UNIVERSITY OF ALGARVE
FACULTY OF SCIENCES AND TECHNOLOGY

DEVELOPMENT AND STANDARDIZATION OF A PROTOCOL FOR SPERM CRYOPRESERVATION OF TWO IMPORTANT COMMERCIAL OYSTER SPECIES

Francisca Félix de Azeredo Pinto e Melo

Thesis for Master degree in Aquaculture and Fisheries

Dr Elsa Cabrita, CCMAR, University of Algarve
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Declaração de Autoria do Trabalho

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

(Francisca Félix Azeredo)

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Abbreviations and acronyms

ADP – Adenosine diphosphate

AFPs – Anti-freeze proteins

ASMA – Assisted Sperm Morphology Analysis

Asw – Artificial sea water

ATP – Adenosine triphosphate

BSA – Bovine serum albumin

CASA – Computer Assisted Sperm Analysis

DMSO – Dimethyl sulphoxide

DNA – Deoxyribonucleic acid

DNAt – Tail DNA

EDTA – Ethylenedinitrilotetraacetic acid

EG – Ethylene glycol

FAO – Food and Agriculture Organization

Gly – Glycerol

LDLs – Low-density-lipoproteins

MDA – Malondialdehyde

MetOH – Methanol

PBS – Phosphate buffered solution

PEG – Polyethylene glycol

PI – Propidium iodide

PVP – Polyvinyl pyrrolidone

RAS – Recirculation system

SCGE – Single cell gel electrophoresis

SCSA – Sperm chromatin structure analysis

SE – Standard error

Spz – Spermatozoa

TCA – Trichloroacetic acid

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

°C – degrees Celsius

µL – microliter

µM – micromolar

cm – centimeters

fps – frames per second

h – hours

hpf – hours post-fertilization

Hz - hertz

L – liter

M – molar

mA – miliamps

min – minutes

mL – milliliter

mM – milimolar

nm – nanometers

nM – nanomolar

s – seconds

V – volts

v/v – volume/volume

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Abstract

Aquaculture activities have a huge contribution for the world food production and their development is extremely necessary to answer to the lack of resources, especially to the demand for seafood. Bivalve production, especially *Crassostrea angulata* (Portuguese oyster) has been practiced from long ago, and although its production suffered several constraints, in recent years it has been increasing the interests in recovering production and in preserving nature populations. In this sense, new research needs to guarantee an efficient and economically viable production, contributing to a relatively new environmental concern: wild population restoration.

Nowadays, pure wild populations of *Crassostrea angulata* are rare to find due to multiple factors that affected this oyster industry. Cryopreservation technology could promote alternative techniques to contribute for the resource management efficiency of the Portuguese oyster and associated economic activity. In this sense, standardization of procedures is important for *Crassostrea* genus. At the present there are no cryopreservation reports on *Crassostrea angulata* sperm, and therefore, one of the objectives of this work is to design a cryopreservation protocol for this species, testing the more adequate cryoprotectant solution, its ideal concentration, different freezing rates and types of containers. In parallel, this stablished protocol was applied in *Crassostrea gigas* and compared to other previously published for this species.

Analysis of motility, viability, agglutination and fertilizations were used as guides for the establishment of the protocol in *C. angulata*. Moreover, ATP content, DNA fragmentation and lipid peroxidation were done in order to standardize the same protocol for both species. Movement analysis were assessed by CASA system, viability through common staining techniques and flow cytometer, agglutination was quantified according to the scale developed by Dong *et al*., (2007), ATP content determined by bioluminescence, Comet assay was performed to quantify the DNA fragmentation and lipid peroxidation determined spectrophotometrically by measuring the absorbance of the malondialdehyde (MDA).

Significant differences were observed ($p<0.05$) for lipid peroxidation and fertilization trials whereas ATP content and fragmentation of DNA of the cryopreserved samples did

not differ significantly from the control. In *C. gigas*, the same analysis were performed and did not reveal post-thaw quality differences in the samples cryopreserved with 10% DMSO.

The established protocol revealed to be effective and with a low degree of cellular damage on *C. angulata* sperm and, at the same time, viable to apply in other species, such as *Crassostrea gigas*.

Key words: cryopreservation; sperm; *Crassostrea angulata*; *Crassostrea gigas*, standardization.

Resumo

As atividades de aquicultura contribuem significativamente para a produção mundial de alimentos, pelo que o seu desenvolvimento torna-se extremamente necessário de modo a responder à falta de recursos, especialmente no âmbito dos alimentos provenientes do meio aquático. A produção de bivalves, nomeadamente de *Crassostrea angulata* (ostra portuguesa) tem vindo a ser praticada ao longo dos anos e embora a sua produção tenha sofrido várias alterações e até mesmo restrições, recentemente assiste-se a um interesse crescente na recuperação da produção, assim como na preservação destas populações naturais. Nesse sentido, a investigação necessita de garantir uma produção eficiente e economicamente viável, contribuindo para uma preocupação ambiental relativamente nova: o repovoamento da população selvagem.

Atualmente, as populações puras de *Crassostrea angulata* são raras de encontrar no meio natural devido a múltiplos fatores que afetaram esta "indústria de ostras". Deste modo, as técnicas de criopreservação podem tornar-se úteis ao promoverem alternativas que contribuam para a eficiência da gestão dos recursos de ostra portuguesa e para a sua atividade económica associada. Neste sentido, a estandardização dos procedimentos torna-se importante de aplicar no género *Crassostrea*. Atualmente, não existem protocolos de criopreservação de sémen de *C. angulata*, pelo que o objetivo deste trabalho consiste em desenvolver um protocolo de criopreservação para esta espécie testando qual a solução crioprotectora mais adequada, a sua concentração ideal, diferentes rampas de congelação e qual o tipo de recipiente mais apropriado. Paralelamente, este protocolo estabelecido foi aplicado em *Crassostrea gigas* e comparado com outro previamente publicado para esta espécie.

Os resultados das análises de mobilidade, viabilidade, aglutinação e fertilizações foram utilizados como linhas condutoras para o estabelecimento do protocolo. Para além disso, o conteúdo de ATP, a fragmentação do ADN e a peroxidação lipídica foram realizados de modo a avaliar a eficácia do protocolo desenhado. Os testes de mobilidade foram feitos através do software CASA, a viabilidade foi obtida usando técnicas de coloração e citometria de fluxo, a aglutinação foi quantificada de acordo com a escala desenvolvida por Dong *et al*., (2007), o conteúdo de ATP foi determinado por

bioluminescência, o “Comet assay” foi utilizado para quantificar a fragmentação do ADN e a peroxidação lipídica foi determinada por espectrofotometria, através da medição da absorvância do malondialdeído (MDA).

Observaram-se diferenças significativas ($p<0,05$) relativamente ao nível da peroxidação lipídica e dos ensaios de fertilização, enquanto que o conteúdo de ATP e a fragmentação do DNA das amostras criopreservadas não revelou quaisquer diferenças significativas em comparação com o grupo controlo. As mesmas análises foram realizadas em *C. gigas*, as quais não revelaram quaisquer diferenças após descongelação na qualidade das amostras criopreservadas com 10% DMSO.

O protocolo estabelecido revelou-se eficaz e com um reduzido grau de danos celulares no esperma de *C. angulata* e, ao mesmo tempo, viável para aplicar a outras espécies como a *Crassostrea gigas*.

Palavras-chave: criopreservação, esperma, *Crassostrea angulata*, *Crassostrea gigas*, estandardização.

State of the art

1. Aquaculture

Aquaculture importance in nowadays primary industry has grown. The wild fish stocks are decreasing and new biotechnologies associated to aquaculture production are in development to answer to this lack of resources. It is an important activity with a huge contribution for the world food production. According to FAO (2015) fish production is forecast to reach 168.6 million tons in 2015, up 2.6% from the previous year, wherein 147.5 million tons was to food proposes. Among others, this is one of the reasons why aquaculture contributes to the world's wellbeing and prosperity.

2. Bivalve production

In order to preserve the continuous growth of bivalve aquaculture, the production of farmed species has to be efficient and economic viable, not only to meet the demand for seafood, but also because it has a crucial role in the wild population restoration.

One-third of all farmed fish production, 13.4 million tons, is achieved by oysters, mussels, clams and other bivalve species. In 2012, the world aquaculture production of mollusks represents 22.8% of the total inland and mariculture production, which means 15.2 million tons, where 4.5 million tons were due to oyster production (FAO, 2014).

Life cycle of shellfishes, as oysters, with a huge commercial interest is quite complex. After the external gamete fertilization, the embryo begins with mitotic division, segmentation and gradually starts to develop into the pelagic larval stages: trocophore, the earlier and ciliated larvae, progressing from veliger or D-larvae, already able to swim, to pediveliger that confer them the ability to crawl due to the formation of a new support structure. The next process, settlement, recruit larvae onto hard substrates or other oyster shells to initiate their sessile benthic life (Collet *et al*., 1999; Thiyagarajan and Ko, 2012). Finally after one to three years old, depending on the species, they are an adult form ready to spawn.

The development time between embryonic phase and the others larval stages is species-specific and temperature dependent. Also factors such salinity, type and availability of

food, quality of seawater and even of the eggs, affects their development, growth and survival (Thiyagarajan and Ko, 2012).

Crassostrea angulata (Lamarck, 1819), known as the Portuguese oyster, is only found in Europe, precisely in Portuguese rivers Sado and Mira and in the south of Spain, in Guadalquivir (FAO, 2012), mainly in river mouths and head of estuaries with suitable hard substrates (Michinina *et al*., 1997). Until 1970s in Europe shellfish industry, *C. angulata* was the major species cultivated. It was introduced from Portugal into France in 1868, where it spread and became the basis of a large coastal industry in the 1950s, producing up to 100 000 tons per year (Boudry *et al*., 1998). In Portugal, the oyster production in Algarve, Sado and Tejo achieved 9 000 tons per year. However, a bad management of the "oyster industry" together with a decrease of water quality exposed the species to several pathologies that almost lead to the Portuguese oyster extinction (Comps *et al*., 1976). The introduction of a new oyster species, *Crassostrea gigas* or Pacific oyster, appears as a solution to restock the wild population of the autochthone species, since it was more resistant to diseases and showed a huge capacity for habitat competition, high growth rates and low mortalities (Haure *et al*., 2003).

Although in recent years we have assisted to a natural restock of the wild populations of the Portuguese oyster, nowadays the oyster production in Portugal is not up to 900 tons per year, being the *C. gigas* the main species produced.

3. Cryopreservation: basic principles

Cryopreservation is defined as a process that permits the storage at mid and long term of cells, tissues or even organs through temperature decreasing, in some cases until reach -196 °C (Day *et al.*, 2008). Under this conditions biochemical reactions, as all biological processes, are interrupted and it is expected that cryopreserved material remains intact until thawing, when recovers their activity. The duration of storage depends not only on the conditions, but also on species and quality of the material. Definitely is a useful tool for conservation programs to create gene banks, as for management of a species reproduction in the dairy industry (Paniagua-Chavez and Tiersch, 2001).

It has been seen as an economic solution for the animal production industry, since it appears to have innumerous advantages: selection of desirable lines, possibility to develop hybrids with a great quality, reduction of the risks in the proliferation of the infectious diseases (Paniagua-Chavez and Tiersch, 2001), costs reduction in breeding management, availability of gametes all year around and an efficient exploitation of the reproduction material from each individual. Moreover, cryopreservation allows the gametes synchronization, avoids displacement of breeders and makes the transport between production sites easier and economical and finally, cryopreservation techniques opens a new market view over the commercialization of gametes stocks (Cabrita *et al*., 2010).

When an organism is submitted to temperatures below the cooling point of the pure water, 0 °C, formation of ice crystals occurs, which can damage the cell membranes or even tissues and compromise the viability of the cryopreserved material. As the temperatures goes down, solutes present in organic material remains intact while water become crystalized, raising the osmotic gradient and lead to cells dehydration (Watson and Fuller, 2001). Recrystallization during thawing is also a problem, reason why thawing rates have to be higher in order to avoid a reorganization of the crystals, which can be lethal to the cells (Watson and Fuller, 2001).

3.1 Freezing systems and storage equipment

There are different methods to freeze the biological material. According to Adams *et al*., (2004), there are two efficient methods for cryopreserve: using a controlled rate freezer program or set the samples at different distances from a liquid nitrogen source. The cheaper and that do not require complex and expensive equipment have a limited control on freezing process and can be classified according to the freezing agent used: dry ice, alcohol or liquid nitrogen (LN_2). These freezing systems were developed to maintain the biological samples at a certain distance from the freezing agent, ensuring that the samples are submitted to an empiric temperature rate. There are some reports using dry ice method for semen of some species, such as Northern pike (*Esox lucius*) (Babiak *et al.,* 1999). However, liquid nitrogen is more widely used due to some advantages such as a high control freezing rate. Sperm of rainbow trout (Cabrita *et al*., 2001b) gilthead seabream (*Sparus aurata*) (Cabrita *et al*., 2005a) and African catfish

(*Clarias gariepinus*) (Viveiros *et al*., 2000) was successfully cryopreserved using liquid nitrogen.

On the other hand the controlled rate freezer devices are able to decrease or increase the temperature of biological samples at a predefined rate. As in the simple methods, there are several kinds of methods to control temperature but most freezers utilize LN_2 from a pressurized tank to cool the samples (Martínez-Pastor *et al*., 2008). For both methods, the biological solution can be stored either in straws or cryovials, with the intended density.

There is a great variety of accessories for cryobanking proposes. To store extended semen, eggs, embryos or larvae, straws or cryovials are the most indicated consumable materials since they permit an individual identification, better organization and dosage than using other methods. Straws are plastic cylinders with short diameter relative to their length which make their heat transfer rate high. Normally in fish farms and in species with high sperm production higher storage volumes are used, such as macrotubes that can hold 5 mL, but it is necessary to take into account the heat transfer rates and adapt the protocols developed for lower volumes. As in the preview case, cryovials are available with different volumes, up to 4.5 mL. They are also plastic cylinders but with a hemispherical base and a lid screwed to the body (Martínez-Pastor *et al.,* 2008).

3.2 Velocities for freezing and thawing

Rates of freezing and thawing are a crucial point for the success of the whole cryopreservation process. If the freezing rate is too slow, the cellular suspension will be more time exposed to a hyperosmotic medium and the risk of dehydrate is higher (Watson and Fuller, 2001), as it was mention before. On the other hand, if the thawing rate is too fast, despite avoiding the exposure to a hypertonic medium, intracellular crystallization can lead to mechanical cell damage (Wolfe and Bryant, 2001). Thus, freezing rates have to be controlled since it cannot be too much fast or slow.

Thawing is a process that allows cells to recover their metabolism and theoretically must be as fast as possible in order to avoid the reorganization of the intra and extracellular ice crystals, as it was mentioned before (Watson and Fuller, 2001). The appropriate

velocity of thawing and their effects on cells are related to the freezing rate applied to the sample. During this process cells can suffer osmotic stress due to rehydration and recrystallization (Grout and Morris, 1987), thus ultrafast thawing have to be carried out to prevent irreversible damage on cells.

3.3 Vitrification

Vitrification is a recent technique to cryopreserve organs and embryos successfully. It consists in solidification of a liquid sample by an increase in their viscosity during cooling. During this process, the solution becomes vitreous, but without suffering any formation of ice crystals (Fahy *et al*., 1984). To achieve the vitrification proprieties it is necessary to use vitrification solutions, which contains high concentration of cryoprotectants, able to avoid the completely crystallization, neither extracellular nor intracellular ice is formed. The liquid solution becomes vitrified when temperature is low enough and high enough solute concentration are present, which make the nucleating rate decrease. It is also possible, according to Meryman *et al*., (2007), to avoid nucleation and achieve vitrification in the absence of solutes through ultrarapid cooling. Regardless of some damage in the sample, Fahy *et al*., (2005), used this technique successfully in cryopreservation of rabbit kidneys.

However, a biological sample can be consider vitrified if during thaw crystallization does not occur. To achieve an effective vitrification process it is necessary to use hydrostatic pressure not only to decrease drastically the freezing temperatures, but also during thaw. These requirements make this technique not available to the average laboratory (Fahy *et al*., 2004) and is more used in complex biological systems than in sperm samples.

3.4 Cryoprotectants

Extender solutions and cryoprotectants all together act in order to protect the cells against damage produced during the cryopreservation process. An extender is a medium used to dilute sperm and, in most part of the fishes, to get a larger amount of diluted sperm because they produce small amount of sperm. On the other hand, cryoprotectants are a material which are added into an extended sperm dilutions (Muchlisin, 2004, 2005).

Cryoprotectants are responsible for protecting and reduce cellular damage associated to dehydration and formation of ice crystals, and can be classified according to their molecular weight (Meryman, 1966). There are permeable and non-permeable agents. The first one are capable to penetrate in the cell and thus, exercise their function inside and outside the cell, such as ethanol, methanol, glycerol and dimethyl sulphoxide (DMSO) (Ieropoli *et al*., 2004). The second ones have a higher molecular weight, as some carbohydrates and proteins, which make them not able to penetrate the cell by passive diffusion and their function focused on the external medium (Parks, 1997). In the following table are described some cryoprotectants commonly used.

Table 1. Cryoprotectants most commonly used in cryopreservation studies

Cryoprotectant	**Abbreviation**	**Permeability**
Dimethyl sulphoxide	DMSO	Permeable
Ethylene glycol	EG	Permeable
Glycerol	Gly	Permeable
Methanol	MetOH	Permeable
Polyethylene glycol	PEG	Permeable
Polyvinyl pyrrolidone	PVP	Non-permeable
Sucrose	Suc	Non-permeable
Trehalose	Trh	Non-permeable

3.4.1 Permeable cryoprotectants

Permeable cryoprotectants act in the membrane lipids, reduce the osmotic shock and inhibit ice formation inside the cell through their colligative proprieties. Once added these amphipathic molecules, the freezing point of the biological solution goes down, the water came out of the cell and the number of crystals formed decreases (Rall *et al*., 1983). Other important point is the protection of the plasmatic membrane through the interaction of these substances with the phospholipids and the proteins present on the membrane (Anchordoguy *et al.,* 1991).

Among all, DMSO has been widely used as internal cryoprotectant since it provides a good protection of the cells, is the case of sperm cells from rainbow trout (Cabrita *et al.,* 2001a) and Pacific oyster (Dong *et al.,* 2007) wherein DMSO showed the best cryoprotective effect in a consider range of concentrations, from 5% to 20% in *C. gigas*.

3.4.2 Non-permeable cryoprotectants

The non-permeable, protects the cells from the ice formation near to the external membranes due to a water flux and an increase in the viscosity of the solution. Thus, they can act in the physicochemical proprieties of the external medium, external cryoprotectants, or acting like a membrane stabilizer, depending on their nature (Cloud *et al.,* 2009). The first ones have high molecular weight and avoid the crystallization in the solution. Are examples the polyvinyl pyrrolidone (PVP) and the sucrose that acts like a dehydration agent reducing the effect of the internal cryoprotectant (due to a reduction in the concentration used), decreasing their toxicity inside the cell. Furthermore, the high viscosity of these kind of substances at very low temperatures elude the formation of ice crystals (Watson and Fuller, 2001). The membrane stabilizers can be lipids, proteins and sugars.

Lipids

The first reference of one of those protectors reports the using of egg yolk by Phillips (1939), after this the egg yolk has been commonly used in sperm cryopreservation of a great variety of species, including bull, ovine (Ansari *et al.,* 2010; Marco-Jiménez *et al.,* 2004) and fishes, such as salmonids and tilapias (Cabrita *et al.,* 2001a; Rana and McAndrew, 1989). However, the use of egg yolk has been associated to sanitary risks, due to the production of metabolites and toxins and the risk of infection, which results in reduced sperm quality (Althouse, 2008; Yildiz et al., 2013).

Proteins

Proteins are mainly used for the seminal cryopreservation. Despite the lack of knowledge about the mechanism behind each substance, the most commonly used are the bovine serum albumin (BSA) and the soy protein concentrates that showed to be very effective against the agglutination in fish sperm cryopreservation (Chauvaud *et al.,* 1995). Furthermore, there are the Anti-freezing Proteins (AFPs), discovered on blood

from the Nototheniidae Antarctic fish's family by DeVries and Wohlschlag (1969). Nowadays, are known different types of AFPs and they are present in plants, insects and bacteria that lives in extremely environmental conditions (Duman *et al.,* 2004). The mechanism of action comes from their capacity to link with the ice crystals preventing their growth, reason why they are also known as ice binding proteins (IBPs). Moreover, when added to the cryoprotective solution, the AFPs promotes a decrease on the freezing temperature, avoid the recrystallization and protects the cell membranes (Duman and Wisniewski, 2014).

Sugars

Lastly, sugars such as sucrose and fructose also can be used for protecting the cell membranes against the injuries provoked by the cryopreservation process by interacting with the phospholipids phosphate group (Labbe *et al*., 1997; Muchlisin, 2005).

3.4.3 Concerns and state of the research

Nowadays, the trend is to change the avian egg yolk for other suitable alternatives with plant-based Low-density-lipoproteins (LDLs), as soybean lecithin that was tested in carp by Yildiz *et al.,* (2013), who concluded that the animal protein-free extender containing 10% soybean lecithin had a similar effect against freezing damages and fertilization and thus it can be used in cryopreservation of fish sperm, reducing the sanitary risks (Andrabi, 2009). Furthermore, despite the protection function of these substances, they can exert a toxic effect on the cells closely related to their exposure time, temperature and concentration, making the efficiency of a cryoprotectant dependent on this relation. Moreover, it does not exist one cryoprotector and their optimal concentration efficient for all species, it is species-specific (Ieropoli *et al*., 2004), reason why dilution and toxicity tests are so important and the choice of the cryoprotectant dependent on these results and the capacity to protect the cells (Hassan *et al.,* 2015).

After words the equilibration time, it means exposure time to the cryoprotectant, became a crucial part on a cryopreservation protocol and has been tested from 5 minutes to one hour or more in several species, including oysters, depending on the type of the cryoprotectant and their concentration (Hassan *et al.,* 2015). Normally the higher the cryoprotectant concentration is, the shorter the equilibration time is or vice versa.

These kind of tests about equilibration and toxicity tolerance have a huge importance in the selection of a cryoprotectant (Tiersch, 2000).

3.5 Cellular damage and quality assessment of sperm

The majority of cells and tissues suffer some degree of damage during freezing and storage time without cryoprotectant substances. Cryoprotectants interact with the cells membrane, increasing their plasticity, protect from the osmotic shock (Hubálek, 2003) and cell disruption due to ice crystals formation. At the same time these substances can provoke some toxicity degree (Ieropoli *et al*., 2004). Moreover, cryopreservation can induce some damage on membranes, mitochondria and DNA. Due to the above mentioned reasons, post-thawing analysis are made to ensure the viability of the cryopreserved material.

Despite the research done to date there is no consensus about which technic is more indicated or more acute for asses the sperm quality. In fact, according to Cabrita *et al*., (2009) sperm quality is about sperm overall "fitness" and not just their milt characteristics.

3.5.1 Motility, fertilization and hatching rates

As mentioned before there are different techniques to assess the spermatozoa quality. Post-thawing motility, fertilization assays and hatching of eggs are the most common methods to evaluate the quality of cryopreserved sperm (Hui *et al*., 2011). Furthermore, and depending on the author, they can be used individually as simple method or together as combined methods providing more and reliable results.

Among all, motility is the most widely used method for determining sperm quality and has been applied in a great variety of species. Sea urchin spermatozoa were one of the first models for studies on sperm movement (Gibbons 1981) but it was the study of fish that have emerged concepts such as motility duration, initiation and also motility patterns (Cosson *et al.,* 2008a). The majority of aquatic species have immotile sperm inside of their male gonads, but after released in the water sperm contacts with a different medium and is submitted to the aquatic environmental conditions, triggering sperm movement (Cosson *et al.,* 2008b; Suquet *et al*., 2010). Furthermore, the duration of the movement is not only species-specific due to the reproductive strategy adapted

for living, but also depends on the sperm cellular characteristics, such as the morphology, and consequent quality (Suquet *et al.,* 2010).

Normally, the initial velocity of these cells is very high which makes visually observations and records especially difficult. Moreover this simple, easy and quick method is very subjective and depends on the operator experience (Cabrita *et al.,* 2009). In order to overcome this higher variability, Computer Assisted Sperm Analysis (CASA) system and other different software's were developed to increase the precision of the motility measurers. CASA system is specialized in quantifying cellular concentration and analysis of the movement patterns (motility, circular velocity, straight line velocity and linearity) of sperm (Hassan *et al*., 2015). Nowadays, due to the contribute of the technology, it has been also used video techniques such as stroboscopic illumination that allows to record frames at frequencies up to 800 Hz and other technics as high-speed video that records up to 10 000 images per seconds which permits to describe in details the flagella characteristics during the motility period (Cosson*,* 2015).

In different species have been found correlations between motility and fertilization capacity (Suquet *et al.,* 2010) but there is also some research reporting low motile cryopreserved sperm able to fertilize, as in the case of the eastern oyster *Crassostrea virginica,* described by Yang *et al.,* (2012). Theoretically, fertilization trials should be the most unequivocal method for determine the sperm quality, although the eggs features, the sperm/egg ratio and the environmental conditions are important and inherent elements in the technique that may influence the results. Moreover, the effects can only be obtained some hours or minutes after the fertilization procedure and it just have in account the cleavage activation, reason why hatching rate is a more precise method but still have the time as a limiting factor (Cabrita *et al.,* 2009).

3.5.2 Mitochondria and metabolic activity

Despite the few available material on aquatic species, also mitochondria can provide precious information about the damage caused by the cryopreservation process (Hassan *et al.,* 2015). Mitochondria is an essential organelle for spermatozoa in the fusion process with the female gamete, oocyte. Mitochondrial integrity and morphology are key-factors for spermatozoa function and any change will be reflected in decrease of

ATP content and in motility of the cell. Usually, morphology is studied using electron microscopy, but also using fluorescent probes that provide faster and simple methods to evaluate the structure and the functional characteristics (Cabrita *et al.,* 2009). For the rest, there are some techniques to evaluate the potential of the mitochondrial membrane while others the ATP content.

It is assumed that a relationship between ATP content and motility is present in several species. However, it is also correlated with other sperm features. As described by Christen *et al.,* (1987) in rainbow trout, the decrease of intracellular ATP is associated to the spermatozoa motility weakening, on the other way, fertilization capacity of Pacific oysters depends on different sperm characteristics and not only on the intracellular ATP content (Suquet *et al.,* 2010). ATP and ADP concentrations could be measured by luminescence and the cryopreservation process and the inherent osmotic stress affect their values (Cabrita *et al.,* 2010).

Fast depletion of ATP content during the movement phase occurs in several fish species: trout, carp, sea bass and turbot (Cabrita *et al.,* 2009), however through the phenomenon called "second motility phase" the ATP levels can be re-established in some species, such as trout that after 15 minutes can recover the initial motility after dilution in a medium without calcium (Alavi *et al.,* 2006). On the other hand, there are some species, especially invertebrates that have been proved to keep their motility phase from hours to days. After a 20 hours incubation in seawater, the percentage of motile sea urchin (*Hemicentrotus pulcherrinus*) spermatozoa is still close to 50% and in American oyster, sperm movement lasts no more than 4 to 5 hours (Suquet *et al.,* 2010). Nevertheless, comparing to fish species sperm movement duration of most invertebrate species is still very high, as is the case of Pacific oyster (20–24 h) related to turbot (3–5 min) or seabass (40–50 s) (Suquet *et al.,* 2012).

3.5.3 Viability and membrane integrity

Plasmatic membrane is responsible for the integrity and permeability of the cell. Viability has a lot of definitions and according to Cabrita *et al.,* (2009) "any cell with an injured membrane should be unable to develop its functions, and any non-functional cell should reveal alterations in membrane structure and/or permeability". In order to

evaluate the damage caused on this cellular structure by freezing-thawing process, some tests have been developed using fluorescent techniques (fluorescence microscopy or flow cytometry) (Hui *et al*., 2011).

One of the most common method based on differential staining is the combined use of SYBR-14 and Propidium Iodide (PI) that allows simultaneous observation of both live and dead cells together with flow cytometry, giving a fast and precisely analysis of the integrity of the membranes (Cabrita *et al*., 2005b; Fauvel *et al,* 2010). This technique has been widely used in different species, including eastern oyster (*Crassotrea virginica*) sperm and eggs by Paniagua-Chávez *et al.,* (2006) and in teleost fishes such as gilthead sea bream sperm *(Sparus aurata)* by Cabrita *et al*., (2005). There are other molecular probes available in the market for detection of acrosome integrity, such as LysoTrack green kit, and mitochondrial membrane damage, as combination of Rhodamine and Propidium Iodide (Hassan *et al.,* 2015). The principle of these techniques is the permeability of the probes, for example PI and Rhodamine are non-permeable substances able to penetrate only in damaged or dead cells contrarily of SYBR-14 that stains the nucleus of the viable cells due to their permeability to this probe.

3.5.4 DNA integrity

The injuries on DNA can also be quantified. Spermatozoa DNA damage has been observed in several mammalian species (Hamamah *et al.*, 1990; Steele *et al.*, 2000), using different techniques. Most of the techniques were developed for other cell types and then adapted for spermatozoa. Is the case of TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP-biotin end-labelling), the sperm chromatin structure assay (SCSA) and the Comet assay single cell gel electrophoresis (SCGE).

One of the most used is the commonly known Comet assay, which determines DNA fragmentation in individual cells. The Comet assay is a method in which DNA lysis and electrophoresis are performed under neutral or alkaline conditions, and a fluorescent dye is used for staining the DNA. In this metaphor from astronomy the distinct head of the “comet” represent the intact DNA, and the tail contains damaged or broken pieces of DNA (Liao *et al.*, 2009). To evaluate individual cells this is a simple, precise and cheap method (Collins, 2004) that has been used in all type of species, cells and for different

proposes. In invertebrates, including mussels and bivalves is widely used to detect variations in water quality caused by chemical pollution (Andem *et al*., 2013).

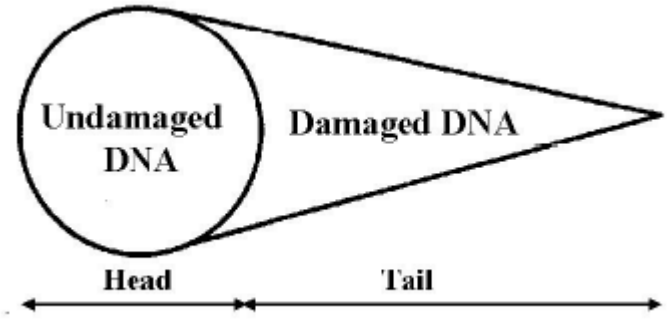

Fig. 1. Definitions of basic comet properties (Gwo *et al*., 2003).

4. Cryopreservation in aquatic organisms

Several protocols have been designed for cryopreservation of gametes and embryos of different aquatic species. Many of these efforts contributed for the fish farming technology, however some concerns remaining about to apply these achievements on farming (Cabrita *et al.,* 2010).

4.1 Sperm cryopreservation: advantages and concerns

It is estimated that spermatozoa of more than 200 species of freshwater and marine fishes have been cryopreserved, including salmonids, tilapias, carps and catfish, which are groups with aquaculture importance among fresh water species (Paniagua-Chavez and Tiersch, 2001). Also other saltwater species such as turbot, gilthead seabream, European seabass, European and Japanese eel and cod have been target species for improvement their cryopreservation protocols due to their huge importance in this aquaculture industry (Cabrita *et al.,* 2010).

There are many advantages on dealing with fish sperm that promotes the amount of studies that exists: in farmed species broods are available in part of the year, sperm is easy to collect and store for short periods, marine fish sperm are easy to cryopreserve, although in some species (with external fertilization) the sperm is immotile in the seminal fluid (SF) when in contact with a proper medium motility is totally activated, it is possible to select good males using different parameters already mentioned before

(by motility, fertilization, viability tests, for example), fish sperm are homogeneous, all spermatozoa can be activated at the same time (Cosson *et al.,* 2008a; Gwo, 2011), among others characteristics which makes the fish sperm an interesting target for study.

There are still some concerns and technical barriers to overcome in the cryopreservation procedures. Osmolarity of the mediums, motility and sperm flagella behavior are examples of the nowadays research topics on going. During spermatogenesis, cells are under controlled environmental conditions: they are surrounded by Sertoli and Leydig cells, essential for the spermatozoa growth, and this process take place in a non-harmful seminal plasma fluid (Cosson *et al*., 2008a). Nevertheless, the problems about sperm and their study starts here, when after released these cells contacts with a different medium, with totally different characteristics.

Moreover, in marine fish, motility activation occurs immediately after contact with sea water (SW), a high osmolarity medium compared with seminal fluid, it happens in species such as cod, *Gadus morhua* (Westin & Nissling, 1991), in sea bass, *Dicentrarchus labrax*, and in sea bream, *Sparus auratus* (Billard, 1978). Recent studies reported by Cosson *et al.,* (2010), focus on a more advance description, such in hake (*Merlucius merlucius*) spermatozoa flagellar wave characteristics and their associated motility parameters.

4.2 Oocytes, embryos and larvae

The possibility of embryos and larvae storage can be very helpful in the management of reproduction. There are still many problems related to fish embryos due to their complex structure: large size, low surface/volume ratio, large yolk content and its osmotic behavior, high chilling sensitivity and high probability of membrane disruption due to ice crystals formation. Permeability is also a crucial propriety for the success and embryos have many different compartments in which permeability differs (Robles *et al*., 2008).

For the reasons above mentioned, at the present, it is not possible to cryopreserve fish embryos and although cryopreservation of oocytes is much more difficult than cryopreserve sperm or embryos, the first successful protocol was reported by Tervit *et*

al., (2005), for Pacific oyster oocytes and can be used in hatcheries and in selective breeding programs.

5. Bivalve cryopreservation

Gamete quality is a prerequisite for the success of any cryopreservation protocol. However research in bivalves in this topic remains limited. Gamete quality can be characterized by fertilization rates and embryo development yields as also other methodologies mentioned before (Song *et al.,* 2009). High fecundity rates are not a gamete quality parameter, as was shown in *Crassostrea gigas* which produced high fecundity rates, but reproductive success remained variable and inconsistent (Suquet *et al*., 2007).

Fertilization success depends on a variety of factors: experimental techniques, gamete concentration and age, contact time between gametes, distance between spawning individuals and hydrodynamic conditions (Song *et al*., 2009). A successful oocyte cryopreservation protocols developed in *C. gigas* was reported for the first time by Tervit and collaborators in (2005), but studies in oocytes quality factors continue. According to Corporeau *et al*., (2012), the production of *C. gigas* oocytes are not equal and they have identified several proteins that are accumulated according to oocyte quality.

Several studies have been made in sperm cryopreservation in oysters. The effects of practical procedures, such as cooling rate and effect of extender composition was studied by Ieropoli *et al.,* (2004) in *Crassostrea gigas* sperm. One year later, Dong *et al*., (2005b) performed a more detailed experiment testing the effects of cooling rate, the use of single or combined cryoprotectants at various concentrations, different equilibration times, straw size, and cooling methods in order to optimize the cryoprotectant protocols for diploid oysters *C. gigas* sperm. Also the agglutination of sperm in the same species was investigated by the same author, due to the erroneous idea that agglutination is a synonym of low fertilization capacity (Dong *et al.,* 2007).

In other species, such as Eastern oyster (*Crassostrea virginica*) and Black-lip pearl oyster (*Pinctada margaritifera*) sperm cryopreservation studies have been published (Yang *et*

al., 2013; Hui *et al*., 2011). In the first species, Paniagua-Chavez and Tiersch, (2001), improved cryopreservation techniques for sperm and trocophore larvae for aquaculture hatcheries proposes. They tested 12 different cryoprotectant solutions and 5 different larval concentrations, concluding that the best results were obtained using 10 or 15% propylene glycol and survival of trocophore larvae were higher when the concentration per macrotube was lower. Also in this species, Yang *et al.,* (2013), studied different methods for sperm collection: natural spawning, anesthesia method that induced the opening of the shell which permits to perform a biopsy as a non-invasive method, and evaluated the mechanical notching for biopsy.

Also Pacific oyster tetraploids were studied, firstly by Dong *et al.,* (2005a) that demonstrated that sperm from these individuals can be collected, frozen, and stored for production of triploid offspring, and after by Suquet *et al.,* (2010), that performed sperm experiments in order to study the changes in the motility and related ATP content, morphology and fertilization capacity, always comparing the results with sperm from diploid males. Contrary to expectations, the sperm from tetraploids revealed lower quality and the cessation of the sperm movement was due to anomalies on the morphology and not to the decrease in the ATP content.

In terms of the analysis of the quality of cryopreserved material, Suquet *et al.*, (2012), studied some laboratory parameters to assess the quality of post-thawed Pacific oyster D-larvae and determined that movement velocity of trocophores (using CASA system) and area of D-larvae (measured by ASMA system) allow an early and reliable estimation of the larvae quality. Two years later, Suquet *et al.*, (2014), presented the first successful post-thawing development to reproduction stage of diploid cryopreserved larvae in *C. gigas*, testing three different stages of trocophore (13, 24 and 48 hpf). Furthermore, survival, growth and reproductive performance did not differ between these three initial larval stages and the results were closed to the respective control groups.

These recent and successful developments arouse for diversifying the research in this area, extended the laboratory studies for different species. Paredes *et al.,* in (2013), made some comparative cryopreservation trocophore larvae studies testing different cryoprotectant solutions and two different freezing rates in two species of bivalves,

Crassostrea gigas and *Mytilus galloprovincialis*, and therefore improved a cryopreservation protocol. Despite Portuguese oyster is closely related with Pacific oyster, there are no available cryopreservation protocols for this species in any larval stage or in each type of gametes, oocytes and sperm.

Objectives

The presented project makes part of the CRIOBIV pilot-project which pretends to develop cryopreservation procedures that guarantee the application of these techniques both at production and conservation programs for the Portuguese oyster (*Crassostrea angulata*).

The first objective of the work is to create a sperm cryopreservation protocol for *C. angulata* and, at the same time, make use of not only the conventional methods for the evaluation of the protocol, but also apply other techniques capable to provide reliable information and allowing a better evaluation of the designed protocol.

The second part of the work is the standardization of the developed protocol, in order to apply it to other oyster species, such as Pacific oyster (*Crassostrea gigas*). For this, the established cryopreservation procedures were applied in *C. gigas* sperm and the results compared to the obtained from *C. angulata*. In addition, the application of this protocol in *C. gigas*, was also compared with other published freezing methods for Pacific oyster sperm.

Material and methods

1. Broodstock conditioning

Crassosstrea angulata broodstock (n= 120) was acquired in Alvor and Mira bivalve farms, while *Crassostrea gigas* (n= 100) was obtained from the Ria Formosa bivalve farm. Both species were maintained in IPMA at Tavira. During conditioning period, Portuguese and Pacific oysters were fed daily with a mixture of two microalgae (*Chaetoceros calcitrans* and *Skeletonema costatum*: 50 cells per µL of each microalgae) and kept in a recirculation system (RAS), one for each species, at temperatures between 18 to 22 °C. A total of 50 males and 20 *C. angulata* females were used in all experiments.

2. Sperm collection and dilution

For each experiment, individuals were opened and a small sample of gonad tissue was removed and the sex identified using a microscope (Nikon 200, Japan). Sperm was collected from the mature males by dry method: gonad area was wiped from any contamination and the sperm was extracted directly from the gonad using a micropipette. Sperm was immediately diluted 1:10 (v/v) in artificial sea water (Gwo *et al*., 2002) or in artificial sea water supplemented with 10 mM of caffeine. Osmolarity of both solutions and sperm was checked using an osmometer (Gonotec osmomat 030, Germany).

After this, the diluted sperm was filtered using two different sieves: 100 µM to retain larger impurities and 20 µM for the smaller debris, passing only the spermatozoa to the beaker.

Taking a sample of 10 µL, sperm movement and concentration were determined using CASA software. For all the motility analysis, motility was assessed in a Makler chamber using a phase-contrast microscope (Nikon 200, Japan) with a 10 x objective, a digital camera (Basler A312f C-mount, Germany) set for 25 fps. The settings for CASA (ISAS, Proiser R+D, S.L., Spain) were adapted for oyster sperm. The parameter settings for CASA were: 1 < Particles Area (in microns^2) < 90; VCL: 1 < slow < 45 < medium < 100 < rapids (microns/sc); progressivity: 80%; connectivity: 6; minimum number images to calculate ALH: 10). Only males showing motility rate values equal or higher to 40% were used.

Sperm concentration was measured using the same software and the values range from 1 to 2 x 10^9 in all analysed samples. Some experiments were performed using individual males and others with pools of, at least, 3 males. After processing, samples were maintained at 21-22 °C until the beginning of the experiments.

3. Establishment of a cryopreservation protocol in *C. angulata* sperm

To establish an ideal cryopreservation protocol for sperm from Portuguese oyster several conditions were tested.

3.1 Long term motility duration

In order to know the motility duration of *C. angulata* spermatozoa, a fresh sample experiment was performed during 7 days. The sperm was collected and diluted in artificial sea water as previously described and storage for one week at 4 °C. In each day the motility was determined as described before at two different conditions: (i) at 4 °C immediately after removing a subsample for activation and (ii) after 30 minutes, at room temperature. Motility was scored as described before using the software ISAS (Proiser, Spain).

3.2 Toxicity test

The aim of the toxicity test was to determine the best cryoprotectant to use and their ideal concentration. For this, four different cryoprotectants were tested, polyethylene glycol (PEG), dimethyl sulfoxide (DMSO), ethylene glycol (EG) and methanol (MetOH), at three concentrations (5, 10 and 20%, final concentration) and in two dilutions mediums, artificial sea water and artificial sea water supplemented with 10 mM of caffeine. The following scheme represent these conditions.

Table 2. Schematic representation of the toxicity tests conditions.

Toxicity test																							
PEG						**DMSO**						**MetOH**						**EG**					
5%		10%		20%		5%		10%		20%		5%		10%		20%		5%		10%		20%	
Asw	Asw + caf	Asw	Asw + caf	Asw	Asw + caf	Asw	Asw + caf	Asw	Asw + caf	Asw	Asw + caf	Asw	Asw + caf	Asw	Asw + caf	Asw	Asw + caf	Asw	Asw + caf	Asw	Asw + caf	Asw	Asw + caf

Sperm was diluted 1:1 (v/v) in each condition and exposed to the cryoprotectant effect for 10 minutes (equilibration time). After, 10 µL of diluted sperm were used to determine the motility as described before, and 15 µL was used for the viability test. The percentage of motile cells and concentration were recorded. Spermatozoa viability was determined mixing 15 µL of diluted sperm, 0.5 µL SYBR Green (final concentration 100 nM) and 1.5 µL Propidium iodide (final concentration 12 µM) and observed in a fluorescence microscope (Nikon E200, Japan). The percentage of live cells (cells with membrane integrity-green cells) was recorded. Both motility parameters and cell viability were also measured in fresh samples (sperm without dilution, control).

3.3 Cryopreservation assays

3.3.1 Freezing rates

Two distinct freezing rates were tried, a fast and a slow freezing rate, using two different cryoprotectants (DMSO and PEG), two concentrations (10 and 20%) and the two mediums above mentioned (according to the previous toxicity tests). For the slow freezing (0.3 °C/min from 0 to -30 °C and then plunged directly into liquid nitrogen) and for the fast freezing (6 °C/min from 0 to -70 °C and then plunged directly into liquid nitrogen) (Ieropoli *et al*., 2004) it was used a portable programed biofreezer (Asymptote Grant EF600, UK). All samples were cryopreserved using 0.5 mL French straws (IMV, France).

Thawing was performed in a water bath at 37 °C for 10 s and samples were immediately used to evaluate sperm quality, through motility and viability tests, as described before, and also through agglutination test. All the experiments were repeated using at least three different males and the same samples were analysed in fresh as control. The agglutination was verified taking photos of cryopreserved samples with a Canon G12 digital camera and classified according to an agglutination scale developed by Dong *et al.*, (2007). This scale have six levels of sperm agglutination: (i) homogeneous suspension, level 0; (ii) few clumps discernable, level 1; (iii) many clumps evident, level 2; (iv) aggregation of clumps, level 3; (v) formation of elongated clumping (“noodles”), level 4; (vi) formation of well-developed noodles, level 5. After the photographic evaluation, viability photos were acquired from the same samples, in order to comprehend if the agglutination affects the survival rate.

3.3.2 Type of containers

The type of container was also tested, using straws and cryovials. To freeze the samples it was used 10% DMSO diluted in artificial sea water and the fast freezing rate, previously described. The comparison was based in the motility and viability results.

3.4 Fertility trials: optimization of sperm to egg ratio

For fertilization procedures the gametes were extracted with two different methods according to the sex. Male gametes were obtained by dry method, as described before, and female gametes were obtained by wet method. In the wet method, the oocytes are released by the gonad, using a scalpel, performing horizontal and vertical small cuts. Artificial sea water was used to wash the gonad and to collect the oocytes into a 1 L beaker. For each fertilization were collected, at least, gametes from four different females. After, the oocytes were filtered using two sieves: 100 µM to retain larges debris and 20 µM to retain the oocytes. Next, oocytes were counted in a counting chamber (Sedgewick rafter, Portugal) and the calculations for the fertilization procedures were done in order to have 200 000 eggs/L for each tank.

For the sperm, 40 straws were thawed using the conditions previously established (section 3.3.1) and an aliquot was removed from the cryopreserved samples to determine, with the CASA system, the concentration after thaw. For fresh samples the same procedure was followed. It was tested four different spermatozoa/egg ratios (1 000, 2 500, 5 000 and 10 000 spz/egg) for the cryopreserved material and for the control (fresh sperm) a ratio of 500 spz/egg was used. Fertilizations were done using replicates under controlled environmental and kept at 21-22 °C. Two to three hours after mixing the gametes the fertilized eggs were counted. An egg was consider fertilized if it was present the polar body or more advanced embryonic stages. The percentage of fertilized eggs was calculated in respect to the total number of incubated eggs.

4. Standardization for the *Crassostrea* genus

After established the cryopreservation protocol for the Portuguese oyster sperm, spermatozoa from Pacific oyster were cryopreserved under the same conditions (Fast freezing rate, 1:1 dilution ratio in artificial seawater + 10% DMSO, 10 minutes of

equilibration time, sperm packaged in straws, thawing at 37 °C for 10 seconds). In addiction, was also performed the same protocol, but with a different cryoprotectant, 10% EG, which was considered by other authors (Ieropoli *et al*., 2004) the best cryoprotectant to use in *C. gigas* sperm. For this experiment a total of 20 *C. gigas* males was used.

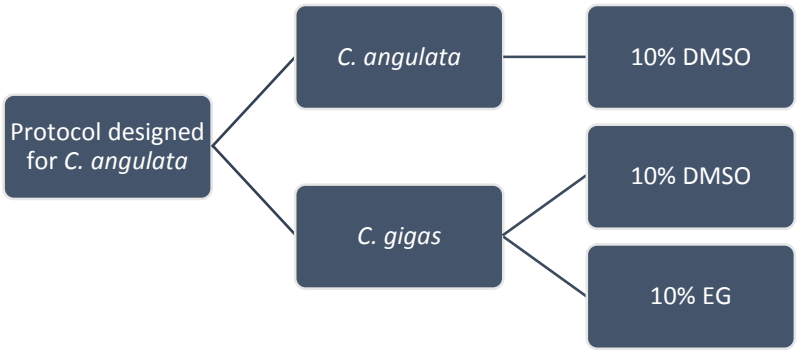

Fig. 2. Schematic representation of the cryopreservation procedures for *C. gigas* sperm.

To evaluate the effectiveness of the established protocol, several post-thaw quality tests were made for Portuguese oyster samples (N=6 pools) and for Pacific oyster sperm (N=3 pools).

4.1 Viability on flow cytometer

Viability of the *C. angulata* and *C. gigas* spermatozoa was performed on fresh and post-thawed sperm samples. Both were maintained in sea water and then prepared for flow cytometry analysis.

For this analysis, propidium iodide (PI-Sigma, Portugal) was added at 4 mg/mL, final concentration, to detect dead cells (cells permeable to the dye). Immediately after, samples were acquired in a flow cytometer (BD FACSCalibur™, Cell Analyzer, BD Biosciences, USA) adjusted for blue excitation (488 nm), which are the excitation lines for the detection of PI (670/30) fluorescence. Data analysis was performed applying Weasel 3.1 free software. A total of 75 000 events were counted for each sample. Flow cytometry settings was previously established for these type of samples.

4.2 Lipid peroxidation (MDA)

Oxidative stress was measured through the determination of a lipid peroxidation subproduct concentration, the malondialdehyde (MDA), using a spectrophotometry assay, following the protocol described by Martínez-Páramo *et al.,* (2012a). This procedure was adapted and modified for oyster sperm. Cryopreserved samples were thawed at 37 °C for 10 seconds. Briefly, 250 µL of spermatozoa (fresh and cryopreserved) were washed with artificial sea water and centrifuged at 1 500 g for 10 minutes. The pellet was resuspended in 100 µL of Phosphate Buffered Saline (PBS), homogenized and sonicated to release MDA and incubated in a 200 µM sodium ascorbate solution containing 40 µM $FeSO_4$ for 30 min at 37 °C in the dark. After that, reagents provided by the kit (Bioxytech, MDA-586 assay, Deltaclon, Spain) were added to 100 µL of the supernatants and the samples incubated for 1 hour at 45 °C, also in the dark. Samples were centrifuged at 10 000 g for 10 min at 4 °C, and 200 µL of supernatants were transferred to a 96-well flat-bottom transparent plate (Nunc, Denmark). Finally, using a microplate reader (BioRad 30, Portugal) at 586 nm, the absorbance was read and MDA concentrations determined from a standard curve (0, 1, 2, 4, 8 and 10 µM MDA) and presented as nM of MDA per million of spermatozoa. Technical replicates were used for each sample and a control was also present. A total of 6 pools was used for *C. angulata* and 3 pools for *C. gigas*.

4.3 ATP content

ATP content was determined by bioluminescence (ATP Bioluminescence Assay, Kit CLS II, Sigma, Portugal). The collection of samples for quantification of ATP required first to determine their cell concentration using the CASA software. After this, samples from fresh and cryopreserved (post/thawed) sperm were centrifuged at 1 500 g for 10 minutes, resuspended in 500 µL of artificial sea water and then 10 µL of fresh sperm and 20 µL of cryopreserved sperm were used for preparation. Always taking attention to the pH of the solutions used, 150 µL of 2% TCA (2% trichloroacetic acid, 2 mM EDTA) were added to the samples, left for 15 min and then mixed in a vortex to rupture the cells and centrifuged at 10 000 g for 10 minutes at 4 °C to pellet the cell debris. From this suspension an aliquot of 100 µL of the supernatant was added to 500 µL of Sorensen

buffer (pH = 7.8) and this final sample was stored at -20 °C until analysis. Finally, the samples were thawed and 150 µL were transferred to a 96-well flat-bottom transparent plate (Nunc, Denmark) to read the luminescence at the microplate reader (BioRad 30, Portugal), wherein 50 µL of luciferase were dispensed automatically. The ATP content was determined from a standard curve (0, 0.01, 0.1, 1 and 10 µM ATP) and the results presented as nM of ATP per million of spermatozoa. Technical replicates were used for each sample and a control was also present. A total of 6 pools was used for *C. angulata* and 3 pools for *C. gigas*.

4.4 Comet assay

For DNA fragmentation test (Comet assay), samples were thawed and the protocol described by Cabrita *et al.*, (2005) was used, once adapted and modified for oyster sperm. Briefly, isolated cells obtained after sperm homogenization were embedded in agarose slides and exposed to a lysis solution (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, 1% Triton X-100, 1% lauryl sarcosine) for 1 hour at 4 °C. After lysis, the slides were placed horizontally in an electrophoresis cube (BioRad, Spain) filled with freshly made electrophoresis solution (0.3 M NaOH, 1 mM Na_2-EDTA, pH 12.8). Alkaline electrophoresis was conducted for 10 min at 25 V and 300 mA at 4 °C and after it, the slides were neutralized (0.4 M Tris, pH 7.5) with two washes of 5 min at 4 °C and then fixed in pure methanol for 3 minutes.

For comet visualization, 10 µL of propidium iodide (0.1 mM) were pipetted into the sample and covered with a coverslip. The samples were observed in an epifluorescence microscope (Nikon TE200, Japan) with an excitation filter of 510-560 nm and a barrier filter of 590 nm. Each slide was randomly analysed by selecting several fields for image recording and approximately 60 cells from each slide were recorded with a digital camera (Nikon DS-Ri1, Japan) and the imaging software NIS Elements v3.01 (Nikon, Spain). Comet analysis was performed with the imaging system Komet software v6.0 (Andor Technology, Ireland). For each cell analyzed the percentage of tail DNA (% DNAt) was used to characterize fresh and cryopreserved samples. A total of 6 pools was used for *C. angulata* and 3 pools for *C. gigas*.

5. Statistical analysis

In all experiments, data were collected, treated and analysed using the software SPSS Statistics v.22 (IBM, USA). One-way ANOVA was performed followed by Student-Newman-Keuls (S-N-K) *post hoc* test for the motility and viability tests of Portuguese oyster sperm and also for the fertilization trials of this species. A Student's t-test was applied for the results from the lipid peroxidation, ATP content, Comet assay and viability (in flow cytometer) for both species, comparing only fresh with cryopreserved from the same treatment.

Results

1. Cryopreservation of *C. angulata* sperm

1.1 Long term motility duration

During this experiment the motility of fresh samples was assessed using the CASA software during one week and the results are described in figure 3.

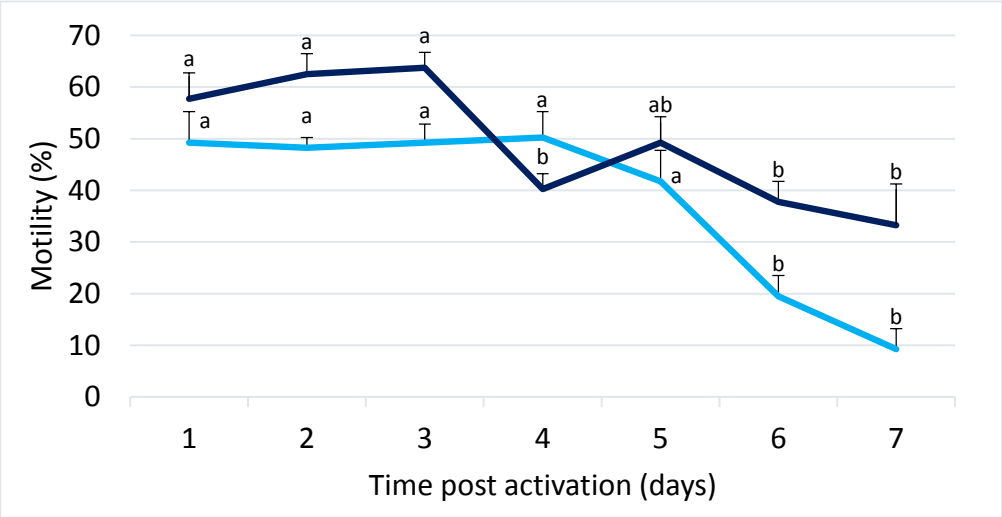

Fig. 3. Motility changes of fresh spermatozoa in relation to time post activation (N=6; mean ± SE) at two different temperatures: 4 °C - storage temperature (light blue) and 30 minutes after at room temperature (dark blue).

The spermatozoa from Portuguese oyster showed movement during the whole experiment (7 days) when stored at 4 °C. Using the same sample, motility (% of motile cells) was also measured 30 minutes after, at room temperature, and higher values of motile cells were obtained. In both conditions, motility rates were decreasing over the time. As shown on the figure by the light blue line, until the 5th day, at a storage temperature of 4 °C, there were no differences in the motility rate, but after this period, there was a strong decrease. According to the dark blue line, the motility of the samples maintained the same values until the 3rd day, after it there was a decrease of 20% in the spermatozoa movement rate (from 60% to 40%) and then the cells recover their movement, in the 5th day, in 10% (from 40% to 50%). From the 5th day until the end of the experience the motility decrease slowly.

1.2 Toxicity test

The potential toxicity effect of four different cryoprotectants, at three different concentrations is described in figure 4.

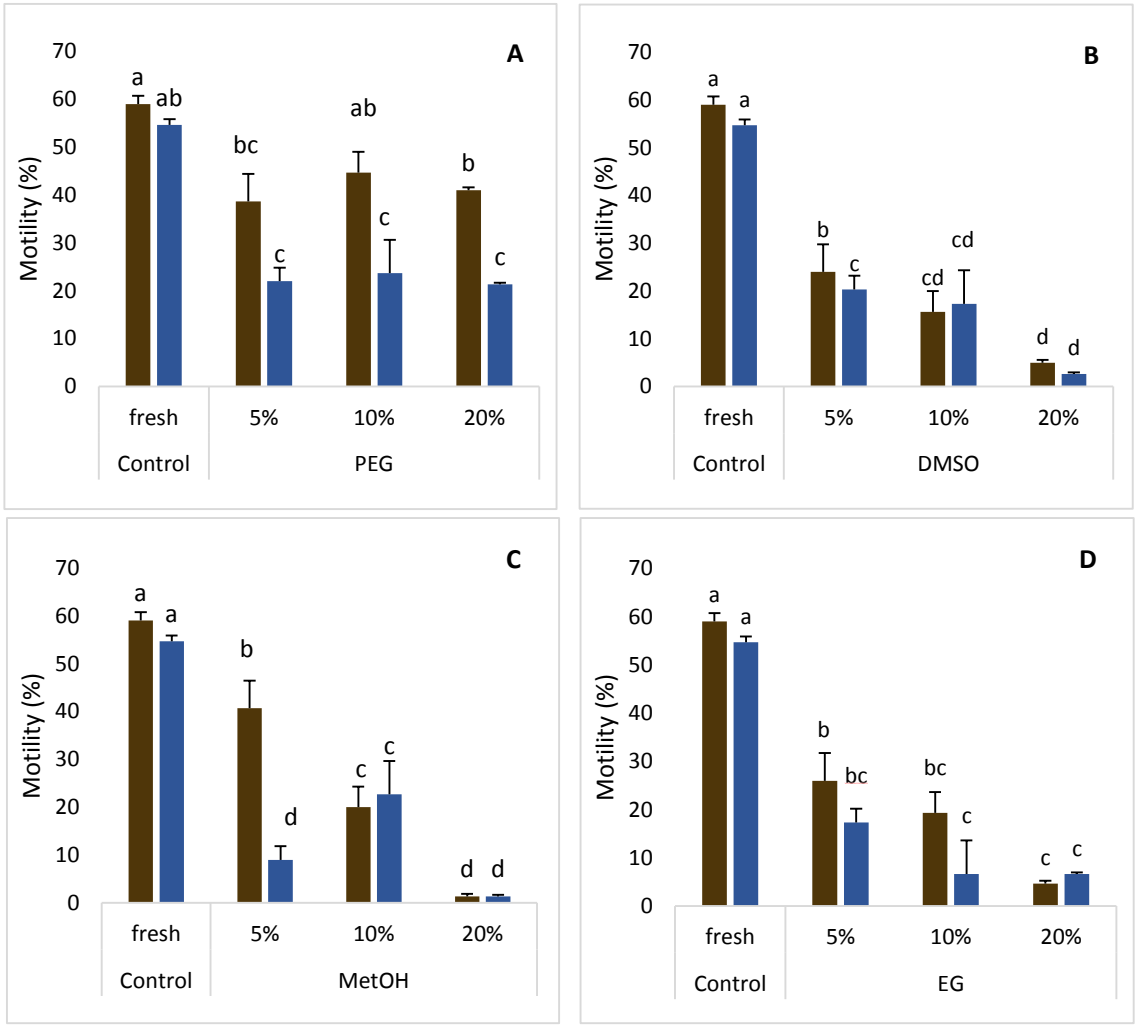

Fig. 4. Sperm motility rates (Percentage of motile cells) after exposure to different cryoprotectants at three different concentrations (5, 10 and 20%) in two mediums, artificial sea water (blue bars) and artificial sea water supplemented with 10 mM of caffeine (brown bars). (A) polyethylene glycol (PEG); (B) dimethyl sulphoxide (DMSO); (C) methanol (MetOH); (D) ethylene glycol (EG); (N=3; mean ± SE; One-way ANOVA; $p<0.05$). Different letters refer to significantly different results.

According to the results and related to the dilution medium, the effect of the caffeine supplementation varied according to the cryoprotectant used. It increased significantly the motility values in: 10 and 20% PEG (A), 5% DMSO (B) and 5% MetOH (C). On the other conditions tested there were no differences between the cryopreserved samples and the respective control. Among cryoprotectants, the one that showed less toxicity effect in terms of motility was the 10% polyethylene glycol diluted in artificial sea water

supplemented with caffeine (A) and the more toxic was the 20% methanol when diluted in both mediums (C).

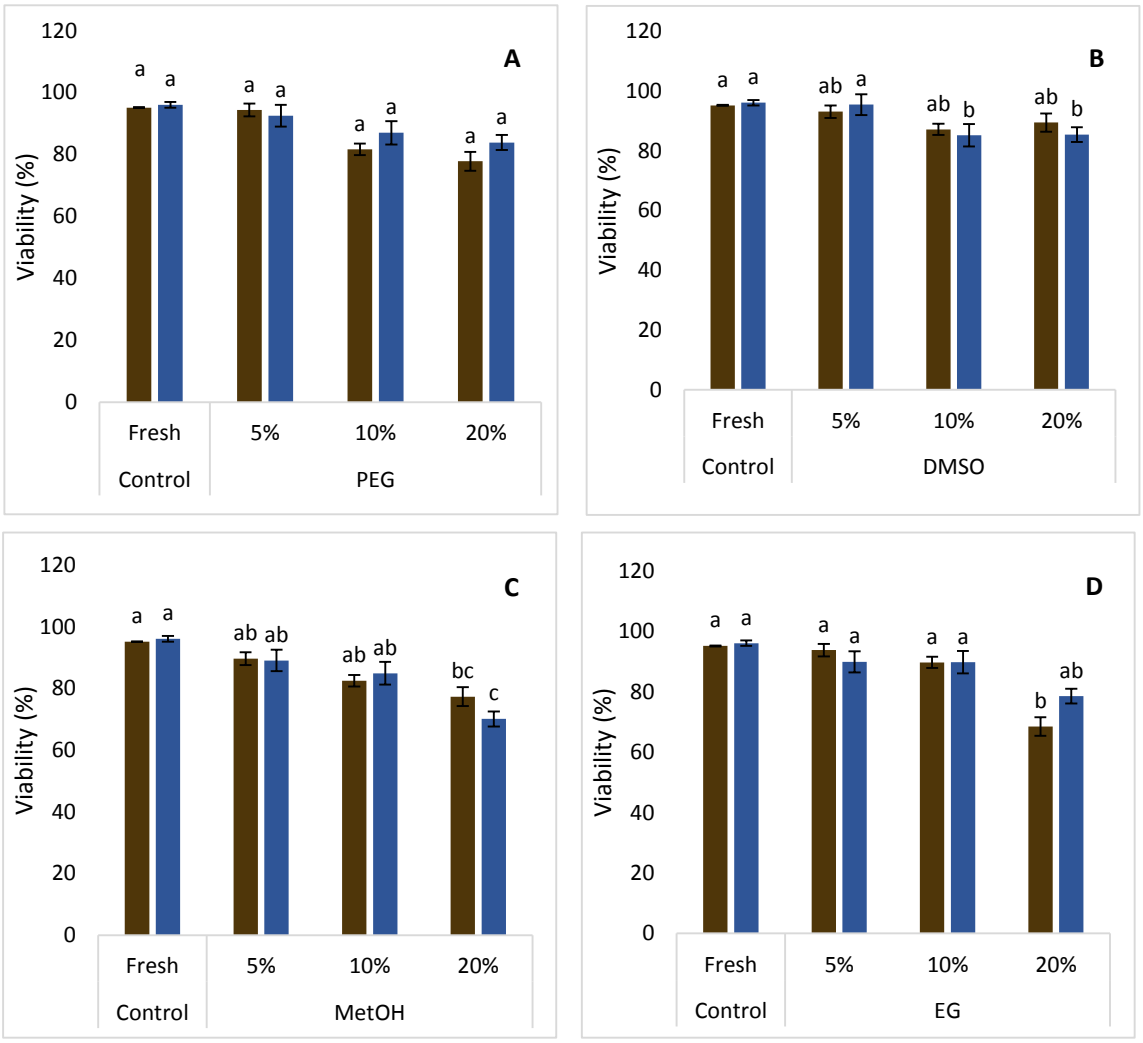

Fig. 5. Viability rates of the samples after exposed to different cryoprotectants, at three different concentrations (5, 10 and 20%) in two mediums, artificial sea water (blue bars) and artificial sea water supplemented with 10 mM of caffeine (brown bars). (A) polyethylene glycol (PEG); (B) dimethyl sulphoxide (DMSO); (C) methanol (MetOH); (D) ethylene glycol (EG); (N=3; mean ± SE; One-way ANOVA; $p<0.05$). Different letters refer to significantly different results.

According to sperm viability showed in the figure 5, the supplementation of caffeine in the dilution medium did not reveal any drastic effect in the viability of the cells. Furthermore, 5% cryoprotectant concentration showed higher viability values for all the cryoprotectants used (A, B, C and D) in both dilution mediums, and the condition most harmful was 20% EG (D) in terms of cells survival rate.

Linking the results from motility and viability, the toxicity test defined 10% PEG diluted in artificial sea water (Fig. 5. A) as the combination less toxic to *C. angulata* spermatozoa (viable sperm > 85%), since demonstrated to produce no significant differences in both parameters (motility and viability), when compared with fresh sperm.

1.3 Freezing rates

As described previously, were tested fast and slow freezing rates to cryopreserve Portuguese oyster semen. The extender media and cryoprotectant was chosen according to the results from the previously toxicity test. It was used the cryoprotectant less toxic for the cells, polyethylene glycol (PEG), and dimethyl sulphoxide (DMSO) due to its published cryoprotectant effectiveness during the freezing process (Hassan *et al.,* 2015).

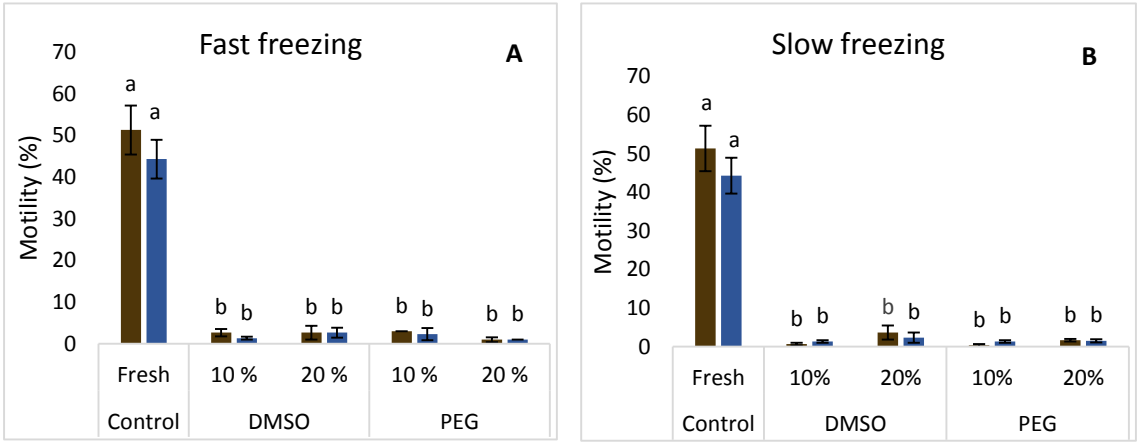

Fig. 6 Post-thaw and control motility rates of the samples after submitted to a fast freezing rate (A) and a slow freezing rate (B) using the cryoprotectants dimethyl sulphoxide (DMSO) and polyethylene glycol (PEG) at two different concentrations (10 and 20%) and in two mediums, artificial sea water (blue bars) and artificial sea water supplemented with caffeine (brown bars). (N=3; mean ± SE; One-way ANOVA; $p<0.05$). Different letters refer to significantly different results.

In both freezing rates the spermatozoa movement after thaw were very low (<5%) and there were no significant differences between cryoprotectants, concentration and dilution mediums used (Fig. 6 A and B). Only fresh samples showed high motility values, but the supplementation of caffeine, even in the control, did not increase the percentage of motile cells.

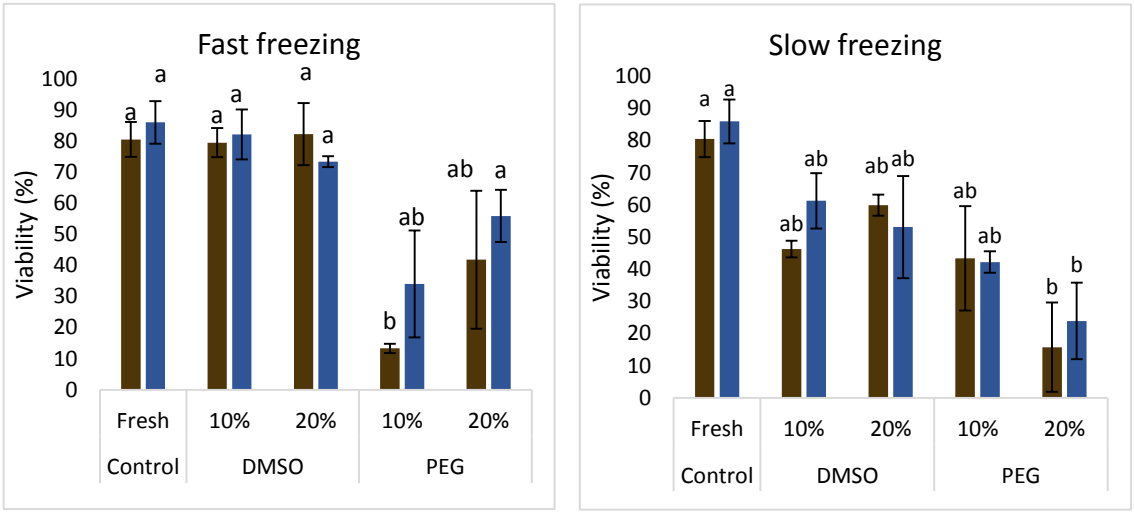

Fig. 7. Viability of the samples after submitted to a fast freezing rate (A) and a slow freezing rate (B) using the cryoprotectants dimethyl sulphoxide (DMSO) and polyethylene glycol (PEG) at two different concentrations (10 and 20%) and in two mediums, artificial sea water (blue bars) and artificial sea water supplemented with caffeine (brown bars). (N=3; mean ± SE; One-way ANOVA; $p<0.05$). Different letters refer to significantly different results.

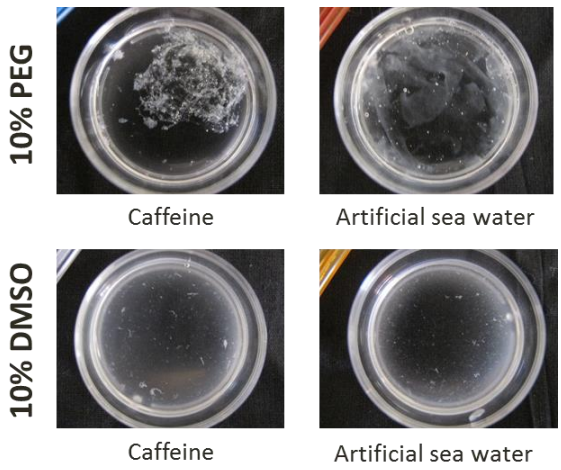

Fig. 8. Photos of thawed sperm, cryopreserved in a fast freezing rate using 10% PEG and 10% DMSO in two dilution mediums, artificial sea water and artificial sea water supplemented with caffeine.

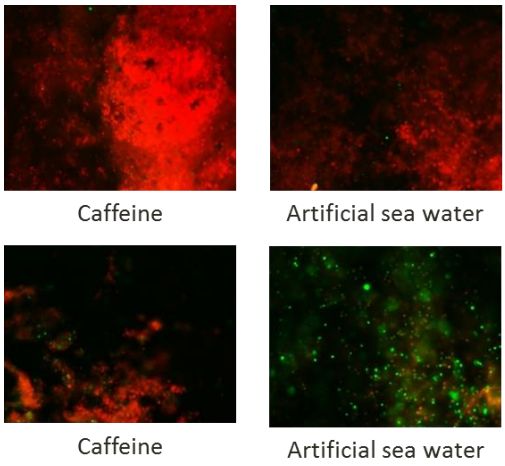

Fig. 9. Viability photos of thawed sperm, cryopreserved in a fast freezing rate using 10% PEG and 10% DMSO in two dilution mediums, artificial sea water and artificial sea water supplemented with caffeine.

According to the results, higher viability rates were obtained using 10 and 20% DMSO using a fast freezing rate (Fig. 7 A). The use of PEG reveled to be inadequate for cryopreserve oyster sperm, since it showed the worst results in both freezing rates and at the two concentrations used (Fig 7. A and B). Together with a fast freezing rate and regarding the data acquired, 10% DMSO diluted in artificial sea water (Fig. 7. A) was the best cryoprotectant to use. The results from the agglutination test were in consonance

with the described results. The use of PEG provoked agglutination on sperm when comparing with DMSO (Fig. 8) and the respective images taken of the viability test, confirmed that the majority of cells cryopreserved with PEG were all non-viable (stained with PI, red cells) (Fig. 9), contrary to what happened with DMSO (SYBR green positive cells).

1.4 Type of containers

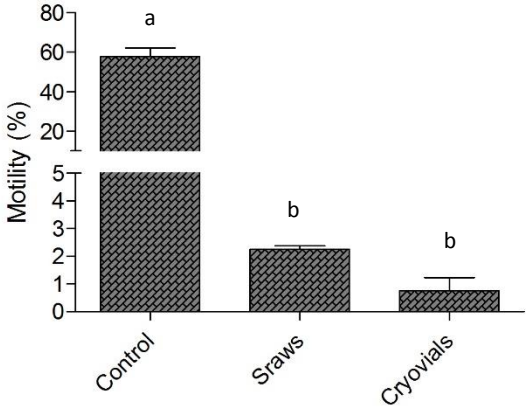

Fig. 10. Sperm motility after thaw using straws and cryovials, 10% DMSO and fast freezing rate (N=3; mean ± SE; One way ANOVA; $p<0.05$). Different letters refer to significantly different results.

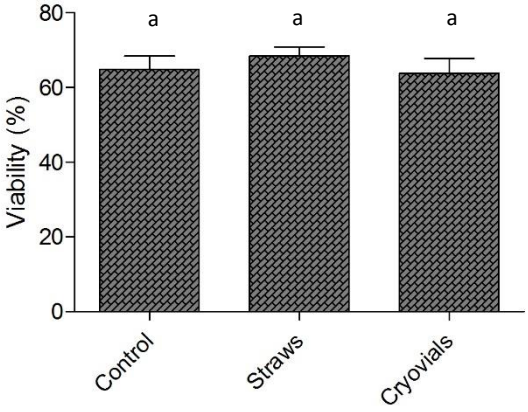

Fig. 11. Sperm viability after thaw using straws and cryovials, 10% DMSO and fast freezing rate (N=3; mean ± SE; One way ANOVA; $p<0.05$). Different letters refer to significantly different results.

With regard to the type of containers tested, there were no significant differences between straws or cryovials in terms of motility and viability. Motility values were lower in cryopreserved sperm (up to 2%) compared with fresh (up to 55%) (Fig. 10) and the viability percentages were up to 60% for both conditions (Fig. 11). In terms of cell viability there were no significant differences between fresh and cryopreserved samples.

1.5 Fertility trials: optimization of sperm to egg ratio

The fertilization results are described in figure 12.

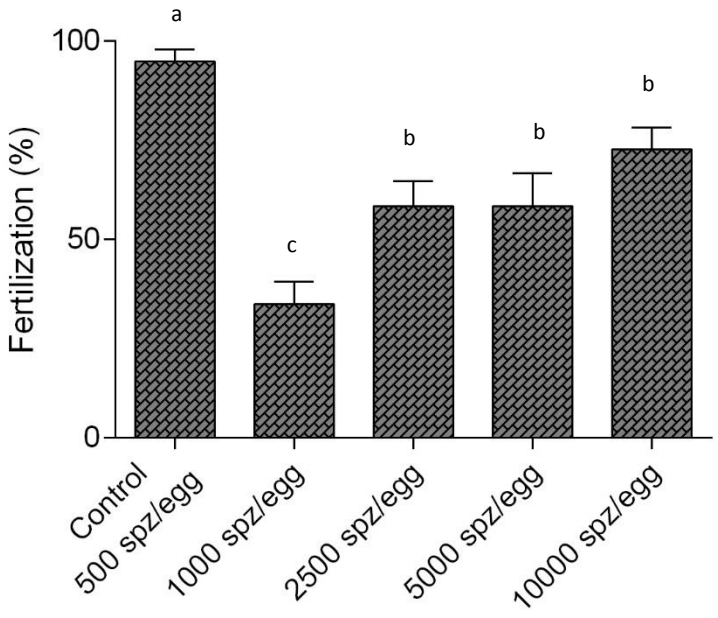

Fig. 12. Percentage of fertilized eggs using fresh and cryopreserved sperm. For the thawed sperm were used four different ratios spermatozoa/egg (N=4 pools; mean ± SE; One way ANOVA; $p<0.05$). Different letters refer to significantly different results.

The fertilization trails performed with four different spermatozoa/egg ratios revealed significant differences between the conditions. The fresh sperm, as expected, demonstrated the higher percentage (>90%) of eggs with polar body (fertilized eggs). As showed in figure 12 up to 50% of fertilized eggs were obtained in a range from 2 500 to 10 000 spz/egg, being the highest ratio the one that showed better results (>70%), even if not significantly different from the rest. The worst ratio was the one with the smallest number of spermatozoa per egg.

2. Comparative post-thaw quality of *C. angulata* and *C. gigas* sperm

The same analysis that was performed to evaluate the protocol designed for Portuguese oyster was made on cryopreserved sperm from Pacific oyster. However, instead of using only the established 10% DMSO, it was also used 10% EG since it was one of the protocols described in literature.

3.1 Viability

Cell viability was performed by flow cytometer, comparing the fresh and cryopreserved samples from both species.

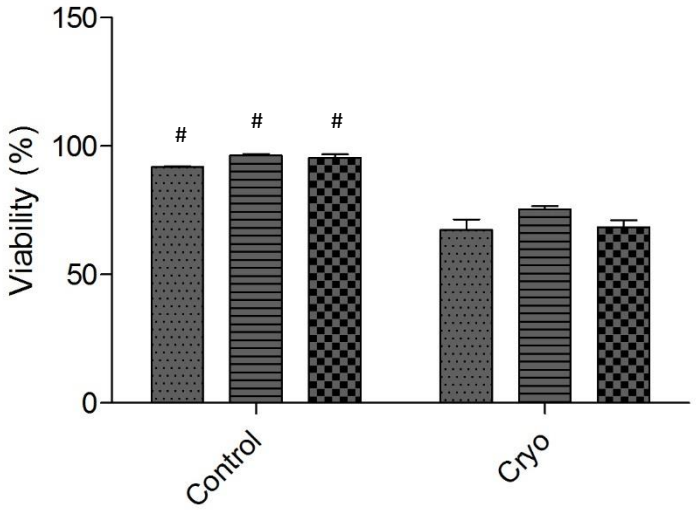

Fig. 13. Viability (%) of fresh and cryopreserved samples of *C. angulata* with 10% DMSO (dots), *C. gigas* with 10% DMSO (stripes) and *C. gigas* with 10% EG (squares). (N=6 pools for *C. angulata*; N=3 pools for *C. gigas*; mean ± SE; T-test; $p<0.05$). Columns with # showed significantly different results when compared cryopreserved samples with their respective control.

According to the results, the fresh samples differ significantly from the cryopreserved ones, in all tested conditions. For both species, among all the conditions, the highest viability percentages (>90%) were obtained in the fresh samples, comparing to the cryopreserved samples which rates were around 60 to 70%. In addition, in *C. gigas*, there was no difference between the effects of the cryoprotectants in the survival of the spermatozoa.

3.2 Lipid peroxidation, ATP content and Comet assay

Lipid peroxidation, ATP content and DNA fragmentation were assessed for the Pacific and Portuguese oyster and are represented in figures 14 and 15.

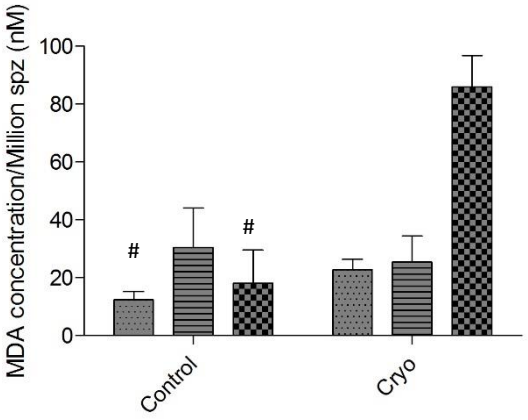

Fig. 14. Lipid peroxidation (MDA concentration) per million spermatozoa (nM) in fresh and cryopreserved samples of *C. angulata* with 10% DMSO (dots), *C. gigas* with 10% DMSO (stripes) and *C. gigas* with 10% EG (squares). (N=6 pools for *C. angulata*; N=3 pools for *C. gigas*; mean ± SE; T-test; $p<0.05$). Columns with # showed significantly different results when compared cryopreserved samples with their respective control.

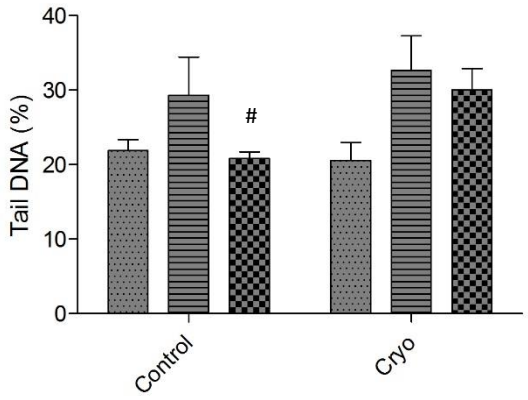

Fig. 15. Percentage of DNA fragmentation (DNAt) in fresh and cryopreserved samples of *C. angulata* with 10% DMSO (dots) *C. gigas* with 10% DMSO (stripes) and *C. gigas* with 10% EG (squares). (N=6 pools for *C. angulata*; N=3 pools for *C. gigas*; ± 60 cells from each slide; mean ± SE; T-test; $p<0.05$). Columns with # showed significantly different results when compared cryopreserved samples with their respective control.

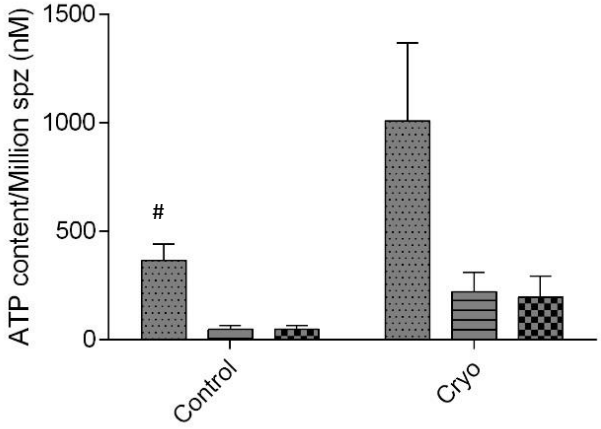

Fig. 16. ATP content per million spermatozoa (nM) in fresh and cryopreserved samples of *C. angulata* with 10% DMSO (dots), *C. gigas* with 10% DMSO (stripes) and *C. gigas* with 10% EG (squares). (N=6 pools for *C. angulata*; N=3 pools for *C. gigas*; mean ± SE; T-test; $p<0.05$). Columns with # showed significantly different results.

The quantification of MDA in *C. angulata* sperm cryopreserved with 10% DMSO and in *C. gigas* sperm cryopreserved with 10% EG, revealed significantly different results from the respective control samples (Fig. 14). Moreover, it was obviously the huge difference (more than 70%) in sperm cryopreserved with 10% EG (85%), comparing with the respective control (18%), producing this protocol higher levels of lipid peroxidation in cryopreserved sperm than the one developed in this study . The use of 10% DMSO in *C. gigas* and in *C. angulata* showed more closely results (Fig. 14).

Regarding sperm DNA fragmentation in *C. angulata* and *C. gigas* using 10% DMSO as cryoprotectant, there was no significant difference between the control and the cryopreserved samples. However, in *C. gigas*, when using 10% EG as cryoprotectant, the percentage of DNAt in cryopreserved samples was higher (30%) and differ significantly from the control (20%) (Fig. 15).

Regarding the ATP content, only *C. angulata* showed differences between the fresh and cryopreserved conditions (Fig.16). The use of ethylene glycol (EG) and dimethyl sulphoxide (DMSO) did not differ from their respective controls and the ATP values were similar (197 nM 10^{-9} for 10% EG and 222 nM 10^{-9} for 10% DMSO) in both types of samples.

Discussion

1. Cryopreservation of *C. angulata* sperm

1.1 Long term motility duration

During this study, several motility and viability tests were made in order to comprehend some basic features of Portuguese oyster spermatozoa. *Crassostrea angulata* sperm revealed to be very resistant and to have long duration movement, since it showed motility for at least 7 days, when stored at 4 °C. According to published literature, the most similar species to *C. angulata* in terms of movement duration, is the American oyster (*Crassostrea virginica*), which motility can reach 180 hours (7 days and a half) (Rose and Heath, 1978). Moreover, until the 5th day the *C. angulata* sperm could be able to maintain their initial motility rate, but after it there was a strong decrease and higher standard errors that could be explained by the decrease in some samples quality. Furthermore, the motility of the samples maintained for 30 minutes at room temperature, did not changed their values until the 3rd day, after it there was a decrease of 20% in the spermatozoa movement rate (from 60% to 40%) and then the cells recover their movement, in the 5th day, in 10% (from 40% to 50%). These results are according to the data obtained by Suquet *et al.,* (2010) wherein similar results were obtained in motility studies on *C. gigas* sperm and they emphasize the long spermatozoa movement of Pacific oyster and, at the same time, the necessity for further research to achieve more detailed information about the description of this changes in the motility, since oysters are marked as species with sperm with a long swimming phase. Contrary, there are some species that shows short movement duration of their spermatozoa, ranging from 2 to 20 minutes, such as fish species like seabass, seabream and turbot (Cosson *et al*., 2008a, Suquet *et al*., 2012). Furthermore, as seen in Fig. 2, measuring the same parameter 30 minutes after, at a room temperature, the motility increased. This difference on the motility in the same samples are explained under the increase of samples temperatures, which variation was enough to activate the metabolism and achieve higher motility rates.

1.2 Toxicity tests

The osmolality of Portuguese oyster is 990 mOsmol Kg^{-1} and thus the more adequate medium to dilute sperm is the artificial sea water (1 100 mOsmol Kg^{-1}), since it represents an isosmotic medium for *C. angulata*. Still within the fresh sperm analysis and regarding the effect of the caffeine, it provoked an increase in motility in some conditions (10 and 20% PEG, 5% DMSO and 5% MetOH), but in others the achieved motility values were not significantly different from the control. The results suggested that the effect of the caffeine can vary depending on the cryoprotectant (in ethylene glycol there was no differences for all the concentrations tested – Fig. 4 D) and also on their concentration (in dimethyl sulphoxide and methanol only increased the movement of the samples cryopreserved with the lowest concentration). Thus, the supplementation of the caffeine in the dilution medium did not reveal the expected result, such in other fish species as in Pacu (*Piaractus mesopotamicus*), in which sperm motility of fresh samples increased with a straight linear relation with the increase of caffeine concentration (Carvalho *et al*., 2014). It was also reported for the "maturation process" of Pacific oyster sperm (Suquet *et al.,* 2012), that can be stimulated through the addition of 10 mM caffeine to sea water, increasing the beat frequency of flagella. This reaction can be obtained also with the addition of theophylline. Caffeine and theophylline act as phosphodiesterase inhibitors and can increase the intracellular cAMP (a derived form from ATP), which stimulates protein phosphorylation and trigger sperm movement (Suquet *et al*., 2012).

After the dilution medium, the choice of a cryoprotectant is also an important factor and depends on its toxicity and the capacity to protect sperm against the freezing effects. In this study, 10% PEG was the cryoprotectant that showed less toxicity effect on the cells, however 10% DMSO revealed the better cryoprotectant effect against freeze damage. This is not a surprising result, since DMSO is one of the most used cryoprotectants due to its ability to penetrate cells quickly and give better protection from freezing and thawing injury (Hassan *et al*., 2015).

1.3 Freezing rates and type of containers

Agglutination is a phenomenon that appears on post-thaw sperm samples and it was previously described on other species, such as common carp (*Cyprinus carpio*), Pacific oyster and Eastern oyster (Dong *et al*., 2007). The results obtained in the experiments permitted to infer that this was also a problem when dealing with Portuguese oyster sperm cryopreservation. In addition, agglutination phenomenon affected drastically the viability of the spermatozoa of *C. angulata* and polyethylene glycol (PEG) revealed to provoke agglutination as well as the presence of caffeine that also contributed to this purpose. This may also help to explain the fact that caffeine was not as effective as expected in fresh samples because it affects their viability. Moreover, the freezing rate also played an important role in this field, since the agglutination was higher in samples cryopreserved with a slow freezing rate and were avoided using a fast freezing. The results showed the same pattern as the ones obtained by Dong *et al*., (2007) in *C. gigas*, where she demonstrated that cryoprotectants at low concentrations, cooling and thawing at slow rates and freezing samples at high sperm concentration led to the formation of high levels of agglutination. Furthermore, according to the agglutination scale developed by Dong *et al*., (2007), *C. angulata* sperm cryopreserved with 10% DMSO was considered as level 1 (few clumps discernible) (Fig. 8), in a scale from 0 (non-agglutinated sperm) to 5 (well-developed "noodles"), being the appearance of the sperm homogeneous.

There were no significant differences between freezing sperm in straws or cryovials. However, although not significant, a lower percentage of motile cells was seen when using cryovials to store sperm. This fact could be attributed to a higher heterogeneity during thawing since the relation volume-surface is lower in cryovials than in straws, leading to lower heat transference. This fact was also observed in rainbow trout sperm frozen at large volumes (Cabrita *et al*., 2001b)

After all, 10% DMSO, fast freezing and the use of straws as container represented the best cryopreservation conditions for freezing Portuguese oyster sperm.

2. Post-thaw quality analysis on *C. angulata*

Motility has been the major parameter to evaluate the cryopreservation protocols and the quality of post-thaw spermatozoa (Cosson *et al*., 2008b). The incorporation of morphological and functional integrity analysis with other quality assessment indicators appears as an improvement of the techniques to identify the sources of sperm defects caused by the freezing process.

2.1 Lipid peroxidation

There were significant differences between the fresh and cryopreserved samples, being the levels of MDA, a subproduct from the lipid peroxidation, higher in the cryopreserved sperm. These results suggest that sperm was sensitive to the cryopreservation process, thus resulting in a decrease in sperm quality after-thawing. According to studies performed by Neild *et al*., (2005), in mammals, lipid peroxidation and the integrity of the membrane seems to be not correlated and to be independent processes, since damage was located in different parts of the spermatozoa. Furthermore, in previous studies in fish sperm, as sea bass, lipid peroxidation was not correlated with other analysed parameters in a cryopreservation protocol. In the case of fish sperm membranes, these are particularly susceptible to lipid peroxidation due to greater amounts of polyunsaturated fatty acids (PUFA) (Martínez-Páramo *et al.,* 2012bc). Similar facts could occur in oyster sperm, but more and different research is fundamental in this field in order to deepen the knowledge of this subject in this species.

2.2 ATP content

Regarding the initial ATP content, there were no significant differences between the fresh and cryopreserved samples, which is a good indicator when it comes to quality evaluation of the cryopreserved material. According to Suquet *et al.*, (2010), there is a huge interspecies diversity in ATP levels. The obtained values of ATP content for *C. angulata*, ranging from 367 to 1009 nM 10^{-9}, were in accordance to the values described for black-lip pearl oyster (ranging from 251 to 1977 nM 10^{-9}) (Demoy-Schneider *et al*., 2012), but were very different from the Pacific oyster values (approximately 50 nM 10^{-9}) (Suquet *et al.,* 2012). Pacific oysters have higher sperm motility duration (20-24 h) when comparing with the black-lip pearl oyster (10 minutes). Both species have

motilities duration lower than Portuguese oyster sperm (7 days), despite the similar ATP content levels found between the Portuguese and the black-lip pearl oyster. This means that the ATP content could not be neither the limiting nor the main factor for the long movement duration of oyster spermatozoa (Suquet *et al*., 2010), suggesting the existence of a mechanism responsible for the regeneration of the ATP. In this sense, and despite this mechanism is not detailed reported, it is known that after 24 h movement, in Pacific oyster, the ATP content only decreases to 94% of its initial rate and thus, this phenomenon of energy restoration is sustained by the limited changes that occur in spermatozoa morphology after the movement period (Suquet *et al*., 2010, 2012). Comparing to fish species, such as rainbow trout and seabass (Christen *et al.*, 1987, Suquet *et al*., 2012) this capacity to restore ATP is higher in oyster species.

Thus, and as suggested by Demoy-Schneider *et al*., (2012), more and complementary studies on other energy rich substrates or on the activity of enzymes involved in the regeneration of ATP should be performed in further researches on *C. angulata*. It will be also interesting to see the final levels of ATP after movement cession in this species.

2.3 Comet assay

DNA is an important parameter to check after cryopreservation since it is known in several species that cryopreservation can induce different levels of DNA fragmentation (single and double strands) (Cabrita *et al.,* 2010). The percentage of tail DNA fragmentation was around 20% in both conditions. The degree of sperm DNA damage provoked by cryopreservation procedures vary within species (Martínez-Páramo *et al*., 2009) and can decrease the sperm fertility capacity and the development of embryos (Pérez-García *et al.,* 2015). The first report of a comet assay performed on Pacific oyster for sperm quality evaluation after thawing was by Gwo *et al.,* (2003), wherein he performed a similar cryopreservation protocol (Asw, 10% DMSO, fast freezing rate, 1.5 mL microtubes), but obtained not only higher values of Tail DNA (DNAt) but also significant differences between fresh (60.65% of DNAt) and cryopreserved (68.60% of DNAt) samples. There are some parameters that can affect the differences in results such as the freezing/thawing rate or type of container used to freeze the samples. Once more this difference with our protocol and results could be associated with the heterogeneity of post-thaw quality when using microtubes for freezing sperm. In this

system sperm is exposed to higher temperatures during thawing and the heat is not homogenously transferred through the entire sample, inducing a quick thawing of the cells near the tube wall and a longer exposure to high temperatures than cells in the inner part of the tube. The effects of this phenomenon are associated with a loss of cell viability and DNA integrity. Taking into account the different type of container this could be one explanation for the 40% DNAt of difference between the two methods used in these two oyster species. Moreover, despite the taxonomic relationship between *C. gigas* and *C. angulata*, the results obtained by Gwo *et al.,* (2003) were in a different species and that fact can be enough for the variance on the results.

2.4 Fertility trials: optimization of sperm to egg ratio

The ultimate part of the protocol evaluation is the success of the fertilizations, comparing the results from eggs fertilized with fresh sperm and with the cryopreserved material. As recommended in the review on oyster sperm cryopreservation from Hassan *et al*., (2015), the optimal spermatozoa/egg ratios must be optimized and fro that different proportions need to be tested, since it could be species-specific. In this sense, it was tested four different sperm/egg ratios and the obtained results showed significant differences between some of them. It was obtained up to 50% of fertilized eggs, in a range from 2 500 to 10 000 spz/egg, being the higher ratio the one that showed better results (>70%) and the lower the worst (>30%), comparing with the results from the fresh sperm (>94%). In other oyster species, such as Pacific oyster, a rate of 96% of fertilized eggs were obtained, using thawed sperm, with a ratio of 10 000 spz/egg. In this way, despite the low post-thaw motility rates registered in Pacific and Portuguese oyster sperm, fertility rates were not compromise if the amount of sperm per egg ratio was adjusted. Hassan *et al*., (2015), assumed that 1 000 to 10 000 sperm per egg is enough to apply in other species to compare the fertility, if the sperm functionality was not affected by cryopreservation, as it was observed by the achieved percentages of fertilized eggs in the present study. Moreover, according to Adams *et al*., (2004), similar fertility rates can be obtained if 30 to 100-fold more sperm is used during the procedures with cryopreserved sperm. In concordance with this information, it remains the possibility in further studies to increase the number of cryopreserved spermatozoa until 50 000 spz/egg, which could increase the number of fertilized eggs. There is also the

problem of male-to-male variability, referred by Yang *et al*., (2013), in terms of motility, membrane integrity and fertility. For this purpose, the quality evaluation techniques used in this study were all performed with pools with three males.

The results obtained in this work can be comparable with others using a similar protocol developed for *Crassotrea virginica* (10% DMSO, 20 minutes of equilibration time, fast freezing rate, use of straws, thawing at 40 °C for 8 s) wherein fertilization trials achieved the 77% of fertilized eggs (Yang *et al.*, 2012), values close to the obtained in this study.

In further experiments it will be necessary to incubate the eggs for longer period to check embryonic development, hatching rates, and larval survival, in order to accept the hypothesis that 20% of DNA damage do not interfere and affect the embryonic development.

3. Comparative post-thaw quality of *C. angulata* and *C. gigas* sperm

3.1 Viability

One of the easiest methods to test the success of a designed cryopreservation protocol is performing viability analysis of the material, through membrane integrity and permeability tests (Cabrita *et al*., 2010). In these cases, sperm evaluation is normally done by microscopy using fluorescent dyes and counting each cell individually to achieve a global percentage of viable cells. This procedure is somehow time consuming and other methods such as the use of flow cytometry can be more reliable and faster in their analysis. In the present study and with the goal of standardizing protocols and quality analysis methodologies for the genus *Crassostrea,* viability analysis was tested by flow cytometry, which has been used in fish sperm and also showed to be useful in oyster sperm, as in *Crassostrea virginica* (Paniagua-Chávez *et al.,* 2006). In the viability test, the cryoprotectants used in *C. gigas* affected the survival of the cells, in the same way that affected the cells from *C. angulata*. Moreover, the fresh samples of both species showed higher percentage of survival (>90%), when compared to the cryopreserved ones (60 to 70%), which was an expected result due to the injuries that all biological material suffer during the freezing and thawing processes (Cabrita *et al*., 2008).

3.2 Lipid peroxidation, ATP content and Comet assay

Regarding the lipid peroxidation in Pacific oyster, sperm cryopreserved with 10% ethylene glycol (EG) revealed significantly different results from the respective control samples (more than 70% of difference). On the other hand, the use of 10% DMSO in *C. gigas* and in *C. angulata* showed more closely results, suggesting that DMSO is better for protecting the spermatozoa of both species against the inevitable process of lipid peroxidation. As mentioned by Hassan *et al.,* (2015), DMSO have a great capacity to penetrate into the cells and thus protect them from the cellular damage.

The analysis of ATP content did not reveal any difference between both cryoprotectants used in *C. gigas*, which means that DMSO can also be applied for this species. In addition, the lower ATP content determined in sperm from *C. gigas* (from 30 nM 10^{-9} to 170 nM 10^{-9}) when compared with *C. angulata* support the idea of longer motility duration for Portuguese oyster when compared with the spermatozoa movement duration of the Pacific oyster. Also, the obtained values of ATP in *C. gigas* are in concordance with the ones obtained by Suquet *et al.,* (2012), despite the different method of determination used.

The acquired results from the Comet assay demonstrated that Pacific oyster sperm cryopreserved with 10% EG (30% DNAt) differ significantly from the control (20% DNAt) and the use of DMSO, as already happened in the previous test, is more viable than EG and should be used in *C. gigas* spermatozoa cryopreservation. With this improved protocol there were no differences respect to the control, contrary to data obtained by other authors who registered high levels of damage in cryopreserved samples (Gwo *et al*., 2003). As above mention, in the section 2.3, this variance in the results are explained through some different techniques that were used, which in fact aware to the important necessity to create a standard protocol and laboratory procedures able to be reproduced by other operators, leading to the same results.

This study permits to infer that 10% dimethyl sulphoxide (DMSO) can be consider a standard cryoprotectant for both species. Nevertheless, for further research the incorporation of a non-permeable cryoprotectant should be tested. It was already proved that the inclusion of 0.45 M of trehalose to DMSO can increase the fertility of

the cryopreserved sperm in Pacific oyster (Adams *et al*., 2004, Hassan *et al*., 2015), and thus could also be interesting to test if produces the same effects in Portuguese oyster.

Conclusions

The present study permitted to conclude that sperm from Portuguese oyster (*C. angulata*) was very resistant and had long duration motility and, at the same time, the supplementation of caffeine in the dilution medium did not increase this parameter.

Regarding the cryoprotectants and their toxicity test, 10% PEG was the cryoprotectant that showed to be less toxic to the cells and 20% MetOH the one with worst results. Agglutination is a phenomenon previously described on other oyster species and it was also a problem when dealing with Portuguese oyster sperm cryopreservation. In this experiment, polyethylene glycol (PEG) revealed to provoke agglutination and also the presence of caffeine seemed to contribute for this purpose. In addition, this agglutination phenomenon affected drastically the viability of the spermatozoa.

The better cryoprotectant for the freezing process of *C. angulata* sperm was 10% DMSO when diluted in artificial sea water. Applying these conditions, the samples submitted to a fast freezing showed to have superior post-thaw viability percentages (>80%) compared to the 60% obtained for slow freezing. This fact was isolated from the type of container used, which showed no influence in terms of motility and viability.

Among post-thaw quality analysis, lipid peroxidation (MDA) was significantly higher in cryopreserved samples, contrarily to the ATP content and Comet assay, wherein there were no significant differences between the control and the thawed material. Subsequently, fertilization trials with the cryopreserved sperm were successfully achieved, being the ratio of 10 000 spermatozoa/egg the more adequate ratio, where up to 70% of the eggs were fertilized. A successful protocol was designed and described for the first time for Portuguese oyster sperm.

Concerning the comparison between the use of the established protocol in Pacific oyster, the three analysis performed (lipid peroxidation, DNA fragmentation and ATP content) did not reveal post-thaw quality differences between the control and the samples frozen with 10% DMSO.

With the designed protocol it was possible to overcome the limits imposed by agglutination, and thus the protocol revealed to be effective and with a low degree of cellular damage on cryopreserved sperm from Portuguese and Pacific oyster.

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