The Portuguese oyster *Crassostrea angulata* and the striped venus clam *Chamelea gallina* are important resources that have suffered production and population decline attributed to pathologies, climate change, and anthropogenic factors. Therefore, it is important to develop strategies to safeguard the genetic material of the target species. In bivalves, it is possible not only to cryopreserve sperm but also complex structures such as larvae. However, cryopreservation methodologies are specific for each genetic material and species. Enhancing cryopreservation methodologies, with a particular emphasis on optimizing cryoprotectant solutions, is crucial to ensure high-quality post-thaw larvae. Cryoprotectants are compounds that protect against ice crystal formation and dehydration, despite having an associated level of toxicity. Designing a cryoprotectant solution requires considering not only the protective effects but also the potentially harmful effects when the larvae are exposed to them, especially of permeant agents.

Therefore, chapter 3 is focused on the development of D-larvae cryopreservation protocols for Portuguese oyster and striped venus clam, through the selection of the most suitable permeant cryoprotectant (DMSO and EG). This chapter originated the first protocols of D-larvae cryopreservation of *C. angulata* and *C. gallina*. In this study, we used new quality assessment tools for bivalve cryopreserved larvae, namely antioxidant enzymes activity (SOD, GPX and GSR).

The content of this chapter corresponds to an article authored by Catarina Anjos and published in the Cryobiology journal.

# ASSESSMENT OF LARVAL QUALITY OF TWO BIVALVE SPECIES, *Crassostrea angulata* AND *Chamelea gallina*, EXPOSED AND CRYOPRESERVED WITH DIFFERENT CRYOPROTECTANT SOLUTIONS

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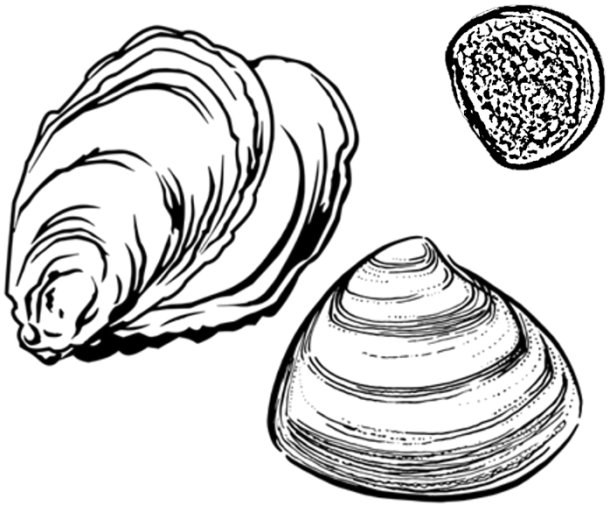

**[Cryobiology 2022, 106: 24-31]**

https://doi.org/10.1016/j.cryobiol.2022.04.007

## 3.1. Abstract

Marine bivalves are valuable resources, however, some shellfish populations are endangered due to factors such as anthropogenic pressure, pathologies or lack of reproduction synchrony. Portuguese oyster (*Crassostrea angulata*) and striped venus clam (*Chamelea gallina*) have high socio-economic value and their endangered natural populations require rehabilitation. Cryopreservation is a valuable method for the preservation and management of genetic resources for aquaculture and restocking. Larvae cryopreservation is particularly valuable since diploid organisms are obtained upon thawing. The objective of this work was the establishment of *C. angulata* and *C. gallina* D-larvae cryopreservation through the selection of permeant cryoprotectant in the freezing solution, namely ethylene glycol (EG) and dimethyl sulfoxide (DMSO). Cryoprotectants exposure showed that, in *C. angulata*, DMSO promoted significantly higher incidence of abnormalities and enhanced glutathione reductase activity when compared to control (larvae without cryoprotectant exposure) or even to EG treatment. However, for both species, EG significantly reduced D-larvae average path velocity (VAP). In *C. angulata* post-thaw D-larvae, EG treatment promoted significantly lower motility and velocity when compared to control and DMSO treatment. Superoxide dismutase (SOD) activity showed a reduction in *C. angulata* post-thaw D-larvae when compared to control, which was compensated by the enhancement of glutathione peroxidase (GPX) activity. In *C. gallina* post-thaw D-larvae, only motility, velocity and SOD activity were significantly lower than control. Therefore, the best treatment to cryopreserve *C. angulata* D-larvae was EG while for *C. gallina* DMSO produced better results. This work established for the first time D-larvae cryopreservation protocols for *C. angulata* and *C. gallina*.

## 3.2. Introduction

Bivalves have a major impact in aquatic environment (Vaughn and Hoellein, 2018) and are valuable resources for fisheries and aquaculture (Cardoso et al., 2013; van der Schatte Olivier et al., 2020). Portuguese oyster (*Crassostrea angulata*) and the striped venus clam (*Chamelea gallina*) are two species with high socio-economic potential (Batista et al., 2015; Öztürk and Altınok, 2021; Yu et al., 2017). However,

in Europe several banks of these native populations reveal signs of degradation, due to climate change and anthropogenic factors (pollution and overfishing) (Fabioux et al., 2002; Joaquim et al., 2016). This is a concerning situation and requires the development of strategic approaches to overcome this loss of resources. One possible solution is the development of tools to support the management of aquaculture production and fisheries or preservation strategies (Anjos et al., 2017; Pogoda et al., 2019; Riesco et al., 2017a).

Cryopreservation can be a helpful tool in reproductive management and preservation of biodiversity. This technique allows the storage of valuable genetic lines or endangered species (Adams et al., 2008) and, in aquaculture production can simplify broodstock management in hatcheries, allowing to extend the spawning out of the natural breeding season for the species (Cabrita et al., 2010; Hassan et al., 2015). Most of the works published in this area concerning bivalves focus on the cryopreservation of gametes and larvae of Pacific oyster (*Crassostrea gigas*) (Labbé et al., 2018; Martínez-Páramo et al., 2017; Paredes, 2015), being sperm the most widely studied (Hassan et al., 2015). Nevertheless, the cryopreservation of bivalve larvae presents a high potential due to the availability of diploid organisms immediately after thawing (Labbé et al., 2018; Suquet et al., 2014). Moreover, larvae cryopreservation allows the storage of genetic material of the progeny, which is particularly advantageous when cryopreservation protocols are not fully developed for gametes of both sexes (Paredes et al., 2021). The development of protocols to cryopreserve larvae of *C. angulata* and *C. gallina* is very useful, however, it can also be very challenging, since embryos and larvae of bivalves have complex internal structures and present several stages of development and metamorphosis (Helm et al., 2004). During development, the specificity and complexity of tissues and cells' structures change greatly throughout time. One of the main constraints in cryopreservation is the full penetration of permeant cryoprotectants in complex cell/tissue structures, such as oocytes and larvae, to fully protect them against the formation of ice crystals or cold damage (Labbé et al., 2018). Despite these constraints, in invertebrates, successful results have been reported in larvae, such as in the case of *C. gigas*, where cryopreserved larvae were able to grow and successfully generate progeny (Suquet et al., 2014).

In the last years, several works developed protocols to cryopreserve larvae of mussels (Y. Liu et al., 2020b; Rodriguez-Riveiro et al., 2019; Rusk et al., 2020), clams (Heres et al., 2021; Simon and Yang, 2018) and oysters (Labbé et al., 2018; Y. Liu et al., 2020a; Suneja et al., 2014). These works focused on the selection of embryo or larval development stage and in the freezing and thawing conditions. Initial stages of larval development (embryos and trochophore), seem to be less tolerant to cryodamage compared to the D-larvae stage (Heres et al., 2021; Y. Liu et al., 2020b; Rodriguez-Riveiro et al., 2019; Rusk et al., 2020). Additionally, a step-wise freezing rate was shown to be more beneficial (Labbé et al., 2018; Paredes et al., 2013). In bivalves, several works combined permeant and non-permeant cryoprotectants in their freezing solution protocols (Labbé et al., 2018; Y. Liu et al., 2020a; Suneja et al., 2014; Suquet et al., 2014), since these combinations proved to be more beneficial than a single agent (Y. Liu et al., 2020b). Permeant cryoprotectants substitute the water of the cells avoiding ice crystal formation and the non-permeant cryoprotectants protect the cellular membrane, avoiding the formation of ice crystals in the cell's surroundings (Elliott et al., 2017). To cryopreserve bivalves larvae, the permeant agents usually applied were ethylene glycol (EG), propylene glycol (PG) and dimethyl sulfoxide (DMSO), while sugars (sucrose, trehalose) and polyvinylpyrrolidone (PVP) were the most used non-permeant agents (Yang and Huo, 2022). Although cryoprotectants interact with the inner and outer biological environment protecting the biological material against ice crystals formation and dehydration, they have an associated level of toxicity, especially the permeant agents (Elliott et al., 2017; Sieme et al., 2016). For this reason, it is crucial to assess their protective effect versus their toxicity to avoid compromising its functionality (Raju et al., 2021).

The research in larvae cryopreservation protocols assesses a few quality parameters such as motility, survival and development status along the time (Gwo, 1995; Labbé et al., 2018; Y. Liu et al., 2020a; Suquet et al., 2012). Only a few works investigated how larvae structures are affected during cryopreservation, throughout organogenesis and neurogenesis (Rusk et al., 2020; Suneja et al., 2014). However, most of the works in bivalve larvae cryopreservation do not explore the cellular changes induced by the freezing and thawing process, except one study that investigated the redox status and gene expression of the *Mytilus galloprovincialis*

post-thaw larvae (Liu et al., 2022a). Bivalves larvae present antioxidant enzymes that enable the maintenance of homeostasis and prevent oxidative damage (Genard et al., 2011; Ragg et al., 2019). These antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase) are a mechanism of defense to suppress or prevent the increase of reactive oxygen species (ROS) levels during cryopreservation (Sandoval-Vargas et al., 2021b). Superoxide dismutase (SOD) is responsible for the dismutation of superoxide ions into oxygen and hydrogen peroxide, while hydrogen peroxide was converted into water and oxygen by catalase and glutathione peroxidase (GPX) enzymes (Ighodaro and Akinloye, 2018). Glutathione reductase (GR) regenerates reduced glutathione from its oxidized form, which is required for GPX operation.

The main goal of this study was to assess the effects of two cryoprotectant solutions (CPAs) in *C. angulata* and *C. gallina* D-larvae and evaluate the protective effect of the CPAs during freezing/thawing. This study allowed the production of viable post-thaw D-larvae, establishing for the first time a successful cryopreservation protocol for both species.

## 3.3. Material and methods

### *3.3.1. Biological material*

Portuguese oyster (*C. angulata*) and striped venus clam (*C. gallina*) were the species of bivalves used in this work. During the natural reproductive season (May to July), adult oysters of *C. angulata* were acquired from the bivalve farm Neptunpearl Lda. (Setúbal, Portugal), while *C. gallina* breeders were harvested from natural beds in the southern coast of Portugal (37°03′ 46.0"N 8°06′ 32.9"W) and transported to the IPMA Experimental Station (Tavira, Portugal) for natural spawning.

### *3.3.2. Gametes' collection and D-larvae production*

Gametes of the *C. angulata* were collected by gonadal stripping, followed by artificial fertilization. The individuals were opened, and the sex was determined by microscopic (Nikon Eclipse 200×, 10× objective, Japan) observation of a small

sample of the gonad. Gametes were individually collected by gonadal incisions and, to eliminate part of agglomerates eggs and tissues aiming to reduce potential contaminations, egg suspensions were filtered at 100 µm and retained in 20 µm mesh screen, while spermatozoa were sieved at 20 µm. Both gametes were maintained in filtered (0.35 µm) and UV-sterilized seawater (FSW) until fertilization. Only males with motile spermatozoa and females with spherical oocytes after 20 min in contact with FSW were selected. Sperm and oocytes concentrations were determined through the computerassisted sperm analysis (CASA) system (Proiser R + D S.L., Valencia, Spain) and microscopic counts, respectively. Fertilization was carried out with a proportion of one thousand spermatozoa per oocyte (Riesco et al., 2017a). One hour after fertilization, the eggs were again filtered at 20 µm mesh screen, to remove the excess of sperm. To obtain a pool of larvae, gametes of three males and two females were fertilized. A total of eight larvae pools were produced and each pool was considered a replicate (n = 8).

To induce *C. gallina* to spawn it was necessary to apply a hydrodynamic stimulus. For that purpose, individuals were maintained in an open circuit during 8 h with the zootechnical conditions (temperature, FSW flow rate and density) established by Joaquim et al. (2016). The breeders spawned naturally in the tank and fertilized eggs from the major spawning event were collected with a 30 µm mesh screen, followed incubation until D-larvae stage. Each tank of breeders originated a pool of eggs. A total of seven pools were incubated separately (n = 7).

For both species, larvae incubation was made in 250 L tanks with FSW at 21 ± 1 °C in a concentration of 100 eggs per ml, with sligh aeration. After an incubation of 24–30 h, the veliger D-larvae (early and middle stage, according to Labbé et al. (2018) was confirmed by microscopy. Afterward, larvae were sieved in a 30 µm mesh and counted to calculate the total number of larvae. D-larvae were concentrated (around 120,000 larvae per ml) in FSW and kept on ice (4 °C), until the experiments started. For each species, the same pools of larvae were used to perform the exposure of the D-larvae to CPAs (experiment 1) and D-larvae cryopreservation (experiment 2).

### *3.3.3. Experimental design*

Reagents used to perform the CPAs were acquired from Sigma-Aldrich (Saint Louis, MO, United States). CPAs were prepared immediately before being used, with twice the final concentration required and maintained at 4 °C.

The methodologies of this work were adapted from those previously employed by Labbé et al. (2018) in *C. gigas* larvae.

#### *3.3.3.1. Experiment 1 – exposure of D-larvae to cryoprotectant solutions*

Two CPAs were prepared in milli-Q water using a combination of one permeant cryoprotectant at a final concentration 10% (v/v) – ethylene glycol (EG) or dimethyl sulfoxide (DMSO) - with two non-permeant cryoprotectants, 1% (w/v) polyvinylpyrrolidone (PVP-40, 40,000 MW) and 0.2 M sucrose. Previously, concentrated D-larvae were exposed (1:1) to each CPAs for 3 min at 4 °C. After incubation, larvae were diluted (1:3) with FSW to dissipate the CPAs. The same procedure was done with a control group but without CPAs exposure. D-larvae quality was evaluated by determining the percentage of normal veliger D-larvae, the percentage of motile D-larvae and the average path velocity (VAP). The activity of superoxide dismutase (SOD) glutathione reductase (GR) and glutathione peroxidase (GPX) were also determined to evaluate antioxidant defense.

#### *3.3.3.2. Experiment 2 – D-larvae cryopreservation*

Pools of D-larvae of each species were exposed to CPAs as described in experiment 1. During the equilibration time, larvae were loaded into 0.5 ml French straw (30,000 per straw) and kept at 4 °C. Subsequently, the straws were cooled in a programable freezer (Asymptote EF600, Grant Instruments Ltd., Cambridge, United Kingdom) using a rate of - 2.5 °C/min from 0 to - 10 °C, hold for 5 min at - 10 °C, followed by a - 0.3 °C/min from - 10 °C to - 20 °C and - 2.5 °C/min down to - 35 °C. Then, straws were plunged into liquid nitrogen and stored in the liquid nitrogen container for 1–2 months. The straws were thawed by immersing in a water bath at 37 °C for 10 s. Afterward, a recovery bath was prepared, where the content of each straw was diluted in 2 L of FSW for 1 h at room temperature. The D-larvae were washed, concentrated in 1.5 ml of FSW and the post-thaw quality was immediately evaluated (percentage of normal and motile D-larvae, VAP and SOD, GR and GPX

activity). The same analyses were performed in the control groups of fresh D-larvae diluted in FSW.

### *3.3.4. Larval quality analysis*

D-larvae quality parameters such as percentage of normal D-larvae, motile D-larvae, VAP and SOD, GR and GPX activity were evaluated in control and treated larvae obtained from both experiments (exposure of D-larvae to cryoprotectant solutions and D-larvae cryopreservation).

Morphology of D-larvae was assessed using a microscope (Nikon Eclipse 200×, 10× objective, Japan), to observe the characteristics of at least 100 D-larvae in control and treated larvae from each experiment. D-larvae were classified as normal when showing more than 70% of the area of the shell filled with body tissues. The percentage of normal D-larvae was calculated as the number of larvae with normal morphology in relation with the total number of D-larvae observed.

D-larvae movement was evaluated based on the method described by Suquet et al. (2012). The number of moving larvae was counted in a microscope (Nikon Eclipse 200×, 10× objective, Japan) and the percentage was calculated as the number of motile larvae over the total number of D-larvae observed. Motile D-larvae individuals exhibited spiral and rotatory swimming. The larvae that had a heartbeat or cilia movement but without any displacement were considered immotile larvae. For the control and treated groups, a minimum of 50 D-larvae were evaluated in triplicate. Larval velocity (average path velocity-VAP) was studied by image analysis using the motility module of the Integrated Semen Analysis System (ISAS, Proiser R + D, S.L., Valencia, Spain). This analysis was recorded with a camera (ISAS 782C, Proiser R + D, S.L., Valencia, Spain) attached to a microscope (Nikon Eclipse 200×, 4× objective, Japan). The settings established were described by Suquet et al. (2012): 25 frames per second, 50–500 $\mu m^2$ for particles area, and minimum VAP of 30 μm per second. For the control and treatments of each pool, the VAP of at least 50 individuals was measured.

D-larvae antioxidant capacity was evaluated through superoxide dismutase (SOD) glutathione reductase (GR) and glutathione peroxidase (GPX) activities. Pools of 20,000 to 30,000 D-larvae were centrifuged (7,400 g, 5 min, 4 °C) to remove the

FSW. Larvae pellets were resuspended in phosphate-buffered saline (0.01 M PBS) with 0.1% (v/v) triton X-100 at a ratio of 1:5 and sonicated for 20 s in an ice bath. After that, pellet solutions were diluted to a ratio of 1:2 with PBS and centrifuged (7,400 g, 5 min, 4 °C). The supernatant was stored at - 80 °C for enzyme activity and protein quantification. SOD, GR and GPX activities were evaluated using assay kits (Randox Laboratories Ltd., Crumlin, United Kingdom), according to the protocols of the manufacturer, previously adapted. Protein quantification was assessed with the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, United States), following producer instructions. Enzymatic activity was expressed as units of enzyme per g of protein (U/g protein).

### *3.3.5. Data analysis*

Statistical analysis was performed through IBM SPSS Statistics 26.0 (IBM, New York, United States) statistical software. Data expressed as percentages were normalized by arcsine transformation. The normality and homogeneity of variances were investigated using Shapiro-wilk and Levene's tests, respectively. One-way analysis of variance (ANOVA, posthoc Student–Newman–Keuls test (SNK)) was applied when normality and homogeneity of variance were observed; Kruskal–Wallis nonparametric test was applied when normality and homogeneity of variance were not observed. Results were assumed as significant at the 5% level ($p < 0.05$) and expressed as mean ± standard deviation (SD).

## 3.4. Results

### *3.4.1. Experiment 1 – exposure of D-larvae to cryoprotectant solutions*

The percentage of normal *C. angulata* D-larvae was significantly lower in DMSO treatment (92.91 ± 6.37%) than the control (93.73 ± 13.16%; larvae diluted in FSW) and EG treatment (Fig. 3.1A). There were no significant differences in the morphology of *C. gallina* larvae when exposed to CPAs, since alterations in the space occupied by the body tissues concerning the shell were similar between control and both CPAs (Fig. 3.1B).

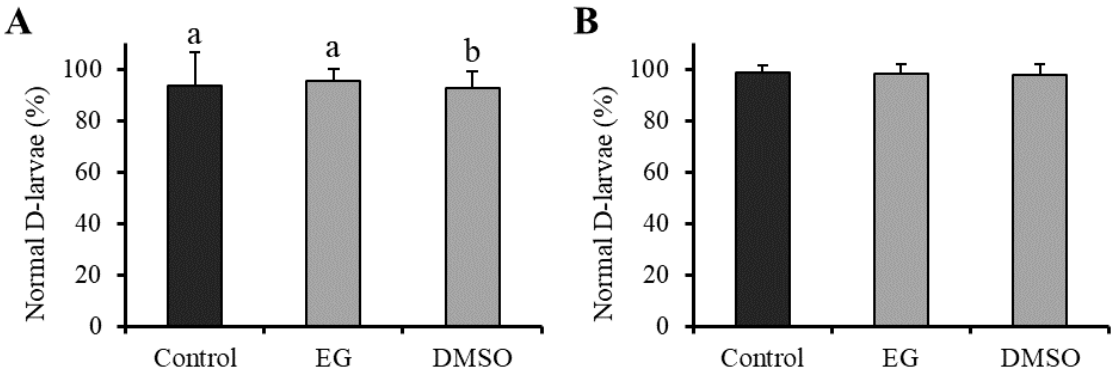


Figure 3.1 Effect of exposure to cryoprotectant solutions on the percentage of normal D-larvae of *C. angulata* (n = 7) (A) and *C. gallina* (n = 6) (B). Control (fresh larvae); permeant cryoprotectant: EG – ethylene glycol and DMSO – dimethyl sulfoxide. Data were expressed as mean ± SD and analyzed with Kruskal–Wallis followed by Dunn's test. Different letters reveal significant differences between treatments ($p < 0.05$).

The percentage of motile D-larvae in both species were identical for samples exposed to control and CPA treatments (Fig. 3.2A and B). However, D-larvae velocity (VAP) was affected by the addition of CPAs in both species (Fig. 3.2C and D). *C. angulata* larvae exposed to EG showed the significantly lowest VAP when compared to control and DMSO treatment (Fig. 3.2C and supplementary Videos S1, S2 and S3). In *C. gallina*, the exposure to the CPAs resulted in a significant reduction of VAP when compared to control (Fig. 3.2D).

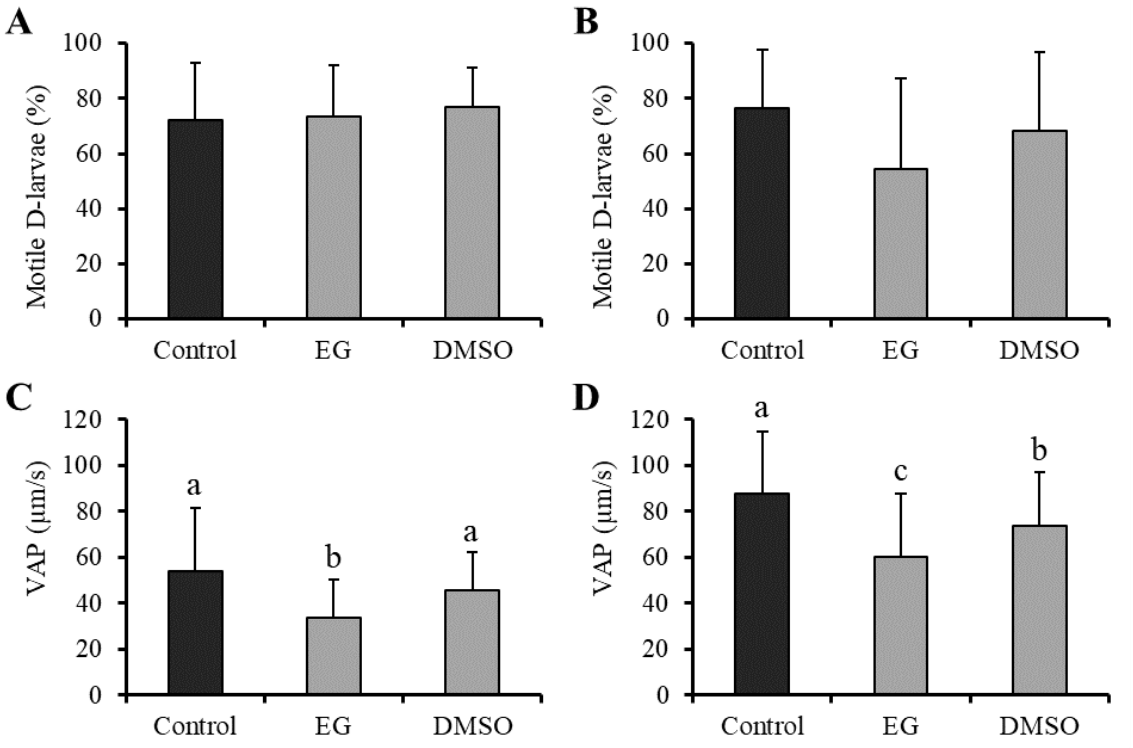


Figure 3.2 Effect of exposure to cryoprotectant solutions on D-larvae movement characteristics *of C. angulata* (A, C) and *C. gallina* (B, D). Percentage of motile D-larvae (A, B) and VAP (C, D) were represented as mean ± SD, of 8 and 7 pools of oyster and clam larvae, respectively. Control (fresh larvae); permeant cryoprotectant: EG – ethylene glycol and DMSO – dimethyl sulfoxide. Data were analyzed applying Kruskal–Wallis and a Dunn's test, except for VAP data, which followed normality and a one-way ANOVA and a SNK test were used. Different letters show significant differences between treatments ($p < 0.05$).

The antioxidant capacity of control larvae and exposed to CPAs was determined by measuring the activity of the SOD, GR and GPX enzymes (Fig. 3.3). For all enzymes, no significant differences in activities were observed between the control and samples exposed to EG and DMSO solutions for both species, except for GR activity in *C. angulata* larvae. In this case, oyster D-larvae diluted in DMSO showed significantly higher activity of GR than control (Fig. 3.3C: Control – 125.79 ± 22.70 U/g protein; DMSO – 163.58 ± 38.83 U/g protein).

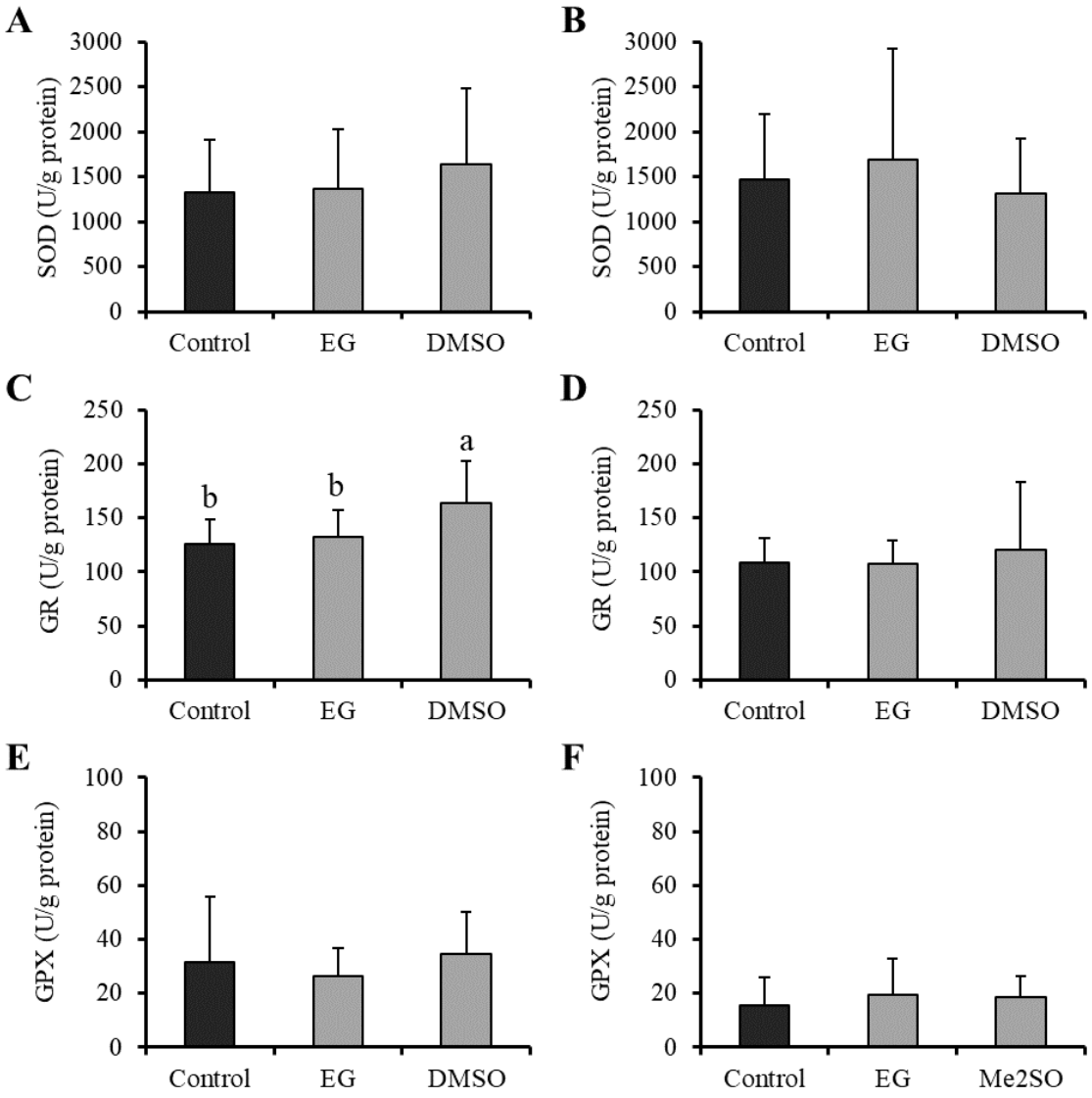


Figure 3.3 Effect of exposure to cryoprotectant solutions on the enzymatic activity of *C. angulata* (A, C, E) and *C. gallina* (B, D, F) D-larvae. Control (fresh larvae); permeant cryoprotectant: EG – ethylene glycol and DMSO – dimethyl sulfoxide. The analyzed enzymes were superoxide dismutase (SOD: n = 8 and n = 7 for oyster and clam larvae, respectively) (A, B), glutathione reductase (GR: n = 8 and n = 7 to oyster and clam larvae, respectively) (C, D) and glutathione peroxidase (GPX: n = 8 and n = 7 to oyster and clam larvae, respectively) (E, F). Data were expressed as mean ± SD and analyzed with one-way ANOVA followed by SNK as post-hoc test (GR and GPX of oyster larvae and SOD and GPX of clam larvae) or Kruskal–Wallis followed by Dunn's test (SOD of oyster larvae and GR of clam larvae). Different letters reveal significant differences between treatments ($p < 0.05$).

### *3.4.2. Experiment 2 – D-larvae cryopreservation*

To understand the effects of CPAs during cryopreservation morphology, movement, and antioxidant enzyme activity of post-thaw larvae were evaluated. *C. angulata* and *C. gallina* D-larvae morphology are exhibited in Fig. 3.4. *C. angulata* post-thaw larvae showed a significant lower percentage of normal D-larvae when

compared to fresh samples designated as control (Fig. 3.4A: Control – 93.73 ± 13.16%; EG – 75.26 ± 26.29%; DMSO – 78.95 ± 26.54%). On the other hand, no morphological differences were detected in *C. gallin*a post-thaw larvae (Fig. 3.4B). Fig. 4C–H shows photomicrographs of post-thaw larvae of oysters (Fig. 3.10C, D and E) and clams (Fig. 3.4F, G and H) with normal (Fig. 3.4C and F) and abnormal (Fig. 3.4D, E, G and H) morphology related to body tissues. The morphological alterations detected were tissue disorganization (Fig. 3.4D and G), reduction of body size in relation to the shell (Fig. 3.4E) and mantle protuberance (Fig. 3.4H).

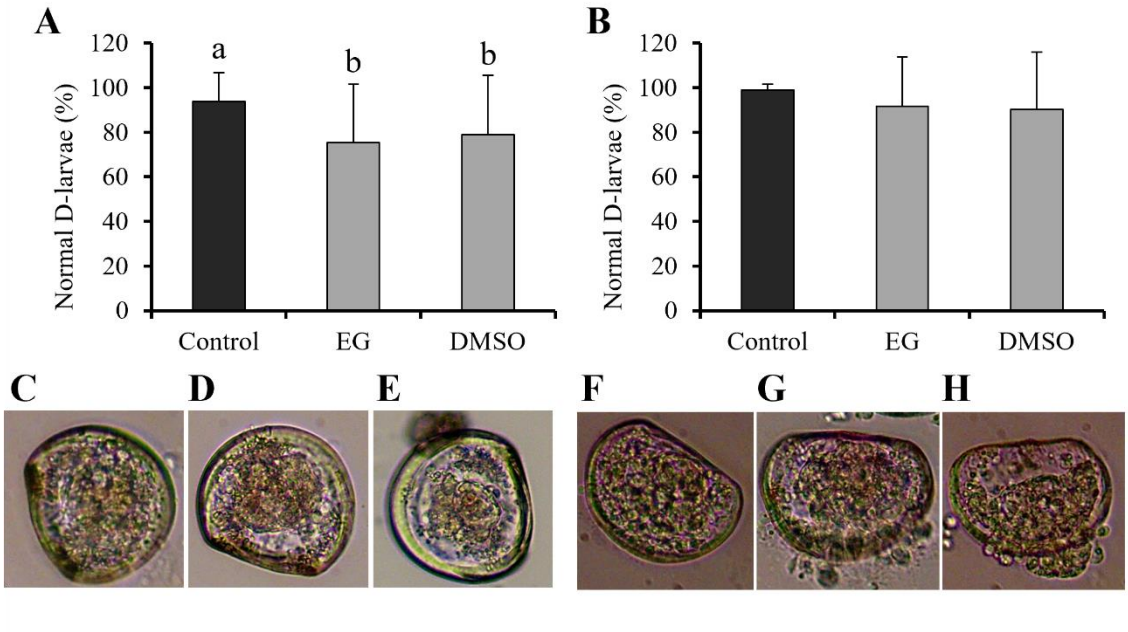


Figure 3.4 Effect of cryopreservation on the percentage of normal D-larvae of *C. angulata* (n = 8) (A) and *C. gallina* (n = 6) (B). Light microscopy images showing normal (C, F) and abnormal (D, E, G, H) D-larvae of *C. angulata* (C, D, E) and *C. gallina* (F, G, H) after thawing (Nikon Eclipse 200×, 20× objective). Control (fresh larvae); permeant cryoprotectant: EG – ethylene glycol and DMSO – dimethyl sulfoxide. Data were expressed as mean ± SD. Different letters reveal significant differences between treatments (Kruskal–Wallis followed by Dunn's test, $p < 0.05$).

Cryopreservation of *C. angulata* and *C. gallina* D-larvae with both CPAs produced significantly lower percentages of motility and VAP values than control, where EG showed the significantly lowest values (Fig. 3.5A, C and D and supplementary Videos S1, S4 and S5) except in clams post-thaw motility where no significant differences were detected within CPA treatments (Fig. 3.5B).

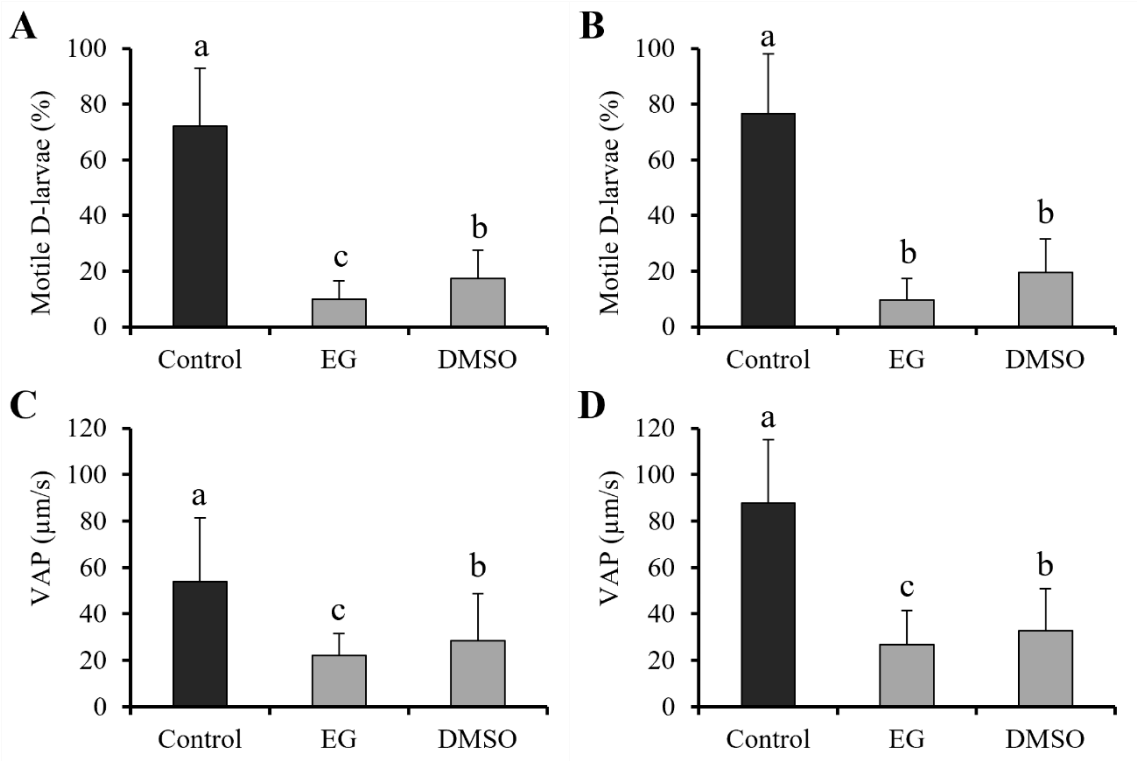


Figure 3.5 Effect of cryopreservation on D-larvae movement characteristics of *C. angulata* (A, C) and *C. gallina* (B, D). Percentage of motile D-larvae (A, B) and VAP (C, D) were represented as mean ± SD, of 8 and 7 pools of oyster and clam larvae, respectively. Control (fresh larvae); permeant cryoprotectant: EG – ethylene glycol and DMSO – dimethyl sulfoxide. Different letters reveal significant differences between treatments (Kruskal–Wallis followed by Dunn's test; $p < 0.05$).

Antioxidant enzyme assays revealed that *C. angulata* and *C. gallina* larvae cryopreserved with DMSO and EG showed significantly lower values of SOD activity than control (fresh larvae) (Fig. 3.6A and B). Regarding the GPX activity in the Portuguese oyster larvae, the values of DMSO were significantly higher than EG and control (Fig. 3.6E: Control – 31.70 ± 24.09 U/g protein; EG – 45.67 ± 18.05 U/g protein; DMSO – 59.27 ± 18.10 U/g protein). For the remaining enzymes analyzed in both bivalve species, no significant differences in activity were found between the control and samples cryopreserved with EG or DMSO (Fig. 3.6C, D and F).

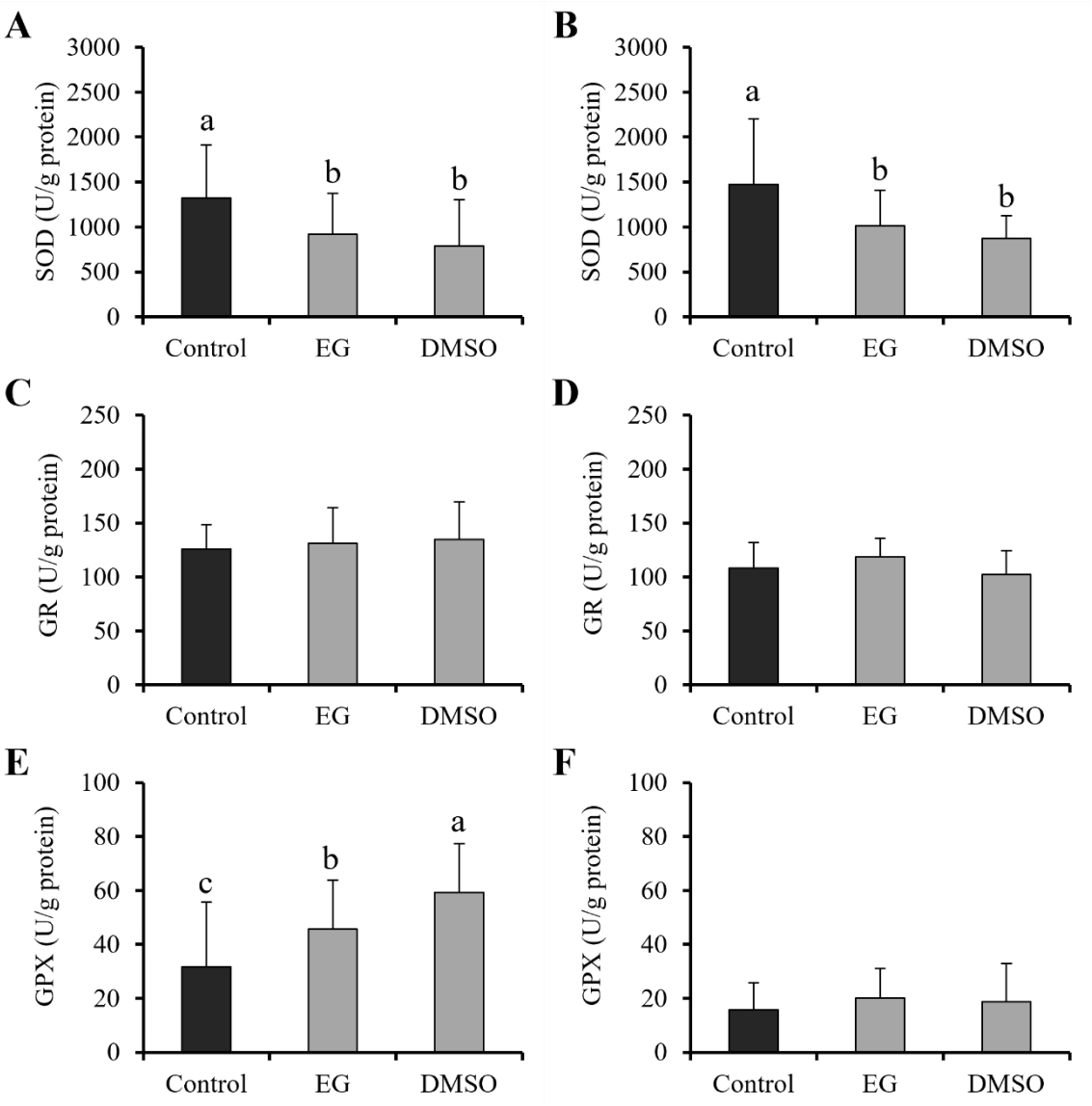


Figure 3.6 Effect of cryopreservation on the enzymatic activity of *C. angulata* (A, C, E) and *C. gallina* (B, D, F) D-larvae. Control (fresh larvae); permeant cryoprotectant: EG – ethylene glycol and DMSO – dimethyl sulfoxide. The analyzed enzymes were superoxide dismutase (SOD: n = 8 and n = 7 to oyster and clam larvae, respectively) (A, B), glutathione reductase (GR: n = 8 and n = 7 to oyster and clam larvae, respectively) (C, D) and glutathione peroxidase (GPX: n = 8 and n = 7 to oyster and clam larvae, respectively) (E, F). Data were expressed as mean ± SD and analyzed with one-way ANOVA followed by SNK as post-hoc test (SOD, GR and GPX of oyster larvae and GR and GPX of clam larvae) or Kruskal–Wallis followed by Dunn's test (SOD of clam larvae). Different letters reveal significant differences between treatments ($p < 0.05$).

## 3.5. Discussion

Cryopreservation of bivalve larvae shows high potential, since it presents several advantages when compared to conventional gametes cryopreservation. *C. angulata* and *C. gallina* are two endangered species that require measures for population restocking. The development of larval cryopreservation methodologies can also support *C. angulata* aquaculture management, since it would allows the availability of diploid organisms immediately after thawing (Labbé et al., 2018; Suquet et al., 2014) and all year around (Gwo, 1995). This tool can be helpful to shellfish species, such as *C. angulata and C. gallina*, to preserve and recover their natural populations, which show signs of depletion, due to poor management strategies, pathologies and lack of reproductive synchrony in the natural environment (Fabioux et al., 2002; Joaquim et al., 2016). To the best of our knowledge, there are only three sperm cryopreservation protocols developed for *C. angulata* (Anjos et al., 2021; Riesco et al., 2019, 2017a) and none developed for larvae. There are no cryopreservation protocols for any cell or tissue in *C. gallina* and the current study constitutes the first attempt in cryopreservation of this species. The present study aimed to develop cryopreservation protocols for D-larvae to establish the most suitable permeant cryoprotectant for both species.

In cryopreservation of bivalve larvae, a freezing solution that combines permeant and non-permeant agents has been reported as a suitable strategy to improve the post-thaw quality (Labbé et al., 2018; Y. Liu et al., 2020a). The freezing solutions used in the current study contained non-permeant cryoprotectants previously used in *C. gigas* larvae cryopreservation (Labbé et al., 2018). Moreover, the concentration of the permeant cryoprotectant solution was selected according to previous studies in *C. gigas* (Labbé et al., 2018; Suquet et al., 2014, 2012). Different permeant cryoprotectants were tested in the freezing solution namely EG and DMSO. EG was selected due to the high success observed in studies in bivalve larvae cryopreservation (Choi and Chang, 2014; Labbé et al., 2018; Y. Liu et al., 2020a; Suneja et al., 2014; Suquet et al., 2014) and DMSO was selected since it is one of the most common cryoprotectants used in cryobiology (Awan et al., 2020; Elliott et al., 2017). The objective was to evaluate their biological performance and select

the most successful permeant cryoprotectant for each species D-larvae cryopreservation.

The exposure of cells or organisms to cryoprotectants is a common practice to evaluate the effect of these agents on larval survival (Gwo, 1995; Horváth et al., 2012). These toxicity tests are a useful tool to perform preliminary prospection during the optimization of cryopreservation protocols (Heres et al., 2021; Simon and Yang, 2018). This method is helpful because cryoprotectants present inherent cellular toxicity that can compromise larval development, growth and survival (Elliott et al., 2017; Simon and Yang, 2018). Moreover, these preliminary tests can reduce the labor associated with full cryopreservation tests when CPAs already demonstrated a high level of toxicity. In the present work, D-larvae were exposed to the solutions containing both permeant CPAs. *C. angulata* revealed that EG promoted significantly lower abnormal larvae when compared to DMSO treatment and showed no significant differences when compared to control. The increase of abnormal larvae observed with DMSO can be related to the cryoprotectant toxicity or dehydration phenomena previously reported in other species, such as *C. gigas* (Gwo, 1995). However, this fact is not observed in *C. gallina* since there were no differences in larval morphology after cryoprotectant exposure. In *C. angulata*, DMSO treatment showed no significant differences in the percentage of motile larvae and VAP when compared with control. In the case of *C. gallina* the same tendency was observed for larval motility, with no effect of CPAs, although VAP registered lower values than control. VAP analysis in bivalve larval toxicity tests seems to be a useful quality parameter, since it demonstrated that a certain degree of cilia impairment should have occurred because changes in motility pattern behavior were registered. These same findings were suggested by Suneja et al. (2014), when analyzing D-larvae velum structure after cryopreservation.

The cellular mechanism used to prevent or suppress damage produced by ROS is through the antioxidant defense mechanism, which relies in part on the activity of several antioxidant enzymes (Ighodaro and Akinloye, 2018). The results of GR activity are in agreement with the incidence of malformed oyster D-larvae in DMSO treatment, since when larvae were exposed to this cryoprotectant there was an increase of the GR activity. This increased activity can be a cellular response of

oyster larvae to tackle the stress promoted by DMSO, which can cause anomalies in larval structure. However, these D-larvae abnormalities caused by DMSO did not seem to affect the structure responsible for larval motility (velum), neither the energetical mechanism responsible for cilia movement, since motility and VAP were not affected. Further studies including larval ultrastructure and ATP production should be fundamental to elucidate some of the causes behind this damage.

Cryopreservation protocols optimization have the ultimate goal to improve samples post-thaw quality and avoid lethal and sublethal damage (Martínez-Páramo et al., 2017; Yang and Huo, 2022). After D-larvae exposure to the freezing solution and confirmation of CPAs toxicity level, the subsequent step was to evaluate the protective effect of CPA during freezing/thawing. In this work, it was observed that cryopreservation produced a significant reduction in the percentage of normal *C. angulata* D-larvae, where no effects were seen in *C. gallina*, and in the percentage of motile larvae in both species. These results are in agreement with data reported in the literature in protocols developed for cryopreservation of bivalve larvae, which showed lower performances in terms of swimming activity, development and growth, in post-thaw larvae than in fresh larvae (Heres et al., 2021; Suquet et al., 2014, 2012). Our results are also in agreement with previous studies that described a reduction of larval motility and lower body area, suggesting a retraction of the larvae body in *C. gigas* D-larvae (Labbé et al., 2018; Suquet et al., 2012). In both studied species, EG treatment showed the lowest values of motility and velocity when compared with control. As mentioned in toxicity trials (experiment 1) these results can be related to injuries inflicted in the velum, consequently affecting larval movement and feeding (Suneja et al., 2014).

Regarding antioxidant enzymes, SOD activity was significantly lower in cryopreserved samples than in control for both species. On the other hand, GPX activity showed a significant increase in both CPAs but only for *C. angulata*. Figueroa et al. (2019) observed a decrease in catalase activity and a GPX increase in the post-thaw Salmo salar sperm. Also, Whitaker and Knight (2008) observed in porcine oocytes that when SOD and catalase activities were hindered, GPX increased. This may indicate, as suggested by these authors, that GPX activity is enhanced to

counteract the effects of certain ROS (e.g. hydrogen peroxide), due to the fact that SOD activity was inhibited.

In this work, we decided to quantify the abnormalities associated with the reduction of body size in relation to the shell, once we cryopreserved larvae with fully formed shell. Overall, our results suggest that the response to cryopreservation was species-specific at least in some aspects of the post-thaw evaluation. The standardization of cryopreservation protocols between species would be ideal in cryobiology, however, it is unusual to obtain high performances with the same protocol between different species and biological materials. It is noteworthy the fact that in our study a similar cryopreservation methodology (except the type of cryoprotectant) obtained good post-thaw larvae quality for both species, *C. angulata* and *C. gallina*. Despite this, *C. gallina* D-larvae seem to be less prone to body tissues retraction than *C. angulata*. The most affected parameters due to cryoprotectant toxicity were D-larvae velocity, whereas after cryopreservation, larval motility, velocity and SOD activity showed significant differences in both species. Therefore, these parameters are potential biomarkers for post-thaw larvae quality in bivalves, especially when long-term development cannot be performed to confirm protocols success. Nonetheless, evaluation of post-thaw larvae survival throughout development is very important to investigate.

As this is the first study in larvae cryopreservation for both species, future experiments should focus on the study of larvae development and growth performance, particularly focusing on the comprehension of how metabolism and cell structures are affected (e.g. morphology, shell growth, shell deformities or modifications of the hinge structure). Different concentrations of the selected CPAs in *C. angulata* and *C. gallina* larvae cryopreservation should also be tested to ameliorate some of the cryodamage encountered in this study.

## 3.6. Conclusion

In conclusion, the knowledge obtained by the present work allowed to select the most suitable permeant cryoprotectants for *C. angulata* and *C. gallina*, namely EG and DMSO, respectively. This work established, for the first time, larvae

cryopreservation protocols for *C. angulata* and *C. gallina* that enable the establishment of natural populations management and restocking methodologies and support *C. angulata* aquaculture management.

## 3.7. Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2022.04.007.

# CHAPTER 4. MOLECULAR ANALYSIS TO STUDY DIFFERENT SUSCEPTIBILITIES TO CRYOPRESERVATION

# PREAMBLE

Cryopreservation is a method that suspends physiological processes, cellular functions and chemical reactions at ultra-low temperatures, allowing to safeguard the genetic resources of endangered populations. However, it is necessary to protect the biological material from the adversities of the cryopreservation steps which can be damaging. Evaluation of cryopreservation steps, including cryoprotectant solutions and the freezing process, is essential for establishing reliable protocols. One of the main constraints of bivalve cryopreservation is that the majority of the studies are based on the assessment of larvae post-thaw quality by swimming activity, morphology, and survival. Therefore, it is important to expand the current methods of assessing post-thaw larvae. Some studies have successfully utilized gene expression analysis to investigate cryodamage in sperm and larvae of various species. So, the use of transcriptomic tools could be useful for full screening of the alterations of larvae molecular networks and biological processes promoted by cryopreservation.

The objective of this chapter was to evaluate the changes that occur during cryopreservation from a molecular perspective, aiming to understand the alterations in gene expression of cryoprotectant-exposed and cryopreserved *C. angulata* larvae using RNA sequencing. The work developed in this chapter, for the first time, enabled the description of molecular alterations and damage occurring in the transcriptome of *Crassostrea angulata* D-larvae during cryopreservation.

Manuscript of this chapter was submitted to BMC Genomics journal on November 6$^{th}$, 2023, with Catarina Anjos as the primary author.

# COMPARATIVE TRANSCRIPTOME ANALYSIS REVEALS MOLECULAR DAMAGE ASSOCIATED WITH CRYOPRESERVATION IN *Crassostrea angulata* D-LARVAE RATHER THAN TO CRYOPROTECTANT EXPOSURE

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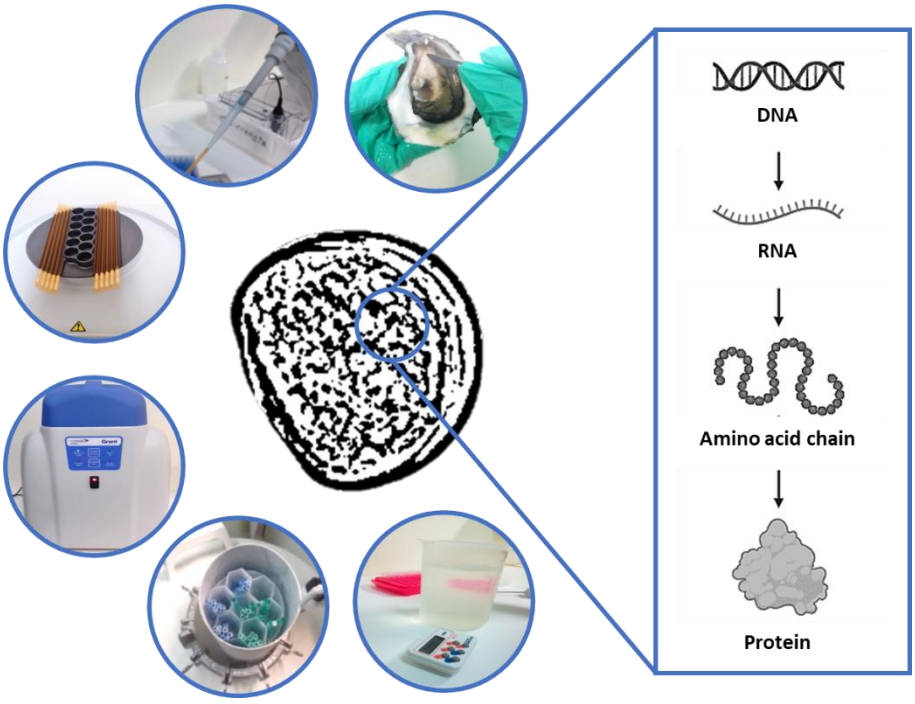


**[BMC Genomics, submitted on November 6th, 2023]**

## 4.1. Abstract

Background: The Portuguese oyster *Crassostrea angulata*, a bivalve of significant economic and ecological importance, has faced a decline in both production and natural populations due to pathologies, climate change, and anthropogenic factors. To safeguard its genetic diversity and improve reproductive management, cryopreservation emerges as a valuable strategy. However, the cryopreservation methodologies lead to some damage in structures and functions of the cells and tissues that can affect post-thaw quality. Transcriptomics may help to understand the molecular consequences related to cryopreservation steps and therefore to identify different freezability biomarkers. This study investigates the molecular damage induced by cryopreservation in *C. angulata* D-larvae, focusing on two critical steps: exposure to cryoprotectant solution and the freezing/thawing process.

Results: Expression analysis revealed 3 differentially expressed genes between larvae exposed to cryoprotectant solution and fresh larvae and 511 differentially expressed genes in cryopreserved larvae against fresh larvae. The most significantly enriched gene ontology terms were “carbohydrate metabolic process”, “integral component of membrane” and “chitin binding” for biological processes, cellular components and molecular functions, respectively. Kyoto Encyclopedia of Genes and Genomes enrichment analysis identified the “neuroactive ligand receptor interaction”, “endocytosis” and “spliceosome” as the most enriched pathways. RNA sequencing results were validated by quantitative RT-PCR, once both techniques presented the same gene expression tendency and a group of 11 genes were considered important molecular biomarkers to be used in further studies for the evaluation of cryodamage.

Conclusions: The current work provided valuable insights into the molecular repercussions of cryopreservation on D-larvae of *Crassostrea angulata*, revealing that the freezing process had a more pronounced impact on larval quality compared to any potential cryoprotectant-induced toxicity. Additionally, was identify 11 genes serving as biomarkers of freezability for D-larvae quality assessment. This research contributes to the development of more effective cryopreservation protocols and detection methods for cryodamage in this species.

## 4.2. Introduction

The Portuguese oyster *Crassostrea angulata*, a bivalve of major economic value and widely distributed around the world (Wang et al., 2010), used to be an important resource for the European aquaculture industry until the 1970s. By this decade it suffered great mortality mainly due to an iridovirus disease (Grize and Héra, 1991) and since then, not only its production but also its natural populations are endangered due to climate and anthropogenic factors (Huvet et al., 2000). To overcome this situation, there is a need to create tools and strategies to preserve this species' natural banks and to improve its production (Anjos et al., 2017).

Cryopreservation presents itself as a valuable strategy to secure the storage of important genetic lines of endangered species, preserve biodiversity, and improve the management of species reproduction (Adams et al., 2008). Many studies show the possibility of cryopreserving both gametes and larvae of different invertebrates, including bivalve species such as oysters (Martínez-Páramo et al., 2017; Paredes, 2015; Yang and Huo, 2022). When compared to sperm storage, the main advantage of larvae cryopreservation is the immediate availability of a diploid organism upon thawing (Labbé et al., 2018; Suquet et al., 2014). However, there are some challenges on the cryopreservation of larvae when compared to gametes, such as the size and complexity of a multicellular organism (Robles et al., 2008; Suquet et al., 2014).

The improvement of cryopreservation methodologies is imperative to obtain high post-thaw larvae quality. For that purpose, the evaluation of the effect of cryopreservation steps, such as cryoprotectants solutions and freezing process, on the biological processes and pathways of the organism is essential for the establishment of a reliable protocol (Wagh et al., 2011).

The most common quality parameters used to evaluate larval post-thaw quality are swimming activity, morphology, and survival (Yang and Huo, 2022). Information is still scarce, but new technologies are finding their way to become valuable tools in these types of studies. From a molecular point of view, the use of techniques such as transcriptomic and proteomic analysis in cryobiology (Ciereszko et al., 2017; Nynca et al., 2015; Yang et al., 2019), may help to identify different

freezability biomarkers and to understand the molecular consequences related to cryopreservation. This will be extremely important for a species such *C. angulata*, where cryopreservation tools can be applied for the creation of genebanks to secure species preservation.

The application of transcriptome analysis technologies such as next-generation sequencing (NGS) is becoming widespread, using tools such as RNA sequencing (RNA-seq) that allow gene identification and their respective expression (Metzker, 2010; Wang et al., 2009). Gene expression have been successfully used in the investigation of sperm and larvae cryodamage in several species including bivalves (Hossen et al., 2021; Liu et al., 2022b, 2022a; Riesco et al., 2019). There are studies in the use of RNA-seq to identify gene alterations in post-thaw sperm and embryos of different species (Niu et al., 2022; H. Wang et al., 2022; Zhang et al., 2022). In blue catfish (*Ictalurus punctatus*) cryopreserved sperm, authors identified an upregulation in genes related to sperm motility-related functions (cilium, motile cilium, and microtubule cytoskeleton) and amide (often used as a cryoprotectant in sperm preservation) biosynthesis pathway (H. Wang et al., 2022). In kelp grouper cryopreserved larvae (*Epinephelus moara*) it was identified downregulation of genes related to eye development, cranial nerve development, sensory light stimulation and neurotransmitter transport, suggesting an impairment of larvae central nervous system development (Zhang et al., 2022). However, information is still scarce regarding invertebrate larvae and the effects are only associated to the last step of cryopreservation not taking into account the possible toxicity of cryoprotectants during exposure. In a previous study performed by our group in cryopreserved *C. angulata* D-larvae it was identified that the use of different cryoprotectant solutions induced an increase in larvae abnormalities incidence and a reduction of larvae swimming velocity after thawing (Anjos et al., 2022). However, there is no information about more in-depth damage resulted from gene alterations that can compromise further survival and development. The use of transcriptomic tools would be useful to investigate the molecular damage induced by all steps of the cryopreservation process and to identify putative cryodamage biomarkers on oyster´s larvae. This can be achieved by the full screening of the alterations of larvae molecular networks and biological processes promoted by cryopreservation. Therefore, this comprehensive investigation would support the selection of the

most successful cryopreservation methodologies and potentially the identification of new analytical methods for the detection of relevant cryodamage in this species.

The objective of this study was to characterize the molecular damage promoted by cryopreservation in *C. angulata* D-larvae, using transcriptomic tools in two critical cryopreservation steps namely exposure to cryoprotectant solution (CPAs) and freezing/thawing process. Additionally, this work aims to identify putative quality biomarkers to understand the potential impact in larval structures, biological, cellular and molecular functions.

## 4.3. Material and methods

### *4.3.1. Biological material*

*C. angulata* breeders were acquired from Neptunpearl Lda. bivalve farm (Setúbal, Portugal) during their natural spawning period, between May and July. These individuals were transported to IPMA Experimental Station of Shellfish Production (Tavira, Portugal) and kept at 4 °C for a maximum of 24 h. Prior to gametes collection, the oysters were wiped to removed debris and fouling organisms.

### *4.3.2. Gamete collection and D-larvae production*

The oysters were opened, and their sex was determined by microscopic observation of gametes obtained by a small incision in the gonad. Each breeder was independently stripped to collect the gametes for posterior fertilization. Egg suspensions were filtered at 100 µm and retain in 20 µm mesh screen, and spermatozoa sieved at 20 µm, to reduce potential contaminations. Both gametes were maintained in filtered and UV- sterilized seawater (FSW), until the fertilization. The sperm motility and concentration were evaluated by computer-assisted sperm analysis (CASA) system (Proiser R + D S.L., Valencia, Spain), while the oocyte sphericity and concentration were confirmed after 20 min of contact with FSW through observation via light microscopy. Only males with motile spermatozoa and females with spherical oocytes were used. A total of three males and three females were crossed to produce each larvae pool. The fertilization was carried out with a

spermatozoon to oocyte proportion of 1000:1. One hour after fertilization, the eggs were filtered at a 20 µm mesh screen, to remove the remaining sperm, and incubated at 21 ± 1 °C for 24 to 30 h until reaching the D-larvae stage. Each pool was incubated in a 250 L tank with FSW at a concentration of 100 eggs per ml, with slight aeration. The larvae were recovered at a 30 µm mesh screen and kept in FSW to determine their concentration. The total number of larvae in each pool was counted in triplicates under microscopic observation, using a Sedgewick Rafter counting chamber. Pooled D-larvae with no apparent malformations were concentrated (around 120,000 larvae per ml) in FSW and kept on ice (4 °C) for a maximum of 2 h. A total of three pools (n = 3) were obtained in this study.

### *4.3.3. Experimental design*

To characterize the transcriptional changes related with the different steps of cryopreservation, the whole transcriptome profile of fresh larvae diluted in FSW (fresh larvae), fresh larvae exposed to a CPAs (cryoprotectant exposed larvae) and post-thaw larvae (cryopreserved larvae) was compared.

For this purpose, each D-larvae pool was exposed to the three following conditions, one control group and two treatments. As a control group, fresh larvae were concentrated in FSW as previously described. In the first treatment, larvae exposed to CPAs, the concentrated D-larvae were diluted with a 1:1 proportion in a CPAs consisting in a final concentration of 10% (v/v) dimethyl sulfoxide (DMSO) (Sigma-Aldrich), 1% (w/v) Polyvinylpyrrolidone (PVP)-40 40000 MW (Sigma-Aldrich) and 0.2 M Sucrose (Sigma-Aldrich) in milli-Q water, and incubated for 3 min of equilibrium time, at 4 °C. After incubation, the pools of D-larvae were diluted in a 1:3 proportion in FSW to dissipate the CPAs. The second treatment intended to evaluate the effects of the freezing/thawing process. Following the same procedure as in the first treatment, the pooled larvae were incubated with the same CPAs. During the equilibrium time, the larvae pools diluted in the CPAs were loaded to 0.5 ml French straws (30,000 per straw) and maintained at 4 °C until finishes the 3 min equilibrium time. Subsequently, the straws were frozen in a programable biofreezer (Asymptote Grant EF600, United Kingdom) according to Labbé et al. (2018) with the following freezing protocol: 2.5 °C/min from 0 to -10 °C, hold for 5 min at -10 °C, 0.3 °C/min from -10 °C to -20 °C and 2.5 °C/min down to -35 °C, and finally, plunged into

liquid nitrogen (LN) and stored in a LN container. After two months of storage, straws were thawed in a water bath set at 37 °C for 10 s. Afterward, a recovery bath was prepared, by diluting the content of each straw in 2 L of FSW during a period of incubation of 1 h at room temperature. This procedure allowed the removal of cryoprotectants. The post-thaw D-larvae were collected in a 30 µm mesh screen, washed and concentrated in 1.5 ml of FSW. Debris were eliminated, and the larvae quality was evaluated according to Anjos et al. (2022).

### *4.3.4. Transcriptome analysis*

#### *4.3.4.1. RNA extraction, library preparation and sequencing*

Whole transcriptome analysis was performed in triplicate (n = 3) for pools of the fresh larvae, cryoprotectant exposed larvae and cryopreserved larvae. For this purpose, pools of 30,000 and 60,000 D-larvae were centrifuged at 7,400 g for 5 min at 4 °C to remove the FSW. Larvae pellets were then resuspended in 1 ml of TRI Reagent® (Sigma-Aldrich) and stored at -80 °C, until the RNA extraction.

RNA was isolated using TRI Reagent® (Sigma-Aldrich), according to the manufacturer's recommendations, and total RNA was posteriorly purified using the NucleoSpin® RNA II kit (Macherey-Nagel, Germany). One treatment of dsDNase was performed to avoid genomic DNA contamination. The concentration and purity of the total RNA samples were measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA). The RNA integrity was assessed on a bioanalyzer in RIN value (RNA integrity number ≥ 8.6), assessed by Experion RNA StdSens analysis kit (BIO-RAD). A total of 9 RNA samples were stored at -80 °C, for further analyses.

Whole transcriptome sequencing and respective bioinformatic analysis were performed by the Lifesequencing S.L.-ADM company (Valencia, Spain).

A total of 9 libraries were prepared starting from 1 µg of total RNA using a TruSeq RNA Library Preparation Kit v2 of Illumina (Illumina, USA). To confirm the quality of these libraries (library size: 276 - 348 bp; concentration: 76 - 162 nM) a HS D5000 Kit of Agilent 4200 was used, in a TapeStation bioanalyzer (Agilent technologies, USA). The libraries were sequenced in the NovaSeq 6000 Illumina instrument generating paired-end 150 bases reads.

#### *4.3.4.2. Reads processing, mapping, and annotation*

The raw reads were filtered using the BBTols v38.75 software (Bushnell B.). The sequencing adapters, low quality sequences (< Q20) and short sequences (< 40 nucleotides) were removed. Additionally, the reads were checked for potential contamination of bacteria, eukaryote, or archaea rRNA with the SortMeRNA v2.1b program (Kopylova et al., 2012). As *C. angulata* whole genome was not available (Qi et al., 2022), the clean reads were then aligned to the reference genome of *Crassostrea gigas* (RefSeq accession: GCF_000297895.1 from the NCBI database). The proteins were then functionally annotated with the gene ontology (GO) terms by Blast against the Bivalvia taxa (taxID 6544) proteins, using OmicsBox v1.2 program (Gotz et al., 2008). Due to the presence of many isoforms, the quantification of the expression was taken to the gene level, a step performed by the Salmon v1.1 software (Patro et al., 2017).

#### *4.3.4.3. Differential expression analysis*

Differences in expression were assessed using the DESeq2 v3.10 R package (Love et al., 2014). Counts were then filtered to remove the unexpressed genes and those with an expression lower than 5 counts in at least 3 samples. Thresholds were set for significant differential expression as False Discovery Rate (FDR) < 0.05 and $|\log2FC| > 1.5$ (FC – Fold Change) for all the comparisons.

#### *4.3.4.4. Gene Set Enrichment Analysis (GSEA)*

The functional enrichment analysis was carried out with all the genes and not only those with significant different expression. GO terms with 10 - 600 genes annotated and 1,000 permutations were the conditions chosen for the GSEA. The threshold defined for the results was a FDR of 0.05.

#### *4.3.4.5. RT-qPCR confirmation*

To confirm our RNA-seq data and define putative cryodamage markers in *C. angulata* cryopreservation, eleven differentially expressed genes (DEGs) were selected for quantitative RT-PCR (RT-qPCR). The genes were selected for their relation to the oyster growth (*adgre3*, dynein beta), structure-mantle and shell formation- (*mp*, *fbn2*, *myob3b*), oxidative stress (*epx*, *hsp70*) and immune response

(*bp10*, *muc19*, *socs5*, Lectin). The 18S gene was used as reference gene. The primers were designed using Primer-Blast (NCBI) (Table 4.1). Eight hundred nanograms of total RNA were reverse-transcribed to cDNA using a Maxima™ First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Scientific™), following the manufacturer instructions. The qPCR reactions were conducted in duplicate on a CFX96 realtime PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using a SsoFast™ EvaGreen® Supermix. In a total volume of 15 µL, the PCR reaction contained 7.5 µL of supermix, 0.75 µL of each 10 µM forward and reverse primers, and 3.75 µL of cDNA. The thermo cycling protocol used was as follow: 3 min at 95 °C for an initial denaturation followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s and extending at 72 °C for 1 min. A final extension step was carried at 72 °C for 5 min. To normalize the data, the expression of the reference gene in a pool of all samples was used. The relative expression of transcripts was evaluated by the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 4.1 List of forward (FW) and reverse (RV) primers used for the different transcripts analyzed through quantitative RT-PCR.

| Gene | Primer | |
|---|---|---|
| adhesion G protein-coupled receptor E3-like | **FW** | GCCTGAGTATGGCGTTGGAT |
| | **RV** | TAAGCACCCGGGACGTTTTT |
| blastula protease 10 | **FW** | TATATCCCTCCGCCCAGGAC |
| | **RV** | CTGAGGTTTCGCAACGGTCT |
| eosinophil peroxidase | **FW** | CCCAGGAGACTGTACGGAGA |
| | **RV** | TCGGGAGGCAGTCAACTCTA |
| heat shock protein 70 B2 | **FW** | GCGCACTCAAACGACGAAAA |
| | **RV** | CCGTGTCTGTGAATGCAACG |
| mucin-19 | **FW** | GAGGTGCCGGAATAGCTCCA |
| | **RV** | ATGCGCTCATTGCGTTGTCA |
| mantle protein | **FW** | ACCCCGTCGATGTTACCAAG |
| | **RV** | CCTTTGGATTCGTAACCGCC |
| suppressor of cytokine signaling 5-like | **FW** | AGTCAGCTTCCGGCGATATG |
| | **RV** | TGTACGATGCAAGGGAGTGG |
| fibrillin-2 | **FW** | CGGAGGATTTCGATGTGAGT |
| | **RV** | TGAATACCCTTCCCAACAGC |
| dynein beta chain X5 | **FW** | AAAGTGACCACTCTCAGCAGC |
| | **RV** | GCATTATCTGTCCAGTGTCCTCA |
| myosin-IIIb | **FW** | TCCGACCAGAAAAATTCTAGCCA |
| | **RV** | GGAATAGGCTTGGCCACTGA |
| lectin | **FW** | GCTCTCCTGGTGGGACTTTT |
| | **RV** | TCGTTGGCTGCATCTGAACA |

## 4.4. Results

### *4.4.1. RNA sequencing and mapping*

A pair end sequencing of 9 libraries was performed using a NovaSeq 6000 Illumina instrument. After a filtering step, 375,187,599 total clean reads were obtained. The average quality of the generated reads ranged from 35.50 to 36.18. The mapping rate of the final reads to the *C. gigas* RefSeq genome (GCF_000297895) was between 66.38% and 79.75%. The distribution of raw and clean reads among the different samples, as well as their mapping rate, are represented in Table 4.2.

Table 4.2 RNA sequencing results for the reads counting and mapping to the reference genome (*Crassostrea gigas*) in each replicate of the three different treatments: Fresh (fresh larvae diluted in FSW), CPA exposure (fresh larvae exposed to CPAs) and Cryopreserved (post-thaw larvae).

| Sample name | Raw reads | Clean reads | Mapping rate |
|---|---|---|---|
| **Fresh 1** | 39,715,992 | 35,869,429 | 78.26% |
| **Fresh 2** | 37,386,071 | 34,327,775 | 71.32% |
| **Fresh 3** | 62,447,666 | 57,490,869 | 78.36% |
| **CPA exposure 1** | 54,670,784 | 48,589,140 | 78.67% |
| **CPA exposure 2** | 48,127,214 | 44,519,020 | 75.57% |
| **CPA exposure 3** | 62,107,727 | 57,668,375 | 79.75% |
| **Cryopreserved 1** | 33,792,962 | 30,215,425 | 74.03% |
| **Cryopreserved 2** | 38,604,604 | 34,370,367 | 66.38% |
| **Cryopreserved 3** | 36,055,724 | 32,137,199 | 76.05% |

### *4.4.2. Differential expression analysis*

By using the DESeq2 R package, a total of 22,787 genes were identified among all the samples. In Fig. 4.1 shows two volcano plots representing the differentially expressed genes in the two different comparisons, cryoprotectant exposed larvae against fresh larvae (Fig. 4.1A) and cryopreserved larvae against fresh larvae (Fig. 4.1B), and a Venn diagram representing the distribution of DEGs between the two comparisons (Fig. 4.1C). For the first comparison, 3 DEGs were identified, 1 downregulated and 2 upregulated. Addressing the second comparison, a total of 511 DEGs were found, 278 of them were considered downregulated and the remaining 233 upregulated. Due to the low number of genes differentially expressed in the first condition (Fig. 4.1A) for further analysis only the second comparison was taken in account.

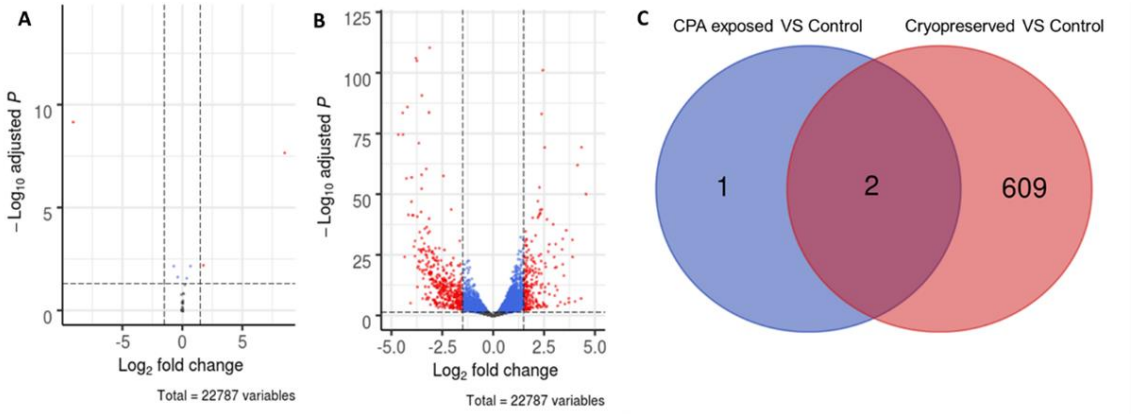


Figure 4.1 Volcano plots representing the results of differential expression analysis between the two comparisons. (A) Cryoprotectant exposed larvae against fresh larvae. (B) Cryopreserved larvae against fresh larvae. (C) Venn diagram representing the distribution of DEGs between the two comparisons.

### *4.4.3. Enrichment analysis*

For a further understanding of the biological meaning of the DEGs represented under cryopreservation of *C. angulata* larvae, a GO enrichment analysis was performed for all the DEGs, using the Database for Annotation, Visualization and Integrated Discovery (DAVID). The most significantly enriched GO terms in biological processes (BP), cellular components (CC) and molecular functions (MF) were "carbohydrate metabolic process" (GO:0005975), "integral component of membrane" (GO:0016021) and "chitin binding" (GO:0008061), respectively.

It is also important to mention the significant enrichment of the "extracellular region" (GO:0005576) and the "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, another compound as one donor, and incorporation of one atom of oxygen" (GO:0016716) (Fig. 4.2).

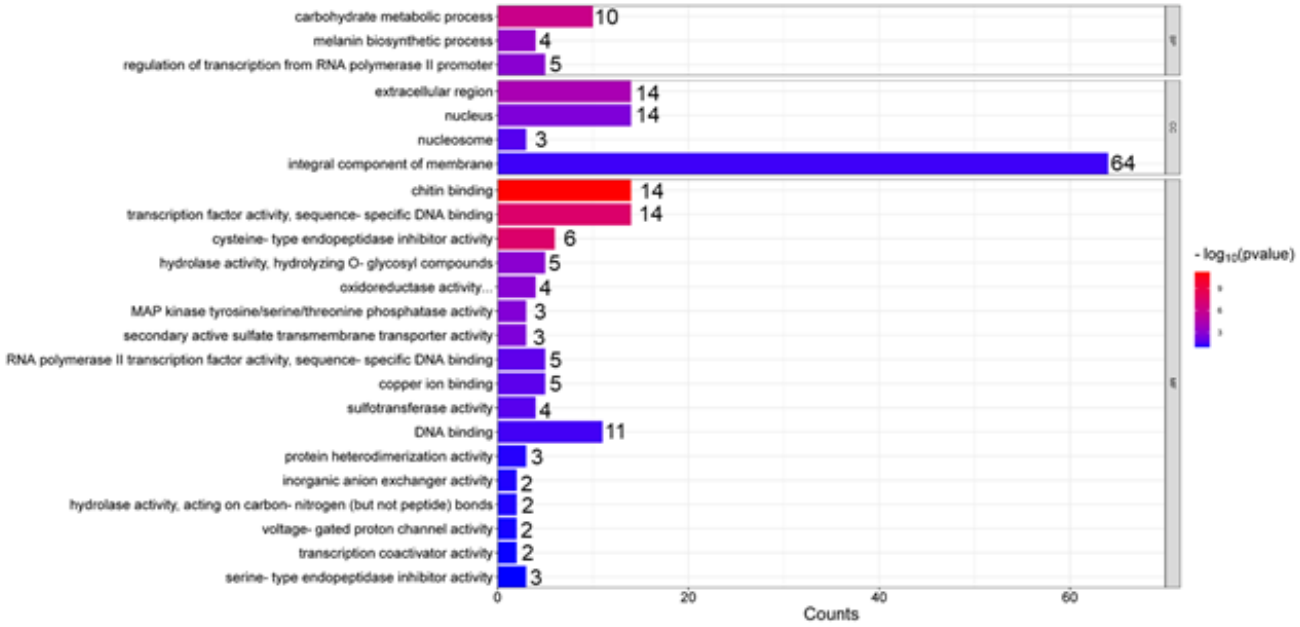


Figure 4.2 Gene ontology (GO) enrichment results for all differentially expressed genes (DEGs) in the comparison between cryopreserved larvae against fresh larvae.

Moreover, a Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis for all the DEGs was performed, to assess the most enriched pathways. The 10 most enriched pathways from a total of 126 are represented in Fig. 4.3. The most enriched pathways were the "neuroactive ligand receptor interaction", "endocytosis" and "spliceosome". However, it is also important to notice the significant enrichment of protein regulation pathways such as "protein processing in endoplasmic reticulum" or "proteasome", and other important developmental pathways as "ribosome" and "FoxO signaling pathway".

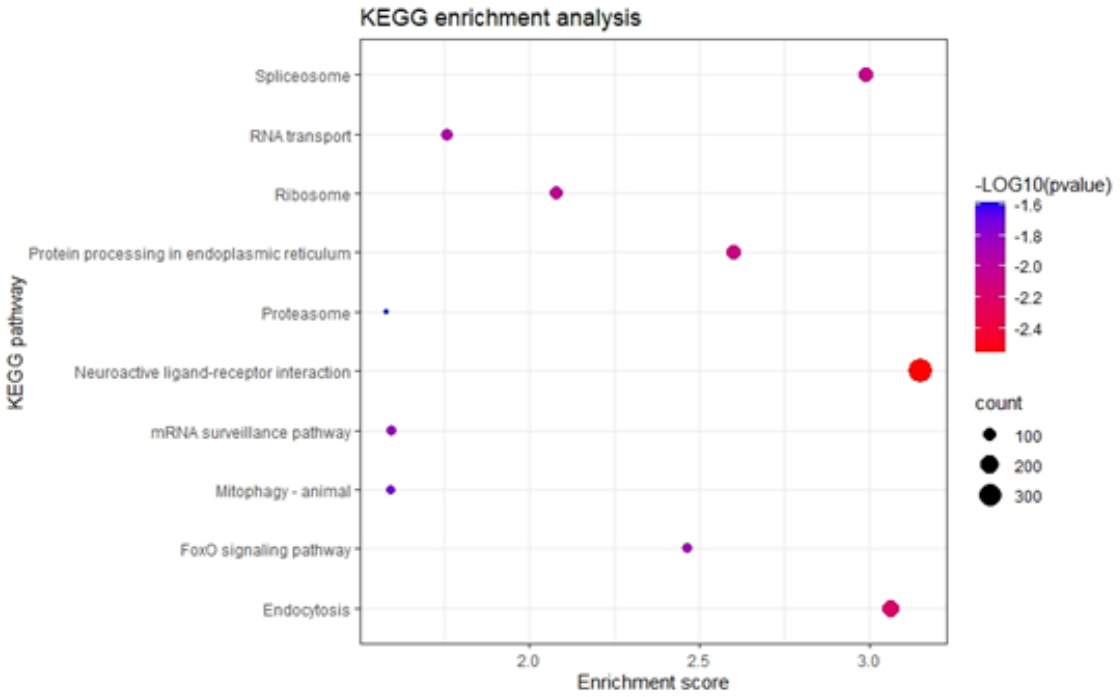


Figure 4.3 Top 10 enriched pathways according to the results of the KEGG enrichment analysis for all differentially expressed genes in the comparison between cryopreserved larvae against fresh larvae.

### *4.4.4. RT-qPCR confirmation*

Through RT-qPCR, the results from the RNA-seq analysis were confirmed using a set of 11 genes related to important mechanisms such as growth (*adgre3*, dynein beta), structure (*mp*, *fbn2*, *myob3b*), oxidative stress (*epx*, *hsp70*) and immune response (*bp10*, *muc19*, *socs5*, lectin). The comparison between the results of gene expression in both techniques presented the same tendency and for that reason the RNA-seq output was considered to be confirmed. More specifically there was a general upregulation of *adgre3*, *socs5*, *hsp70* and *myob3b* regarding control group. Contrarily, dynein beta, *bp10*, *muc19*, lectin, *epx*, *mp* and *fbn2* were downregulated in the two different methods. These results are shown in Fig. 4.4. Table 4.3 summarizes the expression results obtain using both methods for all the 11 selected genes, and their respective previously reported function.

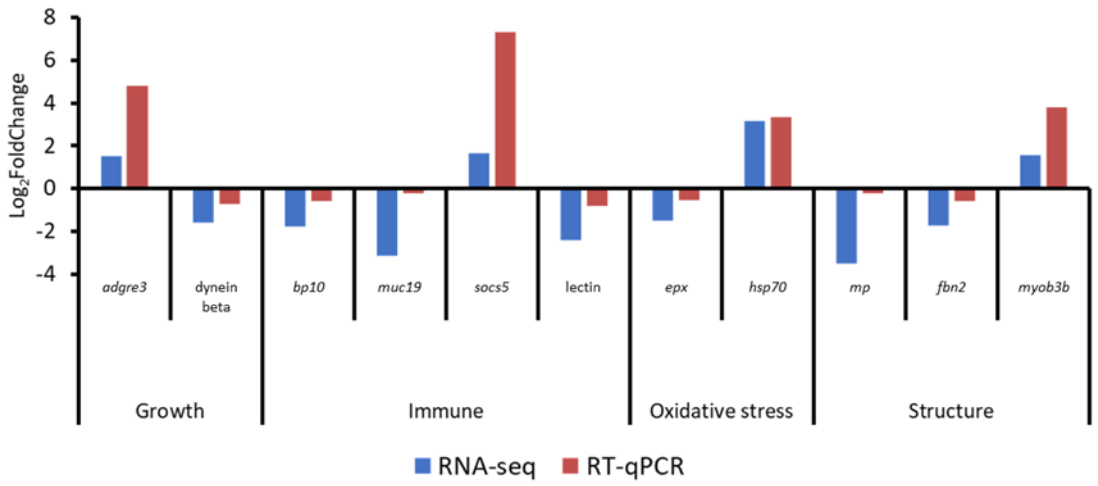


Figure 4.4 Quantitative qPCR validation of RNA-seq results in terms of relative gene expression.

Table 4.3 Functions of the differentially expressed genes selected as putative biomarkers of cryodamage. Relative expression and statistical significance are described.

| Gene name | Gene symbol | RT-qPCR expression | RNA-seq log2Fold change | *p*_value | Description |
|---|---|---|---|---|---|
| **adhesion G protein-coupled receptor E3-like** | *adgre3* | Up | 2.04 | 2,69e-19 | Related to immune function of *Pimephales* sp. (Klymus et al., 2022) |
| **dynein beta chain X5** | dynein beta | Down | -1.60 | 9,72e-06 | Involved in the generation and regulation of the bending of cilia and flagella in eukaryotes (Park et al., 2006) and in the initial shell formation process of *C. gigas* (De Wit et al., 2018) |
| **blastula protease 10** | *bp10* | Down | -1.79 | 4,08e-12 | Applied to assess the toxic effects of metals in *P. lividus* embryos (Bonaventura et al., 2022) |
| **mucin-19** | *muc19* | Down | -3.13 | 1,60e-20 | Detected as an immune effector in *Crassostrea virginica* larvae (Modak and Gomez-Chiarri, 2020) |
| **suppresor of cytokine signaling 5-like** | *socs5* | Up | 1.63 | 4,95e-21 | Involved in intercellular signal pathways related with the immune system (De Zoysa et al., 2009; De Zoysa and Lee, 2009), growth and development of the organisms (Ilangumaran et al., 2004) |
| **lectin** | lectin | Down | -2.41 | 6,42e-13 | Involved in the self and nonself recognitions, innate immunity, reproduction and food capture and ingestion in bivalves (Allam et al., 2021; Pales Espinosa and Allam, 2013; Saco et al., 2023; Wang et al., 2019) |
| **eosinophil peroxidase** | *epx* | Down | -2.06 | 8,33e-23 | Protective mechanism of *C. gigas* for mitigating cellular stress and reducing levels of ROS (Zhao et al., 2016) |
| **heat shock protein 70 B2** | *hsp70* | Up | 3.59 | 3.87e-06 | Considered a stress response in frozen-thawed bovine embryos (Park et al., 2006) |
| **mantle protein** | *mp* | Down | -4.02 | 4.66e-42 | Protein present in the mantle epithelial cells of bivalves (Yarra et al., 2016) |
| **fibrilin-2** | *fbn2* | Down | -1.75 | 9.19e-12 | Down regulated in the mantle and gills of *C. gigas* after hypoxia exposure (David et al., 2005) |
| **myosin-III** | *myob3b* | Up | 1.78 | 1.44e-12 | Important role in *C. gigas* embryo-larval related to the formation of the muscle structures particularly associated with locomotion (Li et al., 2021) |

## 4.5. Discussion

After a few studies in *C. angulata* to establish and improve freezing/thawing protocols, cryopreservation has been identified as a technique with the potential to preserve its genetic lines and enhance its reproduction management (Anjos et al., 2022, 2021; Kuo and Gwo, 2022; Riesco et al., 2019, 2017a). Although *C. angulata*, like other bivalves, had promising results in preserving sperm and larvae (Anjos et al., 2022, 2021; Kuo and Gwo, 2022; Riesco et al., 2019, 2017a), cryopreserving larvae has proven to be challenging due to the multicellular organism's size and complexity (Anjos et al., 2022). In bivalves, the evaluation of the post-thaw quality of larvae has been based on swimming activity, morphology, and survival (Anjos et al., 2022; Paredes, 2015; Yang and Huo, 2022). To elucidate about the molecular changes that occur during the cryopreservation process and identifying the involved molecular mechanisms of cryodamage, the current study has analysed the alterations in gene expression of cryoprotectant exposed and cryopreserved *C. angulata* larvae using RNA sequencing.

### *4.5.1. Transcriptomic, functional and enrichment analysis*

In the present study, it was possible to identify a set of differentially expressed genes in both treatment comparisons. Assessing the comparison between cryoprotectant-exposed larvae and fresh larvae, it was possible to detect 2 upregulated and 1 downregulated DEGs. Since the number of DEGs in this comparison was considered to be low, any possible toxic effect dealt by cryoprotectant exposure was not considered for further analysis. However, the number of DEGs detected when comparing cryopreserved larvae against fresh larvae, was considerably higher. It was possible to identify 233 upregulated and 278 downregulated DEGs.

Following a functional analysis, the relation of all DEGs to specific cellular components (CC), molecular functions (MF) and biological processes (BP) was evaluated through an enrichment analysis. Among these different gene ontologies (GO), the most significantly enriched were integral components of membrane (CC) and Chitin binding (MF). Chitin is the second most abundant natural polysaccharide and it is abundantly present in the shell matrix of oyster species such as *Pinctata fucata martensii* and *C. gigas* (Lee and Choi, 2007; Sukuzi et al., 2007). Chitinase and

Chitin synthases are vastly represented in the mantle, a very important tissue in shell formation and during early oyster larval development stages (Du et al., 2017). The results of the present study showed a downregulation of chitin synthase, chitin binding proteins and different isoforms of acidic mammalian chitinase in cryopreserved larvae, suggesting an impairment in shell formation and corroborating the data presented by Anjos et al. (2022) in previous studies. Also, chitinases are known for their importance in immune response and by helping to maintain normal life cycle functions since, not only synthesis, but also degradation of chitin, are important in the development of chitin containing organisms (Badariotti et al., 2007). Different DNA binding related molecular functions (RNA polymerase II transcription factor activity, sequence specific DNA binding; transcription factor activity, sequence-specific DNA binding; DNA binding) were also significantly enriched. An example of DNA binding functions are the changes occurring in DNA methylation during early development in *C. gigas* as reported by Riviere et al. (2013). Moreover, Zhao et al. (2012) hypothesized that due to its connection to cell proliferation, DNA binding was a key element in stress response. This means that as a stress factor, the cryopreservation process may induce an impairment in the early development of *C. angulata* larvae.

Regarding the KEGG pathway enrichment analysis, the most significantly enriched pathway was the "neuroactive ligand-receptor interaction". This pathway is made up of receptors situated on plasma membranes that are involved in signal transduction from the external environment into cells (Lauss et al., 2007). An analysis of the transcriptome of *C. gigas* and *P.f. martensii* at different stages of development revealed that neuroendocrine pathways such as this one, were implicated in important developmental functions like shell formation, settling, and metamorphosis (Zheng et al., 2019). Furthermore, a previous study by Lu et al. (2022) reported a significant enrichment of this pathway upon induced stress in *P.f. martensii*. Both previous studies and the results of our study are a plausible evidence of the malformations observed by (Anjos et al., 2022).

The second two most significant enriched pathways were the endocytosis and the spliceosome. The first one is an imperative part of the membrane receptors activity and quantity control, regulating the signal transduction mediated by those

receptors (Yarden and Tarcic, 2013). This pathway is also known to be related to cell proliferation and organism growth since it is connected to the degradation of epidermal growth factor receptors (EGFR) (Wu and Zhang, 2020). These receptors were referred to be directly correlated to the development and growth of the pearl oyster (Wang et al., 2018). Also, Li et al. (2023), proposed that endocytosis-induced processes such as signal transduction and plasma membrane proteins degradation, may regulate oyster growth by integrating endogenous signaling pathways and environmental input. These previous studies corroborate the results of the present study and therefore, it is hypothesized that cryopreservation may affect growth in *C. angulata* larvae.

Concerning "spliceosome", as a key tool of genetic information processing, it is extremely important in the survival, adaptation and development of the organism (Mitra et al., 2020). Previous studies have already demonstrated an impairment of this process due to stress factors such as ocean acidification (X. Wang et al., 2022). The same may be assumed by observing the functional analysis results of the present research, more specifically, the significant enrichment of processes like "DNA binding".

In our study, there was also a significant enrichment of the "ribosome" pathway. This molecular organelle is responsible for the translational capacity of a cell and so, directly related to protein synthesis and functions such as cell growth, proliferation and apoptosis (Q. Liu et al., 2020). Moreover, other protein processing related pathways were significantly enriched, the "proteasome" and "protein processing in endoplasmic reticulum", suggesting an increased amount of degraded proteins. Cryopreservation clearly induces a stress response in this organism and apparently leads to a development malfunction, mainly at the shell formation and membrane level.

### *4.5.2. Putative biomarkers of cryopreserved larval quality*

To validate RNAseq data, a selection of 11 genes was done according to their relevance in certain functions. These genes were checked for their putative use as biomarkers of post-thaw larval quality. One of the functions affected by cryopreservation was the oxidative stress system. Heat shock proteins 70, *hsp70*, a

subgroup of chaperones, are important to maintain the homeostasis of the cell and has the capacity of counteract apoptotic mechanisms, interviewing in cell processes such as cell movement and cytoskeleton stabilization (Balogi et al., 2019; Zhang et al., 2012). Our findings indicate that the expression levels of *hsp70* were higher in post-thaw D-larvae than in fresh larvae (larvae dilute in FSW). This result is in line with data reported by Park et al. (2006), where the expression levels of four apoptotic-related genes, including *hsp70*, were observed to be significantly elevated in cryopreserved bovine embryos. This expression may arise, as suggested by the authors, as a stress response and potentially compromised developmental ability. Overall, these proteins have the function of preventing thermal or oxidative stress, which is commonly associated with cryopreservation procedures.

Eosinophil peroxidase (*epx*) is an enzyme released from eosinophils granulocytes, that is essential to maintain the main function and homeostasis of eosinophils (Choi et al., 2021). Eosinophils are made part of a group of cells known as hemocytes that play a crucial role in bivalve immune response to defend against different stressors, such as pathogens, temperature, acidification and pollution (de la Ballina et al., 2022). In the present work, the *epx* expression revealed that this gene was suppressed in D-larvae after freezing/thawing steps. Other studies, conducted in *Anguilla japonica* and *C. gigas* reported that when organisms were exposed to osmotic stress conditions, the levels of *epx* were also suppressed (Tse et al., 2013; Zhao et al., 2016), having been suggested by Zhao et al. (2016) that this could serve as a protective tactic for mitigating cellular stress and lowering levels of reactive oxygen species (ROS). The observed suppression of *epx* expression in our study may be related to our experimental protocol that involved environmental fluctuations due to the freezing/thawing process, which must have induced thermal stress, leading to increased expression of *hsp70* rather than *epx*.

The dynein beta chain flagellar outer arm, *dyh4*, belongs to the dynein protein family. Dynein proteins are divided into two main groups, which are cytoplasmic and axonemal dynein (Toda et al., 2020). Cytoplasmic dynein is responsible for intracellular transport and cell mitosis (Canty et al., 2021; Toda et al., 2020). Axonemal dynein is a microtubule-based molecular motor that is in charge of the generation and regulation of the bending of cilia and flagella in eukaryotes (Aprea

et al., 2021; Toda et al., 2020). Dynein arms convert the chemical energy released upon ATP binding into mechanical force, producing the driving power for the organelles' movement (Aprea et al., 2021). Cilia and flagella play important roles in bivalve sperm and larvae motility. The expression of dynein beta in thawed *C. angulata* D-larvae was lower than in the fresh larvae, suggesting some level of cilia impairment which may affect the larvae swimming performance and, in further larvae development stages, their feeding behavior and sensorial role. Unfortunately in the present study we could not follow larval development but this data corroborated previous findings reported by our group, where a significant lower velocity and motility was observed in thawed *C. angulata* D-larvae when compared with the control group (Anjos et al., 2022). Similar findings were reported by Suquet et al. (2012) and Suneja et al. (2014), where lower performances in swimming activity of *C. gigas* post-thaw D-larvae were reported. Suneja et al. (2014) even explored the organogenesis of these larvae and identified development problems in the velum structure as a result of cryodamage, which suggests that modifications in the structure of the velum can lead to cilia impairment. This can affect the swimming and feeding behaviour and compromise the performance of the larvae or even lead to their death at later stages. Apart from impairing structural modification associated with filtration and movement mechanisms, the downregulation of dynein motor proteins may also contribute to shell malformations, previously detected (Anjos et al., 2022). Since as stated by De Wit et al. (2018) these proteins have an important role as transporters of cellular components during the initial stages of shell formation in this species.

Myosin is the key muscle protein of thick filaments (Li et al., 2021) and its function is to transform chemical energy in mechanical force that travels along actin filaments, resulting in the contraction of the muscle (Asai and Wilkes, 2004). In bivalves, during larval development, the actin filaments or expression profiles of myosin heavy chain are mainly found in the velum retractor and adductor muscle, while in the adult phase in the adductor and mantle muscles (Li et al., 2021, 2019; Yang et al., 2023). Myosin heavy chain seems to have an important role in *C. gigas* embryo-larval developmental phases, especially in the stages of trochophore and D-larvae, due to the formation of the muscle structures during embryogenesis some associated with larval locomotion (Li et al., 2021). Thus, our results revealed that

cryopreservation may have affected certain mechanisms in *C. angulata* D-larvae related to myosin protein production. This is evidenced by the higher gene expression of *myob3b* in the cryopreserved D-larvae compared to the control group, which may suggest a compensatory response to the cryopreservation process.

Fibrillin-2 is a protein encoded by the *fbn2* gene that belongs to the fibrillin family (Zhang et al., 1994). In *C. gigas*, fibrillin is highly expressed in the mantle (Miyamoto et al., 2002), a soft tissue layer that lines the inner shell and covers the visceral mass. This structure provides protection, contributes to shell formation, facilitates respiration and plays a crucial role in the oyster's filter-feeding mechanism (Joubert et al., 2014; Yarra et al., 2016). Our results showed that expression of the *fbn2* transcript was suppressed after cryopreservation. This suggests that there may be an alteration at the level of the extracellular matrix of *C. angulata* larvae. This alteration is likely to have implications in the development and maintenance of tissues, potentially jeopardizing the subsequent larval developmental stages. David et al. (2005) observed a down-regulation of the fibrillin gene in the mantle and gills of *C. gigas* after 24 days of hypoxia exposure. These results together with the ones obtained for *mp*, a specific mantle protein present in the epithelial cells, where expression was also suppressed, demonstrate that these alterations could compromise proper functioning, which can lead to alterations in the mantle tissues, shell formation and feeding capacity, especially during the early stages of development when structures are being formed. Some morphological alterations as mantle protuberance and reduction of body size related to the shell were detected in *C. angulata* post-thaw D-larvae in a prior work conducted by our group (Anjos et al., 2022). These morphological alterations can be related to *mp* gene suppression observed after freezing/thawing steps.

Suppressors of cytokine signaling (*socs*) are a class of inhibitory proteins that negatively regulate cytokine signal transduction (De Zoysa and Lee, 2009; Liu et al., 2013). These inhibitory proteins play an essential role in several intercellular signal pathways that are engaged in the immune system (De Zoysa and Lee, 2009; Li et al., 2015), growth and development of the organisms (Ilangumaran et al., 2004) being identified in several organs of *C. gigas* (Li et al., 2015). In our results, we identified the socs5 gene as being upregulated in cryopreserved larvae when compared with

the fresh larvae. This result suggested that the cryopreservation protocol may have induced physical stress in the D-larvae of *C. angulata*, due to exposure to a combination of hyperosmotic solution (cryoprotectant solution) with temperature variations (freezing/thawing steps). These stress factors may have activated the production of cytokines, and to control an excessive response that can be harmful to the host, *socs5* was activated. Similar results were obtained by De Zoysa et al. (2009) with the *socs2* gene being upregulated in *Haliotis discus discus* during thermal, low-salinity and hypoxic stress.

Blastula protease 10, *bp10*, involved in immune functions and embryonic development (Bonaventura et al., 2022; Hartman et al., 2018) is an enzyme that belongs to the astacin metalloprotease family (Lhomond et al., 1996). This gene has been used to evaluate the toxic effects of metals and other contaminants in *Paracentrotus lividus* embryos being upregulated or downregulated depending on the compound tested (Bonaventura et al., 2022). Our results revealed that cryopreservation suppressed the *bp10* expression in the D-larvae of *C. angulata* when compared to the control. However, little is known about the *bp10* gene in oysters and further studies will be needed to characterize and understand how variation in the expression of this metal binding protein is affected in cryopreserved larvae.

Lectins play a fundamental role in self and non-self-recognition, innate immunity, reproduction and food capture and ingestion in bivalves (Allam et al., 2021; Pales Espinosa and Allam, 2013; Saco et al., 2023; Wang et al., 2019). Particularly in shell formation, these groups of proteins are involved in the extracellular matrix agglutination (De Wit et al., 2018). In our study, the expression of lectins in *C. angulata* cryopreserved D-larvae was lower than in the fresh larvae. Since lectins can bind carbohydrates present on the surface of microalgae, they participate in the recognition of food particles. Therefore, in our study changes in lectin gene expression may affect larvae's digestive functions, impairing their ability to efficiently capture and process food. It is crucial to understand the implications of altered lectin gene expression in *C. angulata* thawed D-larvae as it can have consequences on their feeding selection and further digestive function and ultimately in larval developmental outcomes.

## 4.6. Conclusion

In this study, the analysis of differential gene expression revealed significant changes in genes associated with growth, structural development, oxidative stress response, and the immune system in cryopreserved *C. angulata larvae*, compared to fresh larvae. No effects were seen in larvae exposed to cryoprotectants. Our findings provide valuable insights into the development of reliable cryopreservation protocols and in the detection of relevant biomarkers of cryodamage in *C. angulata* D-larvae. Importantly, they identify the critical step in all the cryopreservation process, revealing that gene expression is not affected by cryoprotectant exposure as it is by the freezing process itself.

## 4.7. Supplementary data

Records were submitted to GEO repository (NCBI) with the accession number GSE246924.

# CHAPTER 5. GENERAL DISCUSSION

# General Discussion

Due to anthropogenic impact, pathologies and asynchronous reproduction, worldwide natural banks of bivalves are experiencing a decline (Oyarzún et al., 2018; Rato et al., 2022). The Portuguese oyster and striped venus clam serve as two examples of important European resources affected by these problems (Chiesa et al., 2021; Joaquim et al., 2016). In this way, it is crucial to implement effective strategies to prevent these losses. Recognizing the importance of preserving the genetic diversity, one possible approach involves the establishment of a cryobank using cryopreservation to store the genetic material (Martínez-Páramo et al., 2017; Yang et al., 2021). However, cryopreservation should be associated with aquaculture practices, not only to store breeding lines but also to optimize the use of resources from the hatcheries. For example, during the breeding season, hatcheries do not have the capacity to grow the total number of produced larvae and must discard some of them. With the implementation of the cryopreservation procedure, the hatcheries can store the surplus larvae and produce out-of-season (Labbé et al., 2018; Yang and Huo, 2022). Indeed, several cryopreservation methodologies have been explored and have shown the potential of this tool to support conservation programs and aquaculture production. For example, genetic banks for sperm from various coral species were established and are being used to help mitigate threats to the Great Barrier Reef and other areas (Hagedorn et al., 2019). A similar approach was developed by other authors who reported a streamlined procedure for the construction of a sperm repository for a breeding program of *Crassostrea virginica* (Yang et al., 2021).

In the field of bivalves, there is the possibility of establishing genetic repositories containing information on progenitors and progeny, through cryopreservation methodologies developed for gametes and embryos or larvae, respectively (e.g. Paredes, 2015; Yang and Huo, 2022). The majority of the research conducted has been focused on sperm (e.g. Demoy-Schneider et al., 2018; Yang et al., 2021), however, there has been a recent tendency to address the cryopreservation of embryos and larvae (e.g. Heres et al., 2023; Liu et al., 2022b), as oocytes have proved to be challenging with limited success (Adams et al., 2009).

The storage of biological material at cryogenic temperatures and the recovery of its functions with success relies on the development of protocols to prepare the samples for the cryopreservation process. Cryopreservation involves multiple steps, including sample collection and preparation, selection of the cryoprotectant solution (type, concentration and combination of cryoprotectant agents), packaging of the sample and, freezing and thawing processes (Paredes, 2015; Yang and Huo, 2022). During this process, damage to the biological material can occur. Some damage can be lethal, resulting in cell death, while other damage may be sublethal, causing injuries that do not lead to immediate death but could render fertilization or developmental functions unfeasible (Pini et al., 2018). The ultimate objective of preserving sperm and larvae to establish a genetic bank for future repopulation programs is compromised when this type of damage occurs. The intricate balance between preserving viability and minimizing damage requires continuous refinement of cryopreservation protocols. Addressing the issues of both lethal and sublethal damage is imperative to ensure the success of genetic banks and the long-term viability of stored biological material.

The present work aimed to provide baselines for an *in vitro* genetic resource repository dedicated to *C. angulata* and *C. gallina*. This was achieved through the improvement of existing cryopreservation methodologies or the development of new protocols for species lacking available information. Additionally, several cryodamage evaluation techniques were explored to understand the cryopreservation process's impact on the biological material of the target species.

## 5.1. Strategies to reduce the cryodamage and improve post-thaw quality by the cryoprotectant solution

Cryoprotectants are substances that interfere with the inner and outer of the cells mitigating the adverse effects caused by ice crystal formation and dehydration (Elliott et al., 2017; Ozimic et al., 2023). In this way, strategies involving the optimisation of the cryoprotectant solution have been identified as one key factor in improving the quality of biological material after thawing. These strategies may include selecting the most suitable type of cryoprotectant agent and concentration,

evaluating their protective impact compared to their harmfulness and, combining different agents (e.g. Heres et al., 2021; Riesco et al., 2017a; Simon and Yang, 2018).

**Chapter 2** of this thesis focused on the cryopreservation of *C. angulata* sperm, where the cryoprotectant solution was addressed. This involved supplementing the permeant agent (10% DMSO) with sugars (0.45M trehalose or sucrose) to improve the post-thaw quality. This work was based on the previous work by Riesco et al. (2017a) where the concentration and type of permeant cryoprotectant, conditions of freezing and thawing and storage method were established. However, this work did not explore the effect of combining different types of cryoprotectants.

The combination of permeant agents with compounds that interact with the outside environment of the cell has demonstrated several benefits. For example, Riesco et al. (2017c) improved the post-thaw quality of *Epinephelus marginatus* sperm through the supplementation of the control solution (1% NaCl + 10% DMSO) with 50 mM taurine. In another study, Zilli et al. (2014), added the antifreeze protein AFPIII in the cryopreservation medium significantly improving the quality of the thawed sperm in *Sparus aurata*. Other types of compounds that can be used as additives are sugars, which are non-toxic compounds, that have been explored as a strategy, namely, in sperm cryopreservation of mammals (Öztürk et al., 2020; Zhu et al., 2017), fish (Nynca et al., 2016; Sandoval-Vargas et al., 2021a) and bivalves (Adams et al., 2004; Hassan et al., 2017a). Liu et al. (2016) developed a protocol for freezing sperm of *Mytilus galloprovincialis* and reported that the addition of sugars (glucose, sucrose or trehalose) in DMSO did not enhance post-thaw sperm fertilization rates, whereas 0.8% glycine significantly improved this parameter. Adams et al. (2004), have investigated the effect of trehalose alone and combined with different concentrations of DMSO during sperm cryopreservation of *Crassostrea gigas*. They found that the combination of trehalose and DMSO resulted in a modest improvement in fertility compared to trehalose alone. In experiments performed by Hassan et al. (2017a), the inclusion of trehalose or glucose with DMSO further increased the post-thaw motility and viability of *Ostrea angasi* sperm.

As trehalose combined with the external cryoprotectant DMSO was successfully tested in oysters, the combination of one sugar (trehalose or sucrose) with DMSO was chosen to assess its effect on sperm of *C. angulata*, where no published studies were available. The protective properties of sugars during cryopreservation can be related to their high molecular and viscosity, helping in cellular dehydration and stabilizing the surrounding environment (Nicolajsen and Hvidt, 1994; Tsai et al., 2018; Woelders et al., 1997). These natural cryoprotective agents are of non-animal origin, making them less prone to promoting bacterial contamination, unlike egg yolk or bovine serum albumin, commonly used as membrane stabilizers. Overall, in the present thesis sugar supplementation, especially trehalose, showed an important role in reducing oxidative stress by improving sperm plasma membrane integrity and reducing ROS levels, acrosome damage, and lipid peroxidation. This approach, inspired by nature and applied in **Chapter 2** of this thesis, has proven effective in mitigating cryodamage related to oxidative stress in the sperm of *C. angulata*. After the article of **Chapter 2** was published, another protocol to freeze sperm of *C. angulata* arose, authored by Kuo and Gwo (2022). In this protocol, a cryoprotectant solution similar to our control was employed, but with faster freezing and thawing rates than ours. Considering the outcomes of both studies, future protocol optimization for *C. angulata* should address the DMSO supplementation with different concentrations of trehalose and faster freezing and thawing rates.

At the outset of this thesis, no cryopreservation protocols were available for *C. angulata* larvae and any type of biological material of *C. gallina*. Hence, the exploration of this subject was necessary, and the details are described in **Chapter 3**. Larvae cryopreservation is advantageous over sperm, once after thawing diploid organisms are available immediately (Labbé et al., 2018; Suquet et al., 2014). This is particularly useful in species where there are no established procedures for the cryopreservation of gametes from both sexes, as in the specific case of *C. angulata*. Furthermore, it may be particularly interesting to establish a genetic bank for *C. gallina*, given the difficulty in collecting gametes separately, considering the small size of breeders and the necessity of a hydrodynamic stimulus for spawning viable gametes (Joaquim et al., 2016).

In **Chapter 3**, cryopreservation protocols for D-larvae of *C. angulata* and *C. gallina* were established. Similarly, to **Chapter 2**, the strategy for designing the cryoprotectant solution was based on the mix of permeant and non-permeant agents. However, due to a lack of information on target species, **Chapter 3** explores the effect of two types of permeants cryoprotectants (10% DMSO or EG) combined with a pre-established external cryoprotectant (1% PVP-40 and 0.2M sucrose) on D-larvae quality during cryoprotectant exposure and after thawing. Non-permeable compounds were chosen for their ability to protect against osmotic stress and reduce the toxicity of permeable agents. Furthermore, PVP-40 has the capacity to increase the viscosity of the solution due to its high molecular weight (Fuller, 2004). The selection of the cryoprotectant types and concentrations, as well as the remaining steps of the cryopreservation, including sample preparation, packaging and the process of freezing and thawing, were adopted from the available literature on *C. gigas*. Labbé et al. (2018) reported the best survivals for post-thaw larvae by frozen at late trochophore (20 hpf) and early-D larval (24 hpf) stages, applying a very slow freezing rate without seeding, and by the use of 10% EG with 1% PVP-40 and 200 mM sucrose as cryoprotectant solution. Additionally, Y. Liu et al. (2020b), showed that the highest post-thaw D-larval rate was achieved with a cryoprotectant medium consisting of 10% EG, 5% Ficoll and 0.2% PVP. Suquet et al. (2014), reported a successful post-thawing development of *C. gigas* until the reproductive stage was achieved, using a cryoprotectant solution composed of 10% EG with 1% PVP and 200 mM trehalose.

Based on the bibliography available for *C. gigas* that was mentioned earlier, **Chapter 3** evaluated also the toxicity of different cryoprotectant solutions, namely 10% DMSO or EG. The initial step in selecting the cryoprotectant solution is to evaluate its impact on the normal functions of the biological material (Heres et al., 2021, 2019). This preliminary experiment is important for excluding treatments that may present high or lethal levels of toxicity during the exposure period. Based on the findings from **Chapter 3**, it was observed that DMSO exposure promoted effects on the morphology and antioxidant enzymatic activity of *C. angulata* larvae when compared to fresh larvae (control) and those exposed to EG. The DMSO treatment resulted in an increased percentage of larvae with a reduced body size concerning the shell and an elevation in the glutathione reductase (GR) activity.

However, the percentage of motile larvae and their velocity were similar to the control. According to Gwo (1995), the reduction in body size can be explained by water loss due to dehydration caused by the cryoprotectants. This process involves initial shrinkage, subsequent cryoprotectant influx until equilibrium is reached, and gradual re-expansion of the embryos. Horváth et al. (2012) investigated the impact of DMSO exposure on two larval stages (trochophore and veliger) of *Ostrea edulis* at different concentrations (5, 10, 15 and 20%). Among the tested concentrations, the trochophore larvae exposed to the highest concentration of DMSO recorded the lowest survival rate. Additionally, Heres et al. (2021) conducted toxicity trials on different larval stages (fertilized egg, trochophore, 48 and 72 h-old D-larva) of three clam species (*Venerupis corrugata*, *Ruditapes decussatus* and *Ruditapes philippinarum*) exposing them to increasing EG, propylene-glycol (PG), DMSO and glycerol (0.5, 1, 1.5, 2 and 3M). The authors observed similar exposure responses across clam species, with the D-larvae stage being the most resistant and EG and PG showing less toxicity than DMSO and glycerol. Contrastingly, the cryoprotectant exposure trials in **Chapter 3**, did not provide conclusive evidence to exclude any of the cryoprotectant solutions.

Conversely, cryoprotectants can promote cell protection, even with a certain level of toxicity. They achieve this by protecting against the formation of ice crystals, aiding in cell dehydration, and reducing the freezing point (Elliott et al., 2017; Ozimic et al., 2023). The process of choosing a suitable cryopreservation solution hinges on finding the right equilibrium between tolerance to toxicity and providing effective cryoprotection. Therefore, it is important to evaluate the protective effect during freezing after the exposure experiments. Upon analyzing the outcomes in **Chapter 3**, it becomes apparent that the quality of the thawed larvae was reduced compared to the fresh larvae. Nevertheless, when comparing the two cryoprotectant solutions applied in **Chapter 3**, the results after thawing were very similar, except for the increased glutathione peroxidase (GPX) enzymatic activity, suggesting a potential stress response promoted by DMSO treatment to *C. angulata* D-larvae.

Taking into account the results shown in **Chapter 2** and **3**, the formulation of the cryoprotectant solution proved to be a crucial factor in the establishment and optimization of cryopreservation methodologies applied to both species. In this

way, the combination of permeant and non-permeant agents improved the post-thaw quality of the biological material for both species addressed in this thesis.

## 5.2. Cryodamage inflicted during cryopreservation: cellular and molecular impacts

Cryopreservation can pose significant challenges, potentially compromising the biological material's post-thaw performance. Therefore, cryodamage assessment is imperative to devise strategies to reduce them. **Chapters 2** and **3** of this thesis explored the structures and functions affected during the cryopreservation process of the sperm and larvae. Meanwhile, **Chapter 4** conducted a comparative transcriptome analysis to assess larval cryodamage from a molecular perspective, providing further insights into the results obtained in **Chapter 3**.

As outlined in **Chapter 2**, the freezing medium was supplemented with sugars to evaluate their effectiveness in protecting the organelles and functions of *C. angulata* sperm during cryopreservation. The post-thaw evaluation considered impacts on DNA fragmentation, plasma membrane, and acrosome integrity. Moreover, essential cell maintenance functions were analyzed, encompassing free radical levels, cell mobility, programmed cell death mechanism (apoptosis), and antioxidant enzyme activity. Upon comparing the three treatments, it was observed that sugars protected the plasma membrane and the acrosome, allowing higher integrity of these structures and reduced levels of ROS and lipid peroxidation. Hassan et al. (2017a), also demonstrated a positive impact in enhancing the post-thaw sperm motility of *Ostrea angasi* through the addition of trehalose to DMSO. Both our findings and the results of the previous study suggest that membrane stabilization and mitigation of oxidative stress can be promoted through sugar supplementation. This may be explained by their colligative properties and ability to form bonds with the phospholipid headgroup (Anchordoguy et al., 1987; Tsai et al., 2018). Furthermore, previous studies by Pezo et al. (2020) demonstrated a reduction in ROS levels and an enhancement in the cell viability in boar post-thawed sperm, cryopreserved with sugars. Similarly, Sandoval-Vargas et al. (2021a) reported a relationship between ROS levels and lipid peroxidation in *Oncorhynchus*

*kisutch* sperm, highlighting that high superoxide anion levels in post-thaw sperm correlated with elevated MDA content and reduced viable sperm. In summary, the results reported in **Chapter 2** highlight the importance of using several approaches to evaluate cryodamage, demonstrating the crucial role of membrane stabilization and oxidative stress mitigation during the cryopreservation of marine bivalve sperm.

**Chapter 3** addressed the effects seen at structural and biochemical levels on *C. angulata* and *C. gallina* D-larvae, during exposure and the cryopreservation with two cryoprotectant solutions, EG and DMSO. Each pool of larvae was divided into five sub-samples: one with the larvae diluted in FSW (fresh larvae or control), the other two used in the cryoprotectants exposure trial, and the remaining two in freezing and thawing trial. The assessment of the quality of D-larvae (fresh, exposed to cryoprotectants and after thawing) included the effects on morphology, movement and activity of antioxidant enzymes. The study in **Chapter 4** follows a similar experimental design to **Chapter 3,** but exclusively employs the cryoprotectant solution DMSO, and was conducted only with *C. angulata* D-larvae.

The results of the cryoprotectants exposure trial in **Chapter 3** showed that, in *C. angulata*, DMSO treatment significantly increased the incidence of abnormalities. This raises two possible explanations: a response to the cryoprotectant's toxicity or dehydration, a phenomenon documented in prior studies involving *C. gigas* (Gwo, 1995). Furthermore, as other potential signs of stress in D-larvae during the exposure trials, an observed enhancement in GR activity of *C. angulata* and a reduction in movement speed, especially in the EG treatment, were noted for both species. In a related study, Heres et al. (2021) conducted cryoprotectant exposure trials before cryopreservation to evaluate the impact of cryoprotectant solutions on the embryos or larvae of several clam species. Their findings revealed distinct stress responses to cryoprotectants among the species, where the older larval stage displayed a lower percentage of abnormality compared with embryos.

While the cryoprotectants exposure trial in **Chapter 3** on D-larvae revealed potential stress, particularly in the DMSO treatment, **Chapter 4** transcriptomic analysis focused exclusively on this treatment, offering clarity on the molecular

damage incurred during the exposure trial. Despite DMSO-apparent toxicity to *C. angulata* larvae during the exposure trials, the results of the work developed in **Chapter 4** clarify that the reduction in D-larvae quality was associated with the freezing/thawing processes, rather than cryoprotectant toxicity. This is associated with the fact that transcriptome analysis showed similar gene expression patterns between DMSO-exposed larvae and fresh larvae (control), having larvae exposed to cryoprotectants a relatively low number of differentially expressed genes (DEGs). Therefore, it is postulated that the effects seen in **Chapter 3** in DMSO-exposed larvae (morphology) could be more associated with an osmotic effect than to the chemical toxicity of the compounds used.

Regarding the cryopreservation trial of *C. angulata* and *C. gallina* conducted in **Chapter 3**, it was observed that this procedure induced damage to the quality of D-larvae. The results revealed a significant decrease in the percentage of normal *C. angulata* D-larvae, as well as in the percentage of motile larvae and velocity in both species. A similar reduction is consistent with findings in *R. decussatus*, *R. philippinarum* and *C. gigas* indicating lower performances in post-thaw larvae than in fresh larvae regarding the growth, development, and swimming activity (Heres et al., 2021; Suquet et al., 2014, 2012). Our findings align with those previously reported by Labbé et al. (2018) and Suquet et al. (2012), suggesting that cryopreservation may lead to a reduction of larval motility and retraction of the larvae body, as observed in *C. gigas* D-larvae. Regarding motility impairment, Suneja et al. (2014) elucidated this issue through post-thaw D-larvae organogenesis assessment, detecting injuries inflicted on the velum structure. These injuries could affect the locomotion and feeding of the larvae which can be a possible explanation for the D-larvae velocity reduction observed in **Chapter 3**.

The assessment of post-thaw D-larvae quality was also focused on the responses to oxidative stress by determining the activity of antioxidant enzymes. Specifically, a significant decrease in SOD activity was observed in cryopreserved samples compared to the control for the target species. In contrast, GPX activity in *C. angulata* demonstrated a notable increase in both cryoprotectants. These findings align with observations in post-thaw sperm of *Salmo salar*, where GPX levels increased when catalase decreased (Figueroa et al., 2019). Also in porcine oocytes,

GPX levels increased when both SOD and catalase activities were inhibited (Whitaker and Knight, 2008). Expanding on these trends, Liu et al. (2022b) work *in C. gigas* revealed an antioxidant scenario, noting that the expression of SOD was significantly upregulated in post-thaw trochophore, whereas GPX showed downregulation at the trochophore stage and upregulation at the D-larvae stage. The observed GPX enhancement suggests a potential compensatory mechanism against specific reactive oxygen species (ROS), such as hydrogen peroxide, especially considering the inhibition of SOD activity, as proposed by Whitaker and Knight (2008). In conclusion, upon assessing the cryodamage in both *C. angulata* and *C. gallina*, it becomes apparent that the latter appears to be less susceptible to cryoinjuries. This observation finds support in the work of Carducci et al. (2020), who, through RNA sequencing, reported that the striped venus possesses a notable ability to adapt to various environmental conditions.

The observed cryodamage to D-larvae of *C. angulata* in **Chapter 3** was further elucidated in **Chapter 4** by transcriptome analyses. A significant higher number of DEGs was observed in the cryopreserved larvae. This suggests a complex molecular response to cryopreservation, indicating potential shifts in the larval transcriptome. The enrichment analysis of gene ontologies revealed the downregulation of genes related to chitin, a vital component in oyster exoskeleton, suggesting an impairment in shell formation. The KEGG enrichment analysis in the context of larval growth revealed significant enrichments in the "neuroactive ligand receptor interaction", "endocytosis", and "spliceosome" pathways. This finding provides plausible evidence for larval malformations observed in **Chapter 3** and supports the hypothesis that cryopreservation may adversely affect growth in *C. angulata* larvae. Moreover, the work in **Chapter 4** enabled the recognition of a set of 11 genes as crucial molecular biomarkers, to provide new cryodamage assessment tools to be applied in future research.

In cryopreservation, a key challenge is to prevent damage to biological structures and cellular functions at both molecular and biochemical levels to enhance post-thaw quality. Strategies addressing this challenge necessitate the use of tools for understanding damage. The observed cryodamage, involving cellular and molecular changes, emphasizes the complex challenges of cryopreservation,

providing insights into its impact on sperm and larval viability and future development.

### 5.3. Assessing cryodamage: techniques, significance and applications

While it is advised to employ diverse analytical methods for evaluating post-thawing quality (Cabrita et al., 2010; Yang and Huo, 2022), it is not assured that all these methods exhibit equal relevance in the assessment process.

A fundamental technique in reproductive biology studies is sperm motility assessment, used to promptly and practically evaluate the performance of males (Waberski et al., 2022). Motility plays a key role in the sperm's journey to the oocyte and the subsequent fertilization process (Boulais et al., 2019). Despite being extensively employed and meaningful in cryopreservation studies of mammals (e.g. Gangwar et al., 2020; Öztürk et al., 2020) and fish species (e.g. Nynca et al., 2016; Sandoval-Vargas et al., 2021a), this approach appears less informative when applied to bivalves, either independently or in combination with fertilization. In **Chapter 2** of this thesis, the total sperm motility of *C. angulata* was evaluated after thawing to study the effect of sugar supplementation. However, the technique proved inconclusive regarding the sugar's role, with values below 2% across all treatments. A similar decrease in post-thawing values has been reported in the studies on *C. angulata* performed by other authors (Kuo and Gwo, 2022; Riesco et al., 2017a). Nevertheless, Riesco et al. (2017a) highlighted that sperm motility may be less critical than other parameters jeopardizing cells since the extended sperm motility duration of *C. angulata* enables fertilization with few motile cells. Furthermore, distinct methodologies to evaluate sperm motility have been employed in the evaluation, based on qualitative measurements, or through automated systems, further complicating result comparisons across studies.

In contrast, the motility assessment technique proved to be informative for the work in **Chapter 3** performed in larvae. Throughout this chapter, the movement of *C. angulata* and *C. gallina* larvae was characterized when exposed to the cryoprotectant and after thawing. The methods to characterize larval movement included determining the percentage of motile larvae through microscope counting

and the quantification of average path velocity (VAP) using the CASA software feature. The VAP quantification, was particularly valuable as a quality parameter in the cryoprotectant exposure trials, as it revealed changes in motility pattern behaviour for both D-larvae species that were not detected by the other techniques. After cryopreservation, both methods used to characterize the movement, namely the percentage of motile D-larvae and VAP, detected changes in the swimming pattern. However, VAP analyses showed to be a potential biomarker for assessing bivalve larvae quality throughout cryopreservation steps, especially in situations where immediate and precise evaluation is needed. This CASA system adapted by Suquet et al. (2012) for *C. gigas* larvae allows objectivity and reliability, aiming for standardization among studies.

The method to assess sperm plasma membrane integrity or viability was crucial in **Chapter 2**, to reveal lethal cryodamage. Given the sugars' role in stabilizing the plasma membrane during cryopreservation (Tsai et al., 2018) and contributing to maintaining cell viability, there was a possibility of improvement in this parameter with trehalose and sucrose treatments. Another method for detecting lethal cryodamage is through the caspase-3/7 pathway. This pathway initiates irreversible apoptosis, resulting in DNA and protein degradation, impacting further post-thaw sperm viability (Diogo et al., 2018; Riesco et al., 2017c). Both plasma membrane integrity and cellular apoptosis indicate whether the cell is alive, in the process of death or already dead. Despite the proven efficiency of membrane integrity as a relatively fast quality indicator to evaluate the success of the procedure, it is not detailed which functionalities of the sperm have been compromised. Therefore, especially during the establishment of new protocols or optimization, this key parameter must be complemented with other specific measures for the identification of sublethal damage.

The most relevant parameters to elucidate the role of sugars in the protection of the *C. angulata* sperm during cryopreservation involved plasma membrane integrity, lipid peroxidation and ROS detection. It is crucial to examine such damage in bivalves because sperm and early-stage larvae are highly susceptible to cold damage, given their elevated lipid content (Riesco et al., 2019; Yang and Huo, 2022). While an imbalance of ROS levels and lipid peroxidation may not lead to immediate

cell death, they can impair the fundamental functions of the biological material, compromising their post-thaw performance. In **Chapter 2**, sugars appeared to mitigate damage related to oxidative stress by safeguarding the lipids present in the oyster sperm plasma membrane. This protective mechanism helped prevent lipid peroxidation, maintaining a balanced ROS environment. The collective evidence underscores the crucial role of sugars in attenuating oxidative stress and preserving sperm functionality during cryopreservation.

The antioxidant enzyme activity of scavengers, namely SOD, GR, and GPX, was evaluated in **Chapters 2** and **3** of the present thesis. This first line of defence system to control ROS levels (Ighodaro and Akinloye, 2018) showed an important role in the protection of D-larvae when exposed to DMSO and after cryopreservation, especially in *C. angulata*. For example, during exposure trials, the GR activity of *C. angulata* was enhanced, while after thawing, the SOD activity of both species was reduced. However, only for *C. angulata* was GPX activity enhanced. Overall, the determination of antioxidant enzyme activity shows sensitivity to changes in larval homeostasis and potential as a biomarker in future cryopreservation studies.

In larvae, the molecular tools applied in **Chapter 4** proved to be the most relevant and elucidating tool for understanding D-larvae cryodamage. This innovative approach allowed for a molecular overview through RNA sequencing, revealing that the damage resulted from the freezing and thawing process rather than exposure to the cryoprotectant. Additionally, this molecular tool validated the morphological and swimming behaviour changes reported in the trials of **Chapter 3**. The findings of **Chapter 4** mark the first application of this novel transcriptomic approach to evaluate the post-thaw quality of bivalve larvae, highlighting its potential to enhance our understanding of molecular damage in bivalves.

# CHAPTER 6. MAIN CONCLUSIONS AND PERSPECTIVES

# Main conclusions and perspectives

The research presented in the works comprising this dissertation offers valuable insights into creating baselines to further establish a genetic resource bank for *Crassostrea angulata* and *Chamelea gallina*, enabling the storage of their biological material by applying cryopreservation techniques. In the case of *C. angulata*, this involved sperm and larvae cryopreservation, while for *C. gallina*, it focused on larvae cryopreservation. Another relevant point resulting from this thesis was the development and application of a wide range of tools for cryoinjury evaluation at subcellular, functional, and molecular levels in both types of biological material (sperm and larvae). These assessment tools were relevant to this thesis, providing insights that enhanced the understanding of what was happening in the sperm and larvae during the all process of cryopreservation. Additionally, their versatility makes them applicable not only within the field of cryopreservation but also across other research areas (e.g., ecotoxicology and reproduction).

As a result of this thesis, a protocol was developed to enhance the post-thaw quality of *C. angulata* sperm by supplementing the freezing media containing DMSO (10%) with trehalose (0.45 M). This medium improved plasma membrane and acrosome integrity while minimizing lipid peroxidation and superoxide ions levels.

Furthermore, cryopreservation methodologies for the D-larvae of *C. angulata* and C. *gallina* emerged for the first time. Results demonstrated the possibility of storing the genetic resources of progenitors through sperm cryopreservation in *C. angulata,* and also safeguarding the progeny via larval cryopreservation. Ethylene glycol (EG) and dimethyl sulfoxide (DMSO) were successfully identified as the most suitable permeant cryoprotectants for *C. angulata* and *C. gallina* larvae, respectively. Additionally, the incorporation of novel quality assessment techniques, measuring antioxidant enzymes' activity (SOD, GPX, GSR) in cryopreserved larvae of bivalves, further enriches our understanding of post-thaw quality.

This thesis introduced a novel approach to the field of bivalve larvae cryopreservation that enhances the knowledge about their quality and provides a tool that could be adopted in future studies. For the first time, a transcriptomic approach was used to investigate the molecular damage induced by

cryopreservation in *C. angulata* D-larvae, focusing on two critical steps: exposure to the cryoprotectant solution and the freezing/thawing process. Results revealed that the freezing process was the critical step in the cryopreservation procedure. Moreover, genes associated with growth (*adgre3*, dynein beta), structural development (*mp, fbn2, myob3b*), oxidative stress response (*epx, hsp70*), and the immune system (*bp10, muc19, socs5,* Lectin) have been identified as relevant biomarkers of freezability for D-larvae quality assessment.

Overall, this thesis provided a baseline on cryopreservation methodologies, covering sperm and larvae, to establish a *C. angulata* and *C. gallina* genetic resource bank to future support aquaculture and restocking programs. The findings emphasize the importance of considering both molecular and physiological aspects in the development of effective cryopreservation protocols, providing useful information for future research in cryobiology.

While this dissertation offers valuable insights, it also raises questions for future exploration, particularly concerning the development of protocols for the successful cryopreservation of sperm from *C. gallina* and oocytes from the target species of this thesis. Additionally, the application of the established cryopreservation protocols in this thesis on a large scale is essential to verify if there is an adequate supply of biological material for future restocking programs and to identify any necessary protocol improvements. Moreover, future research for a successful genetic bank should explore the representation and diversity of the stored material to ensure that there is no loss of genetic variability. Upcoming investigations should prioritize integrating omics technologies (e.g., genomics, transcriptomics, proteomics, and metabolomics) into the cryopreservation field to enhance the understanding of biomolecular changes during the cryopreservation process.

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