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Cryopreservation of zebrafish germ cells: technological improvements and methodological standardization for gene banking and management

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Declaração de autoria do trabalho

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

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Dedicated to my son Duarte

and my grandmother Hirminia

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For as long as I remember I wanted to do a valuable Ph.D., since I love science; and I wanted to have a child, since I love life. The existence of both, started in the same unforgettable week. This journey of 4 years was one of the most transformational moments of my life where both dreams came true at the same time. It was a learning experience in development. Development of my son, of zebrafish (my sons in science), development as an individual and researcher. It was no doubt one of the most challenging times of my life and the accomplishment of my dreams was not possible without the support of my supervisors, colleagues, students, volunteers, friends and family to whom I would like to express my gratitude.

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ABSTRACT

Due to the increasing number of zebrafish (*Danio rerio*) mutant and transgenic lines, there is a high demand for assisted reproductive techniques to support facility management. Efficient zebrafish sperm cryopreservation is a pressing necessity to manage and preserve the valuable zebrafish genetic resources. Although zebrafish sperm cryopreservation was first attempted more than 30 years ago, protocols still lack standardization, which translates into high variability in post-thaw sperm quality and *in vitro* fertilization success. Therefore, the present thesis aims to improve the current methodologies used for zebrafish sperm cryopreservation and broodstock management towards the standardization of procedures in this species.

The introductory context of the present thesis is approached in **chapter 1**. In this chapter the relevance of zebrafish model is discussed as well as this species sperm cryopreservation usefulness. The main factors affecting sperm quality and the application of reliable quality analysis are discussed in this chapter. The final objective of sperm cryopreservation is to obtain high quality offspring and therefore *in vitro* fertilization, early development and offspring quality analysis are important tools for the optimization of sperm cryopreservation methodologies. The current knowledge in sperm cryopreservation fundamentals is approached in this chapter, as well as the main advances and bottlenecks in zebrafish sperm cryopreservation.

In **chapter 2**, the zebrafish sperm motility activation was assessed under different conditions of water temperature and conductivity. The environmental conditions present in the fertilization microenvironment are responsible for the mechanism of spermatozoa motility activation and metabolic modulation that influence the probability of fertilization success. Zebrafish is commonly reared at 28°C, but with variable water conductivity conditions among facilities. However, sperm motility analysis is routinely performed with distilled water at room temperature. We aimed to understand the effect of water temperature and conductivity on sperm motility and fertilization ability. Water at 28°C with lower water conductivity (0 and 700 µS/cm) improve sperm motility parameters. Standardization of the water conditions (of system water and activation medium

used for motility analysis) among facilities is highly relevant to improve the reproducibility of sperm quality analysis and thus, to predict with higher accuracy fertilization ability.

Successful cryopreservation depends on high quality sperm, which depends on the quality of breeders. Consequently, broodstock selection and management is a priority to improve sperm cryopreservation. The broodstock diet has a preponderant effect on gamete quality, particularly in phospholipids and antioxidants content which are known to promote spermatogenesis. Therefore, in **chapter 3** we aimed to determine the effects of a tailor-made purified diet supplemented with phosphatidylcholine (PC) or phosphatidylethanolamine (PE) on the zebrafish reproductive performance, gamete quality and larval skeletal malformations. Both dietary supplementations with phospholipids improved sperm motility and eggs quality, however PC increased the incidence of skeletal malformations on the offspring, as previously observed in other teleosts. Although dietary phospholipids classes have a role in the ossification process of the vertebral column in teleosts, its mechanisms are still to be understood. Therefore, the development and use of a standardized diet for zebrafish broodstock is essential to reduce the variability of the reproductive performance among facilities. In **chapter 4**, the selection of optimal age and minimum sperm collection frequency was evaluated, since these factors are essential to obtain high quality samples. Our results indicate that young males (6-8 months) showed higher sperm quality and require a minimum of 14 days between sperm collections to recover sperm plasma membrane viability.

An important bottleneck in cryopreservation is the liquid nitrogen requirement for storage. Therefore, it was established in **chapter 5** a new cryopreservation method using an electric ultrafreezer (-150°C) as an alternative to liquid nitrogen, for the first time in a teleost species. This protocol reaches a fast cooling rate (-66°C/min) in one single step and yields higher sperm viability and DNA integrity in comparison to the traditional methods (-20°C/min in liquid nitrogen). The synergy obtained by the combination of cryoprotectants is a successful cryopreservation strategy that can be beneficial in the optimization of zebrafish sperm cryopreservation. Therefore, it was selected the most adequate

cryoprotectant combination that generates offspring with normal skeletogenesis. Data show that 15% of DMF with 50 mM of bicine or 10% of egg yolk is beneficial for the quality of zebrafish offspring sired by cryopreserved sperm. To the best of our knowledge, this is the first report on skeletal development of zebrafish offspring sired by cryopreserved sperm performed with different extender compositions.

Zebrafish is especially useful to investigate some of the most prominent human diseases such as diabetes. Among other consequences, diabetes (type I and II) causes disturbances in the male reproductive system, since glucose metabolism is an important event not only in spermatogenesis but also in mature spermatozoa metabolism. In **chapter 6** we aimed to validate zebrafish as a useful model organism to investigate male reproductive dysfunctions mechanisms caused by type I diabetes. In this chapter, sperm cryopreservation was applied to a relevant zebrafish model of type I diabetes. The transgenic zebrafish under diabetic conditions shows higher levels of *insulin a* (*insa*), insulin receptor a (*inra*) and *glucose carrier 2* (*slc2a2*) transcripts in spermatozoa when compared to the controls. This is because gametogenesis occurred under diabetic conditions, changing transcription in the germline. Consequently, spermatozoa carry the imprinted transcripts that will be transmitted during fertilization. Sperm quality (motility, viability and DNA integrity) was lower in the transgenic fish under (transient) diabetic state as observed in human and mouse model. Sperm cryopreservation affects sperm quality of fish both under diabetic and non-diabetic conditions. However, diabetic conditions were detrimental in sperm freezability, which can be explained by the lower initial sperm quality. In this chapter zebrafish was validated as a useful model organism to investigate male reproductive dysfunctions mechanisms caused by type I diabetes.

Relevant differences between different zebrafish lines are evidenced in terms of sperm quality and susceptibility to damage, which suggests that it is an important factor to consider while establishing sperm cryopreservation protocols. This thesis offers new insights and a set of guidelines on breeder's management and sperm cryopreservation to improve zebrafish husbandry practices.

Keywords: Zebrafish, sperm quality, cryopreservation, ultrafreezer, sperm motility activation, diet, type I diabetes

RESUMO

O peixe zebra (*Danio rerio*) tornou-se inquestionavelmente num dos organismos modelo mais proeminentes da atualidade, devido às suas características favoráveis para investigação. O desenvolvimento de técnicas de edição genética e a sequenciação do genoma desta espécie possibilitou o desenvolvimento de milhares de linhas transgénicas e mutantes. Consequentemente, a gestão dos numerosos genótipos trouxe desafios na manutenção de espaço e gestão destes recursos genéticos. A criopreservação de sémen é uma ferramenta valiosa para a gestão destes valiosos recursos genéticos, que pode solucionar este problema. No entanto, apesar do primeiro protocolo de criopreservação de sémen de peixe zebra ter sido desenvolvido há mais de 30 anos, ainda requer otimização e estandardização. Consequentemente, existe elevada variabilidade na qualidade do sémen e sucesso da fertilização *in vitro* entre biotérios. O desenvolvimento de uma técnica de criopreservação de sémen eficiente é atualmente um dos maiores desafios da comunidade de peixe zebra. O objetivo principal da presente tese foi a otimização das técnicas de gestão de reprodutores e criopreservação de sémen de peixe zebra, no sentido da estandardização das práticas e maior reprodutibilidade dos resultados científicos nesta espécie.

A introdução ao contexto da presente tese é abordada no **capítulo 1**. Neste capitulo a importância do peixe zebra como organismo modelo é abordado assim como a utilidade da criopreservação do sémen nesta espécie. Os factores que afetam a qualidade do sémen assim como a aplicação de análises de qualidade robustas são discutidos neste capítulo. O objetivo final da criopreservação de sémen é a produção de progenia com elevada qualidade. Consequentemente, neste capítulo a fertilização *in vitro*, o desenvolvimento embrionário e a análise da qualidade da progenia é discutida. Neste capítulo são explorados os fundamentos de criobiologia, principais avanços e dificuldades no desenvolvimento de protocolos de criopreservação de sémen de peixe zebra.

As condições do ambiente de fertilização são responsáveis pela ativação da mobilidade dos espermatozóides e pela modulação do seu metabolismo, afetando consequentemente o sucesso da fertilização. O peixe zebra é estabulado a 28°C com

parâmetros de condutividade da água variáveis entre biotérios. No entanto, as análises de mobilidade espermática são realizadas rotineiramente com água destilada a temperatura ambiente. Consequentemente, no **capítulo 2** o objetivo do nosso trabalho foi caracterizar o efeito da temperatura e condutividade da água na mobilidade de sémen de peixe zebra. Adicionalmente, foi estudado o efeito da condutividade da água no sucesso da fertilização. A água a 28°C e com baixa condutividade (0 e 700 μS/cm) melhorou os parâmetros de mobilidade. A estandardização das condições da água (dos sistemas de cultivo e do meio de ativação usado na análise da mobilidade) entre biotérios é essencial para a otimização das análises de qualidade, reprodutibilidade científica e maior precisão na estimação do potencial de sucesso de fertilização de uma amostra de sémen.

O sucesso da criopreservação depende da qualidade do sémen que, por sua vez, depende da qualidade dos reprodutores. Consequentemente, a seleção e gestão de reprodutores é uma prioridade, de forma a assegurar o sucesso do método de criopreservação. Um dos factores mais importantes na gestão de reprodutores é a sua dieta. A nutrição dos reprodutores tem um importante efeito na qualidade dos gametas já que afeta a gametogénese, particularmente a composição da dieta em fosfolípidos e antioxidantes. O objetivo do **capítulo 3** foi determinar o efeito de dietas purificadas suplementadas com fosfatidilcolina (PC) e fosfatidiletanolamina (PE). A suplementação em fosfolípidos melhorou a mobilidade do sémen; no entanto, a suplementação em PC provocou um aumento da incidência de malformações esqueléticas na progenia. Estes resultados estão de acordo com estudos de nutrição anteriores em teleosteos. O desenvolvimento e utilização de dietas estandardizadas nos reprodutores de peixe zebra é essencial para optimizar a performance reprodutiva e reduzir a variabilidade entre biotérios.

A seleção da idade ótima dos machos e a frequência mínima adequada para recolha de sémen é essencial para obter amostras com elevada qualidade. No **capítulo 4** foi determinado o efeito da idade e da frequência de extração na qualidade do sémen. O nosso estudo mostrou que machos jovens (6-8 meses) de peixe zebra revelam maior qualidade de sémen e necessitam de um mínimo de 14 dias de repouso para recuperarem a viabilidade da membrana plasmática dos espermatozóides.

Uma das maiores desvantagens da criopreservação é a necessidade de azoto líquido para armazenamento de amostras. Considerando esta questão, foi desenvolvido no **capítulo 5** o primeiro protocolo de criopreservação de sémen de teleósteos utilizando um ultracongelador (-150°C). Este protocolo é realizado num só passo, sem a utilização de azoto líquido, sendo as amostras criopreservadas a -66°C/min. Este protocolo melhorou a viabilidade e integridade do ADN dos espermatozóides em comparação com o método convencional (-20°C/min armazenado em azoto líquido). A combinação de diferentes crioprotetores é uma estratégia de criopreservação com elevado sucesso. Consequentemente, um dos objetivos do nosso trabalho foi selecionar a combinação de crioprotetores mais adequada para o protocolo de criopreservação estabelecido anteriormente. Os resultados deste trabalho indicam que a utilização de 15% de DMF com 50 mM de bicina ou 10% de gema de ovo produzem sémen de elevada qualidade do sémen e maior sucesso em fertilizações *in vitro*, assegurando também o adequado desenvolvimento esquelético da progenia. Este foi o primeiro estudo de caracterização de malformações esqueléticas desenvolvidas na progenia de peixe zebra produzido com sémen criopreservado com diferentes composições de crioprotetores.

O peixe zebra é particularmente útil na investigação de doenças humanas com elevada prevalência na população mundial tal como a diabetes. Entre outras complicações geradas por esta patologia, a diabetes tipo I e II afeta o sistema reprodutor masculino. Estas perturbações ocorrem devido à alteração do metabolismo da glucose, essencial durante a espermatogénese e no metabolismo dos espermatozóides. Comparando com outros modelos, o peixe zebra tem gerações mais curtas, consequentemente seria uma ferramenta útil para esta investigação. O objetivo do **capítulo 6** foi validar o peixe zebra como organismo modelo para o estudo dos mecanismos de ação da diabetes tipo I pelos quais afetam o sistema reprodutor masculino. Tal como observado em humanos e no organismo modelo de diabetes roedor, a qualidade do sémen (mobilidade, viabilidade, integridade do ADN) é reduzida na estirpe transgénica sob estado transiente de diabetes tipo I em relação ao controlo. O sémen do modelo transgénico em estado diabético revela aumento dos níveis de transcriptos de *insulina a* (*insa*), *receptor de insulina a* (*inra*) assim como de um transportador especifico de glucose *GLUT 2* (*slc2a2*). Este facto é

devido a uma alteração dos níveis de transcrição destes genes na linha germinal durante a gametogénese. O sémen criopreservado de ambos os tratamentos (controlo e diabético) revelou um decréscimo na qualidade, tal como esperado. O tratamento diabético aumentou a susceptibilidade das células à criopreservação, o que se pode dever à sua qualidade seminal inicial inferior. Assim, evidenciamos neste modelo transgénico para a diabetes tipo I os mesmos efeitos na qualidade seminal observados em humanos e em rato, validando desta forma esta linha para a investigação dos efeitos desta doença no sistema reprodutor masculino.

A presente tese propõe o estabelecimento de medidas de seleção e maneio de reprodutores e análise de qualidade seminal. Adicionalmente propomos um método inovador de criopreservação de sémen, prático e económico, através do uso de um ultracongelador. Verificou-se o impacto de diferentes combinações de crioprotectores na qualidade e na esqueletogénese da progénie gerada com sémen criopreservado. Resumindo, esta tese propõe procedimentos e metodologias para a gestão de reprodutores de peixe zebra relevantes para o estabelecimento de medidas de estandardização, promovendo desta forma a reprodutibilidade de metodologias científicas.

Palavras chave: Peixe zebra, qualidade de sémen, criopreservação, ultracongelador, activação da mobilidade de sémen, dieta, diabetes tipo I

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LIST OF ABBREVIATIONS AND ACRONYMS

µl	Microliter
µWs/cm^2	UV radiation
a.m.	*ante meridiem*
ALH	Amplitude of lateral displacement
ANOVA	Analysis of variance
ARA	Arachidonic acid
BCF	Beat cross frequency
Bici	Bicine
BSA	Bovine Serum Albumin
CART	Classification and regression
CASA	Computed assisted sperm analysis
CD	Commercial diet
Ct	Cycle threshold
Ctrl	Control
CWL	Central wavelength
D	Dark
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpf	Days post fertilization
e.g.	*exempli gratia*
EPA	Eicosapentaenoic acid
EY	Egg yolk
FSC	Forward-scattered light
g	Gram
GFP	Green fluorescent protein
Gly	Glycine
h	Hour
HBSS	Hank´s balanced salt solution
hpf	Hours post fertilization
Hz	Hertz
Ins	Insulin
ISAS	Integrated System for Semen Analysis
Kg	Kilogram
L	Light
l	Liter
LIN	Linearity

LL	Lower left
LN	Liquid nitrogen
LR	Lower right
M	Meter
M	Molar
Min	Minute
ml	Millilitre
Mm	Millimeter
mOsm	Miliosmolar
MS-222	Tricaine methanesulfonate
OF	Ovarian fluid
PBS	Phosphate buffer solution
PC	Phosphatidylcholine
PCA	Principal component analysis
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PI	Propidium iodide
PM	Progressive motility
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
PUR	Purified diet;
qPCR	Quantitative real-time PCR
RNA	Ribonucleic acid
Runx	Runt-related transcription factor
S	Second
S	Siemens
SD	Standard deviation
SNK	Student–Newman–Keuls
SSC	Side-scattered light
STR	Straightness
Tg	Transgenic
TM	Total motility
UF	Ultrafreezer
UL	Upper left
UR	Upper right
VAP	Velocity according to smoothed path
VCL	Curvilinear velocity
VSL	Straight-line velocity
WOB	Curvilinear path wobble

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PREAMBLE

This thesis is organized into eight main chapters where chapter 4 is divided into two sub-chapters. All chapters and sub-chapters, except chapter 1 and 7, are organized according to the format of scientific articles and at the beginning of each chapter, a preamble will describe the main objectives of the work that it precedes, to improve the comprehension of this dissertation. **Chapter 1** is a general introduction to fish reproduction, spermatology and cryopreservation. **Chapter 2** focus on the optimization of zebrafish sperm motility activation conditions to improve the standardization of sperm motility analysis. **Chapter 3** addresses the effect of breeders diet on reproduction, gametes quality and cryopreservation. In **chapter 4** a study on the effect of age and sperm collection frequency on sperm quality is performed, to select high quality male breeders for cryopreservation through repeated non-invasive samplings. **Chapter 5** approaches a teleosts novel method for sperm cryopreservation and storage in an ultrafreezer, which is an advantageous alternative to liquid nitrogen. In addition to this cryopreservation method, a study was conducted on the optimization of the extender through the modulation of cryoprotectant concentration and the use of protein-based additives. **Chapter 6** focus on the application of sperm quality analysis and cryopreservation on a diabetes type I zebrafish model, to apply the know-how gathered during this thesis on a relevant biomedical research model. Finally, in **chapter 7** the discussion of the results and final conclusions of this dissertation are organized in an integrative way, in order to propose guidelines for the standardization of zebrafish sperm quality analysis and cryopreservation procedures.

The author conceived, designed and performed the experiments and analytical procedures, and wrote all the manuscripts included in this dissertation. Throughout this thesis, the experiments were supported and enriched through collaborations with students and colleagues.

CHAPTER 1. GENERAL INTRODUCTION

1.1. Zebrafish model in research

1.1.1. Zebrafish (*Danio rerio*) domestication

Zebrafish is a member of teleost infraclasses, a monophyletic group that is estimated to have diverged approximately 340 million years ago from a common ancestor (Amores et al., 2011). This ancestor underwent an additional round of whole genome duplication denominated teleost-specific genome duplication (Meyer and Schartl, 1999). Zebrafish was first described by Hamilton (1822) as a small freshwater species found naturally in rivers, small streams, channels and paddy fields from Myanmar, Pakistan, India, Bangladesh and Nepal (Arunachalam et al., 2013). Zebrafish is a species tolerant to a wide range of environmental conditions and food resources. Its natural habitat has a wide range of temperatures from 12.3-28.4 ˚C, pH 6.2-9.8 (Arunachalam et al., 2013) and water conductivity 10-280 µS/cm (Engeszer et al., 2007). This species feeds naturally on allochthonous materials such as ants and other insects falling into streams, secondary channels and pools (Arunachalam et al., 2013).

Zebrafish colonies in research facilities are maintained in recirculation systems with controlled environmental parameters such as photoperiod, water temperature, pH and conductivity. Although this species is established in research centers worldwide, the rearing procedures still lack methodological standardization (Lawrence, 2016). As a consequence, high variability is observed on biological and reproductive performances (Lawrence, 2016; Robles et al., 2009; Torres and Tiersch, 2018; Torres et al., 2017; Tsang et al., 2017). Due to its high tolerance and robustness, different protocols and methodologies have been employed between facilities, according to each laboratory specific needs, influenced by personal biases and traditions of specific laboratories (Tsang et al., 2017).

The domestication of wild species requires the ability to control the organism´s husbandry and the comprehension of its specific nutritional and reproductive requirements (Duarte et al., 2007; T. Gjedrem, 2005). This control enables the closure of the species life cycle under captivity. Aquacultured species have high commercial value, therefore requiring high efficiency of fish production to improve the profit (Duarte et al., 2007). Since most aquaculture species breed in

specific times of the year and are much more vulnerable when compared to zebrafish, the efforts in the optimization and standardization of procedures by the scientific community were very efficient (Martínez-Páramo et al., 2017). There are several zebrafish strains considered domesticated such as AB, TU, SJA or TM1, that reveal genomic variations in relation to wild populations, which are typically observed in domesticated species (Whiteley et al., 2011). Therefore, the zebrafish scientific community would benefit greatly by investing in the optimization and standardization of procedures for this species to obtain higher biological efficiency and scientific replicability.

1.1.2. History of *Danio rerio* model species

Zebrafish (*Danio rerio*) have become a widely used model species established in research centres worldwide to study vertebrate mechanisms on areas such as development, regeneration, toxicology and pathologies (Driever et al., 1996; Gemberling et al., 2013; Haffter et al., 1996; Meyers, 2018; Patton and Tobin, 2019; Sieber et al., 2019; Tanguay, 2018). Zebrafish is a robust small bodied vertebrate, cheap to maintain, with high fecundity and year-round reproduction, external fertilization, rapid development, transparency of embryos and ease of experimental manipulation (Lieschke and Currie, 2007; Ribas and Piferrer, 2013). In addition to all these advantageous characteristics, it is a species with the genome fully sequenced and there are feasible genome editing technologies established for this species (Howe et al., 2013; Liu et al., 2017). Altogether, these characteristics make zebrafish a particularly useful model species.

During the 1970's at the University of Oregon (USA), George Streissinger chose for the first time zebrafish as a vertebrate model organism, since it is easy to genetically manipulate and have several advantages over mouse such as a shorter life cycle (Varga, 2018). During the 1980's zebrafish was first characterized as a genetically tractable organism. In the 1990's two large scale screenings for mutants were carried out, one by the Nobel prize winner Christiane Nüsslein-Volhard in Tübingen (Germany) and the other by Wolfgang Driever and Mark Fishman in Boston (USA). Thousands of zebrafish families were investigated to detect

mutations affecting early development. In 1996, around 4000 mutants were identified (Driever et al., 1996; Haffter et al., 1996; Meyers, 2018). Those mutations, when driven to homozygoty, can produce defects and generate pathologies similar to human diseases (Howe et al., 2013). The identification of mutants is a highly relevant strategy for biological research because they can provide the first insight into the role of a gene during normal development. Moreover, it was possible to observe the effect of the mutations, but at that time it was not easy to attribute the mutation to a given gene or to know which gene was affected (Driever et al., 1996; Haffter et al., 1996). Therefore, the scientific community joined efforts to start the zebrafish genome sequencing project in 2001 at the Wellcome Trust Sanger Institute in Cambridge (UK) (Meyers, 2018). The earliest assembly became public in 2002 and the zebrafish genome sequencing is now complete (Howe et al., 2013). The zebrafish genome sequencing was performed according to two strategies: 1) whole genome shotgun (WGS) assembly (Mullikin and Ning, 2003), with subsequent automated annotation in EnsEMBL (Clamp et al., 2003; Hubbard et al., 2002); 2) classical clone mapping and clone-by-clone sequencing with subsequent manual annotation (Potter et al., 2004), which is displayed by the Vega Web browser. The sequencing was also obtained by a hierarchical mapping and clone-by-clone sequencing (Lander et al., 2001; Waterston et al., 2002), therefore validating the sequencing with the highest genome sequence quality (Jekosch, 2004). In 2013, Howe et al. (2013) evidenced more than 26 000 protein-coding genes annotated in zebrafish, where approximately 74% are orthologues to human genes (Howe et al., 2013; Vilella et al., 2009), therefore increasing the validation of zebrafish as a useful model.

The methodological tools to generate zebrafish lines are continuously expanding. There are two main methods namely forward and reverse genetics. Forward genetics are the tools that use mutagenesis screenings either from the random induction of mutations in the genome and subsequent screening for individuals displaying mutant phenotypes, through the induction of chemical mutagenesis using N-ethyl-N-nitrosourea (ENU) treatment in spermatogonia or through insertional mutagenesis (e.g. transposons, retroviruses). After zebrafish genome sequencing, reverse genetics techniques have been developed successfully. The reverse genetics methods use specific knock-out or knock-down of genes of

interest, allowing a rapid understanding of the functions of the predicted/known genes. The main reverse genetics techniques employed are morpholino knock-down, TILLING (targeting induced local lesions in genomes), transgenesis gene-editing technology [zinc-finger nucleases (ZFNs), CRISPR/Cas9 system and transcription activator-like effector nucleases (TALENs)] (Dahm and Geisler, 2006; Housden et al., 2016).

The research performed with zebrafish contributes to the understanding of basic vertebrate biology and development as well as to the understanding of factors controlling the specification of cell types, organ systems and body axis (Lleras Forero et al., 2018; Resende et al., 2014; Talbot et al., 1995). As a consequence, zebrafish is now considered a valuable model organism to investigate human diseases (Lieschke and Currie, 2007) and aging (Gerhard, 2003a; Gerhard, 2003b). Additionally, zebrafish is relevant in research areas such as aquatic toxicology (Hill et al., 2005; Laizé et al., 2014), drug screenings (Vaz et al., 2018), regenerative medicine (Azevedo et al., 2012; Cardeira et al., 2016; Goessling and North, 2014), neuroscience and behaviour (Oliveira et al., 2016).

The practical applicability of zebrafish as a model is more evidently demonstrated through the use of established transgenic and mutant lines that model prominent human diseases such as diabetes (Pisharath et al., 2007; Zang et al., 2017), osteoporosis (Barrett et al., 2006) or cancer (Fior et al., 2017). A transgenic zebrafish model has additional information on its genome due to the artificial insertion of DNA. Through this method, the inserted DNA can produce an overexpression of a gene or it can be associated with a fluorochrome, thus allowing the visualization of cells and organs where the gene is being expressed (Lieschke and Currie, 2007). With the establishment of these lines, it is possible to investigate the disease onset, development and progression, followed by pharmacological methodologies for its control, treatment and cure. A model of chemo-genetically inducible ablation was developed in zebrafish, to address developmental and regeneration processes, involving the targeted expression of a bacterial nitroreductase (NTR) under a cell-specific promoter, when exposed to a prodrug (e.g. metronidazole). The prodrug becomes reduced by NTR into cytotoxic products leading to the ablation of targeted cell types (Bergemann et al., 2018). An example

of this method is the use of zebrafish to investigate type I diabetes, a prominent human pathology. For this purpose, there are NTR zebrafish transgenic lines to model this disease such as Tg(*ins:nfsb*:mCherry) and Tg(*ins:NTR**-P2A:mCherry) (Bergemann et al., 2018; Pisharath et al., 2007). There are transparent mutant zebrafish lines such as *transparent* (*tra*) and *casper* (*mitfa*$^{w2/w2}$;*roy*$^{a9/a9}$) that facilitate developmental and cell transplantation studies through direct observation (D'Agati et al., 2017; Krauss et al., 2013; Presslauer et al., 2016; White et al., 2008). There are numerous zebrafish reporter lines suitable for *in vivo* observation such as the marker for cell differentiation *runt-related transcription factor 2* [Tg(*runx2:*eGFP*)*] (Cardeira et al., 2016; Pinto et al., 2005), *vasa* transgene [Tg(*vasa:*EGFP)] with cellular germline labelling (Krøvel and Olsen, 2002) and *osterix* line (signalling *sp7* gene) [Tg(*Ola.sp7*:mcherry)] marking early osteoblasts (Tarasco et al., 2017).

The exponential generation of newly established zebrafish lines for research brought space and management constraints for the maintenance of all these valuable resources. The cryopreservation of germ cells, particularly spermatozoa, is a valuable methodology for the adequate preservation of these valuable genotypes. Germ cells are responsible for the transmission of genetic information from one generation to another. Mature germ cells, such as mature spermatozoa, are uniquely specialized to overcome the challenges associated with the fertilization process and to provide a single chromosomal complement to offspring (Murphy et al., 2014). Despite the efforts of the scientific community, no cryopreservation protocol for teleosts oocytes and embryos have been successfully developed. Therefore, the cryopreservation methodologies are targeting primordial germ cells (PGCs) and spermatozoa. Male germ cells include spermatozoa and their previous stages of maturation such as primordial germ cells (PGCs) and spermatogonia. Several cryopreservation protocols are developed for testis, PGCs (Marinović et al., 2018a; Marinović et al., 2018b) or spermatozoa (Draper and Moens, 2009; Matthews et al., 2018; Yang et al., 2007). Spermatozoa have small size and have a relatively high resistance to chilling, therefore being more advantageous when compared to other cell types. For this reason, the cryopreservation of spermatozoa is the most widely used technique in aquatic species (Martínez-Páramo et al., 2017). Spermatozoa are the most practical cells to be routinely cryopreserved in zebrafish facilities to

reconstitute their different lines. The main advantage of sperm over PGCs cryopreservation is the fact that sperm collection can be performed through a non-lethal method (Jing et al., 2009a) and does not require laborious cell transplantation methodologies.

1.2. Fish reproduction principles

Sexual reproduction involves the union of two separate gametes with parental genome remixture, allowing thus the diversification of genetic variability of the species and accelerating evolution (Lehmann, 2018). Sexual reproduction occurs through the process of fertilization, which is the union of gametes produced by the two sexes (Lehmann, 2018). Gametes are haploid cells; one sex produces a lower number of gametes -oocytes- that carry nutritional resources for the embryo to develop; the other sex produces substantially smaller gametes in higher numbers –spermatozoa- which are highly specialized motile cells (Schulz et al., 2010). The union of the gametes is a species-specific phenomenon (Herberg et al., 2018) which produces a zygote that inherits a mixture of the parents genomes that will develop into a new unique organism (Lehmann, 2018).

Sperm was first observed by optic experts Nicolaus Artsuican and Antonie van Leeuwenhoek in 1674 (Netherlands). The term spermatozoa, from the Greek σπέρμα "seed" and ζῷον "living being", was first used by Karls Hernest von Boer (Birkhead and Montgomerie, 2009). The production of spermatozoa occurs through spermatogenesis, which is a developmental process during which a small number of diploid stem cells produce a large number of highly differentiated spermatozoa. Spermatozoa are haploid flagellated cells with a recombined genome (Schulz et al., 2010). During spermatogenesis, the regulation of germ cell development occurs according to extrinsic (hormone and growth) and intrinsic (autonomous systems) factors (Schulz et al., 2010). The spermatogenesis events relevant for mature sperm quality will be described elsewhere in this thesis.

Fish are the most diverse and numerous group of vertebrates, having a wide variety of reproductive strategies, in both marine and freshwater species (Gallego

and Asturiano, 2018). Most of them are external fertilizers, where mature spermatozoa are released into a hostile environment to reach the oocyte and perform fertilization (Cabrita et al., 2014; Cosson et al., 2008a; Gallego and Asturiano, 2018). Under captivity, many teleost species experience reproductive constraints such as mismatching of the releasing of gametes between sexes or incomplete gametogenesis. To overcome these issues several strategies can be performed such as artificial reproduction, induction through environmental factors (such as manipulating water temperature and photoperiod) or hormonal induction (Mañanós et al., 2008). In zebrafish there are methods available for hormonal induction (Pang and Ge, 1999; Tokumoto et al., 2011; Wang et al., 2016), however, they are still poorly investigated. The fertilization success is determined by the husbandry practices applied, breeders quality, the quality of gametes and environmental conditions during fertilization (Cabrita et al., 2011a; Rurangwa et al., 2004).

1.2.1. Factors affecting sperm quality

Broodstock selection has been considered one of the most relevant factors to support adequate domestication of a species (Gjedrem, 2005). By artificial selection, the individuals with beneficial characteristics are chosen to generate offspring with improved genotype, towards a specific objective. One of the main objectives is to obtain a population with high reproductive performance and quality under captivity. As a consequence, "good" males characterized by high reproductive performance and sperm quality are selected according to a Mendelian perspective (Gjedrem, 2005). This selection is performed assuming that high-quality offspring will be generated by the inheritance of the genomic information responsible for the desired phenotype. In the past decade, the research community made efforts to further understand the genomic and non-genomic paternal basis of inheritance during sexual reproduction of teleosts, through the novel technological tools available such as transcriptome and epigenome investigation (Herráez et al., 2017; Labbé et al., 2017).

Reproduction is the second most important metabolic effort in adulthood. If an adult organism finds its health or survival at risk, its metabolism will invest preferably on health and survival instead of reproduction, thus decreasing the

efficiency of gametogenesis (Fox et al., 1997). Therefore, an individual can only be at its highest reproductive potential under optimal welfare conditions. The main factors that support adequate broodstock reproduction and gametogenesis process, are environmental conditions and husbandry practices (Cabrita et al., 2011a; Migaud et al., 2013). The main husbandry conditions and practices affecting gametes quality have been extensively reviewed (Alavi and Kazemi, 2006; Cabrita et al., 2009; Cabrita et al., 2011a; Migaud et al., 2013; Rurangwa et al., 2004) and consist of: 1) photoperiod and temperature, 2) nutrition, 3) water and food contamination, 4) stress, 5) diseases 6) broodstock biological characteristics (heritage, age, length, weight, behavior and hierarchical status), 7) type and duration of spermatogenesis, 8) spawning season, 9) methods and sperm collection frequency, 10) methods of spawning induction, and, 11) duration of broodstock participation in the spawning program (Figure 1.1 A). Given that high-quality sperm is related to high-quality breeders, the selection of male donors is essential for broodstock management and cryopreservation programs.

In zebrafish, the optimal temperature and photoperiod are standardized among rearing facilities. Zebrafish is a very useful model for toxicology due to its high reproductive susceptibility to contaminants (He et al., 2014; Kollár et al., 2018; Tanguay, 2018). With the exception of toxicological studies, zebrafish is reared under controlled and uncontaminated conditions. The presence of diseases and their impacts on zebrafish health are reviewed in Kent et al., (Kent et al., 2012). The type and duration of spermiogenesis in zebrafish is discussed in the following sections of this thesis.

The duration of broodstock participation in the spawning program is highly relevant. Although some studies were performed in zebrafish senescence (Gerhard, 2003a; Gerhard and Cheng, 2002), a deeper understanding of the impact of intensive reproduction programs in zebrafish senescence is necessary. Due to the high relevance for the present thesis context, we will address in further detail three main factors affecting sperm quality namely broodstock nutrition, sperm collection frequency and broodstock biological characteristics. On the biological characteristics of broodstock and its relation to sperm quality, special attention will

be given to male aging, social behavior and heritage in terms of genetic and epigenetic inheritance, and maternal contribution.

Nutrition is an essential factor to optimize broodstock fitness, gamete quality and reproductive ability. Therefore, the optimization of fish nutrition can improve fertilization rates and larval development (Izquierdo et al., 2001). The maintenance of fish body homeostasis is determined by the interaction of nutrition, metabolism, gene expression, and epigenetic changes. Altogether, these factors modulate intracellular signaling pathways, thereby producing different physiological responses (Elsamanoudy et al., 2016). Consequently, a possible biological mechanism of nutritional ''imprinting'' able to modulate gene expression and epigenetic patterns that could be inherited by the offspring has been proposed (Elsamanoudy et al., 2016; Lucas, 1998; Rocha et al., 2014; Symonds et al., 2009; Waterland and Jirtle, 2004). Therefore, the understanding of zebrafish nutritional requirements and the development of standardized diets for this species is essential to reduce experimental variability and to obtain high-quality sperm and offspring. In zebrafish, extruded diets with controlled nutritional composition show improved larval quality and growth performance when compared to flaked diets (Siccardi et al., 2009). Early weaning with microdiets significantly improves zebrafish growth and reproductive performance, decreasing the incidence of vertebral anomalies on the offspring when compared to fish fed exclusively with *Artemia* nauplii (Martins et al., 2018). Moreover, the nutritional composition in fatty acids (such as docosapentaenoic acid (DHA) and eicosapentaenoic acid (EPA)) are known to improve health and reproduction in teleosts such as European sea bass and rainbow trout (Sorbera et al., 2001; Asturiano et al., 2001). Although freshwater and marine species are known to have different fatty acids requirements since marine species are not able to synthesize *de novo* phospholipids (Tocher et al., 2008), the dietary supply of fatty acids is beneficial in both cases, because they incorporate into the plasma membranes. This is especially important in spermatozoa membranes where PC and PE count for 50% and 40%, respectively, of total phospholipid content (Martínez-Páramo et al., 2012a). These findings suggest a relevant role of zebrafish broodstock nutrition on gametes quality and offspring health. Although the nutritional requirements of zebrafish are poorly understood, studies point to the fact that this species, as other teleost species, can improve its reproductive

performance through dietary supplementation with fatty acids (Meinelt et al., 1999; Nowosad et al., 2017).

In teleosts it is commonly accepted that inappropriate sperm collection frequency affects sperm quality, however, few studies investigated this subject (Büyükhatipoglu and Holtz, 1984; Hochman et al., 1974; Suquet et al., 1992a). Considering that each species has different types and duration of spermatogenesis cycles, as well as different stress susceptibilities, the adequate sperm collection frequency is species specific and should be optimized. The premature collection of spermatocytes or spermatids, as well as aging spermatozoa, results in low-quality samples. In fact, a negative effect of stripping frequency has been reported on the duration and intensity of sperm movement in rainbow trout (Büyükhatipoglu and Holtz, 1984), but not in turbot or Senegalese sole (Beirão et al., 2015a; Suquet et al., 1992b), where sperm could be collected monthly or fortnightly, respectively (Beirão et al., 2019). Therefore, it is important to respect spermatogenic cycles for each species to obtain high-quality samples, thus avoiding the biases associated with inappropriate sperm collection frequency.

Male aging affects sperm production and quality (Amaral et al., 2008; Ramalho-Santos, 2009) being associated with decreased sperm volume, motility and proportion of morphologically normal spermatozoa (Kidd et al., 2017)). Sperm quality is expected to decline with age due to the accumulation of *de novo* mutations in the germline cells (Kidd et al., 2017). If there are deleterious mutations in sperm mediated by paternal aging, lower sperm quality and fertilization success are expected. Therefore in a competition context young males are predicted to be favored over old males (Pizzari et al., 2008). The accumulation of mutations in germline cells is more problematic in male germ cells since they undergo a higher number of divisions to generate a spermatozoon when compared to the egg (Baker and Aitken, 2005; Herráez et al., 2017). Therefore, male age has a preponderant contribution to germ line mutations (Crow, 2000). Although spermatozoa produce reactive oxygen species (ROS), excessive exposure can lead to sperm genetic integrity damage, therefore reducing the fertilization ability (Baker and Aitken, 2005; Wang et al., 2015) and contributing to genomic alterations that can impact negatively the offspring. Spermatozoa DNA damage is mainly oxidative and is

associated with negative outcomes such as impaired conception rates, increased abortion incidence and offspring defects (Herráez et al., 2017). The detrimental effects of paternal genotoxicity in the offspring are caused by aberrant repair of oxidative DNA damage in the newly fertilized zygote (Baker and Aitken, 2005; Fernández-Díez et al., 2015; Fernández-Díez et al., 2018; González-Rojo et al., 2018).

In the past, spermatozoa were considered mainly carriers of the genomic information necessary to form the zygote, therefore husbandry methodological improvements were focused on oocyte quality improvement. It is now accepted that spermatozoa have a deeper role in embryogenesis (Herráez et al., 2017). Beyond the genomic information provided by spermatozoa to the zygote, there are non-genomic processes that will take part in embryo development. Some of the most relevant spermatogenesis events with a specific role on embryo development are: 1) chromatin processing, 2) chromatin packaging, 3) reorganization of the contacts between DNA and nuclear matrix, 4) remodeling of the epigenetic pattern, 5) cessation of transcription and 6) presence of a set of remaining RNAs in the cytoplasm of mature sperm (Herráez et al., 2017). Zebrafish genes are packaged in blocks of structurally diversified chromatin and their cytoplasmatic RNA profile correlate with reproductive success (Wu et al., 2011). Additionally, spermatozoa epigenetic marks limit the timing of gene expression, as also observed in mammalian sperm (Wu et al., 2011). It is still to be understood if there is parental epigenetic imprinting in teleosts (Labbé et al., 2017), although there are zebrafish studies pointing in that direction (Martin and McGowan, 1995).

The maternal genes present in spermatozoa (mtDNA) can influence sperm quality and therefore fertilization success (Cabrita et al., 2011a; Evans and Simmons, 2007; Zeh and Zeh, 2005). Mitochondria regulate sperm motility since they control oxygen consumption, which is necessary for spermatozoa normal metabolism (Amaral et al., 2008; Ramalho-Santos, 2009). Although these studies are scarce in teleosts, in *Gallus gallus domesticus*, the selection of "good" and "bad" breeders, according to the sperm motility profile (regulated by mtDNA in this species), showed divergent mitochondria function (Froman and Kirby, 2005).

Social behavior dynamics and the hierarchical structure of a population is known to impact reproductive features in many species (Fox et al., 1997; Parker and

Pizzari, 2010). Personality traits, such as boldness and aggressiveness, are often related to reproductive success and offspring survival (Vargas et al., 2018). Dominant individuals generally have bolder behavior and the dominance is established to control critical resources such as food, shelter and reproduction (Sloman and Armstrong, 2002). In several species, including zebrafish, proactive individuals with bold behavior show greater reproductive success and growth rates than reactive individuals (Ariyomo and Watt, 2012; Larson et al., 2006; Paull et al., 2010; Vargas et al., 2018).

Zebrafish are social animals that form shoals and dominance hierarchies in both sexes (Paull et al., 2010). Interestingly, zebrafish males change sperm quality investment according to their social status and breeding population density (Larson et al., 2006; Spence et al., 2006). Moreover, there are evidences of sperm competition in this species (Zajitschek et al., 2014), in the form of a postcopulatory selection that occurs when females breed with multiple males in a single reproductive episode (Parker, 1970). Mating with some degree of sexual selection and sperm competition can influence the metabolic investment in sperm production and quality (Parker and Pizzari, 2010).

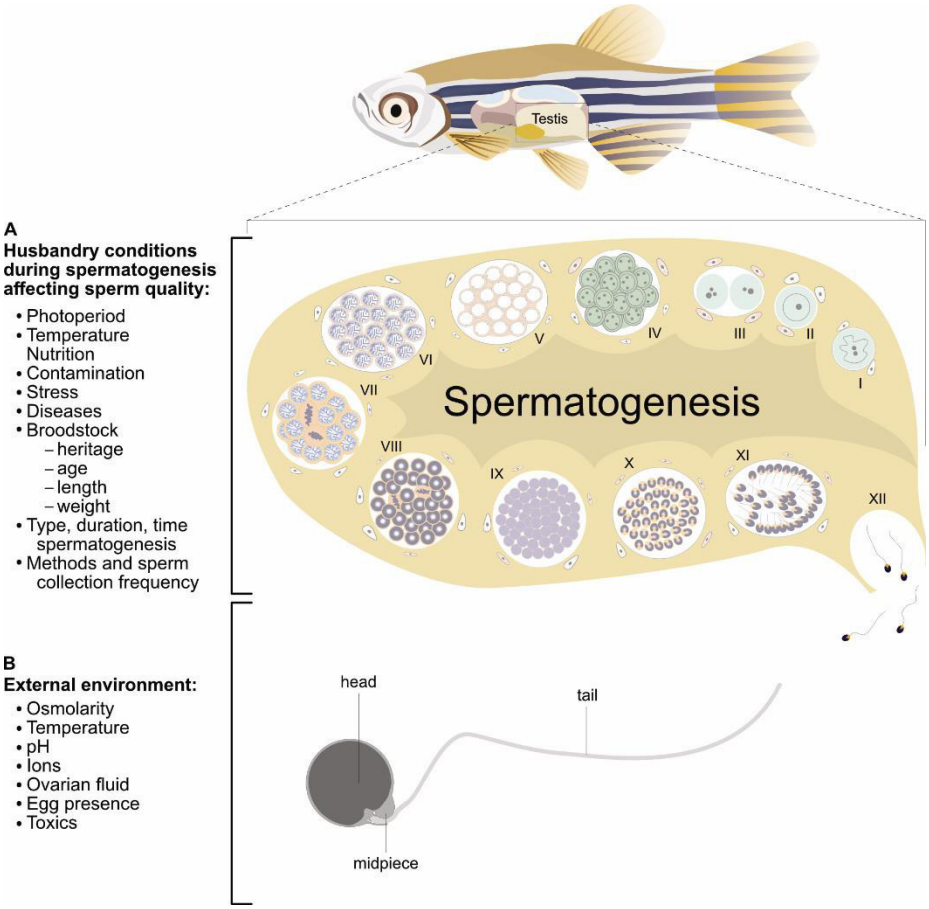

Figure 1.1 Factors affecting zebrafish sperm quality. Schematic representation the two main factors affecting sperm quality: A) the husbandry conditions affecting sperm quality during spermatogenesis and B) external environment conditions into which spermatozoa are released and schematic representation of zebrafish spermatozoon. The zebrafish testis with cystic spermatogenesis is represented according to the description of (Schulz et al., 2010) namely: (I) Type A undifferentiated spermatogonia germ cell (II) type A undifferentiated spermatogonia, (III) type A differentiated spermatogonia, (IV) spermatogonia type B, (V) leptotenic/zygotenic primary spermatocytes, (VI) pachytenic primary spermatocytes, (VII) diplotenic spermatocytes/metaphase I, (VIII) secondary spermatocytes/metaphase II, (IX) early spermatid, (X) intermediate spermatid, (XI) final spermatids and (XII) spermatozoa.

1.2.2. Sperm motility activation and metabolism

Spermatozoa from teleost fish are flagellated single cells adapted to external fertilization, that undergo a period of spermatogenesis. In the testis, sperm is in a safe environment surrounded by seminal plasma, Sertoli and Leydig cells that nourish them (Figure 1.1 I-IV). At this point, spermatozoa are under physical-chemical conditions similar to the body environment (eg. osmolarity 300

mOsm/Kg) (Billard, 1986; Schulz and Miura, 2002; Schulz et al., 2010). In these conditions, spermatozoa are immotile in the testis and only acquire motility when in contact with the hazardous external medium (Cabrita et al., 2014; Cosson et al., 2008b; Gallego and Asturiano, 2018).

The triggering of sperm motility occurs through an osmotic differential. In freshwater species, sperm motility is activated when in contact with the hypotonic environmental medium. Kraznai et al., (2000) proposed for carp (*Cyprinus carpio*) a sperm cell signaling cascade that promotes sperm motility activation. He suggested that the hypoosmotic shock causes the opening of K^+ channels due to the low ionic concentration of the environment, promoting an influx of K^+ from the cell that hyperpolarizes the plasma membrane. This mechanism would be followed by plasma membrane depolarization, and as a consequence, an influx of Ca^{2+} ions into the cell promotes flagellar beating. Once the movement starts, teleost spermatozoa have only a few seconds or minutes (depending on the species) to reach the oocytes and penetrate the micropyle before it closes up or spermatozoa ceases its movement (exhausting their reduced ATP reserves) (Rurangwa et al., 2004). Immediately after motility activation, spermatozoa from teleost species reveal the highest ATP content and motility efficiency, declining progressively through their lifespan. Interestingly, the expenditure of ATP and sperm motility parameters can be modulated by the characteristics of the motility activation medium (Cosson et al., 2008a). The environmental factors affecting sperm motility are water temperature, osmolarity, pH and specific ions on the medium. The biological factors affecting sperm motility are the presence of ovarian fluid and chemoattractant properties of the eggs (particularly on the micropyle) (Figure 1.1 B) (Beirão et al., 2015b, 2019; Butts et al., 2017; Diogo et al., 2010; Lahnsteiner, 2002).

Water temperature affects the beating frequency of spermatozoa flagella and the physiological response is related to the adaptation of each species to the natural environment conditions (Alavi and Cosson, 2005). In general, higher temperature increases the initial motility and, as a consequence of this energetic expenditure, an earlier motility cessation occurs. On the other hand, lower temperature generally results in a prolongation of spermatozoa lifespan with reduction of velocity and flagellar beating frequency (Dadras et al., 2017).

Water temperature also affects spermatozoa biochemical composition and motility characteristics (Ishijima, 2012) since it influences ions availability in cellular compartments, enzyme activity and metabolic pathways (Dadras et al., 2017) as well as dynein motors of the flagellum (Cosson et al., 2008a,b).

Water pH is considered to have a low impact on sperm motility (Cosson, 2004). However, in rainbow trout (*Oncorhynchus mykiss*) (Gatti et al., 1990), Senegalense sole (*Solea senegalensis*) (Diogo, 2011), and burbot (*Lota lota*) (Bokor et al., 2018) the external pH has an effect on motility characteristics. In the fertilization microenvironment, sperm and oocytes are released into the environment along with ovarian fluid. The presence of ovarian fluid in the solution is known to improve sperm motility parameters in several species such as longevity (Dietrich et al., 2007; Lahnsteiner et al., 1995; Wojtczak et al., 2007), speed, trajectory and motility pattern (Beirão et al., 2015b; Butts et al., 2017; Diogo et al., 2010). The mechanism through which ovarian fluid may affect sperm motility is still unknown, although several factors have been proposed as beneficial, such as favorable osmotic environment (Ingermann et al., 2008) or the presence of nutrients, hormones and metabolites that spermatozoa are able to metabolize (Lahnsteiner et al., 1996). Moreover, the egg micropyle contains chemoattractants that guide spermatozoa through chemotaxis. Chemotaxis is the process of modulation of the direction of motile cells movement in response to a gradient stimulus. This motility alteration results in the approach to the chemoattractant or retreat from a repellent. Teleosts sperm chemotaxis is still poorly studied, nevertheless, these facts imply an important female role during the fertilization process and in the modulation of sperm motility (Yanagimachi et al., 1992; 2017).

Spermatozoa from most teleost species have few mitochondria as well as ATP reserves stored prior to the onset of motility (Christen et al., 1987; Lahnsteiner et al., 1993; Perchec et al., 1995). In addition, in these species, the period of motility is very short, around 60 s (Cabrita et al., 2014; Cosson et al., 2008b; Lahnsteiner et al., 1999). In fact, motility duration is not only related to the low ATP content and low capacity to *de novo* generate ATP, but also to the deleterious effect of the hypoosmotic shock on sperm structure and function (Christen et al., 1987).

Despite the fact that zebrafish is extensively used for research purposes, the sperm motility activation mechanism and metabolism is still poorly understood. In zebrafish, the osmolarity for complete motility inhibition (≥ 300 mOsm/Kg) is similar to blood plasma (315 mOsm/Kg) as in most teleosts (Jing et al., 2009a). Tsakai and Morisawa (1995) showed that a decrease in intracellular K^+ is associated to the onset of zebrafish sperm motility, which is in agreement with the model previously described by Kraznai et al. (2000). Ingermann et al. (Ingermann et al., 2011) showed that at the beginning of motility activation, zebrafish sperm relies on stored ATP, but prolonged motility relies on oxidative phosphorylation. The energetic acquisition through oxidative phosphorylation was associated not only with sample total motility (TM), but also to spermatozoa straightness and wobble. Consequently, ATP generation and availability affects not only the number of motile cells but also their trajectory patterns, therefore suggesting that inadequate ATP delivery to flagellar dynein ATPase result in changes in motility characteristics (Ingermann et al., 2011) as observed in other cyprinids (Alavi and Cosson, 2006).

Osmolarity is one of the major factors contributing to sperm motility activation (Morisawa et al., 1983). In addition, the osmolarity of the activation medium influences the number of waves and curvatures of the flagellum (Alavi et al., 2009; Cosson et al., 2008b). In zebrafish, ion-free solutions containing only sugars show the same motility triggering pattern as HBSS, suggesting that osmolarity is the most important factor controlling this species motility initiation (Jing et al., 2009a). Nevertheless, it is possible that after the initial osmolarity stimulus, K^+ efflux may contribute to the process of sperm motility (Takai and Morisawa, 1995).

Environmental conditions have profound impacts on sperm motility initiation and metabolism, which means that the variability of procedures between zebrafish facilities becomes an important source of biases both in quality analysis and in *in vitro* fertilization success. Therefore, it is clear that optimal and standardized sperm motility activation conditions should be established.

1.2.3. Sperm quality evaluation

Sperm quality is defined as the spermatozoa ability to successfully fertilize the oocyte and subsequently progressing to embryo development (Migaud et al., 2013). In the reproductive context, sperm quality evaluation is important to evaluate male status, to perform sperm sample selection for assisted reproduction purposes and for broodstock selection. Most of the sperm quality analyses used are related to the sperm ability to reach the oocyte instead of the analysis of normal embryo development (Herráez et al., 2017). What makes sperm quality particularly useful for the previously mentioned purposes is not only its correlation with fertility, but more importantly, the reason behind that correlation.

Spermatogenesis is a highly regulated process where the final spermatozoa depend on the conditions to which cells were exposed during spermatogenesis, such as nutrition, stress or toxic exposure (Figure 1.2). Spermatogenesis specific genes may function during the mitotic and spermatogenic phases in the adult fish (Schulz et al., 2010). The configuration of zebrafish spermatozoa is similar to aquasperm of external fertilizing teleosts (Mattei, 1991), however, it shows similarities to the spermatozoa of other cyprinids (with some inter-specific differences between them) (Zhang et al., 2014). Zebrafish spermatozoa have an asymmetric shape due to the lateral location of the nuclear fossa, and because of this feature the head is lopsided and the flagellum inserted eccentrically (Figure 1.2 B, Figure 1.2 A and B). A sperm sample is a heterogeneous mixture of cells originated from different spermatogonia, where each spermatogonium produces haploid cells with different genotype and characteristics (Cabrita et al., 2011a). Additionally, fish spermatozoa display highly variable chromatin organization, which is responsible for the protection of the DNA they carry.

Sperm quality analysis can be performed on its constituents, namely seminal plasma and spermatozoa. Seminal plasma can be evaluated according to its enzymes, metabolites, sugars, vitamins, amino acids, lipids, fatty acids, glucose, lactate and other inorganic compounds (Cabrita et al., 2011a). Spermatozoa status can be evaluated by several parameters such as spermatozoa morphology, plasma membrane viability and resistance (functionality, composition and resistance to osmotic shock) (Figure 1.2 F), mitochondria viability and functionality, spermatozoa

motility (Figure 1.2 C and D), spermatozoa metabolism (ATP consumption), DNA integrity (Figure 1.2 E) and fertilization ability (Figure 1.2 G and H) (Cabrita et al., 2009). Additionally, other useful sperm quality markers have emerged in the past decade such as quantification of reactive oxygen species (ROS), total antioxidant status, lipid peroxidation, mitochondrial dysfunction and protein oxidation, transcript markers of sperm quality, proteome analysis (Cabrita et al., 2014; Dietrich et al., 2019; Figueroa et al., 2017; Martínez-Páramo et al., 2017) and epigenetic markers (Labbé et al., 2017). Moreover, the evaluation of fertility and progeny quality are highly relevant (Figure 1.2 I and J), since it demonstrates not only spermatozoa fertilizing ability but also its capacity to generate healthy offspring. However, fertility and progeny analysis are evaluated less frequently than the previously mentioned sperm quality predictors. In fact, the importance of paternal contribution in the progeny development is one of the most challenging topics in reproduction at the present moment (Herráez et al., 2017; Labbé et al., 2017). Nevertheless, sperm motility is the most used sperm quality predictor. The use of computed assisted sperm analysis (CASA) systems allowed the thorough quantification and qualification of spermatozoa motility (Cabrita et al., 2009; Fauvel et al., 2010; Kime et al., 2001). The characterization of sperm quality has become a useful tool not only for sperm sample selection but also for broodstock selection and management, sperm cryopreservation and assisted reproduction techniques (Gallego and Asturiano, 2018). Although many sperm quality methods have been developed, there are no universal biomarkers to characterize a sperm sample quality and therefore, it is recognised that several methods should be employed for an accurate evaluation of spermatozoa status.

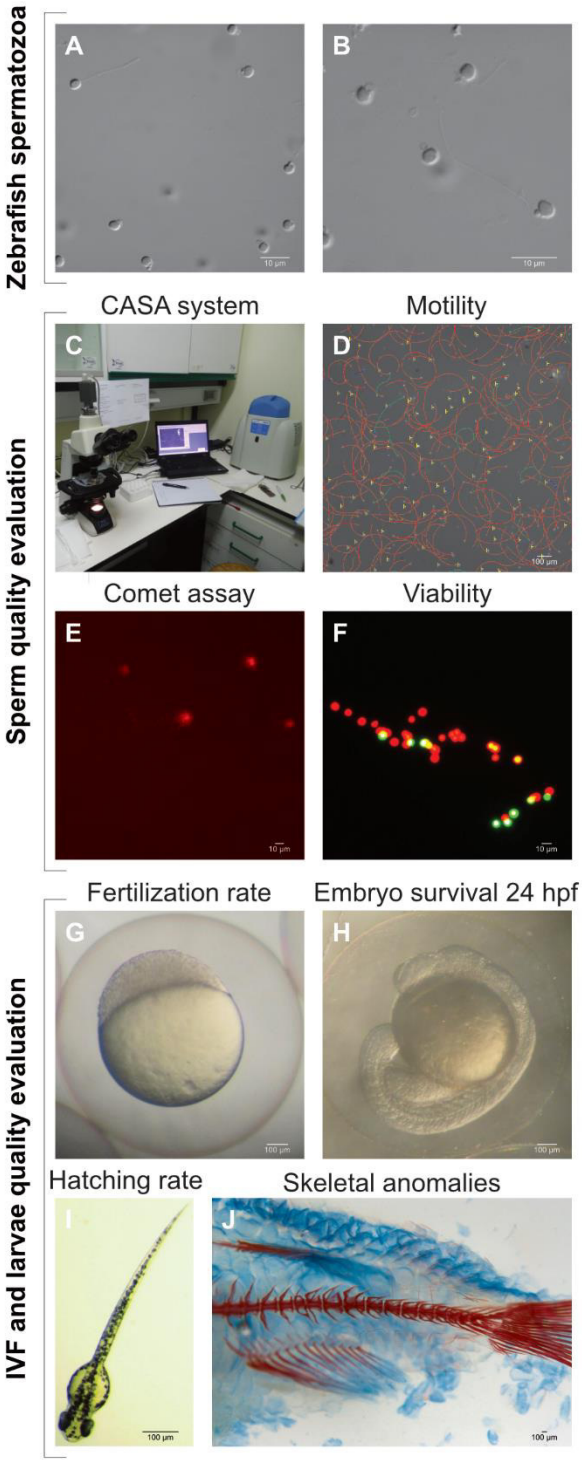

Figure 1.2 Zebrafish sperm quality evaluation. A) zebrafish spermatozoa, B) spermatozoon tail, midpiece and head, C) CASA system and programable biofreezer for cryopreservation, D) sperm motility analysis through CASA system, E) DNA fragmentation analysis through comet assay, F) plasma membrane viability through PI (red cells) and SYBR green (green cells) labelling, G) fertilization rate analysis at 3 hpf, H) embryo survival analysis 48 hpf, I) Hatching rate evaluation at 78 hpf and I) skeletal malformation analysis of the offspring though alcian blue and alizarin S staining.

1.3. Fundamentals of sperm cryopreservation

Cryopreservation is the use of very low temperatures to preserve structurally intact living cells and tissues (Pegg, 2007). Most cryopreservation applications are related to the ability to stop the biological clock at very low temperatures (Benson et al., 2013; Mazur, 1984). However, the cell must be able to survive the extreme environmental shifts that cryopreservation produces (Mazur, 1984). Cryobiology is a multidisciplinary science that studies the biological and physical behavior of cells and tissues at low temperatures (Gao and Critser, 2000). Cabrita et al., (2010) summarized the benefits of cryopreservation in teleost species as follows: 1) synchronization of gametes availability of both sexes, 2) sperm economy, 3) simplification of broodstock management, 4) simpler transport of gametes, 5) simplification of broodstock management and 6) germplasm storage for genetic selection programs or species conservation.

One of the most important theoreticians of cryopreservation was James Lovelock. He suggested that osmotic stress was the main factor contributing to blood cells damage during freezing. Spermatozoa were the first mammalian cells to be successfully cryopreserved (Polge et al., 1949; Walters et al., 2009). The cellular biophysics response during freezing is essential to the development of successful cryopreservation protocols. The cryopreservation methodology is specific for each species cell type, depending on cell properties such as morphometry, density, organelles, compartmentalization of the cell, plasma membrane structure and components (Mazur, 1963; Mazur, 1984). Because of these biophysical characteristics, the water permeability of a type of cell is species specific and therefore the cell requires different cryopreservation methodological improvements, such as different cryoprotectants and cooling/thawing rates (Mazur, 1984; 2008). Gao and Cristser (Gao and Critser, 2000) considered as cryopreservation key points the addition of cryoprotective agents to cells/tissues before cooling, the cooling rate towards the temperature at which the biological material will be stored and the warming of the biological material. Sperm is a particularly challenging cell type to cryopreserve under optimal conditions due to its small size and non-spherical shape (Hagiwara et al., 2009).

As mentioned previously, attempts in cryopreservation of teleosts oocyte and embryo failed (Asturiano et al., 2017). Two types of male germ cells are possible to cryopreserve in zebrafish, specifically the spermatogonia and mature spermatozoa (Harvey et al., 1982a; Marinović et al., 2018a 2018b; Matthews et al., 2018; Wang et al., 2015). The development of new technological methodologies of spermatogonia cryopreservation constitutes a great advance in research (Asturiano et al., 2017). After thawing the cells, they are transplanted onto a surrogate host that will spawn part of the progeny with the desired genotype and, if a triploid surrogate parent can be used all the offspring will carry the desired genotype (Yoshizaki and Lee, 2018; Yoshizaki et al., 2016). This technique is particularly useful for complex double mutant lines such as *casper*. Nowadays these technologies are being successfully developed in zebrafish (Franěk et al., 2019; Marinović et al., 2018a, 2018b). The main disadvantage is that it requires an experienced practitioner, particularly for cell transplantation and therefore is mainly used experimentally. Alternatively, zebrafish sperm cryopreservation is easier and has therefore, higher practical applicability in bioterios worldwide.

In research the reporter zebrafish lines are generally heterozygotic, however mutant lines frequently require homozygoty to express the desired phenotype. The *in vitro* fertilization must be performed with freshly collected oocytes. Consequently, if homozygous or heterozygous females of the same zebrafish line are available in the facility, an *in vitro* fertilization can be performed with cryopreserved sperm to obtain heterozygous and homozygous offspring, according to Mendelian principles of inheritance. In this scenario, freshly collected oocytes from wild-type females can be used to perform *in vitro* fertilization with the cryopreserved sperm from the target zebrafish line. This method allows to obtain heterozygous offspring in a Mendelian proportion.

The ultimate objective of a cryopreservation methodology is to obtain an integrated approach that promotes an adequate balance of the bio-physical events during cryopreservation, protecting successfully the cell throughout the cryopreservation process. Therefore, this methodology should ensure spermatozoa structural and genomic integrity as well as its fertilization competence and offspring health.

1.3.1. Cryoprotectants

The first successful sperm cryopreservation methodology proposed by (Polge et al., 1949) was possible due to the discovery of glycerol cryoprotectant properties. Cryoprotectants are substances that allow the protection of cells and tissues from freezing damage (Elliott et al., 2017). There are several bacteria, fungi, plants, fish, insects and amphibians able to produce natural cryoprotectant compounds such as antifreeze proteins, and sugars (e.g. sucrose, trehalose and raffinose) which can be employed in the extender composition (Elliott et al., 2017). Both compounds allow them to endure very low temperatures. Antifreeze polypeptides bind to small ice crystals and inhibit their growth and recrystallization, avoiding cellular disruption (Elliott et al., 2017; Robles et al., 2019). These natural proteins are used in cryopreservation protocols in several species (Beirão et al., 2012; Martínez-Páramo et al., 2008) and are considered a cryoprotectant class by itself. Additionally, improvements were achieved in post-thaw sperm quality by supplementing the extender composition with sugars such as glucose in salmonids (Judycka et al., 2018) and with amino acids such as taurine in European sea bass (*Dicentrarchus labrax*) (Cabrita et al., 2011b).

Cryoprotectants are classified as permeating or non-permeating according to their ability to permeate the cell membrane. Permeating cryoprotectants are substances with high molecular weight and viscosity that are able to permeate the plasma membrane and substitute intracellular water though osmotic differential (Elliott et al., 2017; Mazur, 1984; Morris et al., 2006). Consequently, the intracellular solute concentration increases (through the cryoprotectant cellular inclusion) avoiding the formation of intracellular ice crystals, which are lethal to the cell (Pegg, 2007). The cryoprotectant concentration must be enough to permeate all cellular compartments. However, most of the compounds used are toxic to the cells in high concentrations (Elliott et al., 2017). Therefore, a compromise must be achieved between cryoprotectant toxicity and effective cellular permeability. A compilation of cryoprotectants named Karows “list of 56” (Karow, 1969), was reduced by Ashwood-Smith (Ashwood-Smith, 1987) to 20 of the most successful cryoprotectants, which was updated by Elliot et al. (Elliott et al., 2017).

Non-permeating cryoprotectants do not permeate the cell plasma membrane (or permeate it at very low rates) and are non-toxic with very few exceptions (Elliott et al., 2017). These compounds are added to the extender medium and improve cell protection on the outer surface of the cell plasma membrane. The mixture of both permeating and non-permeating agents in extenders is often successful (Cabrita et al., 2001, 2011b; Martínez-Páramo et al., 2012a, 2013), however the mode of action of most non-permeating agents is unclear (Elliott et al., 2017). One interesting feature of non-permeating cryoprotectants is the fact that they can allow to decrease the concentration of permeating cryoprotectant in the extender composition, and therefore its toxicity (Elliott et al., 2017). Additionally, their presence can reduce the formation of ice crystals on the cell surroundings, decreasing its susceptibility to extracellular ice formation, cell disruption and cold damage (Cabrita et al., 2001). In optimized human sperm cryopreservation, the presence of egg yolk is able to form an electron dense freeze-concentrated matrix which substituted ice crystals. The matrix which surrounds the frozen spermatozoa showed a normal shape in relation to fresh sperm (Morris et al., 1999). Considering that the sperm of each species has specific cellular and biophysical properties, the cryoprotectants selection concentration and combinations that accommodate the cell requirements are extremely relevant for the success of a cryopreservation protocol. To predict accurately the thermodynamic effects on post-thaw cell quality is one of the most challenging objectives in cryobiology and currently new methods, such as machine learning techniques, are being applied is this area (Cheng et al., 2019).

1.3.2. Cooling and thawing rate

There are two main methods to successfully refrigerate cells and tissues after exposure to cryoprotectants, namely cryopreservation and vitrification. Conventional cryopreservation is considered a slow freezing method, in opposition to vitrification, which is a fast-freezing method. Through the vitrification process, the formation of ice is prevented by producing a glassy state where viscosity reaches such high levels that water behaves like a solid without crystallizing. Vitrification is often performed by plunging the vial directly into liquid nitrogen and requires high concentrations of cryoprotectants. Although it is very useful to avoid ice formation,

the toxic effect of the permeating cryoprotectant is often a handicap in this protocol (Pegg, 2007). Mazur et al. (Mazur, 1984) described thoroughly the biophysics during the cryopreservation process. The temperature decreases until -5°C, where both cells and its surroundings are unfrozen due to supercooling and the depression of the freezing point by the protective extender solutes. At approximately -15°C, ice is formed in the extracellular medium (spontaneously or by seeding/artificial nucleation method). At this point cell contents are supercooled and unfrozen, and the intracellular supercooled water has higher chemical potential than the external medium. Because of this difference in chemical potential, the water flows out of the cell and freezes externally. After this point, all biophysical events depend on the cooling rate. If cells are cooled too fast, they are not able to lose water fast enough to maintain equilibrium, becoming increasingly supercooled and eventually acquiring equilibrium by freezing intracellular space. If the cooling rate is adequate, the cthe ells lose water rapidly enough to concentrate the intracellular solutes sufficiently to eliminate supercooling. Consequently, cells lose their intracellular water through osmotic gradient, and crystallthe ization does not occur inside the cell. If the cells are cooled too slowly, there will be severe cell shrinkage and long-term exposure to high solutes concentration before eutectic temperature is reached (Mazur, 1984). The optimal cooling rate is dependent not only on the cell type but also on the extender composition and volume of the packaging used. Therefore, for each methodological modification, the optimal cooling rate should be investigated and adjusted to improve post-thaw results.

The thawing temperature is the last step of the cryopreservation process and it is specific for each protocol since the extender composition and the cooling rate to which cells were exposed will affect the final outcome. The thawing rate is typically very fast to avoid ice recrystallization. In zebrafish, several thawing temperatures were proposed ranging from 36°C to 40°C in a water bath. However, considering that all methodologies reported are highly different, this information is not useful and for each protocol, an optimization of the thawing temperature should be performed.

1.4. Zebrafish sperm cryopreservation

An effective method to perform zebrafish sperm cryopreservation has been one of the most important and difficult objectives to achieve in the past years by the zebrafish scientific community. Zebrafish sperm cryopreservation methodologies lack standardization, and therefore there is high variability in post-thaw sperm quality and *in vitro* fertilization success. The sources of variability reported in literature are not only related to key factors affecting cryopreservation success, but also to zebrafish management features such as nutrition and dietary protocols, sperm collection method, sperm quality analysis method, *in vitro* fertilization procedures (Martins et al., 2018; Paull et al., 2008; Robles et al., 2009; Torres et al., 2017; Varga et al., 2018).

Since the development of the first zebrafish sperm cryopreservation protocol (Harvey et al., 1982b) more than 30 years ago, there have been many attempts to improve and standardize the methodology (Bai et al., 2013; Diogo et al., 2018; Draper and Moens, 2009; Hagedorn et al., 2009; Hagedorn et al., 2012; Harvey et al., 1982b; Matthews et al., 2018; Morris et al., 2003; Wang et al., 2015; Yang et al., 2007; Yang et al., 2016) (Table 1.1). Several extenders and cryoprotectants have been tested in zebrafish sperm with similar results, but it has been difficult to identify the specific effects of cryoprotectants on cryopreservation success (Robles et al., 2009). When reviewing the literature, it is remarkably difficult to compare studies of zebrafish sperm cryopreservation, since all steps of the methodologies used vary considerably. This fact is more evident in studies where the application of different solutions and methods are used, without studying their individual effect on freezing success. Because of this, the beneficial or detrimental effect of the solution and cryopreservation steps are not evident, due to biases associated with the interactions between them. Therefore, a consistent strategy to develop protocols according to cryobiological principles is necessary.

The first step in sperm cryopreservation protocols is the sperm collection, being an important source of variability among the cryopreservation protocol. There is a non-lethal technique to collect sperm by abdominal massage and a lethal method through testis dissection. Jing et al. (2009a) observed that sperm collection through abdominal massage yielded lower sperm volumes when compared to testis

dissection. Although testis dissection improves one of the major bottlenecks of sperm cryopreservation, which is the low sperm volumes yielded per male, it implies the sacrifice of the fish. Additionally, using this sperm collection method a heterogeneous mixture of cells in different maturation stages will be obtained, therefore declining the overall sperm quality of the sample. To sacrifice highly valuable transgenic and mutant fish is, in many cases, not a viable option.

After sperm collection, the sample is immediately diluted in a solution with composition and osmolarity similar to seminal plasma, thus preventing motility activation and maintaining the normal cell functions. In zebrafish, several solutions were tested, such as Ginsburg Ringer solution and Hank's Balanced Salt Solution (HBSS) (Jing et al., 2009a). Previous studies considered HBSS the most suitable diluent for zebrafish sperm (Jing et al., 2009a). Since the development of the first zebrafish sperm cryopreservation protocol, a variety of extender media and permeating cryoprotectants were tested such as methanol, DMSO dimethyl sulfoxide (DMSO), N,N-dimethylformamide (DMF), dimethylacetamide (DMA) glycerol and ethylene glycol at concentrations ranging 4 to 20%. The use of non-permeating cryoprotectants is potentially a powerful tool if used in a comprehensive form.

One of the most relevant and practical applications of cryopreservation in zebrafish facilities is the germplasm conservation of valuable zebrafish mutant and transgenic strains. There are numerous situations where the facility manager has very few individuals of a valuable strain, as in the case of buying or receiving a few adults to establish a cohort (but requiring the proper safeguard of the germplasm) or in the case of a sudden pathological outbreak.

Since one zebrafish male produces only 0.8 to 3 µl of sperm, the packaging must meet this species sperm volume to be useful in the challenging situations mentioned above. Considering zebrafish average sperm volume per male, the use of French straws (250 or 500 µl) is a high investment since it requires high sperm volume to be used in a single *in vitro* fertilization. The first cryopreservation protocols used capillary tubes (Harvey et al., 1982b; Morris et al., 1999). There were several interesting approaches with French straws (Bai et al., 2013; Hagedorn et al., 2012; Wang et al., 2015; Yang et al., 2016) (Table 1.1) which are useful for large

facilities and central repositories, however they are not useful for small facilities and challenging situations. Nevertheless, several protocols were proposed using cryovials (Diogo et al., 2018; Draper and Moens, 2009; Matthews et al., 2018) (Table 1.1) for smaller sperm volumes and lower number of males. This is a useful packaging method with high applicability to zebrafish facilities. Therefore, the development of a successful cryopreservation protocol must meet both cryobiological principles as well as the practical and objective applications in zebrafish facilities.

The cooling rate is one of the major factors affecting sperm cryopreservation as discussed previously. In zebrafish, both the dry ice method (Draper and Moens, 2009; Harvey et al., 1982b; Morris et al., 2003) and controlled cooling through biofreezers (Table 1.1) have been performed. The cooling rates tested with programable biofreezers ranged from -5 to -35°C/min in one step (Hagedorn et al., 2009; Matthews et al., 2018; Yang et al., 2007) or two-step protocols (Bai et al., 2013; Wang et al., 2015). Interestingly, throughout all the highly variable cooling rates and solutions used, the post-thaw sperm quality seemed similar with average sperm total motility values around 20%.

One of the most relevant bottlenecks in germ cell cryopreservation is the necessity for liquid nitrogen. Liquid nitrogen is a refrigerator medium able to store samples at very low temperatures, and therefore traditionally used for cryopreservation purposes and germ cell storage. However, it is hazardous with a high evaporation rate, requires appropriate equipment and management and requires a continuous supply, which is one of the main costs of the methodology. Although tissues and bacteria can be stored without significant loss of cell viability at higher temperatures (-80°C), germ cells are more vulnerable and require temperatures below -153°C (Mazur, 1984). Nowadays, ultrafreezer storage systems refrigerated at -150°C are commonly found in research facilities dedicated to zebrafish research. Some attempts in mammalian sperm cryopreservation in ultrafreezers (Álamo et al., 2005; Batista et al., 2006, 2009; Medrano et al., 2002; Yavaş and Daskin, 2012) with encouraging results. Since this temperature is below the threshold necessary for germ cell storage stability it can be potentially an alternative storage method for zebrafish sperm.

Table 1.1 Summary of sperm cryopreservation protocols in zebrafish and a brief description of the methodologies used in this species.

	Harvey et al. 1984	Morris et al. 2003	Yang et al. 2007	Draper and Moens, 2009	Carmichael et al, 2009	Hagedorn et al. 2012	Bai et al. 2013	Wang et al. 2015	Yang et al. 2016	Diogo et al. 2018	Matthews et al. 2018	Silva et al. 2019
Sperm collection	Stripping	Stripping/ Dissection	Dissection	Stripping	Stripping/ Dissection	Dissection	Dissection	Dissection	Dissection	Stripping	Stripping	Stripping
Extender	GRS	GRS/HBSS300/ BSMIS	HBSS300	GBS/ HBSS300	HBSS300	HBSS300	HBSS300	HBSS300	HBSS Ca^{2+} free,NaCl HBSS300, Glucose	HBSS300	E400	Grayling extender
Permeating cryoprotectant	Methanol	DMA, Etylene glycol, Methanol, DMSO,Glycerol	DMA, DMSO, Glycerol, Methanol	Methanol	Methanol	DMA, DMSO, Methanol	DMSO, Methanol	DMSO	Methanol	DMF	Methanol	Methanol
Non-permeating cryoprotectant	Skim-milk	Skim-milk	-	Skim-milk	-	-	-	-	-	-	Skim-milk, bicine, raffinose	-
Vial	Capillary	Capillary	0.25 mL french straws	Cryovials	Cryovials	0.25 mL french straws	0.25 mL french straws	0.25 mL french straws	0.25 mL french straws	Cryovials	Cryovials	0.25 mL french straws
Freezing method	Dry ice	Dry ice	Programmable freezer	Dry ice	Dry ice	Programmable freezer	Programmable freezer	Programmable freezer	Programmable freezer	Programmable freezer, Ultrafreezer	Programmable freezer, Dry ice	Dry ice
Freezing rate	16°C/min	-	10-20°C/min	-	-	10°C/min	1-130°C/min	25°C/min until -30°C; 5°C/min until -80°C	10°C/min	20°C/min, 66°C/min	5-35°C/min	-
Thawing	RT	37°C	40°C during 5 s	33°C during 8-10 s	-	30°C during 20-40 s	-	40°C during 7 s	40°C during 5 s	40°C during 8 s	38°C during 10-15 s	40°C during 5 s
IVF activation media	100 µL of 40 40% Ringer´s with H_2Odd	1mL of BSMIS in 1 mL of egg water	~20 µL of freshwater	70 µL of HBSS in 750 µL of fishwater	-	-	-	100 µL of sperm in 100 µL of system water	~20 µL of fish water	360 µL of system water (700 µS/cm)	150 µL of SS300 in 150 µL of distilled water	1 mL of system water (500-800 µS/cm)
Motility analysis media	H_2Odd	Egg water	H_2Odd	-	-	H_2Odd	-	Tap water	H_2Odd	System water (700 µS/cm)	H_2Odd	-
Motility analysis	Estimation	Estimation	Estimation	-	-	Estimation	CASA	CASA	Estimation	CASA	CASA	-
Fresh motility (%)	43.0±12.3	-	90	-	-	80	84±3	80-90	80±6	55	71.7±19.2	-
Post-thaw motility (%)	43.0±12.3	12.0±6.0	1-35	-	-	20-40	46.8±6.4	46.8±6.4	7-55	2-24	20.1±12.9	-
Viability (%)	-	-	0-10	-	-	75-55	30-70	30-70	35-66	2-25	-	-
Fertilization rate (%)	-	14.0±10.0	-	28.0±18.0	-	-	-	-	4-83	60-95	68±16	16±9
Embryo survival at (24 hpf)	-	-	-	-	-	-	-	-	4-100	2-8	-	-
Hatching rate (%)	51.0±35.6	-	-	-	-	-	-	-	-	2-8	-	-
Other analysis	-	-	-	-	-	-	-	ROS, MDA, ATP,WB, Survival 5 dpf	-	Annexin V	-	-

1.4.1. Zebrafish *in vitro* fertilization and offspring quality

The final objective of sperm cryopreservation is to successfully fertilize the oocyte through *in vitro* fertilization, resulting in healthy offspring. Sperm cryopreservation and *in vitro* fertilization are assisted reproduction technologies used to accomplish the fusion between male and female gametes. For successful fertilization, sperm must have high-quality parameters, such as motility to reach the oocyte and DNA integrity to form a normal zygote. Other factors affecting the success of fertilization are the oocyte quality and the *in vitro* fertilization methodology.

Egg quality is defined as its ability to be fertilized and subsequently develop into a normal embryo (Bobe and Labbé, 2010). Egg quality estimation for zebrafish *in vitro* fertilization purposes is a relevant source of biases since it is estimated by its appearance and color (good quality, hyaline and yellow), lacking a reliable quantification. The quantitative analyses of egg quality that can be performed, require considerable time expenditure (Yilmaz et al., 2017). Therefore, these analyses are not suitable for the selection of the samples for *in vitro* fertilization, which must be performed within a limited period of time, since after oocyte collection their fertilization ability decreases continuously (Poleo et al., 2001). There are reports on maximum fertilization ability during the first 20 minutes after oocyte collection, using HBSS with BSA or Aquaboost® overcoat (Cryogenetics, USA) (Poleo et al., 2001; Sakai et al., 1997). Moreover, detailed descriptions of zebrafish oocyte activation, fertilization, and early developmental phases show a narrow time window of 1 minute after oocyte activation to perform fertilization. In this species, the micropyle becomes blocked 60 s post-activation (Wolenski and Hart, 1987).

Female zebrafish oogenesis requires 10 days to complete the cellular division and differentiation (Clelland and Peng, 2009). An important source of low egg quality is the post-ovulatory aging, where the oocyte undergoes a decrease in its ability to be fertilized (due to morphological and biochemical alterations) and to further develop into a normal embryo. Post-ovulatory aging can induce a decrease in egg developmental capacities without visible changes in egg appearance (Bobe and Labbé, 2010). Overall, oocyte quality variations in zebrafish are a relevant source of biological variation and are difficult to overcome. Zebrafish eggs have many individual yolk globules connected to the membrane, homogeneously

distributed with ooplasm on the mature oocyte (Beams et al., 1985). When zebrafish oocytes come in contact with the spawning medium, they activate (Hart and Yu, 1980).

The description of zebrafish oocyte activation and fertilization was described in detail by several authors (Lee et al., 1999; Hart and Donovan, 1983; Hart and Yu, 1980; Hart et al., 1992; Wolenski and Hart, 1987)(Figure 1.3). Egg activation begins with a rise in intracellular calcium levels, that propagates as a wave of free cytosolic calcium throughout the egg (Lee et al., 1999). The zebrafish spermatozoon is expected to attach to the micropyle within 5 s post activation (Wolenski and Hart, 1987). The observation of asymmetric divisions in zebrafish embryos is, therefore, an important feature to record for a deeper understanding of the fertilized sample.

Fertilization success is one of the earliest parameters used to accurately estimate egg quality, being also the most integrative estimator of sperm quality (Bobe and Labbé, 2010). In teleosts, oocytes generally have only one entry site called the micropyle (Hart and Yu, 1980). Sperm is attracted towards the egg micropyle through chemotaxis (Dzyuba et al., 2017; Ishimoto et al., 2016; Yanagimachi et al., 2017). The chemoattractants present both in ovarian fluid (Beirão et al., 2015b; Diogo et al., 2010) and egg micropyle (Litvak and Trippel, 1998; Oda et al., 1998) guide sperm towards the micropyle.

Adequate sperm-egg binding is a species-specific phenomenon. However, the molecular mechanism by which it occurs is still poorly understood. In zebrafish, the penetration of the spermatozoon into the oocyte cytoplasm is accompanied by the formation of a fertilization cone beneath the micropyle (Hart et al., 1992) similarly to *Cyprinus carpio* (Kudo, 1980) (Figure 1.3 I-IV). Zebrafish sperm incorporation by the oocyte and early post-fertilization events are well described (Hart and Yu, 1980; Wolenski and Hart, 1987). Soon after fertilization, there is an elevation of the chorion (Figure 1.3 I-IV). Forty-five seconds after the sperm-egg binding moment, the region of the egg surface surrounding the bound spermatozoon is swollen and slightly elevated (1.3 II). The fertilization cone (Figure 1.3 III) shortens 2 min after fertilization and becomes enlarged in a blister shape. The formation of the fertilization cone in zebrafish is independent of fertilization by the spermatozoon or binding to the micropyle (Wolenski and Hart, 1987).

The management of assisted reproductive techniques has relevant impacts on oocyte fertilization and embryo development (Ramos-Ibeas et al., 2019). Therefore, the understanding of the fertilization process in zebrafish is essential for the establishment of adequate *in vitro* fertilization methodologies in this species. Moreover, the correct identification of normal developmental steps in the embryo (Figure 1.4 A-O) is essential in the detection of abnormal divisions or embryo malformations. This knowledge is particularly valuable for accurate quality analysis of the offspring sired by cryopreserved sperm.

In assisted reproduction techniques the fertilization success depends on the sperm to egg ratio (Butts et al., 2009). However, sperm concentration is often disregarded both in aquaculture hatcheries (Żarski et al., 2017) and zebrafish facilities (Torres and Tiersch, 2018). When using cryopreserved sperm, a percentage of spermatozoa die during freezing and thawing, thus the effective sperm population decreases (Cabrita et al., 2009). Therefore, it is often employed a high sperm-egg ratio to improve the probability of oocyte fertilization. Zebrafish ensures its monospermy with a morphological strategy in which the micropylar entry site is slightly larger than the spermatozoa spherical head (2.5–2.8 μm in diameter) (Hart and Donovan, 1983). This zebrafish feature is advantageous for its *in vitro* fertilization methodologies since negative events of polyspermy are prevented.

Of the 12 published articles in zebrafish sperm cryopreservation protocols (Bai et al., 2013; Carmichael et al., 2009; Diogo et al., 2018; Draper et al., 2004; Hagedorn et al., 2012; Harvey et al., 1982b; Matthews et al., 2018; Morris et al., 2003; Silva et al., 2019; Wang et al., 2015; Yang et al., 2007; Yang et al., 2016) 8 perform *in vitro* fertilization (Diogo et al., 2018; Draper et al., 2004; Harvey et al., 1982b; Matthews et al., 2018; Morris et al., 2003; Silva et al., 2019; Wang et al., 2015; Yang et al., 2016), its success being measured through fertilization rates (Figure 1.4 A-D), and the hatching rates have been evaluated only in 4 of those reports (Diogo et al., 2018; Harvey et al., 1982b; Silva et al., 2019; Wang et al., 2015). To compare those results is extremely challenging, considering that the methodologies of each step of cryopreservation vary greatly in features such as types of cryoprotectants used and their concentration, freezing rates (and methodologies to achieve them), type and volume of the packaging, and thawing rates (Table 1.1). Similarly, the *in vitro*

fertilization methodologies are markedly different in features such as a number of spermatozoa per egg, volume of water, activation medium, water temperature, oocyte collection method, etc. Additionally, there are relevant concerns in terms of the standardizations of the analytical methods, for example, the fertilization rate is measured in different times post-fertilization (and therefore embryo stages) ranging from 2-18 hpf. Altogether, these variables hinder the comparison between studies and pose challenges to the improvement of sperm cryopreservation methodologies in this species.

The quality of offspring sired by cryopreserved sperm is still poorly investigated in teleosts since the fertilization rate is considered a valid evidence of the sperm fertilization ability and embryo viability. In fact, as stated previously only 4 articles in zebrafish sperm cryopreservation report hatching rates and only Wang et al (2015) reported larvae survival at 5 dpf. However, this data is of the utmost importance considering that in zebrafish the paternal genotoxic damage results in lower survival of the embryos (Fernández-Díez et al., 2015).

The tolerance of unrepaired sperm DNA is associated with an evolutionary mechanism in fish, enabling the introduction of new mutations potentially advantageous to environment changes (Fernández-Díez et al., 2018). However, from the point of view of assisted reproduction techniques, this characteristic means that we can be overlooking relevant factors. If cryopreservation promotes DNA injuries on sperm and the oocyte cannot repair this extend of damage, there can be relevant consequences on offspring health. A consequence of embryo survival inheriting genetic damage is its lower larval quality, which is observed through the occurrence of larvae malformations (Fernández-Díez et al., 2015). In teleosts, the evaluation of skeletal malformations is an established fish quality evaluation criteria, since it is related to the animal health and welfare conditions (Coutteau et al., 1997). Studies performed by Fernández-Díez et al., (2015) showed that zebrafish embryos sired with damaged sperm resulted in a high degree of malformations that affected processes such as skeleton morphogenesis, chondrogenesis, pigmentation and angiogenesis, as a result of an impairing or incorrect embryo organogenesis. The occurrence of embryonic or larval malformations is a valuable tool to fully characterize the developmental potential of fertilized eggs (Bobe and Labbé, 2010; Labbé et al., 2001, 2017). Therefore, the offspring health and quality analysis of

zebrafish sired by cryopreserved sperm is relevant, since this species is particularly permissive to paternal genotoxicity damage, such as injuries due to cryopreservation. The offspring quality can be a particularly useful tool for improving sperm cryopreservation methodologies since it allows a more accurate observation of the effects of the assisted reproduction methodologies employed.

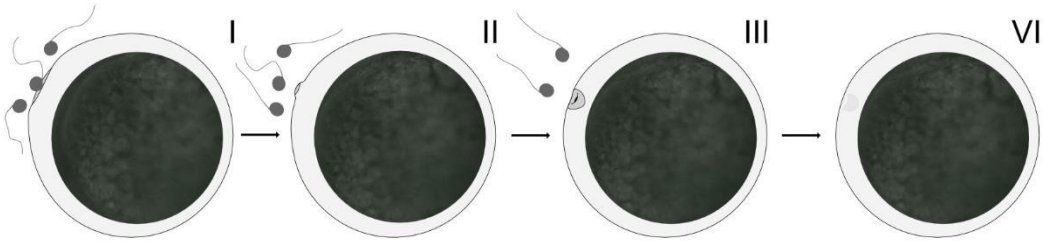

Figure 1.3 Zebrafish sperm-egg binding and fertilization**.** Schematic representation of zebrafish fertilization with I) sperm-egg binding, II) fertilization cone initial upward protrusion, III) fertilization cone formation and VI) disappearance of fertilization cone.

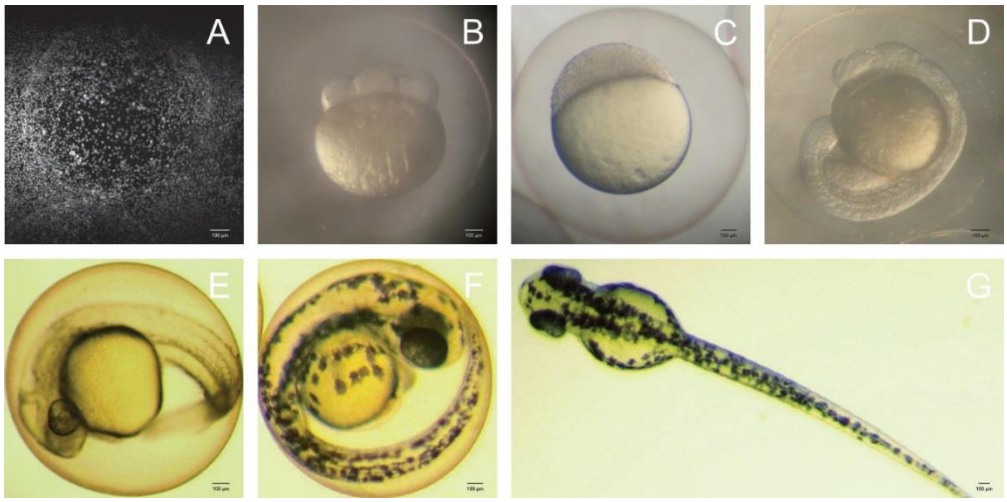

Figure 1.4 Zebrafish embryogenesis. A) oocyte, B) oocyte activation, C) fertilization moment with fertilizing spermatozoon on the micropyle, D) perivitelline space and cortical granules exocytosis on early zygote, E) , F), G), H), I) eight cell embryo stage, J) blastula stage, K) gastrula stage, L) embryo somitogenesis stage, M) embryo prim-25 stage N) embryo high-pec stage, O) early hatched larvae.

1.5. Objectives

Zebrafish have become undoubtedly one of the most important research model organisms in the past decade. Thousands of new mutant and transgenic strains were developed, posing problems in terms of facilities space and management. Therefore, the development of an efficient zebrafish sperm cryopreservation method is a pressing necessity to manage and preserve the exponentially growing number of zebrafish lines. Even though the first zebrafish sperm cryopreservation protocol was developed more than 30 years ago, there is a lack of standardization of the assisted reproductive methodologies, which results in high variability on post-thaw sperm quality and *in vitro* fertilization success.

The main objective of this thesis was to establish guidelines to support the management and storage improvement of zebrafish genetic resources. Ultimately, this project proposes to establish, through a multidisciplinary approach, the improvement of zebrafish sperm cryopreservation methodologies and management tools that ensure offspring viability. The strategy used consisted on the establishment of broodstock and quality standards for the selection of high-quality sperm to be used in cryopreservation. Furthermore, it targets the improvement of protocols for the preservation of mutant and transgenic zebrafish lines through germplasm cryobanking. Therefore, the specific objectives of this thesis were to:

1) Identify the specific constraints in zebrafish male donors affecting reproductive performance;

2) Optimize the cryopreservation of zebrafish germ cells for the implementation of standardized procedures in zebrafish rearing facilities;

3) Establish an analytical tool set for standardized quality assessment of cryopreserved material;

4) Develop specific procedures for the cryopreservation of germ cells from laboratory animal models showing reproductive constrains.

To accomplish the mentioned objectives three main tasks were established, namely: 1) Development of standardized methods for cryopreservation of zebrafish germ cells, 2) Development of an analytical tool set for improving sperm quality

assessment and 3) Application of methodologies to zebrafish lines. Considering these specific tasks, the improvement of the current knowledge about the optimum broodstock and germplasm characteristics in zebrafish were addressed. The characterization of zebrafish fresh and cryopreserved sperm and the establishment of a standard analytical tool set for zebrafish sperm analysis were essential to accomplish the objectives of this work. Moreover, the improvement and standardization of the current zebrafish cryopreservation technologies through cryobiological principles was one of the main concerns. Ultimately, this thesis aimed to improve the understanding of sperm quality from different zebrafish lines and to establish a feasible and practical protocol that ensures the adequate genetic inheritance of the progeny.

CHAPTER 2. SPERM MOTILITY ACTIVATION

PREAMBLE

This thesis project is rooted in the standardization of zebrafish sperm quality analysis and cryopreservation in order to reduce the variability of assisted reproduction techniques intra and inter-facilities. For this purpose, the establishment of a quality tools set was proposed in the present project. Sperm motility is the most used quality assessment techniques due to its relation to cell fertilization ability.

The use of CASA automated systems allowed an accurate quantification and qualification of sperm motility. Teleosts spermatozoa acquire motility through a difference in the osmolarity of the medium and the physicochemical properties of these medium modulate sperm motility and metabolism. Surprisingly, zebrafish sperm motility activation studies activate motility with distilled water and without temperature control. Therefore, the current method for sperm quality analysis in zebrafish can be a relevant source of biases in research. Consequently, it was highly pertinent to investigate the effect of water temperature and conductivity on zebrafish sperm motility and *in vitro* fertilization success.

This study contributed to the technical improvement of zebrafish sperm quality analysis and *in vitro* fertilization protocols avoiding overestimation of spermatozoa fertilizing ability. This chapter represents a manuscript to be submitted to Journal of Experimental Biology first authored by Patricia Diogo.

2.1. TEMPERATURE AND CONDUCTIVITY OF FERTILIZATION MICROENVIRONMENT IMPACTS ZEBRAFISH SPERMATOZOA METABOLISM AND EXPERIMENTAL REPLICABILITY

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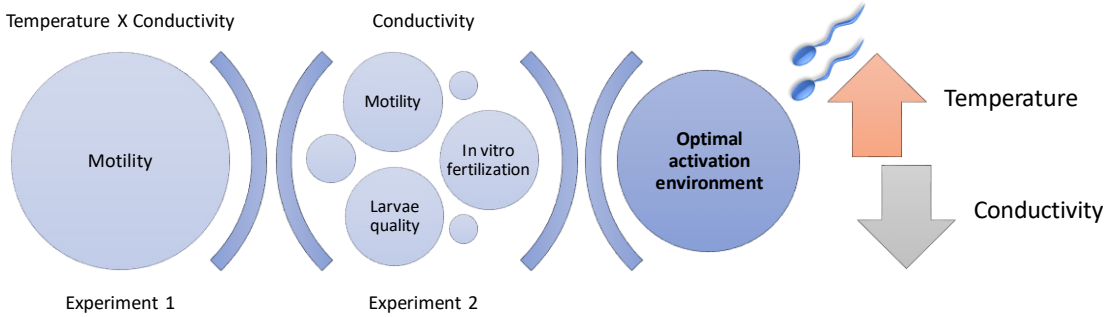

[Theriogenology, submitted]

2.1.1. Abstract

Sperm motility is essential to accomplish fertilization and is an established tool to estimate sperm fertilizing ability. When spermatozoa are released to the environment, temperature and osmolarity modulate motility activation and metabolism. In zebrafish recirculation systems, water temperature and conductivity are stable and natural reproduction occurs under these conditions. However, sperm motility analysis is commonly performed at room temperature with distilled water. Across zebrafish facilities, there are highly variable water conductivities and consequently different osmolarities. Therefore, this study aimed to assess the effect of water temperature and conductivity on zebrafish sperm motility of wild type AB line and *mitfa*$^{w2/w2}$*;roy*$^{a9/a9}$ line (*casper*). Water at 28°C improved motility and high conductivity reduced sperm total and progressive movement in AB males. In *casper*, water at 28°C improved motility parameters on the end of spermatozoa lifespan. *In vitro* fertilizations performed with AB line showed no significant differences when sperm activation is performed using water at 28°C with conductivity 0, 700 and 1200 µS/cm. The use of distilled water for motility activation promoted an overestimation of sperm motility compared to fertilization conditions in the system water. Our study demonstrates that 28°C and low water conductivity conditions are the most suited for zebrafish sperm activation, resembling its natural environment.

2.1.2. Introduction

Zebrafish, *Danio rerio, (*F. Hamilton 1822) is a freshwater species with natural habitat in shallow rivers and ponds of Myanmar, Pakistan, India, Bangladesh, and Nepal (Arunachalam et al., 2013). Due to its natural characteristics, such as small body, high fertility and environmental tolerance, it has been successfully established as a model species. It is maintained in laboratories worldwide and extensively used in numerous research fields including biology, development and biomedical research. However, assisted reproduction techniques, like sperm cryopreservation and *in vitro* fertilization, still lack standardization. As a consequence, there is high variability on sperm quality and fertilizing ability between facilities, as well as low experimental replicability (Robles et al., 2009; Tsang et al., 2017). To the best of our knowledge all the studies reporting zebrafish

sperm motility with the CASA system, used distilled or tap water to activate sperm (Hagedorn et al., 2012; Ingermann et al., 2011; Matthews et al., 2018; Park et al., 2012; Wilson-Leedy and Ingermann, 2007; Yang et al., 2007), with the exception of the reports by Diogo et al. (2015, 2018) where sperm motility was activated using filtered system water. Furthermore, the temperature of the activation solution is commonly not controlled, with the exception of the report by Diogo et al. (2018).

Spermatozoa from teleost's are flagellated single cells, adapted to external fertilization, that undergo a period of spermatogenesis in a safe environment, surrounded by seminal plasma and Sertoli cells in the testes, with physicochemical conditions similar to the body environment (Billard, 1986; Schulz et al., 2010). Zebrafish, as other teleost species, perform a metabolic effort to maintain body fluids with constant osmolarity (~300 mOsm/kg) through osmoregulation mechanisms, despite the variable external ionic environment conditions (Boisen et al., 2003). Under these conditions, spermatozoa are immotile in the testes and only acquire motility when in contact with the external medium. In freshwater species, sperm motility is activated when in contact with the hypotonic external environment (Cosson, 2004). Motility is acquired under the regulation of many extrinsic and intrinsic factors, thus reflecting the specialization of flagellum structure (Dadras et al., 2017).

Sperm motility is the most studied parameter of sperm quality assessment in fish due to its proven correlation with fertility (Cabrita et al., 2014; Gallego and Asturiano, 2018; Kime et al., 2001). Although sperm motility is an incomplete physiological analysis and needs other quality assays to guarantee the status of spermatozoa (Bobe and Labbé, 2010), it is associated with the probability of fertility success. Due to its high physiological response to the environmental conditions, it is a useful tool to analyze the effects of different treatments (Kime et al., 2001), also being used as an ecotoxicological biomarker (Kollár et al., 2018).

Osmolarity is considered one of the most relevant factors in teleost's motility activation. The evaluation of the ions present in the water can be performed according to osmolarity, conductivity or salinity. The ions present in the fertilization microenvironment regulate sperm movement patterns (Alavi and Cosson, 2006; Alavi et al., 2009) and are related to offspring phenotypes (Ritchie and Marshall,

2013). In zebrafish rearing facilities the amounts of ions in the water are controlled through water conductivity, however, this parameter is not standardized, and a wide range of water conductivities are used among facilities (400-1500 μS/cm) (D´India, 2018).

The rearing temperature for zebrafish is universally standardized at 28°C, however, sperm motility analysis and *in vitro* fertilization are commonly performed at room temperature. This fact may be a relevant source of biases in the determination of zebrafish sperm motility. The temperature of the activation solution is known to affect spermatozoa since it increases cell metabolism (Dadras et al., 2017). The adequacy of the activation medium is species specific, since temperature affects the beating frequency of spermatozoa flagella differently, which is physiological related to the adaptation of each species to natural environment conditions (Alavi and Cosson, 2005). Generally, higher temperature increases sperm movement and decreases longevity, while the opposite happens with lower temperatures (Dadras et al., 2017). The increase of metabolic activity with higher temperature depletes faster the limited energetic resources available on spermatozoa (Dadras et al., 2017). In opposition, lower temperatures generally prolong sperm motility (Billard and Cosson, 1992; Bombardelli et al., 2013; Lahnsteiner, 2011; Lahnsteiner and Mansour, 2012; Lahnsteiner et al., 1999) with a reduction in velocity and flagella beating frequency (Cosson et al., 1985; Dadras et al., 2017). Moreover, both pre-fertilization (spermatogenesis) and sperm activation thermal environments are known to affect sperm quality, offspring phenotype and post-hatching performance (Fenkes et al., 2017; Kekäläinen et al., 2018). Therefore, the temperature of the fertilization microenvironment may have relevant transgenerational effects that must be considered both in the context of environmental climate changes, with ecological and evolutionary implications (Marshall, 2015), and under rearing conditions, to improve colony management and fish welfare.

Apart from the wild type lines, there are some zebrafish mutant and transgenic lines that present reproductive constraints which are poorly investigated (Lawrence, 2016). In our study, we used a wild type (AB) line and a transparent double mutant *casper* (*mitfa*$^{w2/w2}$/*roy*$^{a9/a9}$) line with reproductive constraints

(D'Agati et al., 2017; Lawrence, 2016; White et al., 2008). The use of AB and *casper* zebrafish lines in sperm activation with different environmental conditions enable to understand their practical consequences under zebrafish facilities rearing conditions.

The objective of this study was to evaluate the effect of water temperature and conductivity on sperm motility, activation and metabolism on AB and *casper* zebrafish lines. Additionally, we evaluated the effect of water conductivity on *in vitro* fertilization success of AB zebrafish.

2.1.3. Methods

2.1.3.1. Fish husbandry

A population of wild-type AB line (ZFIN ID: ZDB-GENO-960809-7) was maintained at the Centre of Marine Sciences (CCMAR, Faro, Portugal) for more than 10 generations and used to generate the adults necessary for all experimental designs. The genetic variability of this colony is maintained through a yearly import of AB zebrafish established in different laboratories worldwide. The mutant *mitfa*$^{w2/w2}$;*roy*$^{a9/a9}$ (*casper*) line was obtained from a breeding stock kept at Champalimaud Foundation (Lisbon, Portugal).

Adult AB zebrafish and *casper* males (8-12 months old) were selected according to similar size and maintained separated from females in 3.5 l tanks, with 15 fish each. Fish were reared in a ZebTEC® (Tecniplast, Buguggiate, Italy) recirculation system with 980 l of water, as previously described in Diogo et al. (2018). The water was maintained at 28.2±0.5°C, 700±75 µS/cm and pH 7.5±0.2 in the system. The fish were fed *ad libitum* twice a day, from the larval stage until adulthood, with *Artemia* nauplii (AF480, INVE, Dendermonde, Belgium) and ZEBRAFEED® diet (Sparos Lda, Olhão, Portugal).

All animal manipulations were performed in compliance with the Guidelines of the European Union Council (86/609/EU) and transposed to the Portuguese law for the use of laboratory animals on research by "Decreto Lei n° 129/92 de 06 de Julho, Portaria n° 1005/92 de 23 de Outubro", and according to the European

parliament council directive's for protection of animals used for scientific research (2010/63/EU). All animal protocols were performed under a "Coordinator-researcher" license from the Direção-Geral de Veterinária, Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, Lisbon, Portugal, under the "Decreto Lei n°113/2013 de 7 de Agosto" relative to the protection of animals used for scientific research. All the fish sampling procedures were performed by licensed researchers.

2.1.3.2. Sperm collection and motility analysis

On the day prior to sperm collection, males and females were placed in 1 l breeding tanks in 1:1 sex-ratio (Tecniplast, Buguggiate, Italy) and maintained separated while sharing the same water for 16 h (Diogo et al., 2018). This methodology is used to promote hormonal stimulation, improving the release of gametes. Sperm collection was performed, within 1 h after the lights turned on. Zebrafish males were anesthetized with 0.168 mg/ml tricaine sulfonate solution (MS-222, Sigma-Aldrich, Madrid, Spain) according to Westerfield (2007). The males were rinsed with Phosphate Buffered Saline (PBS) solution and carefully dried with a paper towel. An abdominal massage was performed to AB (n=8) and *casper* (n=8) males to collect the sperm using a glass capillary tube attached to a mouth piece. After collection, the sperm was immediately diluted to a final volume of 10 µl using sterilized and filtered (0.20 µm) Hank´s Balanced Salt Solution (HBSS) at 300 mOsm/Kg (8.0 g NaCl, 0.4 g KCl, 0.16 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.06 g Na_2HPO_4, 0.06 g KH_2PO_4, 0.35 g $NaHCO_3$, 1.0 g $C_6H_{12}O_6$ in 1000 ml of mili-Q water, pH 7.5) (Jing et al., 2009a).

Sperm motility analysis was performed using computer assisted sperm analysis (CASA) system (ISAS Integrated System for Semen Analysis, Proiser, Valencia, Spain). Briefly, 0.5 µl of each sperm sample was immediately diluted with 5 µl of the activation solution and placed in a Makler chamber (Microptics S.L., Barcelona, Spain) under a phase contrast microscope (Nikon E-200, Nikon, Tokyo, Japan) with a ×10 negative phase contrast objective. Sperm motility images were captured with a ISAS 782C camera (Proisier, Spain) and processed with CASA software. The settings of CASA system were adapted for this species namely 25 frames $^{s-1}$, connectivity 14, 1 to 90 mm for head area. For sperm concentration a

dilution (1:19) was performed with HBSS and analyzed in CASA system, to calculate the sperm concentration to be used in *in vitro* fertilization. Sperm motility was characterized each 10 s post-activation during 1 minute, according to total motility (TM; %), progressive motility (PM; %), curvilinear velocity (VCL; μm s^{-1}), straight line velocity (VSL; μm s^{-1}), velocity according to smoothed path (VAP; μm s^{-1}), linearity (LIN; %), straightness (STR; %), curvilinear path wobble (WOB; %), amplitude of lateral displacement of sperm head (ALH; μm) and beat-cross frequency (BCF; Hz). LIN (VSL/VCLx100). STR (VSL/VAPx100) and WOB (VAP/VCLx100) were calculated by the CASA system using other analyzed parameters. All motility parameters were described by Boyers et al. (1989). Sperm samples with VCL > 10 μm s^{-1} were considered motile.

2.1.3.3. Experiment 1 – Effect water temperature and conductivity on sperm motility activation and metabolism

System water conductivities were obtained by programming ZebTEC® rearing system settings with a wide range of water conductivities commonly used in zebrafish facilities. This was done to verify the relationship of water conductivity with osmolarity. The conductivity probe was calibrated with a TDS calibration solution 700 μS/cm (HM digital Inc, California, USA) at 25°C, according to the product specifications. The device accuracy was tested by an external probe TDS Meter – COM-100 (HM digital Inc, California, USA). Both devices were calibrated with the same solution. The osmolarity of the water conductivities used in zebrafish facilities (300-1400 μS/cm) (D´India, 2018) was measured with a semi-micro osmometer K7400S (Knauer, Berlin, Germany) (Supplementary figure).

Sperm was collected from individual males from wild type AB (n=8) and *casper* (n=8) lines. Both collection and motility analysis were performed as previously described. Sperm was activated at 20°C or at 28°C, either with distilled water (0 μS/cm) or with system water set at 700 μS/cm or 1200 μS/cm. All activation solutions were filtered (0.20 μm) and sterilized. For sperm motility activation, two of the most common water conductivities established in zebrafish rearing facilities were selected namely 700 and 1200 μS/cm (D´India, 2018). In

addition, distilled water with 0.067 µS/cm (0 µS/cm) was used since it is commonly applied in zebrafish sperm activation in the CASA system (Figure 2.1.).

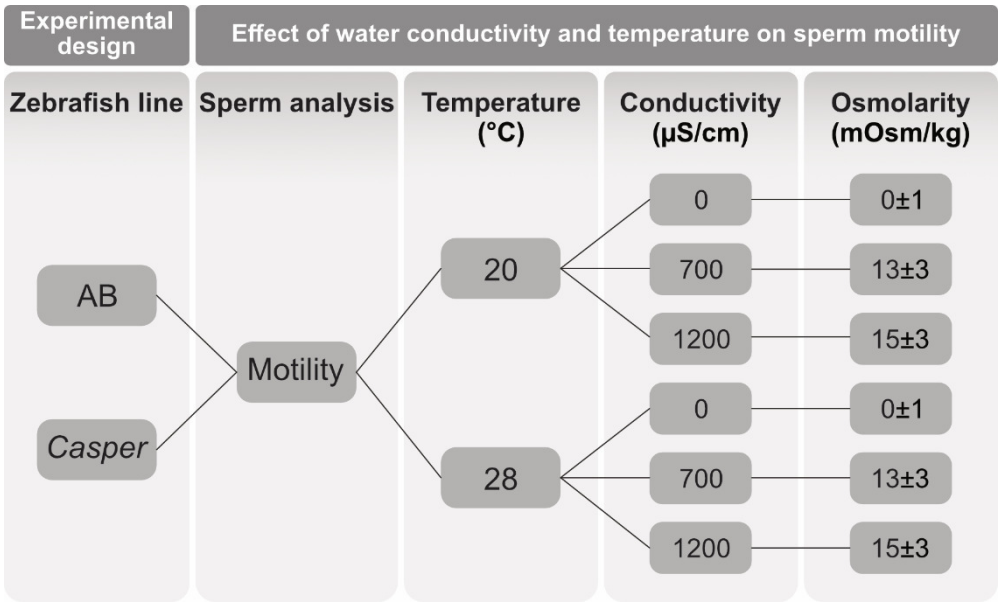

Figure 2.1 Experimental design to evaluate the effect of water conductivity and temperature on sperm motility of AB and *casper* zebrafish lines

2.1.3.4. Experiment 2 – Effect of the water conductivity on in vitro fertilization

To complement the previous study an experiment was set up to study how water conductivity affects *in vitro* fertilization. Adult AB (n=70) males with 6-8 months of age were sampled for sperm collection. This experiment was performed only using the AB line since sperm activation with different water conductivities in *casper* line showed no significant differences (Table 2.1). The male selection to perform sperm pools was performed by motility analysis at 10 s post activation. Males with total motility higher than 50% and cell concentration over 3 x 10^7 cells/ml were selected to perform the pools. Each sperm pool (n=5) contained sperm from 7 males. Sperm concentration was evaluated with the CASA system and motility was activated with distilled and system water set at 0, 700 and 1200 µS/cm of conductivity. All activation mediums were at 28°C for sperm motility analysis according to the results from experiment 1. The motility of the sperm pools was recorded each 10 s post activation for one minute (Figure 2.1.2).

Females used for *in vitro* fertilization (6-8 months) were maintained in a 1 l breeding tank separated from males for a period of 16 h prior to oocyte collection. Females were anesthetized with MS-222, rinsed with sterile PBS (pH 7.4) and placed in a 35 mm Petri dish (Falcon® by Corning, New York, USA). The oocytes were collected by abdominal massage, carefully performed to avoid touching the oocytes. If the clutch had good quality characteristics (Bobe and Labbé, 2010; Carmichael et al., 2009), the oocytes were immediately used for *in vitro* fertilization. *In vitro* fertilization was performed with AB line, however in Figure 2.3 (A and B) oocyte collection and a good quality clutch are illustrated with the *casper* line, taking advantage of its transparency, which improves the observation of oocyte collection. Only good quality clutches (n=35) with 100-200 oocytes were selected to test all treatments. Fertilization was performed within 1 minute after oocyte collection by adding 3-3.5 x 10^6 spermatozoa to the oocytes (3-3.5x10^3 spermatozoa/oocyte). Sperm motility activation was immediately performed with 360 µL of activation medium at 28°C. After 5 min, when all sperm is immotile, 5 ml of system water (700 µS/cm) at 28°C were added to the Petri dish. The embryos were maintained in an incubator at 28°C with the same photoperiod (14L: 10D, lights on at 09:00 a.m.) of the zebrafish facilities. Survival rate was calculated at 24 hpf (hours post fertilization) and hatching rate at 72 hpf. Additionally, the hatching rates were calculated at 48 hpf, according to Kimmel et al. (1995), and at 72 hpf due to delays in hatching. These calculations were performed according to the initial number of oocytes of each clutch. Each sperm pool was used to fertilize 2-3 clutches of oocytes with each activation medium (n=35 fertilizations). At 120 hpf, 40 larvae of each treatment resulting from each sperm pool were photographed with a MZ 7.5 stereomicroscope (Leica, Wetzlar, Germany) equipped with an F-View II camera (Olympus, Hamburg, Germany). The analysis of larvae standard length was performed using Fiji software (Schindelin et al., 2012).

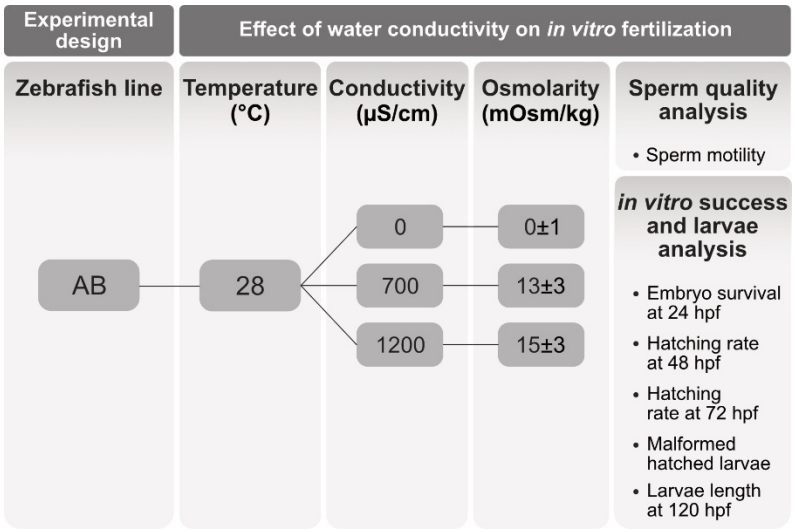

Figure 2.2 Experimental design to evaluate the effect of water conductivity on *in vitro* fertilization success of AB zebrafish line.

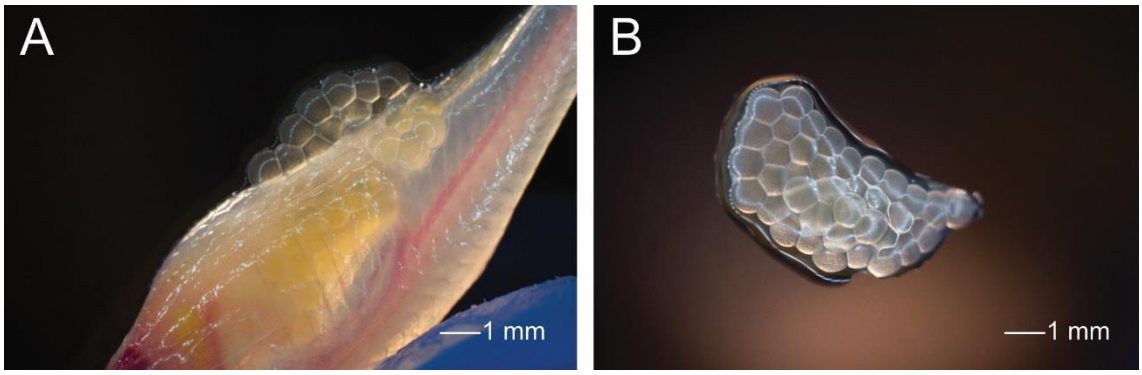

Figure 2.3 Representation of oocyte collection with *casper* zebrafish. A) oocyte collection, B) good quality clutch example to use in *in vitro* fertilization.

2.1.3.5. Data analysis

IBM SPSS Statistics 25.0 (IBM, New York, USA) software was used for statistical analysis. Data were expressed as mean±s.d. (Standard Deviation) and normalized by logarithmic, or arcsine transformation when results were expressed as percentages, to meet assumptions of normality and homoscedasticity. Sperm motility parameters in experiment 1 were analyzed through ANOVA (repeated measures, $P<0.05$). The correction of Greenhouse-Geisser was used since the sphericity of the data was not assumed. Post hoc test Bonferroni ($P<0.05$) was used to investigate differences in water conductivity and independent t-test for water temperature ($P<0.05$). For the second experiment, one-way ANOVA (post hoc

Student–Newman–Keuls, $P<0.05$) was applied on sperm motility, *in vitro* fertilization parameters and larvae length.

2.1.4. Results

2.1.4.1. Experiment 1 – Effect water temperature and conductivity on sperm motility activation and metabolism

To investigate the optimal environmental conditions for zebrafish sperm activation, two temperatures (20 and 28°C) and three water conductivities (0, 700 and 1200 µS/cm) were tested in AB and in *casper* zebrafish lines.

Through an initial analysis the effect of the zebrafish line, water temperature and conductivity on sperm motility parameters were analyzed through repeated measures ANOVA ($P<0.05$). The zebrafish line and time post activation were the factors with the largest main effect on sperm motility parameters (Table 2.1). The AB line displayed significantly higher results in all motility parameters when compared to *casper* line (Table 2.1). This fact is most evident in the motility recorded in the last seconds (Table 2.2). As expected, time significantly decreased all motility parameters throughout post activation time in both zebrafish lines (Table 2.1, Table 2.2, Figure 2.4, 2.5 and supplementary figure 2).

The differences displayed between both zebrafish lines and the occurrence of significant interactions between factors impaired the accurate analysis of the effect of water temperature and conductivity on sperm motility. Therefore, repeated measures of ANOVA was applied for each zebrafish line independently (Table 2.1). Higher water temperature improved significantly sperm velocities, LIN and ALH for both zebrafish lines.

Independent t-test samples were used to study the effect of water temperature on sperm motility parameters, due to the reduced number of groups (Table 2.2). Higher water temperature (28°C) significantly improved all motility parameters in the AB line, except ALH (Table 2.1, Figure 2.1.5 E and Supplementary figure 2). The detailed analysis with t-test shows that this effect of temperature is increasingly relevant in the last seconds of motility (Table 2.2). The water

conductivity affected significantly sperm velocities in the AB line, however, this was not observed in *casper* line (Table 2.1). The interaction between water temperature and conductivity is significant, where 28°C with 0 and 700 µS/cm increases significantly ALH (Table 2.1, Figure 2.1.5 F). In the *casper* line, higher water temperature improved motility parameters related to velocity and trajectory namely VCL, VSL, VAP, LIN, STR, WOB (Table 2.1 and 2.2, Figure 2.1.5 and Supplementary figure 3). The positive effect of higher water temperature in *casper* line is particularly important at 60 s post activation in all motility parameters except PM, ALH and BCF (Table 2.2).

In the AB line, water conductivity significantly affects progressive motility (Table 3). Water conductivity affected significantly sperm velocities, LIN, STR, WOB and BCF in AB line, however, water conductivity showed no significant effects on *casper* line motility parameters. (Table 2.1 and 2.3).

Table 2.1 Zebrafish sperm motility analysis (recorded for 1 minute each 10 s post activation) of AB and *casper* line when activated with different water temperatures (20°C and 28°C) and conductivities (0, 700 and 1200 µS/cm) and their interactions (P values).

	TM (%)	PM (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
Repeated measures ANOVA										
Time	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Line x time	<0.001*	<0.001*	0.001	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Temperature x time	<0.001*	0.153	0.001	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	0.007	0.001
Conductivity x time	<0.001*	0.432	0.210	0.772	0.871	0.677	0.604	0.517	0.296	0.055
Line x temperature x time	0.004	0.178	0.209	0.302	0.263	0.207	0.200	0.108	0.099	0.106
Line x conductivity x time	0.649	0.004*	0.574	0.211	0.721	0.646	0.447	0.315	0.038*	0.188
Line x temperature x conductivity x time	0.112	0.027*	0.674	0.573	0.842	0.673	0.666	0.609	0.374	0.577
AB Repeated measures ANOVA										
Temperature x time	0.009*	0.072	0.049*	0.036*	0.043*	0.044*	0.075	0.065	0.029*	0.091
Conductivity x time	0.061	0.113	0.022*	0.015*	0.019*	0.021*	0.002*	0.002*	0.077	0.033*
Temperature x conductivity x time	0.018*	0.111	0.031*	0.053	0.037*	0.015*	0.004*	0.003*	0.113	0.040*
Casper Repeated measures ANOVA										
Temperature x time	0.186	0.001*	0.015*	0.042*	0.006*	0.021*	0.021*	0.126	0.027*	0.012*
Conductivity x time	0.129	0.064	0.310	0.330	0.609	0.509	0.353	0.67	0.055	0.141
Temperature x conductivity x time	0.119	0.114	0.737	0.350	0.546	0.330	0.414	0.050*	0.056	0.061

Time means post-activation time TPA – Time post activation (s), TM- Total motility, PM- Progressive motility, VCL – curvilinear velocity, VSL- straight line velocity, VAP- velocity according to smoothed path, LIN- linearity, STR- straightness, WOB- curvilinear path wobble, ALH- amplitude of lateral displacement of sperm head, BCF- beat-cross frequency. Significant differences (repeated measures ANOVA, $p < 0.05$) are represented with an asterisk.

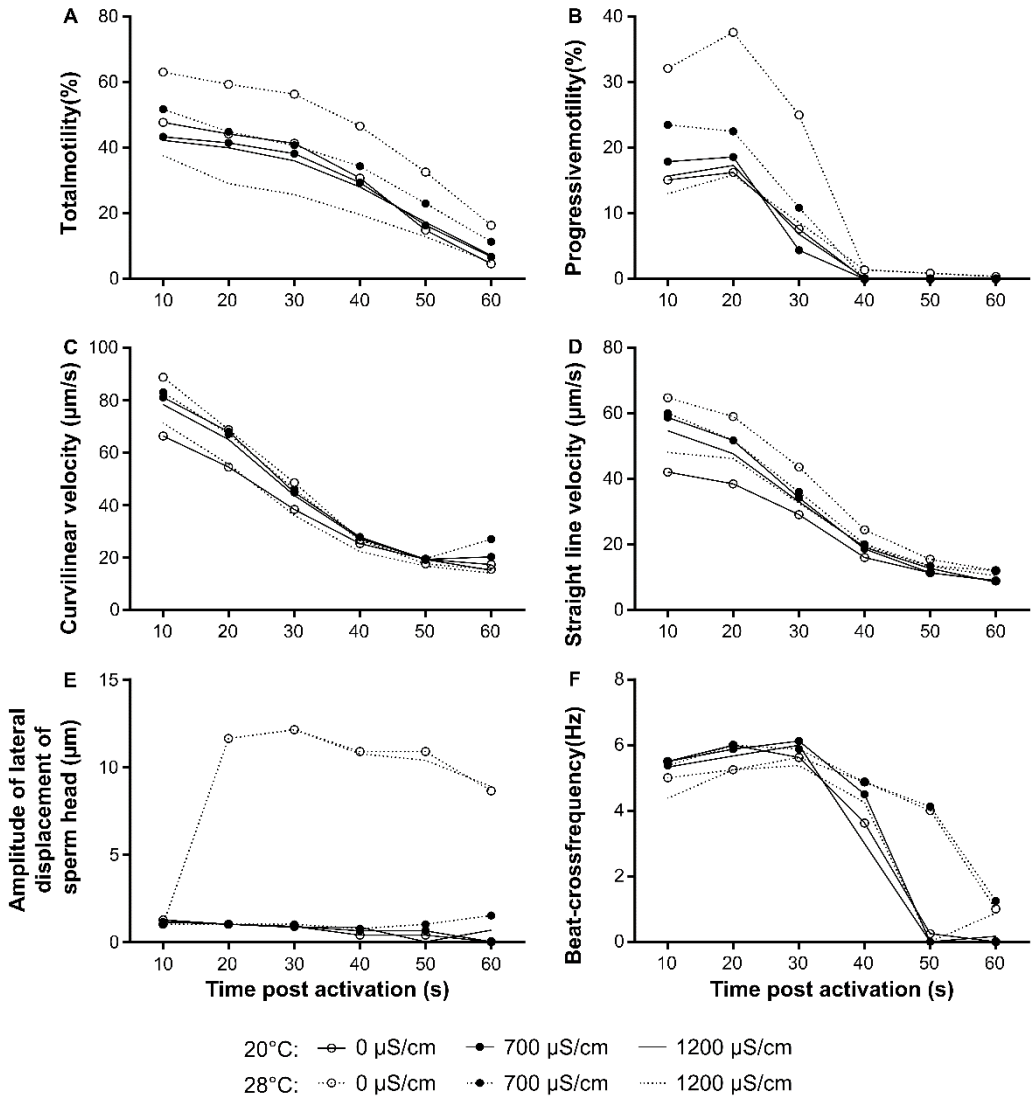

Figure 2.4 Effect of water temperature (20°C and 28°C) and water conductivity (0, 700 and 1200 µS/cm) on sperm motility parameters of zebrafish AB line (n=8). Sperm was activated, and motility parameters were recorded each 10 s for 1 min in terms of: A) TM, B) PM, C) VCL, D) VSL, E) ALH and F) BCF. The values plotted represent mean, continuous line represent 20°C and dashed line represent 28°C of the activation medium. Activation medium with 0 µS/cm is represented with a white circle, 700 µS/cm with a dark circle and 1200 µS/cm without the symbol.

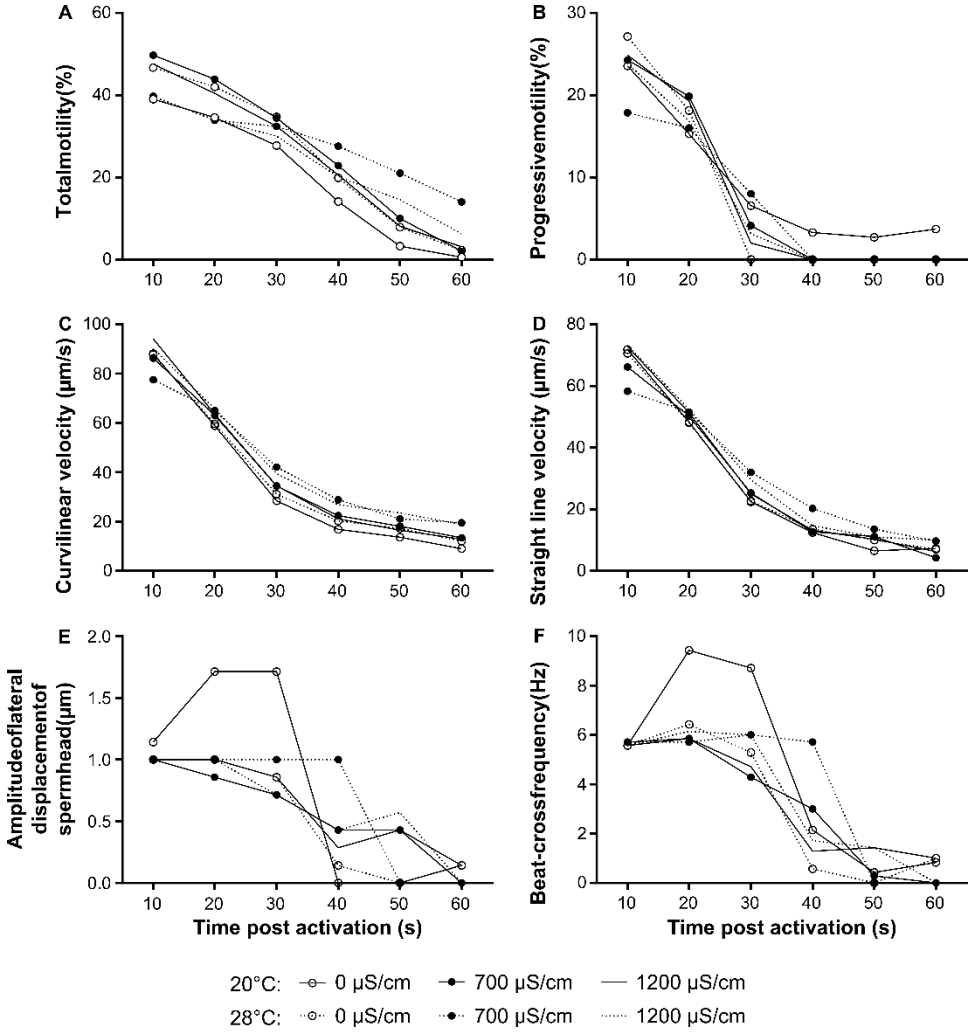

Figure 2.5 Effect of water temperature (20°C and 28°C) and water conductivity (0, 700 and 1200 µS/cm) on sperm motility parameters of zebrafish *casper* line (n=8). Sperm was activated, and motility parameters were recorded each 10 s for 1 min in terms of: A) TM, B) PM, C) VCL, D) VSL, E) ALH and F) BCF. The values plotted represent mean, continuous line represent 20°C and dashed line represent 28°C of the activation medium. Activation medium with 0 µS/cm is represented with a white circle, 700 µS/cm with a dark circle and 1200 µS/cm without the symbol.

Table 2.2 Statistical differences (P values) of AB and *casper* zebrafish lines sperm motility when activated with different water temperatures throughout post-activation time.

Zebrafish line	AB						*Casper*					
Time post-activation	10	20	30	40	50	60	10	20	30	40	50	60
TM (%)	0.053	0.466	0.965	0.221	0.021*	0.019*	0.482	0.551	0.739	0.342	0.010*	0.002*
PM (%)	0.028*	0.067	0.140	0.301			0.611	0.734	0.908	0.323	0.312	0.323
VCL (μm/s)	0.134	0.157	0.133	0.703	0.670	0.083	0.391	0.706	0.078	0.003*	0.041*	0.028*
VSL (μm/s)	0.123	0.098	0.118	0.056	0.009*	0.041*	0.520	0.878	0.100	0.182	0.062	0.025*
VAP (μm/s)	0.089	0.125	0.117	0.268	0.053	0.064	0.449	0.782	0.231	0.026*	0.075	0.031*
LIN (%)	0.504	0.255	0.384	0.009*	0.024*	0.061	0.890	0.734	0.479	0.750	0.210	0.009*
STR (%)	0.812	0.277	0.366	0.034*	0.016*	0.061	0.783	0.443	0.799	0.563	0.088	0.023*
WOB (μm)	0.062	0.097	0.426	0.020*	0.092	0.073	0.985	0.325	0.249	0.253	0.066	0.016*
ALH (μm)	0.133		0.063	0.832	0.440	0.145	0.323	0.442	0.396	0.086	0.676	0.316
BCF (Hz)	0.192	0.810	0.555	0.114	0.013*	0.022*	0.989	0.416	0.764	0,603	0.692	0.650

Significant differences (independent samples t-test, $P<0.05$) are represented with an asterisk

Table 2.3 Statistical differences (P values) of water conductivity on zebrafish sperm motility parameters.

Zebrafish line		AB			*Casper*		
Motility	Conductivity (µS/cm)	0	700	1200	0	700	1200
	0		0.204	0.007*		0.437	0.999
TM (%)	700	0.204		0.505	0.437		0.999
	1200	0.007*	0.505		0.999	0.999	
	0		0.069	0.013*		0.999	0.999
PM (%)	700	0.069		0.999	0.999		0.999
	1200	0.013*		0.999	0.999	0.999	
	0		0.999	0.999		0.999	0.114
VCL (µm/s)	700	0.999		0.680	0.999		0.688
	1200	0.999	0.680		0.114	0.688	
	0		0.999	0.999		0.362	0.471
VSL (µm/s)	700	0.999		0.913	0.362		0.999
	1200	0.999	0.913		0.471	0.999	
	0		0.999	0.999		0.551	0.447
VAP	700	0.999		0.675	0.551		0.999
	1200	0.999	0.675		0.447	0.999	
	0		0.999	0.999		0.999	0.999
LIN (%)	700	0.999		0.999	0.999		0.999
	1200	0.999	0.999		0.999	0.999	
	0		0.999	0.999		0.588	0.935
STR (%)	700	0.999		0.999	0.588		0.999
	1200	0.999	0.999		0.935	0.999	
	0		0.999	0.999		0.663	0.581
WOB (%)	700	0.999		0.999	0.663		0.999
	1200	0.999	0.999		0.581	0.999	
	0		0.423	0.999		0.999	0.768
ALH (µm)	700	0.423		0.867	0.999		0.710
	1200	0.999	0.867		0.768	0.710	
	0		0.999	0.354		0.999	0.999
BCF (Hz)	700	0.999		0.483	0.999		0.999
	1200	0.354	0.483		0.999	0.999	

Significant differences (Repeated measures ANOVA, post hoc Bonferroni, $P<0.05$) are represented with an asterisk.

2.1.4.2. Experiment 2 – Effect of the water conductivity on in vitro fertilization

To understand the effect of water conductivity (0, 700 and 1200 µS/cm) not only on sperm motility activation but also in *vitro* fertilization, 5 sperm pools of AB

line were analyzed with activation medium at 28°C. The activation medium conductivity had a significant effect on TM and PM, where 0 µS/cm improved these parameters when compared to 1200 µS/cm. However, at 0 µS/cm TM and PM were not significantly different when compared to 700 µS/cm (Figure 2.1.6 A and B). Sperm velocities showed no significant differences between treatments (Figure 2.1.6 C and D). Supplementary figure 2.2 illustrates the effect of water conductivity on sperm motility on the same sperm pool, additionally, the respective sperm motility videos are available as supplementary data for 0 (Video S1), 700 (Video S2) and 1200 µS/cm (Video S3).

No significant differences were observed on any of the biological performance of larvae resulting from *in vitro* fertilization using different water conductivities (Figure 2.1.7 and Table 2.4). Sperm activated with 0, 700 and 1200 µS/cm during *in vitro* fertilization did not show significant differences in parameters such as embryo survival at 24 hpf, hatching rate (Figure 2.1.7), hatched larvae at 48 hpf and at 72 hpf, malformed hatched larvae (Table 2.4) and larvae length at 120 hpf.

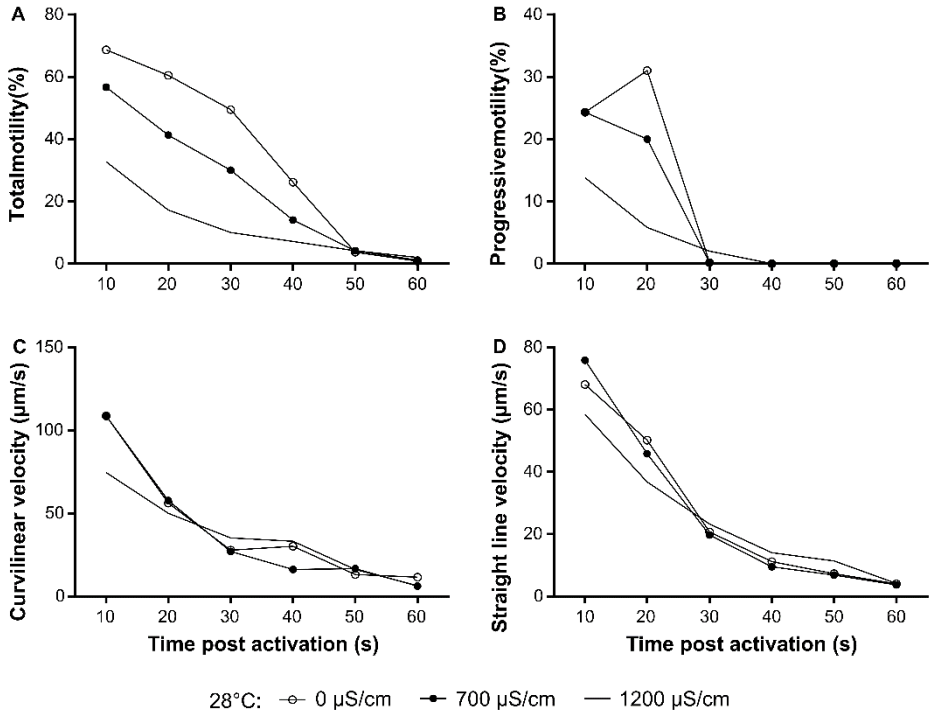

Figure 2.6 Effect of water conductivity (0, 700 and 1200 µS/cm) on sperm motility parameters of zebrafish AB line pools of sperm (n=5 containing sperm of 7 males). Sperm was activated, and motility parameters were recorded each 10 s for 1 min in terms of: A) TM, B) PM, C) VCL and D) VSL. The values plotted represent mean. Activation medium with 0 µS/cm is represented with a white

circle, 700 µS/cm with a dark circle and 1200 µS/cm without the symbol. Different letters represent statistical differences (one-way ANOVA, post hoc SNK P<0.05).

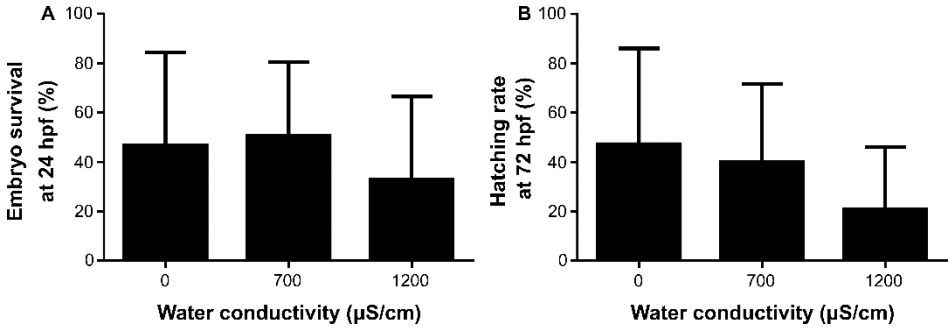

Figure 2.7 *In vitro* fertilization performed with sperm pools (n=5 pools) of AB zebrafish line activated with water conductivities 0 (n=11 clutches) 700 (n=13 clutches) and 1200 µS/cm (n=11 clutches). The fertilization success was measured through: A) embryo survival at 24 hpf, B) hatching rate at 48 hpf, C) total hatching rate at 72 hpf. Additionally, it was evaluated D) malformed larvae at hatching and larvae standard length (5 dpf). The values plotted represent means±SD.

Table 2.4 Biological performance of larvae sired by the sperm of AB (n=8) zebrafish line, activated with different water conductivities.

Water conductivity	0 µS/cm	700 µS/cm	1200 µS/cm
Hatched larvae at 48 hpf (%)	14.16 ± 18.74	5.27 ± 8.34	5.76 ± 15.01
Hatched larvae at 72 hpf (%)	30.13 ± 28.92	35.28 ± 26.86	11.80 ± 15.01
Malformed hatched larvae (%)	13.86 ± 16.43	12.89 ± 12.24	15.75 ± 20.76
Larvae length at 120 hpf (mm)	3.16 ± 0.08	3.15 ± 0.11	3.16 ± 0.08

2.1.5. Discussion

Each species is adapted to specific environmental conditions, where cellular metabolism functions run under optimal conditions. This fact is also true for teleost's spermatozoa cellular metabolism, especially since they are released into an external hazardous environment. Zebrafish is a freshwater species adapted to warm shallow waters of rivers and ponds (Arunachalam et al., 2013). However, this species is established in research centers with higher water conductivities (400 to 1500 µS/cm) when compared to its natural environment (10-270 µS/cm) (Arunachalam et al., 2013). This is a prophylactic measure to control pathogens adapted from aquaculture (Fashina-Bombata and Busari, 2003; Martins et al., 2016)

considering this species high tolerance to salinity variations (Boisen et al., 2003; Uliano et al., 2010), however there are no studies to our knowledge on its effectiveness. Moreover, zebrafish sperm motility activation is routinely performed with distilled water without temperature control. Altogether, the variable environmental conditions used in zebrafish can be a relevant biases source in this species research.

Sperm motility is the most studied sperm quality parameter, it is commonly used as a tool to select and characterize sperm samples and its ability to fertilize the oocyte. However, sperm motility is dependent on several factors that modulate its activation, duration and motility such as medium osmolarity (Alavi and Cosson, 2006), temperature (Dadras et al., 2017), pH (Alavi and Cosson, 2005), ions (Alavi and Cosson, 2006) and presence of ovarian fluid (Butts et al., 2017; Diogo et al., 2010). Spermatozoa motility depends on the energy released with ATP hydrolysis to produce flagellum beating (Alavi and Cosson, 2005). Water temperature affects motility characteristics (Dadras et al., 2017) and dynein motors of the flagellum (Cosson et al., 2008a).

Progressive motility is considered to be the parameter most related to sperm fertilizing ability (Rurangwa et al., 1998). In our study, we observed at 10 s post activation that 28°C showed significantly higher progressive motility when compared to 20°C in the AB line. The duration of total motility and the forward movement are reported to decrease when the temperature of the activation medium increases, along with initial beat frequency (Alavi and Cosson, 2005). However, this was not observed in the AB line since spermatozoa activated with 28°C do not show earlier loss of motility in comparison to 20°C. Zebrafish optimal rearing temperature is 28°C (Avdesh et al., 2012), consequently, the results obtained with our study are in agreement with this species physiological adaptation to warm temperatures (28°C). Our work suggests that 28°C can be the optimal temperature for an enzymatic activity to produce ATP and flagellum beating. The optimal environmental temperature at which this species is adapted to (28°C) is therefore especially relevant in the last seconds of motility, to prolong sperm longevity and improve fertilization. Therefore, the use of controlled water temperature (28°C) in zebrafish rearing, sperm motility analysis and during *in vitro* fertilization

procedures is essential to reduce variability and improve research replicability in this species.

Osmolarity is one of the major factors contributing to sperm motility activation through cell signaling cascade (Morisawa et al., 1983), where the hyposmotic shock causes plasma membrane alterations that promote flagellar beating (Krasznai et al., 2000). In zebrafish rearing systems, the ions are added automatically to the system water and the high variability of water conductivities among zebrafish facilities (Avdesh et al., 2012) may interfere with the sperm activation mechanism. The osmolarity of the activation medium influences the number of spermatozoa flagellum waves and curvatures (Alavi et al., 2009). This fact was observed in marine species such as Atlantic cod (*Gadus morhua*, Linnaeus, 1758), European sea bass (*Dicentrarchus labrax*, Linnaeus, 1758) and hake (*Merluccius merluccius*, Linnaeus, 1758) (Cosson et al., 2008a; Cosson et al., 2008b). In opposition, our study in a freshwater species showed that higher osmolarity decreased motility parameters in the AB line. In our work, sperm activated with distilled water at 20°C showed low motility parameters in this line, however, there is an interaction between water temperature and conductivity. In fact, spermatozoa motility parameters were improved throughout their lifespan when activated with distilled water at 28°C. In our study, the longer spermatozoa motility duration is observed at 28°C with 0 and 700 µS/cm of system water. Therefore, our data indicate that this species is physiologically well adapted for high water temperatures with low conductivities.

Mutant *casper* line was used as an example of lines that are considered more vulnerable when compared to wild type lines (Lawrence, 2016). Overall, the motility parameters of this line were lower when compared to the wild type AB line, especially velocities, total and progressive motility. In *casper line*, the sperm activation medium temperature has significant interaction with post activation time, both in sperm total motility and curvilinear velocity. *Casper* results from the conjugation of *nacre* line, that harbors an inactivating mutation of the *mitfa* gene impairing melanocyte pigmentation (Lister et al., 1999), and the *roy* mutation (Ren et al., 2002). The gene responsible for *roy orbison* phenotype remained unknown until recently. *Roy orbison* mutation causes a loss of function of the mitochondrial

inner membrane protein *mpv17*, due to a perturbation of the first intron of *mpv17* and consequent aberrant splicing (D'Agati et al., 2017). Therefore, *casper* has a loss of function of the mitochondrial inner membrane protein *mpv17* that causes a reduction in mitochondrial folate pools (Alonzo et al., 2018), which impairs oxidative phosphorylation (Morscher et al., 2018). Mitochondrial metabolism disorders are known to affect sperm motility (Demain et al., 2016), which is in agreement with our observations. Zebrafish sperm relies on oxidative phosphorylation in the last seconds of motility (Ingermann et al., 2011), which is defective in *casper* line. Therefore, *casper* is highly dependent on the adequate sperm activation medium temperature to optimize oxidative phosphorylation for energy production and extend their lifespan, improving thus the probability of oocyte fertilization. Although the effect of water conductivity was clear in AB line, in *casper* line there was a lack of response to different water conductivity environments. The characterization of sperm quality in zebrafish lines is a matter poorly addressed and our study evidences the high relevance of this investigation.

The results obtained in our first experiment were potentially relevant to improve zebrafish *in vitro* fertilization conditions. Despite the fact that in our second experiment the *in vitro* fertilization parameters had no significant differences, it is interesting to notice that 1200 µS/cm is the treatment with lower hatching rate. In addition, this treatment showed a high percentage of malformed hatched larvae, although not significant. The embryos resulting from all treatments showed a delay in hatching (Table 2.4) in relation to the standard development (Kimmel et al., 1995). This fact can be a stress-related consequence of assisted reproduction methodologies (Ramos-Ibeas et al., 2019). Therefore, we suggest that zebrafish should be reared at 28°C and low water conductivity (0-700 µS/cm). It is striking that the sperm motility analysis should be performed with system water to avoid biased studies. Moreover, for *in vitro* fertilization purposes, 700 µS/cm of system water at 28°C is the most adequate environmental conditions for sperm motility activation on zebrafish facilities. These conditions will avoid overestimation of sperm motility in relation to natural spawns and to *in vitro* fertilization performed with system water.

Our work emphasizes not only the necessity to standardize sperm motility activation methodology and zebrafish water conductivity in the systems, but also allows the comprehension that different zebrafish strains have specific reproductive features. As in *casper* line, other zebrafish lines have similar lesions in *mpv17*, such as *transparent* (*tra*^{b6}) (Krauss et al., 2013) spontaneous mutant. The specific reproductive features of zebrafish lines are essential to consider while planning experimental design and assisted reproduction methods, such as sperm cryopreservation. Additionally, the use of zebrafish lines with metabolic disorders can be a powerful tool for the comprehension of their consequences on reproductive performance.

The most suitable temperature for zebrafish to breed is 28°C, coinciding with this species natural environmental adaptations. It is striking that sperm motility activation should be performed with system water to avoid the overestimation of sperm motility obtained when the activation is performed with distilled water. These guidelines contributed not only to the improvement of the sperm motility as a tool to predict spermatozoa fertilizing ability under natural spawning conditions, but most importantly support the standardization of zebrafish husbandry procedures and analysis.

Chapter 3. BROODSTOCK NUTRITION

PREAMBLE

Broodstock nutrition has direct implications on teleosts gamete quality and on the resulting progeny health. This fact is well studied in many aquaculture species; however, in zebrafish, the dietary effect on reproduction only recently started to be a focus of attention. Zebrafish nutritional requirements are still poorly understood, therefore a deeper understanding is required. Dietary phospholipids are an important source of energy in teleosts. Moreover, phospholipids are relevant sources of fatty acids involved in fish growth and reproduction. Altogether, the lack of knowledge on zebrafish nutritional requirements and the lack of standardized feeds in this species can lead to variable sperm quality and post-thaw results.

It is increasingly evident the necessity to use a standardized diet to improve experimental reproducibility among the facilities dedicated to zebrafish research. The effect of dietary phospholipids was assessed with purified diets fed to zebrafish. The dietary supplementations were evaluated in terms of zebrafish growth, gamete quality, reproductive performance and offspring skeletal malformations to understand the overall effect on fish fitness. This chapter represents an article published in the Journal of Applied Ichthyology first authored by Patricia Diogo. This work was performed under the ZEBRAFEEDS project, which resulted in the development of a standardized diet for zebrafish by SPAROS Lda, now commercially available worldwide.

3.1. ASSESSMENT OF NUTRITIONAL SUPPLEMENTATION IN PHOSPHOLIPIDS ON THE REPRODUCTIVE PERFORMANCE OF ZEBRAFISH, *Danio rerio* (Hamilton, 1822)

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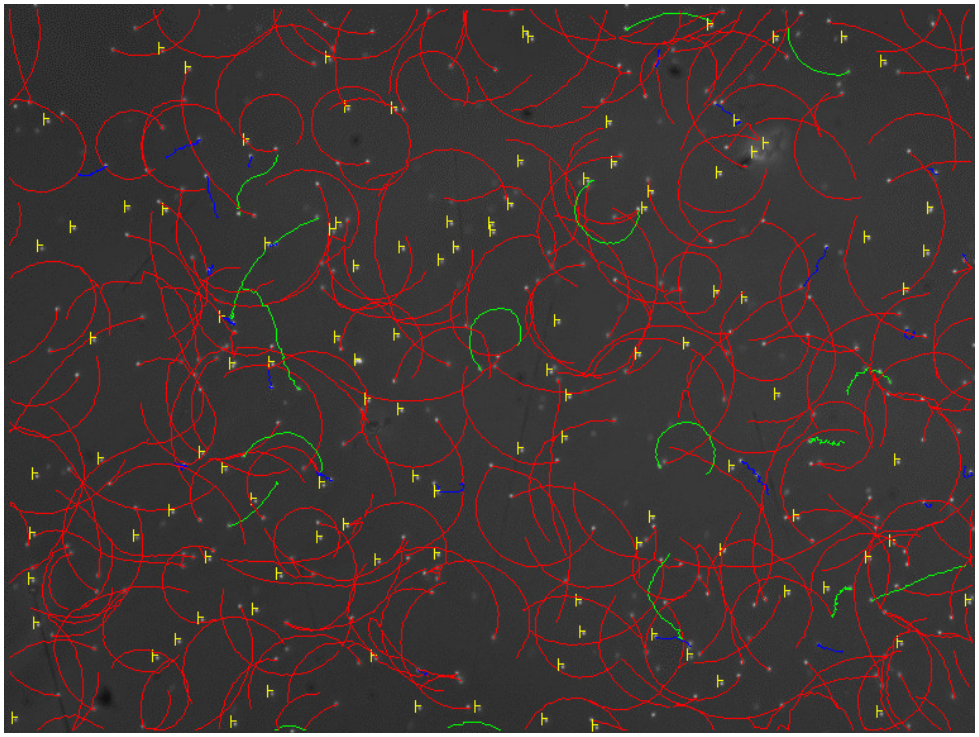

[J Appl Ichtyol 2015, 31(S1): 3-9]

3.1.1. Abstract

The objectives of this study were to determine the effects of a tailor-made purified diet (PUR) supplemented with phosphatidylcholine (PC) or phosphatidylethanolamine (PE) on the reproductive performance, gamete quality, embryo development and growth as well as skeletal malformations of the larvae in zebrafish, *Danio rerio*. The PUR diet was tested against a commercial diet as a negative control (CD). For each diet, one experimental group of eight adult zebrafish (three males, five females) was maintained in 3 l tanks in a water recirculating system under continuously controlled conditions (photoperiod 14 h L: 10 h D, water flow rate 7.3 l/h 1; 28°C; pH 7.5; 700 µS/cm). Two trials were performed to confirm the results. The CD treatment resulted in the highest final weight of the breeders (0.60±0.18 g); however, the fish failed to spawn. The PC supplemented diet promoted a reasonable final weight of the breeders (0.48±0.10 g) similar to the CD and with a mean hatching rate of 93.80±6.9%. Nevertheless, the PC treatment induced the highest rate of skeletal malformations in progeny (82.40±4.1%), which could be related to the low larval survival at 28 days post-fertilization (dpf) (48.00±1.3%). In comparison to the control groups, broodstock fed the PE diet showed the best results in sperm quality, which was revealed by higher total and progressive motility and higher velocities than sperm from males fed the CD and PUR diets. Furthermore, the PE group produced significantly higher egg diameters (1.20±0.05 mm) when compared to the PUR diet (1.14±0.03 mm). This study highlights the importance of phospholipids in zebrafish gamete quality, and that supplying PE in broodstock diets can improve the reproductive performance of zebrafish

3.1.2. Introduction

Zebrafish is an established model species and a powerful tool to study the modulation of reproductive processes through broodstock nutrition. It has already been used successfully to assess the role of specific nutrients such as fatty acids and vitamins on the reproductive performance of other zebrafish (Jaya-Ram et al., 2008; Miller et al., 2012). The role of broodstock nutrition is increasingly considered to be an essential factor to optimize fitness and reproductive ability of the breeder and

improve gamete quality, ensuring higher fertilization rates and an adequate larval development (Izquierdo et al., 2001). However, feeding fish larvae is still challenging, remaining a major concern and bottleneck in marine aquaculture production. This is mainly due to the poor knowledge of larval nutritional requirements and digestive physiology, which complicates the adaptation to inert microdiets (Rønnestad et al., 1999). The quality of maternal nutrition is directly related to good larval development during the endogenous feeding period (Rainuzzo et al., 1997). Broodstock nutrition is highly relevant for gonad growth, fecundity (Watanabe, 1985) and fertilization rates (Fernández-Palacios et al., 1997), where lipids are important nutrients (Watanabe, 1985). In both marine and freshwater fish, the phospholipids are abundant in the spermatozoa membranes and include phosphatidylcholine (PC) and phos- phatidylethanolamine (PE) (Drokin, 1993a). In European sea bass (*Dicentrarchus labrax*) spermatozoa membrane, PC and PE represent about 50 and 40%, of these substances, respectively (Martínez-Páramo et al., 2012a), and accounts for 57 and 12% of the total yolk lipid content, respectively, in halibut egg yolk PC and PE (Rønnestad et al., 1995). This high content in the plasma membrane highlights the importance of supplying these nutrients in the zebrafish diet to improve sperm quality, enhance reproduction and support progeny development. The essential fatty acids present in phospholipids are required for embryo cell membrane development, which is vital for successful larval development (Migaud et al., 2013).

The composition of essential fatty acids in broodstock diets and their metabolites can play a physiological role in the reproductive physiology, steroid production and oocyte maturation, as observed by (Sorbera et al., 2001) in European sea bass, where arachidonic acid metabolism seems to participate in oocyte maturation, and thereby suggesting the involvement of other polyunsaturated fatty acids (PUFAs) and prostaglandins in the oocyte maturation process. Furthermore, the broodstock dietary composition in fatty acids can alter the sperm fatty acid composition, as demonstrated in several fish species such as rainbow trout (*Oncorhynchus mykiss*) (Labbé et al., 1995), European sea bass (*Dicentrarchus labrax*) (Asturiano et al., 2001) and Eurasian perch (*Perca fluviatilis*) (Henrotte et al., 2010). In European eel (*Anguilla anguilla*) sperm, PC has a protective effect against osmotic and cold stress (Asturiano, 2008). The fatty acid

contents present in PC and PE are variable, depending on the species. Thus, since the ratio of polyunsaturated to saturated fatty acids in PC does not differ in marine and fresh water species, in PE this ratio is generally higher in sperm membrane of marine than in freshwater species (Drokin, 1993b). Furthermore, PC has been mentioned as having an important role in sperm cryopreservation, acting as a protective agent and increasing cryoresistance (Cabrita et al., 2010). In fish spermatozoa the plasma membrane is responsible for the reception of the environmental stimulus, triggering responses such as motility activation (Cabrita et al., 2008). Plasma membrane is composed of a lipid bilayer highly sensitive to environmental stress (Cabrita et al., 2008), and its composition and integrity is essential to ensure sperm functionality (Lahnsteiner et al., 2009), and to guarantee correct interaction spermatozoa–egg, necessary for the successful fertilization process (Hart, 1990). The PUFAs influence spermatozoa cell membrane fluidity, whereas permeability to water and ions is determined by membrane lipids and proteins (Bobe and Labbé, 2010).

The objectives of the study were to determine the influence of specific phospholipids (phosphatidylcholine, phosphatidylethanolamine) supplemented in purified diets specifically formulated for broodstock zebrafish feeding, with the aim to modulate broodstock reproductive performance, gamete quality and larval growth and skeletal quality.

3.1.3. Methods

3.1.3.1. Experimental diets

Four dietary treatments, chemically controlled formulated with purified ingredients, were tested in this experiment. As a control diet, a high quality commercial marine fish larval diet (CD; GEMMA Micro 300; Sketting) with 60% crude protein, 14% fat and known to contain high levels of marine proteins and marine phospholipids that had been commonly used to feed zebrafish. A purified diet (PUR) was used as an experimental control, containing 60% crude protein and 12% crude fat (Table 3.1). For dietary supplementation, phosphatidylcholine (PC; 5 g/Kg) and phosphatidylethanolamine (PE; 5 g/Kg) were added to the PUR diet.

Table 3.1 Dietary and chemical composition of the purified diet (PUR) fed to zebrafish broodstock and larvae

Ingredient, %	PUR
Fish gelatin	16.0
Casein	40.0
Wheat gluten	5.0
Dextrine	14.3
Cellulose	4.1
Fish oil	11.5
Vit & Min Premix PV1	2.0
Betaine	0.1
Soy lecithin	0.2
Binder (sodium alginate)	2.0
Antioxidant	0.2
MCP	2.7
L-Lysine	1.0
DL-Methionine	0.5
L-Arginine	0.4
Proximate composition	
Crude protein (g/Kg DM)	60.4
Digestible protein (g kg-1 DM)	56.2
Crude fat (g/Kg DM)	12.1
Fiber (g/Kg DM)	0.0
Starch (g/Kg DM)	14.5
Gross Energy (Kj/g DM)	21.2

3.1.3.2. Fish rearing and sampling

Young adult zebrafish (3 months of age) were selected according to similar size and separated into four groups each composing three males and five females. The experiment was repeated in two trials. For each experimental group, males and females were isolated in 3 l tanks connected to a water recirculation system with 980 l of water containing a ceramic biofilter and mechanical filtration (50 μm), UV sterilization and a granular activated carbon filter (Tecniplast ZebTEC). The rearing system water was partially (10%) replaced daily and the temperature kept at 28.5±0.5°C, 700±50 μS/cm and pH 7.5±0.1. After 2 days of acclimation, each experimental group was fed *ad libitum* twice a day for 68 days, with each

corresponding experimental diet. Food consumption was visually controlled, and any remains were removed daily. The broodstocks were sampled to determine the weight and standard length. length at the beginning (day 1), middle (day 25) and end (day 68) of the experiment. After 3 weeks of experimental feeding, fish from each group were regularly mated every week to guarantee a washout of matured eggs originating from prefeeding conditions. Embryos from two successful spawns per trial of each broodstock were collected and incubated in 1 l of system water (28.5±0.5°C, 700±0 µS/cm and pH 7.5±0.1) containing 50 ppt of methylene blue (Sigma-Aldrich, Saint Louis, MO) in static conditions with A daily renewal of 95% water volume. Egg diameter and perivitelline space (distance from the egg membrane to the embryo) were measured 2 h (64-cell stage) after fertilization (n=20) and hatched larvae (n=20) were sampled for standard length and dry weight at 5, 10 and 28 days post-fertilization (dpf). Measurements were performed through photographic images of samples (Canon power shot G12, Japan) using a stereomicroscope (Leica MZ6, Germany), and posterior analysis in AXIO VISION software (Carl Zeiss, Germany). The larvae dry weight was determined after dehydration for 24 h at 60°C.

In order to evaluate larvae skeletal deformities, 20 larvae per treatment were sampled at 28 dpf, anesthetized with a lethal dose of MS-222 (Sigma-Aldrich) and stained. For that purpose, larvae were washed with a phosphate buffer saline 0.1 M, pH 7.4 solution and stored in 75% ethanol. The acid-free double stain protocols were done using alcian blue 8GX for cartilage detection and Alizarin red S (both from Sigma-Aldrich) for bone detection (Walker and Kimmel, 2007b). Samples were stained in alcian blue for 1.5 h and stained overnight with alizarin red S in a 1% KOH solution. The tissues were cleared with a 0.5% KOH solution. Samples were stored in a solution of 50% glycerol (Merk Millipore, Billerica, MA) at 20°C until the evaluation of the skeleton was performed.

After 68 days of feeding, to avoid stress during the mating trials zebrafish males from each treatment were sampled for sperm motility analyses at the end of the experiment. Males were anesthetized in tricaine methane sulfonate solution (MS-222) prepared according to (Westerfield, 2005), and sperm collected by an abdominal massage using a micropipette. 0.5 µl of sperm were placed on a Makler

chamber and activated immediately by mixing with 10 µl of water from the system. Motility parameters were recorded at 15 s after activation, using the CASA software (Proiser, Spain). Two replicates of each sperm sample were analyzed for each individual male (n=6 males per treatment) (Table 3.1). The images were captured with a Basler camera A312f (Basler Afc, Germany) and processed with the ISAS software (Proiser). TM (%); PM (%), VCL (µm/s), VSL (µm/s) and LIN (%) were determined to assess sperm quality in each treatment. Only those spermatozoa with VCL>10 µm/s were considered motile.

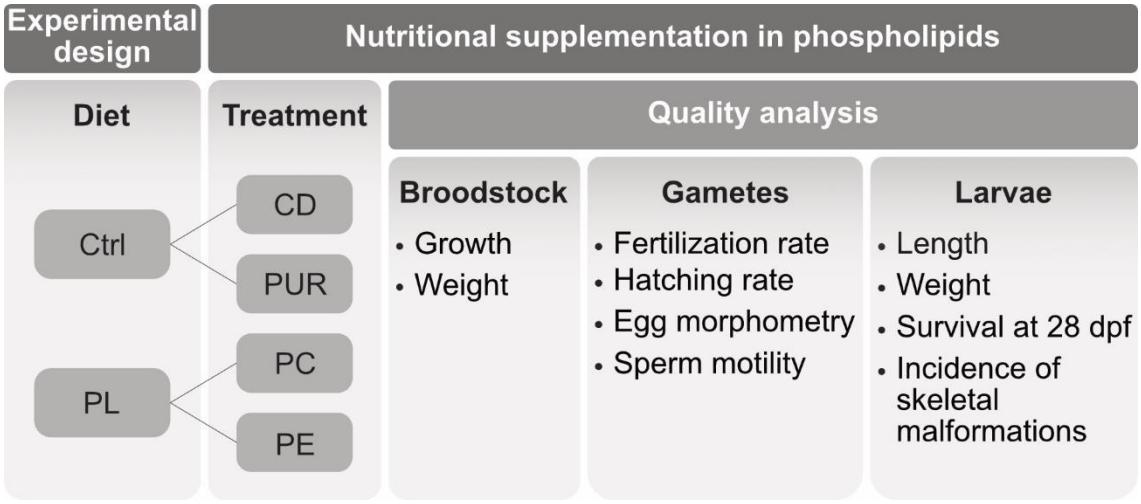

Figure 3.1 Experimental design to evaluate the effect of control diets (CD, PUR) and phospholipid (PL) supplemented diets (PC and PE) on zebrafish growth, reproduction, gametes quality and skeletal development.

3.1.3.3. Data analysis

SPSS 18.0 software was used for statistical analysis. Data were expressed as means±SD, and normalized by logarithmic or arcsine transformation when results were expressed as percentages. All data were subjected to one-way ANOVA at each sampling. Statistical differences between treatments were detected using Tukey´s multiple comparison tests ($P<0.05$).

3.1.4. Results

After 25 days from beginning the experimental feeding period, the broodstock fed commercial control diet (CD) revealed a significantly higher weight

than PUR and PE, but it was not significantly different from the PC treatment. At the end of the experiment (68 days), the final weight of the breeders fed the CD treatment was significantly higher than the PE treatment, but not significantly different from the PUR and PC treatments (Table 3.2). However, fish from the CD treatment did not reproduce successfully and no eggs were obtained. PUR treatment obtained an average of 173 eggs per spawn, while in PE the yield was 183 eggs, and 80 eggs per spawn in the PC treatment (Table 3.3). There were no significant differences in the standard length among the different broodstock groups throughout the experiment (Table 3.2).

Egg diameters were significantly higher in the PE treatment relative to PUR, but no different from the PC treatment (Figure 3.3 A). The PUR treatment results showed a significantly smaller perivitelline space (0.20±0.02 mm) in the developing egg (64 cells stage) compared to PC and PE treatments (Figure 3.3 B).

Table 3.2 Zebrafish broodstock mean standard length and mean wet weight for dietary treatments (CD, PUR, PC, PE) at experiment days 1, 25, and 68. Data expressed as means±SD (n=16 broodfish, including males and females).

	Broodstock standard length (mm)						Broodstock weight (g)					
Treatment	n	Day 1	n	Day 25	n	Day 68	n	Day 1	n	Day 25	n	Day 68
CD	16	26.3 ± 1.2	16	35.9 ± 2.0	16	36.4 ± 2.0	16	0.24 ± 0.03	16	0.53 ± 0.17^{a}	16	0.60 ± 0.18^{a}
PURE	16	27.6 ± 2.6	16	32.9 ± 3.2	16	36.3 ± 2.7	16	0.24 ± 0.06	16	0.40 ± 0.10^{b}	16	0.41 ± 0.09^{ab}
PC	16	27.2 ± 1.8	16	32.8 ± 2.4	16	35.5 ± 2.9	16	0.24 ± 0.05	16	0.45 ± 0.11^{ab}	16	0.48 ± 0.10^{ab}
PE	16	26.0 ± 1.8	16	30.4 ± 2.5	16	33.2 ± 2.5	16	0.24 ± 0.05	16	0.38 ± 0.14^{b}	16	0.38 ± 0.16^{b}

Different superscripts in the same column=significant differences between treatments (one-way ANOVA-Tukey, $P<0.05$).

Table 3.3 Total number of fertilized eggs, hatched larvae, larvae hatching rate, larvae standard length (5, 15 and 28 dpf) and survival for dietary treatments (CD, PUR, PC, PE).

					Larvae standard length (mm)						Larvae survival (%)	
Treatment	n	Total fertilized eggs	Total hatched larvae	Hatching rate (%)	n	5 dpf	n	15 dpf	n	28 dpf	n	Day 28
CD		–	–	–		–		–		–		–
PURE	4	173 ± 35.0	135 ± 36.0	76.7 ± 5.0	20	3.7 ± 0.2	20	4.8 ± 0.3	20	5.8 ± 0.5	4	61.9 ± 13.7
PC	4	80 ± 26.0	75 ± 16.0	93.8 ± 6.9	20	4.0 ± 0.1	20	4.8 ± 0.3	20	6.2 ± 0.9	4	48.0 ± 11.3
PE	4	183 ± 86.3	145.7 ± 64.6	81.1 ± 7.8	20	3.9 ± 0.2	20	4.8 ± 0.2	20	5.5 ± 0.8	4	62. 5 ± 17.2

Different superscripts in the same column=significant differences between treatments (one-way ANOVA-Tukey, $P<0.05$).

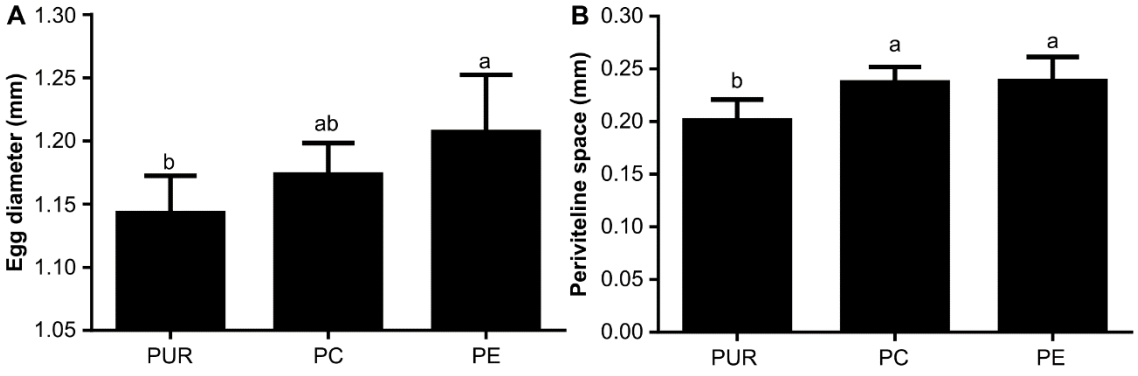

Figure 3.2 Effect of dietary treatments (CD, PUR, PC, PE) on zebrafish (64 cell stage) A) egg diameter and B) perivitelline space. Columns represent means±SD deviation (n=20 eggs). Different letters represent significant differences (one-way ANOVA-Tukey, $P<0.05$).

Sperm total motility, progressiveness, and curvilinear and straight-line velocities were significantly improved by PE treatment when compared to PUR and CD treatments, but the values obtained were not significantly different from the data of the PC treatment (Figure 3.3 A–D), 15 s after activation. The PC supplemented diet resulted in higher curvilinear velocity of spermatozoa than those obtained in the CD test (Figure 3.3 C). Straight-line velocity was also improved by the PE treatment at 15 s post-activation when compared to data from the PUR and CD treatments; however, it was not significantly different from the PC treatment (Figure 3.3 D). Linearity did not reveal any significant differences among treatments (Figure 3.3 E).

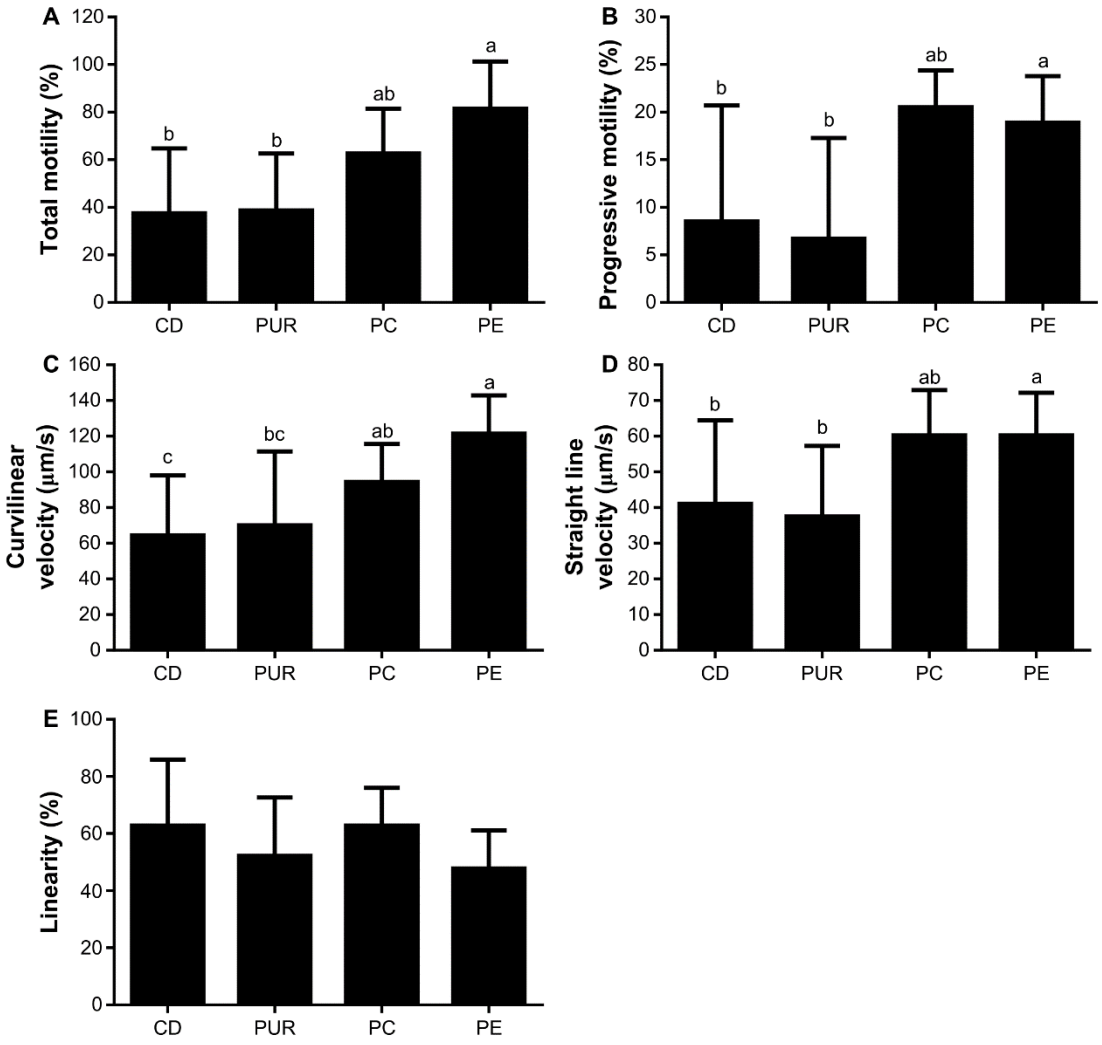

Figure 3.3 Zebrafish breeders: effect of dietary treatments (CD, PUR, PC, PE) on sperm quality after 68 days of feeding. Figures represent average sperm motility parameters: A) TM, B) PM, C) VSL, D) VCL, and E) LIN. Columns represent means±SD bars (n=6 males). Different letters represent significant differences (one-way ANOVA-Tukey, $P<0.05$).

Although there were no significant differences, the highest hatching rate was observed for PC the treatment, followed by progeny obtained from PE and PUR-treated broodfish (Table 3.3). The highest larval survival rate observed was for fish derived from the PE treatment, followed by PUR and PC treatments, without statistical differences (Table 3.3). There were no significant differences in larval standard lengths among treatments ($P<0.05$) throughout the experiment (Table 3.3). Larvae dry weight showed no significant differences among treatments at 5 and 15 dpf. However, at 28 dpf larvae from PE treatment had significantly higher dry

weights when compared to larvae from the PUR treatment, although the data were not significantly different from the PC test (Figure 3.4).

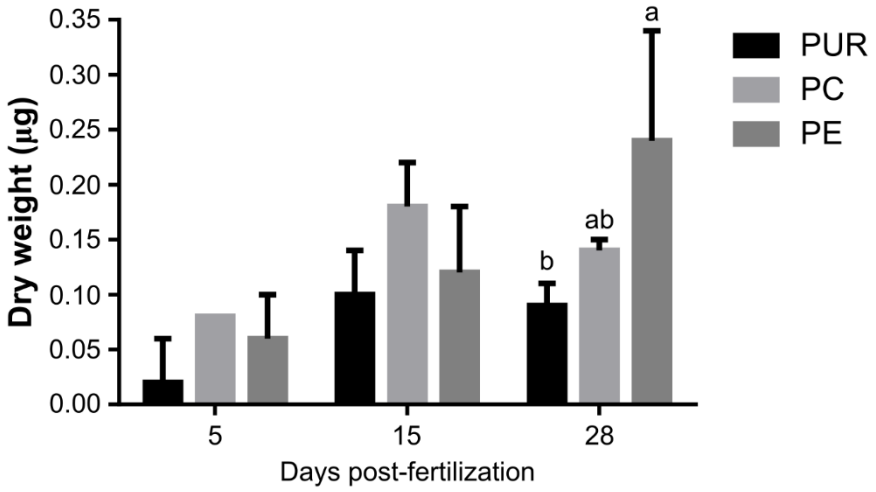

Figure 3.4 Zebrafish larvae: dry weight at 5, 15 and 28 dpf for dietary treatments (CD, PUR, PC, PE). Columns represent means±SD (n=20). Different letters represent significant differences (one-way ANOVA-Tukey, P<0.05).

The PC treatment produced a significantly higher incidence of skeletal deformities in the progeny (as defined in Figure 3.5), when compared to those malformations in larvae from the PE and PUR treatments (Figure 3.5).

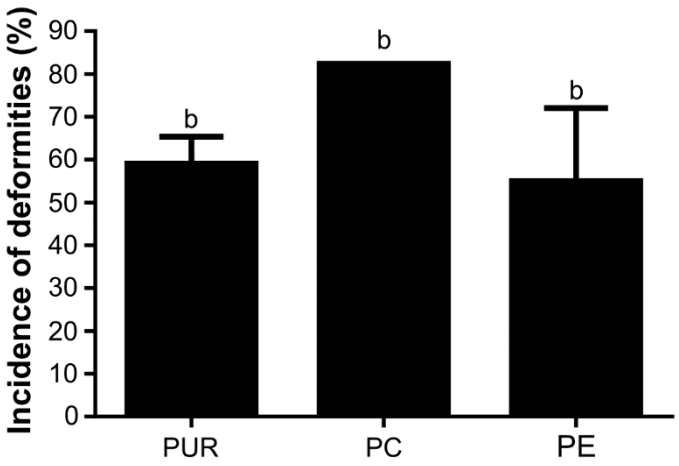

Figure 3.5 Effect of dietary treatments (CD, PUR, PC, PE) on zebrafish larvae skeletal deformities (28 dpf). Columns represent means±SD (n=20). Different letters represent significant differences (one-way ANOVA-Tukey, P<0.05) between treatments.

The main bone deformities observed in the PC treatment were fused and compressed caudal fin vertebrae (29–30 vertebrae) (Figure 3.6 A) and fused caudal and caudal fin vertebrae (Figure 3.6 B). Deformities in the caudal fin arches and the presence of ectopic elements were also observed (Figure 3.6 C).

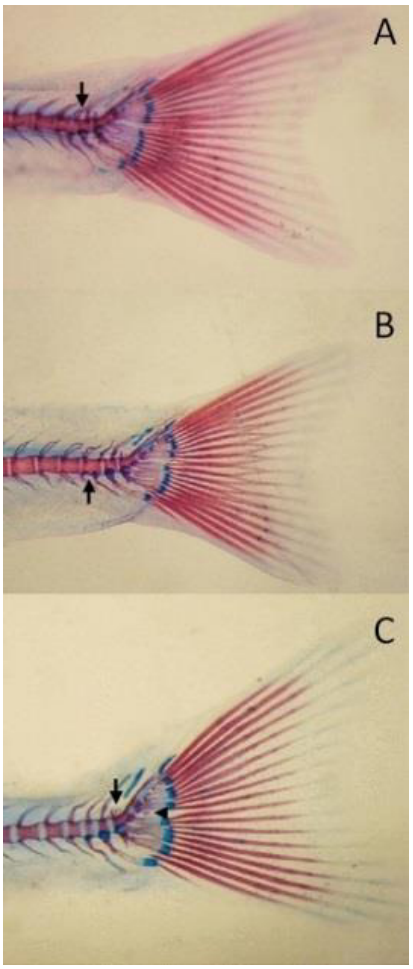

Figure 3.6 Deformities affecting the vertebral column of zebrafish at 28 dpf A) fused and compressed caudal fin vertebrae 28-29 (arrow), with malformed neural and haemal arches. B) Fused caudal and caudal fin vertebrae (arrow), with malformed neural and haemal arches. C) Presence of an ectopic element in the caudal fin (arrowhead) and ectopic arches over urostyle (arrow).

3.1.5. Discussion

Phospholipids and, in particular, fatty acids are the structural components participating in the regulation of plasma membrane properties. The phospholipids phosphatidylcholine and phosphatidylethanolamine and their plasmalogens are the most abundant forms present in the semen of both freshwater and marine fish species (Drokin, 1993b; Martínez-Páramo et al., 2012a).

Nutrition is known to be highly important for successful broodstock spawning (Izquierdo et al., 2001; Migaud et al., 2013). Specific diets are commonly supplied to broodstock in commercial aquaculture during the reproduction period to enhance reproductive potential and gametes quality (Fernández-Palacios et al., 1997). In our work, the use of a commercial diet (CD) led to a significant weight improvement however, at the expense of a dramatic reproductive impairment. The other treatments were formulated with purified ingredients to allow detailed control over its composition. The fatty acid composition of phospholipid classes determines the effect of these phospholipids. While PC is known to contain PUFAs (Izquierdo et al., 2000), PE has a higher proportion of docosahexaenoic acid (DHA) than PC, destined for membrane formation (Bruce et al., 1999). An adequate modulation of the n- 3/n-6 HUFA ratio in broodstock diets is known to improve the levels of arachidonic (ARA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids transferred to eggs, improving the hatching rate and progeny survival (Bruce et al., 1999). Although final weights of the PC and CD broodstock were not significantly different in the zebrafish, a major difference in spawning success was evident, indicating that particular nutrients such as phospholipids and essential fatty acids also have a great effect on their reproduction success. These results demonstrate the necessity of a dietary formula that fulfills the zebrafish broodstock nutritional requirements for growth and gametogenesis.

Using purified diets to study fish nutritional requirements is a powerful research tool since it allows precise and controlled manipulation of nutrients in the diet (Carvalho et al., 2006). Our study of the effects of PC and PE in the diet. resulted in a better understanding of their role in the reproductive process; this diet is an adequate tool to assist in the study of the nutritional requirements and dietary effects on gametes and progeny of zebrafish.

Fecundity and egg hatching success and larval quality are known to improve through optimization of the n-3 and n-6 long-chain polyunsaturated fatty acids (LC-PUFA) intake (Pavlov et al., 2004). In our study, the PC treatment group achieved the highest hatching rate when compared to the other treatments. PC supplementation is known to promote the highest growth rates in several fish species (Hamre et al., 2013) when compared with other phospholipid classes;

however, it is highly associated with the occurrence of skeletal deformities (Geurden et al., 19971998) as also observed in the results of the present study. PC is the main product of phospholipid synthesis in fish enterocytes, inducing enhanced lipoprotein synthesis that can be responsible for the growth promotion effect of PC through the energy flux increment from the intestinal mucosa into the blood (Hamre et al., 2013). In our work, larvae resulting from the PC supplemented parents showed no significant differences in growth when compared to the other treatments. However, a significantly higher incidence of skeletal deformities was observed, which may help to explain the lowest survival rates observed in this group. Since PC is an important source of essential fatty acids (DHA and EPA) for embryos (Hamre et al., 2013), the optimal PC demand in larvae should be assessed in future studies. Rønnestad et al. (1995) observed in larvae at 5 and 10 dpf a better growth performance with a PC supplemented diet, since at first feeding there is PC catabolism and PE net synthesis to body development, and consequently there is a high demand on PC. In our work at 28 dpf there was a dramatic improvement of larval dry weight in PE treatment, a fact that could be explained by the important role of this phospholipid on larval body development (Rønnestad et al., 1995). Lipid metabolism and turnover are associated with cell death initiation and progress (Tyurina et al., 2000) through signaling mechanisms, where cells expose PE on the cell surface in the early stages of apoptosis (Emoto et al., 1997). PE is the primary phospholipid reported to delay oxidative degradation in animal cells (Reiss et al., 1997), thus it may be responsible for better sperm quality. Larval survival rates of PE (62.5%) were also the highest when compared to the other treatments and to the work of Carvalho et al. (2006) (55%) using purified diets for zebrafish during larval growth.

The spermatozoa plasma membrane typically has a high content of polyunsaturated fatty acids, being thus more susceptible to lipid peroxidation caused by reactive oxygen species (ROS) (Nagasaka et al., 2004). This oxidative stress may compromise spermatozoa functionality (Lahnsteiner et al., 2009), since it affects membrane integrity, fluidity and permeability (Nagasaka et al., 2004). Furthermore, sperm motility in European sea bass (*Dicentrarchus labrax*) was found to be negatively correlated with lipid peroxidation and cholesterol/phospholipid ratio (Martínez-Páramo et al., 2012a). The broodstock dietary supplementation in

phospholipids seems to improve sperm total motility, progressiveness and velocity mainly in the first seconds of sperm motility. This fact could be due to the incorporation of these phospholipids in the spermatozoa membrane, as have been demonstrated in other species (Asturiano et al., 2001; Henrotte et al., 2010; Labbé et al., 1995). However, further studies are needed to confirm this hypothesis in our study.

It has been suggested by Pickova et al. (1999) that the dietary lipids during gonad maturation can alter fatty acid egg composition and disturb subsequent embryonic development. This could be observed in our work where PE diet supplementation improved the egg diameter and perivitelline space compared to the control (PUR). Both phospholipids are significantly similar, revealing that the maternal nutrient supplementation successfully enhances egg dimensions. Moreover, it is important for further studies to check the lipid and fatty acid profile of the zebrafish egg yolk as well as the spermatozoa plasma membrane. The perivitelline space develops after egg activation and is shown in zebrafish that a large perivitelline space is related to spawning quality, with unhealthy eggs having a narrow perivitelline space (Kwon et al., 2015; Otani et al., 2009). However, for a better understanding of egg quality, further biochemical analyses should be performed as a complement to the mor- phological parameters, perivitelline space and egg diameter (Cabrita et al., 2008).

In conclusion, PC fed fish showed good sperm quality parameters and the highest hatching rate of eggs; however, the survival, growth and incidence of skeletal deformities revealed some deleterious effects on the larvae. On the other hand, diets with PE promoted not only the production of good quality sperm but also did not affect the growth or quality of larvae. This work proves the importance of dietary control on the reproductive success of zebrafish and shows that supplementation in phospholipids may help to improve sperm and egg quality. Further studies are needed to understand the role of these phospholipids in larvae growth, which highlights the importance of different diet formulations in specific periods of the zebrafish life cycle for optimization of rearing.

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CHAPTER 4. MALE SELECTION CRITERIA

PREAMBLE

One of the major sources of variability in previous studies focusing on zebrafish reproduction was the high variability of gametes quality between males. This is a common issue in broodstocks, especially in teleost species. This fact is particularly relevant in terms of assisted reproduction techniques since they require high-quality gametes to avoid increased labor, animal manipulation and costs related to low-quality samples. The fact that assisted reproduction techniques can reduce the initial quality of gametes due to manipulation, refrigeration and/or cryopreservation, increases the necessity of the selection of high-quality breeders.

In addition, high-quality zebrafish breeders are valuable assets, especially from transgenic and mutant zebrafish lines. Therefore, the application of the 3R´s principles is essential in zebrafish facilities and a non-lethal sperm collection method that allows repeated non-invasive samplings is an important methodology to apply in the facilities. Male aging is considered a factor related to poor sperm quality. Moreover, the use of an inadequate sperm collection frequency can lead to low sperm sample quality in teleost species. Consequently, it was highly relevant to understand the time required for zebrafish males to recover baseline sperm quality after the first sperm collection.

The objective of this chapter was to evaluate the optimal broodstock conditions in terms of age and sperm stripping frequency related to the highest sperm quality to support sample donor's selection for cryopreservation. This chapter represents an article published in the Zebrafish journal first authored by Patricia Diogo.

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4.1. SELECTION CRITERIA OF ZEBRAFISH MALE DONORS FOR SPERM CRYOPRESERVATION

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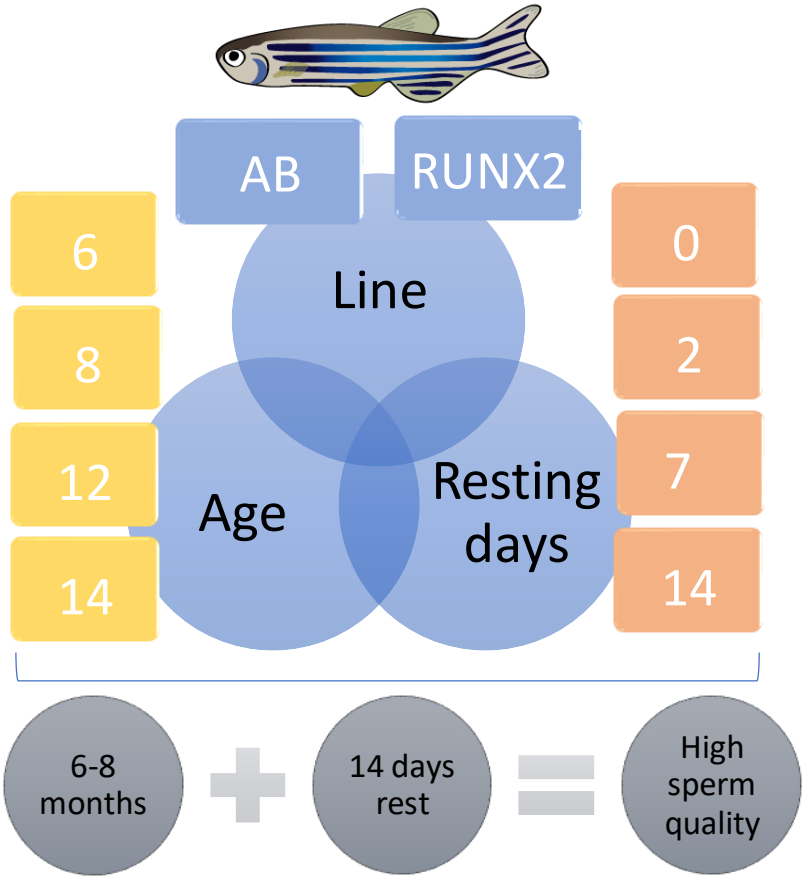

[Zebrafish 2019, 16 (2): 189-196]

4.1.1. Abstract

Selection criteria for sperm cryopreservation are highly relevant in zebrafish since sperm quality is particularly variable in this species. Successful cryopreservation depends on high-quality sperm, which can only be ensured by the selection of breeders. Consequently, male selection and management are a priority to improve cryopreservation, and therefore, this study aimed to characterize optimal age and sperm collection frequency in zebrafish. For this purpose, males from wild-type (AB) and from a transgenic line (Tg*(runx2:*eGFP*)*) were sampled at 6, 8, 12 and 14 months. For each age, sperm was collected at time 0 followed by samplings at 2, 7 and 14 days of rest. Sperm quality was assessed according to motility and membrane viability parameters. The quality assessment showed that Tg*(runx2:*eGFP*)* displayed significantly higher motility than AB and younger males showed higher motility in both lines. Sperm collection frequency affected membrane viability. While AB fish recovered sperm viability after 14 days of rest, Tg*(runx2*:eGFP*)* could not recover. Consequently, it may be important to study the sperm quality of each zebrafish line prior to sperm cryopreservation. Taking into consideration the results achieved in both lines, sperm collection should be performed between 6 to 8 months of age with a minimum collection interval of 14 days.

4.1.2. Introduction

Successful cryopreservation depends on several factors, the selection of high-quality sperm is one of the most important since the freezing process induces damage that decreases sperm quality significantly (Cabrita et al., 2010). Since high-quality sperm is related to high-quality breeders (Cabrita et al., 2010), the selection of male donors is essential for broodstock management and cryopreservation programs. Zebrafish is an established model species maintained in laboratories worldwide and extensively used in numerous research fields, including biological and biomedical research (Howe et al., 2013; Lieschke and Currie, 2007). As a consequence, in the past years, abundant and valuable wild-type, mutant and transgenic zebrafish lines were established, posing problems in terms of space and management. To solve this issue, sperm cryopreservation can be used to support

zebrafish facility management and to safeguard all those valuable genetic resources (Hagedorn et al., 2012). Zebrafish sperm cryopreservation was achieved for the first time more than 30 years ago by Harvey et al. (1982). However, until today, the most relevant issue for successful and reproducible results using cryopreservation is the lack of methodological standardization among laboratories and rearing facilities, which translates into high variability in post-thaw sperm quality and *in vitro* fertilization success.

Sperm quality is defined by the ability of sperm to successfully fertilize an egg (Bobe and Labbé, 2010; Migaud et al., 2013), which is dependent on factors such as heritage (Hansen and Price, 1999), spermiation period, favorable environmental conditions for activation of sperm motility (Pizzari et al., 2008), parental age (Gasparini et al., 2010; Kidd et al., 2017) and sperm output frequency.

It has been reported that the age of males affects both sperm production and quality (Gasparini et al., 2010; Johnson et al., 2015), resulting in lower reproductive success. This phenomenon is associated to the accumulation of *de novo* mutations in germ cells (Hansen and Price, 1995; Hansen and Price, 1999), thus decreasing the genetic quality of gametes (Hansen and Price, 1995; Pizzari et al., 2008) and altering sperm functionality. It has been reported that in humans, age is associated with lower sperm volume, motility and percentage of normal sperm cells (Johnson et al., 2015; Kidd et al., 2017). Furthermore, advanced parental age in several species is associated with a decline in sperm competition (Pizzari et al., 2008; Radwan, 2003). However, the decrease of sperm competition with age was not observed in teleosts, such as reported for sockeye salmon (*Oncorhynchus nerka*) (Hoysak et al., 2004) in *in vitro* fertilization experiments and for guppy (*Poecilia reticulata*) natural spawns (Rowe and Pruett-Jones, 2011). Consequently, the effect of age on sperm quality is not similar in all vertebrates and should be investigated thoroughly in zebrafish to ensure the highest sperm quality for cryopreservation purposes.

From animal welfare and practical point of view, the most convenient technique for sperm collection in a zebrafish facility is through abdominal massage, since it is a non-lethal technique (Buchanan-Smith et al., 2005). In this way, sperm collection can be performed repeatedly on the same male (Pruneda et al., 2005).

The influence of sperm collection frequency on sperm quality has been assessed in teleost species such as trout (*Salmo trutta*) (Billard et al., 1971; Büyükhatipoglu and Holtz, 1984) turbot (*Scophthalmus maximus*) (Suquet et al., 1992a), European sea bass (*Dicentrarchus labrax*) (Zohar et al., 1984) , and white fish (*Coregonus peled*) (Hochman et al., 1974). However, it is commonly accepted that an inappropriate sperm collection frequency affects sperm quality (Migaud et al., 2013) and this must be determined for each species. Consequently, the assessment of an appropriate sampling frequency that allows full recovery of sperm quality in zebrafish is essential for assisted reproduction purposes.

Motility is the most widely studied quality parameter in fish sperm (Gallego and Asturiano, 2018; Rurangwa et al., 2004) and although other analyses are needed to guarantee the status of spermatozoa, it is a useful tool to infer the probability of successful fertilization and to assess the previously mentioned factors (Fauvel et al., 2010; Gallego and Asturiano, 2018). Still, there are no universal sperm quality biomarkers, therefore, besides motility, other parameters are needed or accurate quality analysis (Bobe and Labbé, 2010; Rurangwa et al., 2001). The viability of the plasma membrane is an important feature in spermatozoa since it characterizes the integrity of the cell (Rurangwa et al., 2004). Membrane alterations in spermatozoa can affect motility initiation (motility is triggered by membrane signaling), motility maintenance (loss of intracellular ATP) and the ability of the sperm nucleus to produce the first embryonic cell after fertilization (Fauvel et al., 2010; Herráez et al., 2017; Pérez-Cerezales et al., 2010). In this way, analysis of sperm quality is a useful tool to select the most appropriate conditions to collect zebrafish sperm for cryopreservation and assisted reproduction purposes.

This study aimed to characterize the optimal age for sperm collection in zebrafish and to evaluate the effect of the frequency of non-invasive sperm sampling on motility and plasma membrane viability.

4.1.3. Methods

4.1.3.1. Zebrafish maintenance

Zebrafish AB wild-type and transgenic Tg*(runx2:*eGFP*)* (Knopf et al., 2011), lines, with an AB background, were housed in a standard aquatic recirculation system (Zebtec®, Tecniplast, Italy) with 980 L of water and containing a biological filter (ceramic beads), mechanic filtration (50 µm), granular activated carbon filter and UV sterilization (180 000 µWs/cm^2) to maintain water quality. The water temperature (28±0.5°C), conductivity (750±70 µS/cm) and pH (7.5±0.2), parameters were constantly monitored through automatic probes and water was partially replaced daily (10%) through an automatic mechanism. The fish room had a controlled photoperiod with a 14:10 h light: dark cycle, an independent air conditioning system (26±1°C) and an air extraction system to guarantee the air renewal in the room, maintaining the humidity close to 60%. Males and females were maintained separately in 3.5 l tanks. The fish were fed twice a day *ad libitum* with ZEBRAFEED® (Sparos Lda, Portugal) and *Artemia* nauplii (AF 480; INVE, Belgium) and fish debris were removed daily.

4.1.3.2. Sperm collection and quality analysis

On the day prior to sperm collection (16 h before the sampling) (Diogo et al., 2018), males and females were placed in 1 l breeding tanks at a 1:1 sex-ratio (Tecniplast, Italy) and maintained separated while sharing the same water, in order to promote hormonal stimulation for improved release of gametes. Males were anesthetized with 0.168 mg/ml of tricaine methane-sulfonate solution (MS-222) (Sigma Aldrich, Spain) prepared according to Westerfield (Westerfield, 2005) and sperm was collected (1 h after the lights turn on) by an abdominal massage using a glass capillary tube connected to a mouth piece. Sperm was immediately diluted with 10 µl of sterilized and filtered (0.20 µm) Hank's Balanced salt solution (HBSS) at 300 mOsm/Kg (Hagedorn and Carter, 2011) to prevent motility activation, in accordance with previous studies (Yang and Tiersch, 2009). After sperm collection, the samples were maintained at 4°C in the dark until quality analysis was performed (between 1-2 hours after collection). Meanwhile, the males recovered from the

anesthesia in clean system water and were returned to the rearing tanks. Sperm motility was evaluated using CASA (Proiser, Spain). Only samples with a concentration higher than $3x10^7$ sperm cells/ml were analyzed (Diogo et al., 2018). To evaluate motility parameters, 0.5 µl of sperm at room temperature was placed on a Makler chamber under a 10 x negative phase-contrast objective (Nikon E200, Tokyo, Japan) and immediately activated with 5 µl of filtered and sterilized system water at 28±1°C. Motility was recorded every 10 s post-activation, for 1 min for each sample. The images were captured with a Basler camera A312f (Basler Afc, Germany). Total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s) and linearity (LIN, %) were determined to assess sperm quality. Only those spermatozoa with VCL>10 µm/s were considered motile.

To evaluate spermatozoa membrane viability, the percentage of viable cells was quantified using the fluorescent dyes propidium iodide (PI) (Invitrogen, Spain) and SYBR 14 (Invitrogen, Spain). Before the addition of the fluorescent dyes, the sperm sample was re-diluted (1:10) in HBSS, to reduce cell concentration. Incubation with 5 µM SYBR 14 and 220 µM PI was performed in the dark at 4°C for 5 min. Cell viability was quantified under an epifluorescence microscope (Nikon E200, Tokyo, Japan), equipped with triple excitation filter block DAPI-FITC-Texas Red (excitation filter wavelengths: 395–410 nm (bandpass, 403 CWL), 490–505 nm (bandpass, 498 CWL), and 560–580 nm (bandpass, 570 CWL)). Dead cells with disrupted membrane labeled in red (PI-stained cells) and live cells labeled in green (SYBR 14 stained cells) were counted, and the percentage of viable cells was determined. At least 100 cells per slide were counted, and two slides per sample and per condition were observed.

4.1.3.3. Effect of zebrafish line, male age and sperm collection frequency on sperm quality

Males from wild-type (AB n=90) and Tg(*runx2:eGFP* n=85) zebrafish lines were sampled for sperm collection as previously described. To study the effect of age, sperm motility index was evaluated from: a) AB line at 6 (n=26), 8 (n=13), 12 (n=27) and 14 (n=24) months of age; b) Tg(*runx2:eGFP*) line at 6 (n=23), 8 (n=17),

12 (n=18) and 14 (n=27) months of age. The analysis of membrane viability was performed for the same males: a) AB line at 6 (n=20), 8 (n=13), 12 (n=27) and 14 (n=21) months of age; b) Tg(*runx2*:eGFP) line at 6 (n=23), 8 (n=12), 12 (n=18) and 14 (n=27) months of age.

Sperm collection frequency was evaluated in terms of motility in AB (n=90) and Tg(*runx2:eGFP*) (n=85) zebrafish lines. From the 90 AB and 85 Tg(*runx2:eGFP*) males a total of 78 (AB) and 69 (Tg(*runx2:eGFP*)) males had sperm samples above 3x107 sperm cells/ml and were used to establish 3 treatment groups (AB n=26; Tg(*runx2:eGFP*) n=23). The first group was sampled 2 days after the first sampling (AB n=13; Tg(*runx2:eGFP*) n=17). The second group was sampled after 7 days of rest (AB n=26; Tg(*runx2:eGFP*) n=18). The third group was sampled after 14 days of rest (AB n=24; Tg(*runx2:eGFP*) n=23).

The analysis of membrane viability was performed for the same males in: a) AB line at the first sampling (n=23), 2 (n=18), 7 (n=23) and 14 (n=11) days of rest; b) Tg(*runx2:eGFP*) line at the first sampling (n=22), 2 (n=13), 7 (n=22) and 14 (n=22) days of rest. Not all the samples analyzed for motility were analyzed for membrane viability due to the low sperm volume.

Sperm motility parameters of the individual males were assessed through CASA system every 10 s post-activation during 1 min, to determine TM, PM, VCL, VSL and LIN. Viability of the plasma membrane was evaluated as previously described (Figure 4.1).

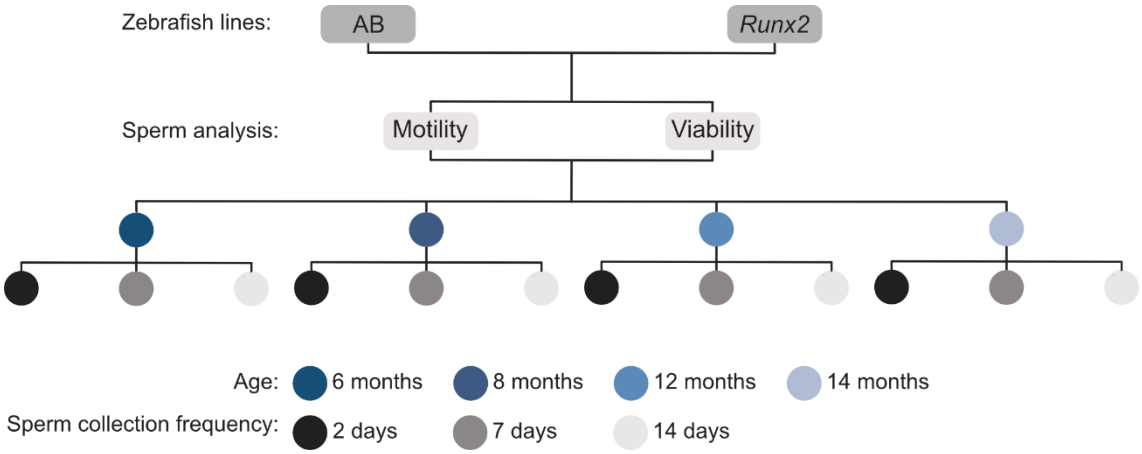

Figure 4.1 Experimental design to evaluate the effect of male age and sperm collection frequency on sperm motility and viability of AB and Runx2 zebrafish lines.

4.1.3.4. Data analysis

Due to the high number of variables related to sperm motility measured for each sample (5 motility parameters × 6 post-activation times = 30 motility-related variables), we started by evaluating their degree of redundancy. Principal Component Analysis (PCA) was used in order to assess whether it was possible to aggregate all 30 variables into a small number of variables, without significant information loss. After a preliminary exploratory analysis, we observed that the LIN variables displayed very low variance, except for sperm samples with extremely low motility and no particularly relevant linear correlation with the other variables. As such, we have no longer considered the LIN parameters for analysis. In contrast, all other parameters (TM, PM, VCL and VSL) displayed a high degree of positive correlation among them, which was reflected by the fact that it is possible to aggregate these 24 variables into a single variable (PC1), that still retains 52% of observed variation (after mean-centering and auto-scaling of these variables, to ensure that PCA does not give preference to higher variance variables) and which can be interpreted as a general "motility index".

This motility index (i.e. the first component of the PCA analysis) consisted of a weighted mean of these motility measurements (after standardization), which was used for further ANOVA analysis.

SPSS 18.0 software was used for statistical analysis. Data were expressed as means±95% C.I. (95% of the confidence interval of the mean), and normalized by arcsine transformation when results were expressed as percentages. Statistical differences between treatments were detected by ANOVA and Student-Newman-Keuls (SNK) multiple comparison post hoc tests ($P<0.05$). A three-way ANOVA (SNK, $P<0.05$) was performed on all motility (PC1) data. For each zebrafish line, a two-way ANOVA (SNK, $P<0.05$) was applied to evaluate the effect of age and sperm collection frequency on sperm quality.

4.1.4. Results

Sperm motility parameters from CASA analysis are presented in supplementary table 1 (AB) and 2 (Tg(*runx2:eGFP*)) where we could observe a decrease of motility with aging and with low post-stripping recovery times. A three-way ANOVA (SNK, $P<0.05$) was performed on motility index (PC1) data which showed that zebrafish line is the factor with the largest main effect on motility (Table 4.1), since Tg*(runx2:eGFP)* had significantly higher sperm motility when compared to AB (Figure 4.2 A and B, 4.3 A and B). Given the high number of observed significant interaction effects between factors (line, age and stripping frequency) (Table 4.1), which impair interpretation of the main treatments effects, a two-way ANOVA (SNK, $P<0.05$) was applied for each line independently, to study the main effects of age and stripping frequency (along with possible interactions between these factors).

Table 4.1 Zebrafish sperm motility analysis of Principal component related to age, stripping frequencies and their interactions in AB and Tg*(runx2:eGFP)* line.

Three-way ANOVA (p value < 0.005)	Motility (PC1)
Line	<0.001*
Age	<0.001*
Frequency	0.437
Strain*age	0.480
Strain*frequency	0.212
Age*frequency	<0.001*
Strain*age*frequency	0.024*
AB (two-way ANOVA) (p value < 0.005)	
Age	0.025*
Frequency	0.825
Age*frequency	0.002*
runx2 (two-way ANOVA) (p value < 0.005)	
Age	0.001*
Frequency	0.179
Age*frequency	0.001*

Significant differences (three-way ANOVA (SNK, P<0.05)) are represented with an asterisk.

Both lines had a consistent main effect of age, where younger males (6 and 8 months) had significantly higher sperm motility when compared to older males (12 and 14 months) (Figure 4.1.4.1 A and B).

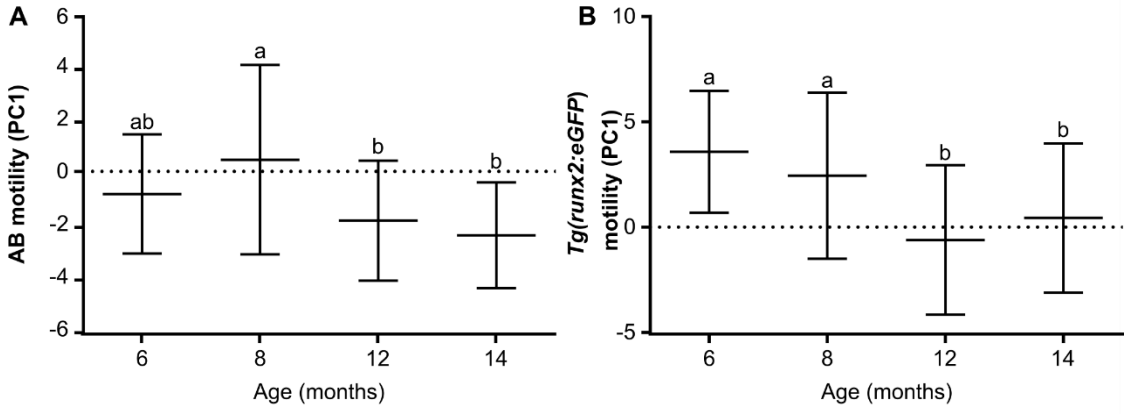

Figure 4.2 Zebrafish sperm motility index according to age of: A) AB line at 6 (n=26), 8 (n=13), 12 (n=27) and 14 (n=24) months of age. B) Tg(*runx2:eGFP*) line at 6 (n=23), 8 (n=17), 12 (n=18) and 14 (n=27) months of age. The analysis was performed with the baseline data of the first sampling. Bars represent means±95% of the confidence interval and statistical differences (two-way ANOVA-SNK, P<0.05) between fish age are represented with letters.

The results of stripping frequency on sperm motility were also consistent between both lines, with no significant main effect being observed (Table 4.1; Figure 4.3 A and B). In both lines, there was an age interaction with frequency effect, which means that stripping frequency had an effect on sperm motility that depends on age (Figure 4.4), though stripping frequency seems to have little effect on sperm motility on younger fish, older fish tend to display lower motility when the recovery time is below 14 days.

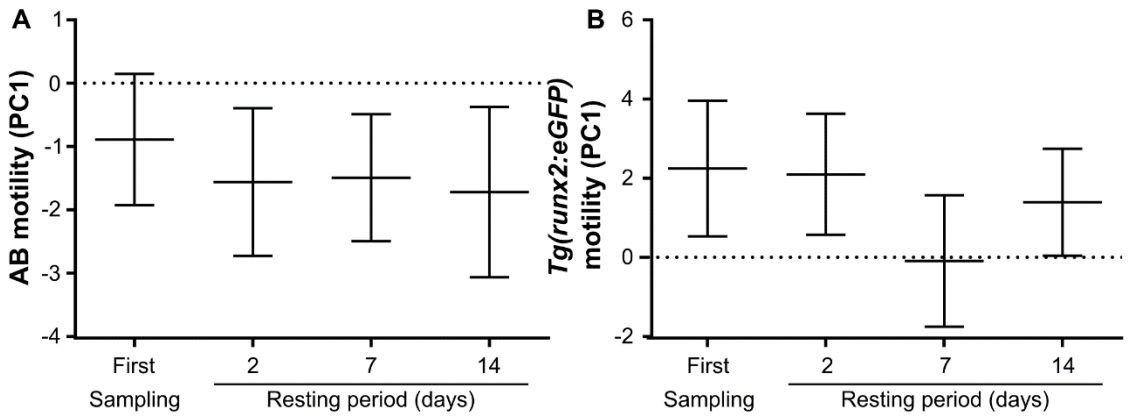

Figure 4.3 Zebrafish sperm motility index after different sperm stripping frequencies. The number of the stripping frequency is related to the time of rest between sperm collections. After the first sampling sperm was collected after 2, 7 and 14 days of rest between samplings. The analysis was performed in: A) AB line at first stripping (n=26) and after 2 (n=13), 7 (n=27) and 14 (n=24) days of rest, B) Tg(*runx2:eGFP*) line at first sampling (n=23) and after 2 (n=17), 7 (n=18) and 14 (n=27) days of rest. The analysis was performed with males with 6 months of age. Bars represent means±95% of the confidence interval and statistical differences (two-way ANOVA-SNK, $P<0.05$) between sperm collection frequencies are represented with letters.

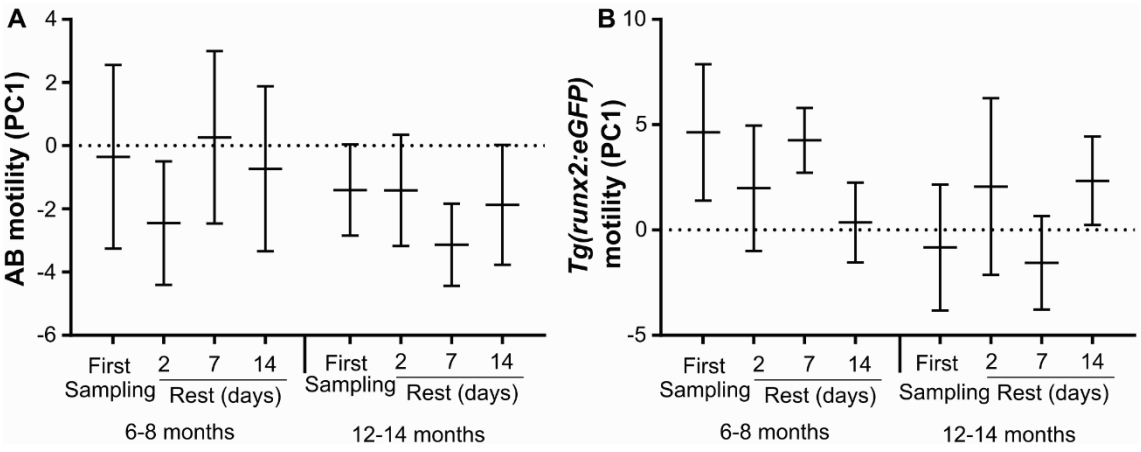

Figure 4.4 Effect of the interaction in zebrafish sperm motility index between males age and sperm stripping frequency in (A) AB wild-type line and (B) Tg(*runx2:eGFP*) line. The number of the stripping frequency is related to the time of rest between sperm collections. Analysis was performed in the first sampling [AB n=24; Tg(*runx2:eGFP*) n=27] and after 2 [AB n=14; Tg(*runx2:eGFP*) n=23], 7 [AB n=33; Tg(*runx2:eGFP*) n=22], and 14 [AB n=19; Tg(*runx2:eGFP*) n=13] days of rest between samplings. This analysis is related to males with 6–8 months [AB n=39; Tg(*runx2:eGFP*) n=40] and 12–14 months [AB n=51; Tg(*runx2:eGFP*) n=45] of age. Bars represent 95% of confidence interval.

The percentage of viable cells was analyzed with a three-way ANOVA (SNK, $P<0.05$) after arcsin transformation, with zebrafish line displaying no main effect on sperm viability (Table 4.2). However, the high number of interaction effects impaired a clear interpretation of the effect of age and stripping frequency on sperm viability, so, as previously, a two-way ANOVA (SNK, $P<0.05$) was applied to each line independently to study the effect of age and stripping frequency. The AB line showed no significant differences between all the studied ages in terms of sperm viability (Figure 4.5 A and B), while Tg*(runx2*:eGFP*)* line had significantly higher sperm viability at 8 months of age.

Table 4.2 Zebrafish sperm membrane viability analysis related to age, stripping frequencies and their interactions in AB and Tg*(runx2:eGFP)* line.

Three-way ANOVA (p value < 0.005)	Viability
Line	0.267
Age	0.007*
Frequency	<0.001*
Strain*age	0.083
Strain*frequency	<0.001*
Age*frequency	<0.001*
Strain*age*frequency	0.346
AB (two-way ANOVA) (p value < 0.005)	
Age	0.145
Frequency	<0.001*
Age*frequency	0.003*
runx2 (two-way ANOVA) (p value < 0.005)	
Age	<0.001*
Frequency	<0.001*
Age*frequency	0.001*

Significant differences (three-way ANOVA (SNK, P<0.05)) are represented with an asterisk.

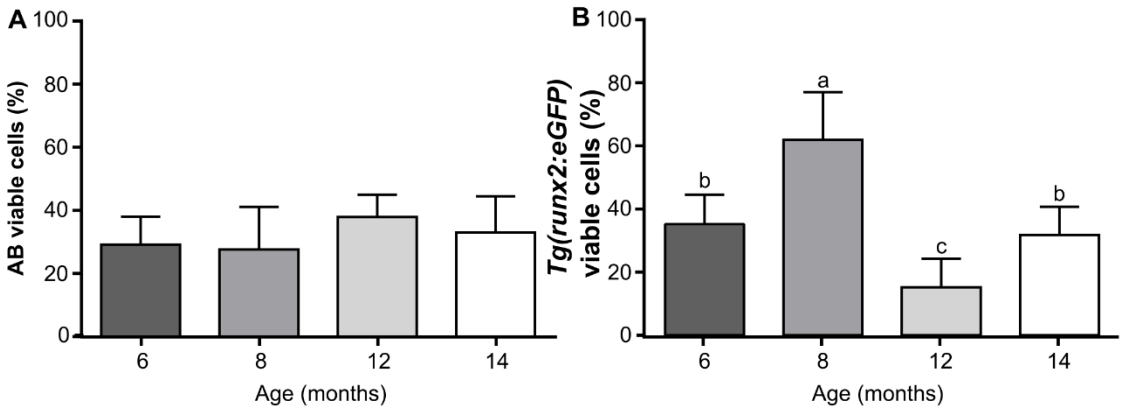

Figure 4.5 Zebrafish sperm viability (%) according to age of: A) AB line at 6 (n=20), 8 (n=13), 12 (n=27) and 14 (n=21) months of age and B) sperm viability (%) of Tg*(runx2:eGFP)* line at 6 (n=23), 8 (n=12), 12 (n=18) and 14 (n=27) months of age. The analysis was performed with the baseline data of the first sampling. Bars represent 95% of confidence interval and statistical differences (two-way ANOVA-SNK, P<0.05) between fish age are represented with letters.

In the AB line, males sampled for the first time had significantly higher sperm viability when compared to 2 and 7 days after stripping, but it was not significantly different from 14 days after stripping (Figure 4.6 A and B). Consequently, AB males

were able to recover membrane viability 14 days after stripping. On the other hand, Tg*(runx2:eGFP)* males were not able to recover membrane viability 14 days after stripping, since males in the first sampling point had significantly higher sperm viability compared with males after 7 and 14 days of recovery (Figure 4.6 B).

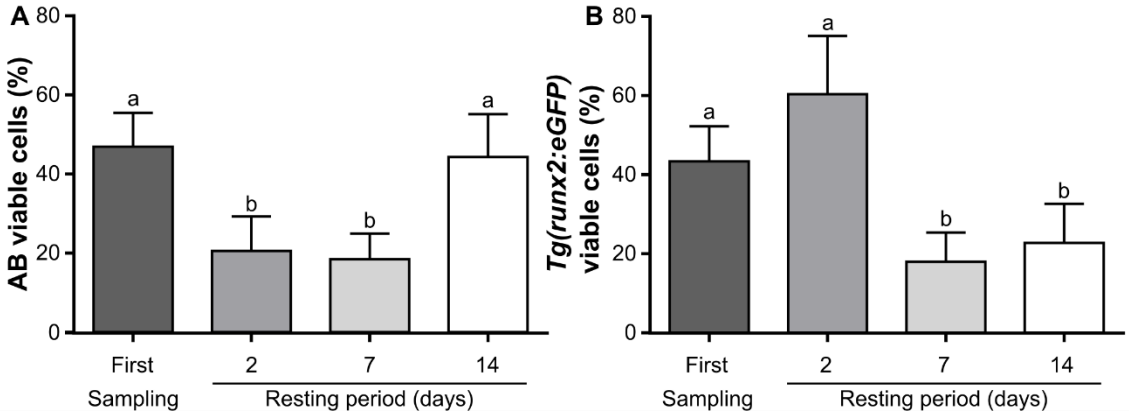

Figure 4.6 Zebrafish sperm viability after different sperm stripping frequencies. The number of the stripping frequency is related to the time of rest between sperm collections. After the first sampling sperm was collected after 2, 7 and 14 days of rest between samplings. The analysis was performed in: A) AB line at the first sampling (n=23) and 2 (n=18), 7 (n=29) and 14 (n=11) days of rest, B) Tg*(runx2:eGFP)* line at the first sampling (n=22) and 2 (n=13), 7 (n=22) and 14 (n=23) days of rest. The analysis was performed with males with 6 months of age. Bars represent 95% of the confidence interval and statistical differences (two-way ANOVA-SNK, $P<0.05$) between sperm collection frequencies are represented with letters.

4.1.5. Discussion

The selection of sperm donors is highly relevant for cryopreservation since it can help in reducing post-thaw variability in zebrafish sperm used for *in vitro* fertilization. In captivity, zebrafish has natural longevity of 42 to 66 months, depending on reproductive effort and caloric intake (Gerhard and Cheng, 2002). In our study, younger zebrafish males (6 to 8 months) showed significantly higher sperm motility when compared to older males (12-14 months). In agreement, Johnson et al. (2018) observed that older zebrafish males had a decline in sperm production and motility, although displaying higher offspring survival. In guppy, male age affected negatively sperm morphology, velocity and sperm number but not membrane viability. Older males had slower sperm, with longer flagellum and higher sperm volume than younger males (Gasparini et al., 2010), but these differences did not affect sperm competition success when compared to younger

males. It is interesting to observe that both in zebrafish and in guppy there is a decrease in sperm motility in older males, which might be associated to the accumulation of oxidized proteins and a decline in mitochondrial functionality (Payne and Chinnery, 2015). Mitochondria aging-related alterations are among the most remarkable features observed in senescent cells. Mitochondrial oxidation is the major source of oxidation lesions accumulated with age, affecting mitochondrial energy metabolism, which is essential for male reproductive function. The damage produced by excessive reactive oxygen species (ROS) in the sperm membrane cause reduced sperm motility and impairs its ability to fuse with the oocyte (Ramalho-Santos, 2009). Oxidative stress reduces sperm motility and viability (Fujihara and Howarth, 1978; Hagedorn et al., 2012; Sanocka and Kurpisz, 2004; Wishart, 1984) and could be one of the possible explanations for the lower motility in older zebrafish males.

Zebrafish are hierarchical fish with dominant-subordinate relationships, which are related to body size and levels of aggression, associated with reproductive success (Paull et al., 2010). The hierarchical relationships established among zebrafish are strongly connected to sperm competition, which is a post-copulatory selection that occurs when females breed with multiple males in the same reproductive episode. In this process, sperm from rival males compete to fertilize the oocytes (Parker, 1970). In sperm competition for fertilization, there is a strong selection for spermatozoa quality parameters that enhance fertilization success, such as sperm quantity and quality (Parker and Pizzari, 2010; Rowe et al., 2010). Therefore, the reproductive set-up established to determine sperm quality is extremely important since changes in the social environment rapidly affects sperm competition and therefore sperm quality (Zajitschek et al., 2014) found that under high sperm competition environment (two males and one female), males display higher sperm motility and velocity than reproductive set-ups where one male was available to two females (low sperm competition environment). In our study, males of each age were permanently maintained separated from females and the reproductive set-up was established in breeding tanks in a sex ratio of 4:4 to stimulate reproduction and collect the sperm on the following day.

Therefore, the results obtained with our experimental design emphasize the effect of age and sperm collection frequency in sperm quality, reducing biases associated with sperm competition effects.

Considering this information, it largely explains the differences observed between studies on the effects of zebrafish age on reproduction and sperm quality. Not only are the samples highly heterogeneous, but they also manifest adaptations and different investments in gamete production according to the social environment and hierarchical relationships. Characterization of the effect of zebrafish age on sperm competition should be undertaken in the future.

Spermiogenesis is a complex and highly regulated process, where diploid cells called spermatogonia proliferate and differentiate onto mature spermatozoa through mitosis, meiosis, and spermiogenesis (Leal et al., 2009). Zebrafish spermatogenesis has a cystic pattern with one of the teleosts fastest spermiogenesis cycle taking only 6 days to reach spermatozoa full maturation (Leal et al., 2009). Reinardy et al. (Reinardy et al., 2013a) observed that with stripping frequencies with a maximum of 7 days of rest, the DNA integrity was not altered, despite the fact that sperm concentration was affected. Consequently, it was necessary to determine the adequate stripping frequency that allows the full recovery of sperm quality. Our data showed that stripping frequency does not affect sperm motility, though it does affect membrane viability. The AB zebrafish line was able to recover the initial membrane viability after 14 days of rest. However, this recovery was not observed in the transgenic line, where sperm membrane viability was still decreased after 7 and 14 days of rest.

In our study recovery time in younger fish is seemingly faster than in older fish, which is observed through the interaction effects of age and stripping frequency: older fish display lower sperm motility when the recovery time between collections is short, while sperm motility for younger fish seems insensitive to the recovery time between collections. Consequently, it is highly advisable to respect 14 days of rest between sperm collection events, particularly in older fish. The fact that younger fish are less susceptible to cellular distress, related to sperm collection events, reinforces the selection criteria of using younger fish for sperm collection.

The experimental design used in our work allowed a better comprehension of the interaction between treatments, that would not be possible otherwise. Both zebrafish lines are commonly used in zebrafish facilities and the transgenic model was used as a comparison between wild-type fish breeders with genetically modified zebrafish lines. The Tg*(runx2:eGFP)* line has an AB background and expresses the Tg*(runx2:eGFP)* transcription factor which is related to osteoblast differentiation but also to the regulation of cell proliferation. Although most studies on zebrafish sperm cryopreservation and assisted reproduction are performed with wild-type lines, its application is most useful in transgenic and mutant lines, and their particularities are generally unknown or disregarded.

Throughout our experiments the Tg*(runx2:eGFP)* transgenic line had systematically higher sperm motility when compared to AB line. The fact that Tg*(runx2:eGFP)* fish displayed significantly higher sperm motility, but lower capacity to recover membrane viability at 7 and 14 days after sperm collection, suggests the existence of relevant differences between zebrafish lines in terms of sperm quality and susceptibility to damage. Consequently, each zebrafish transgenic and mutant line should be investigated prior to the establishment of sperm cryopreservation programs.

The knowledge obtained by this work allows the determination of suitable zebrafish age and sperm collection frequencies to obtain the highest sperm quality possible to facilitate cryopreservation procedures, respecting the 3 R´s principle. Therefore, we consider that males between 6 to 8 months of age have the highest sperm quality and at least 14 days of rest should be respected between sperm collection events.

CHAPTER 5. SPERM CRYOPRESERVATION AND STORAGE

PREAMBLE

One of the main constrains in cryopreservation techniques is the necessity for liquid nitrogen, since it is expensive and hazardous. Nowadays, research facilities dedicated to investigation with zebrafish have available electric ultrafreezers (-150°C). Since this temperature is below the limit considered optimal for germ cell storage (-135°C), these equipment's are suitable candidates for an alternative to liquid nitrogen storage of cryopreserved samples. Zebrafish sperm cryopreservation freezing rates have been the focus of attention by the scientific community, however, the maximum freezing rate tested was -25°C/min. The direct placement of cryovials on electric ultrafreezer (-66°C/min) would simplify the method of zebrafish sperm cryopreservation and storage and would be extremely practical in zebrafish facilities. The objective of the first part of this chapter was to develop an alternative method to liquid nitrogen for the storage of cryopreserved samples in ultrafreezers and the improvement of the freezing rate for this species. This study constitutes the first sperm cryopreservation method with ultrafreezers in a teleost species.

To improve the cryopreservation protocol developed in this chapter the optimization of the cryoprotectants composition in the extender was performed in the second part of this chapter. The concentration of permeating cryoprotectant and the combination of permeating and non-permeating cryoprotectants was evaluated not only on post-thaw sperm quality and *in vitro* fertilization success but also on the skeletal development of the offspring.

Overall this chapter proposed a fast and simple method of zebrafish sperm cryopreservation in ultrafreezers which constitutes a technological improvement highly relevant for this species gene banking. The first part of this chapter represents an article published in Journal of Fish Physiology and Biochemistry and the second part represents a manuscript submitted on Cryobiology journal; both works are first authored by Patricia Diogo.

5.1 ELECTRIC ULTRAFREEZER (-150°C) AS AN ALTERNATIVE FOR ZEBRAFISH SPERM CRYOPRESERVATION AND STORAGE

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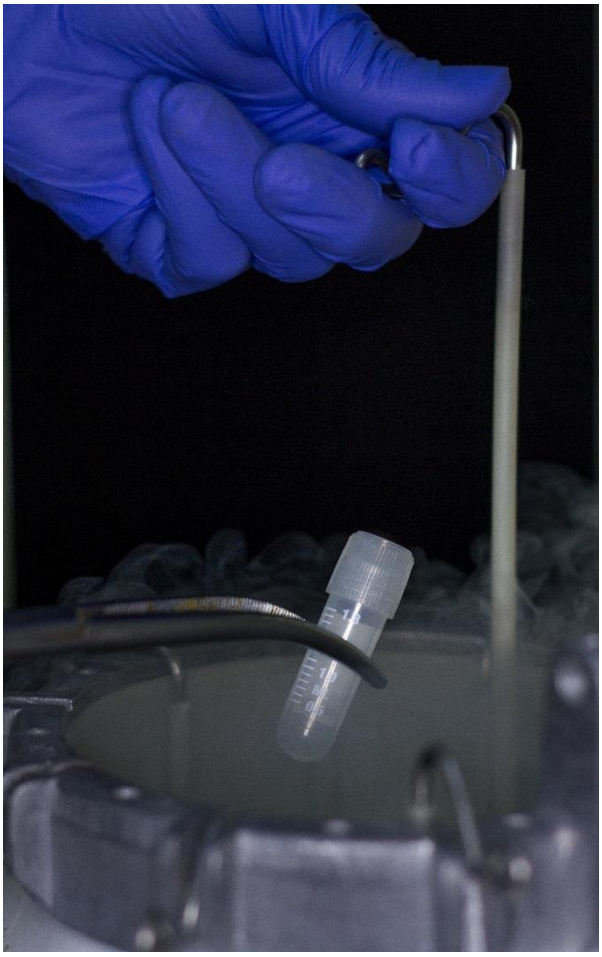

[J Fish Physiol Biochem 2018, 44(6): 1443–1455]

5.1.1. Abstract

Zebrafish sperm cryopreservation is a fundamental methodology to manage and back-up valuable genetic resources like transgenic and mutant strains. Cryopreservation usually requires liquid nitrogen for storage, which is expensive and hazardous. Our objective was to evaluate if electric ultrafreezers (−150°C) are a viable alternative for zebrafish sperm storage. Zebrafish sperm was cryopreserved in the same conditions (−20°C/min), stored either in liquid nitrogen or in an ultrafreezer, and thawed after 1 week, 1 month, and 3 months. Sperm motility, membrane integrity, and fertilization ability were assessed. There were no significant differences in motility and hatching rate throughout storage time. Additionally, we aimed at understanding if cryopreservation directly in an ultrafreezer (−66°C/min) could improve post-thaw sperm quality. Freezing at −20°C/min was performed as before, and compared to samples cryopreserved with a fast cooling rate by placing directly in an ultrafreezer (−66°C/min). Sperm quality was assessed according to motility, viability, DNA fragmentation, and apoptosis (annexin V). The −66°C/min cooling rate showed significantly higher membrane and DNA integrity, and lower number of cells in late apoptosis in comparison to the other treatments. This study showed that zebrafish sperm cryopreservation and storage in an ultrafreezer system is possible and a fast cooling rate directly in ultrafreezer improves post-thaw sperm quality.

5.1.2. Introduction

Sperm cryopreservation is a useful tool applied in assisted reproduction in over 200 species (Tiersch et al., 2007). It constitutes a long-term storage technique that preserves structurally intact living cells (Tsai and Lin, 2012). Cryopreservation usually requires liquid nitrogen for storage since it has high thermal stability in ultra-low temperatures (−196°C); however, it is expensive, with limited availability, can be hazardous for users, and carry the risk of cross-contamination of the stored samples (Grout and Morris, 2009; Larman et al., 2014). Consequently, the use of liquid nitrogen has been considered one of the most relevant bottlenecks for cryopreservation practical application (Larman et al., 2014; Yuan et al., 2016). Although mammalian tissue cultures and microbial suspensions can be stored in

−80°C freezers (Esteves-Ferreira et al., 2013; Polak and Pitombo, 2011), these conditions are not adequate for storage of gametes, since the traces of liquid water observed at this temperature are considered to be responsible for the low stability of samples stored under these conditions (Mazur, 1984).

Nowadays, with the technological advances in the past years, electric ultrafreezer systems (−150°C) are easily available (Álamo et al., 2005; Medrano et al., 2002; Yavaş and Daskin, 2012) in the facilities dedicated to zebrafish research. Liquid water does not exist below −135°C where diffusion rates are negligible (Mazur, 1984); therefore, theoretically, gametes could be stored indefinitely using these systems. Batista et al (2009) summarize the advantages of electric ultrafreezer systems over liquid nitrogen storage. It has higher storage capacity, easier sample manipulation, unlike liquid nitrogen storage it does not require periodic reposition and the global costs of cryopreservation are more cost-efficient. The disadvantages of ultrafreezer are the limited mobility, which in zebrafish facilities is not required, and for cryopreservation, the cooling rate is not programmable.

Altogether, electric ultrafreezers have the potential to be an alternative to liquid nitrogen for sperm cryopreservation and storage. There are few studies on sperm cryopreservation and storage in an ultrafreezer and the only reports found using this technique were in canine (Álamo et al., 2005; Batista et al., 2006), caprine (Batista et al., 2009; Medrano et al., 2002), and bull sperm (Yavaş and Daskin, 2012), reporting encouraging post-thaw quality results. However, there are no studies on this subject using sperm from teleost species.

Zebrafish is an important model species with increasing interest to the scientific community in the past years. Since the development of feasible genome editing technologies in the past two decades, such as Tol2 transposon and CRISPR/Cas9 (Liu et al., 2017; Suster et al., 2009), thousands of new mutant and transgenic strains were developed, posing problems in terms of facilities space and management, which cryopreservation can solve (Cabrita et al., 2010; Harvey et al., 1982a; Robles et al., 2009). However, despite the fact that the first zebrafish sperm cryopreservation protocol was developed more than 30 years ago (Harvey et al., 1982a), there is a lack of standardization of the methodologies (e.g., sperm collection and analysis, cryopreservation procedure), which results in high variability on post-

thaw sperm quality and *in vitro* fertilization success (Robles et al., 2009). The objective of this work was to evaluate if ultrafreezer is a viable alternative to liquid nitrogen for zebrafish sperm storage. Furthermore, we aimed to understand if a fast and simple cooling rate (−66°C/min) directly in an ultrafreezer is beneficial for zebrafish post-thaw sperm quality.

5.1.3. Methods

5.1.3.1. Fish rearing and sperm collection

Adult AB zebrafish males (n=110) and females (n=363) (6–8 months old) were selected as main broodstock, according to similar size and maintained separated by sex into 3.5 l tanks. Males were distributed in 10 aquariums, in a density of 11 males per tank. The 363 females were distributed in 33 aquariums at the same density. The fish were maintained in a ZebTEC® (Tecniplast, Italy) recirculation system. The fish room had a controlled photoperiod with a 14:10 h light/dark cycle, an independent air conditioning system (26±1°C) and an air extraction system to guarantee the air renewal in the room, maintaining the humidity close to 60%. The water rearing system was partially replaced (10%) daily and the water system maintained at 28.5±0.5°C, 700±50 µS/cm and pH 7.5±0.1. The fish were fed twice a day with *Artemia* nauplii (AF480, INVE, Belgium) and ZEBRAFEED® diet (Sparos Lda, Portugal) *ad libitum*. Food consumption was visually controlled, and the debris removed daily.

For sperm collection, males were anesthetized in 0.168 mg/ml tricaine sulfonate solution (MS-222) (Sigma-Aldrich) according to Westerfield (Westerfield, 2005), rinsed with phosphate buffered saline (PBS) solution and the abdominal massage was performed to collect the sperm, using a glass capillary tube attached to a mouth piece. The collected sperm from each individual (1 to 3 µl) was immediately diluted in 10 µl of sterilized and filtered (0.20 µm) HBSS (Hagedorn et al., 2012; Jing et al., 2009a) and pooled after quality analysis and sample selection. The sperm samples were maintained at 4°C in the dark until analysis and cryopreservation were performed (no longer than1 h). Two sets of experiments were performed.

5.1.3.2. Experiment 1 — effect of zebrafish sperm storage throughout time in an ultrafreezer (−150°C) and in liquid nitrogen

To understand the viability of zebrafish sperm storage in an ultrafreezer, we conducted an experiment where sperm samples from the broodstock established previously (n=110), with total motility over 50% (at 10 s post-activation) and cell concentration over 3×10^7 cells/ml were selected to perform3 pools (n=12), each pool contained sperm from 4 males. A control cooling rate (−20°C/min) (Yang et al., 2007) was applied to all samples in a programmable biofreezer (Asymptote Grant EF600, UK). Sperm was cryopreserved with a final concentration of 10% N-N dimethylformamide (DMF) in HBSS (Asturiano et al., 2015), with a dilution rate of pre-diluted sperm to extender of 1:1, in a final volume of 10 µl and stored in 2 ml cryovials (VWR® Low Temperature Freezer Vials). The samples were stored either in a liquid nitrogen tank (LN) or in an ultrafreezer (UF). Thawing was performed in a 40°C water bath during 8 s (Yang et al., 2007). Samples (n=3 pools) were thawed after 1 week, 1 month, and 3 months post-storage. Sperm quality was evaluated through sperm motility, membrane integrity and *in vitro* fertilization success (Figure 5.1).

5.1.3.3. Experiment 2 — effect of a fast cooling rate (−66°C/min) on zebrafish post-thaw sperm quality

To understand if zebrafish sperm can be directly cryopreserved using an ultrafreezer system, an experiment was set up where sperm samples from the broodstock established previously (n=110), with total motility over 50% (at 10 s post-activation) and cell concentration over 3×10^7 cells/ml, were selected to perform 7 pools (n=35). Each pool contained sperm from 5 males. The first treatment was a fast cooling rate of −66°C/min performed by placing the samples directly in the ultrafreezer. This method is not programmable, the cooling rate obtained was verified through a thermocouple (Hanna Instruments, USA). Two control treatments were performed, both had a −20°C/min cooling rate in a programmable biofreezer. However, one treatment was stored in a liquid nitrogen tank and the other in an ultrafreezer system. The samples were thawed in a 40°C

water bath during 8 s. Sperm quality was evaluated in terms of terms of sperm motility and membrane integrity. Additionally, other cell quality tests such as cell apoptosis (annexin V assay) and DNA integrity (Comet assay) were performed to ensure the viability of this process (Figure 5.2).

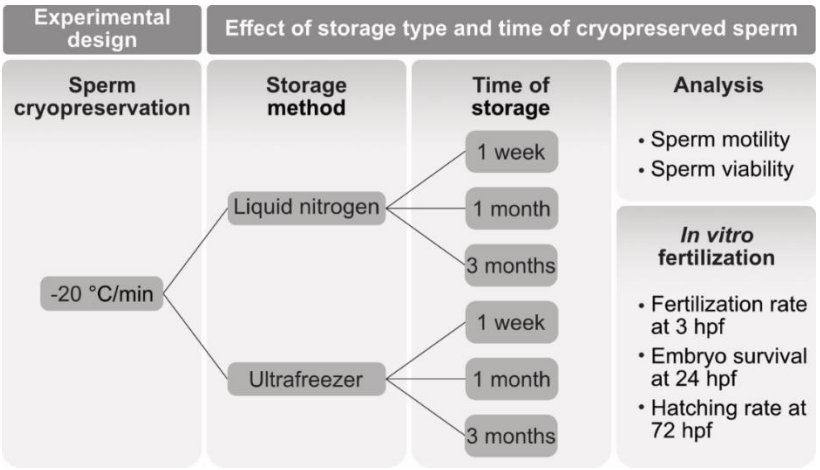

Figure 5.1 Experimental design to evaluate the effect of the storage type (LN, UF) of cryopreserved zebrafish sperm throughout storage time on sperm motility, viability and in vitro fertilization success.

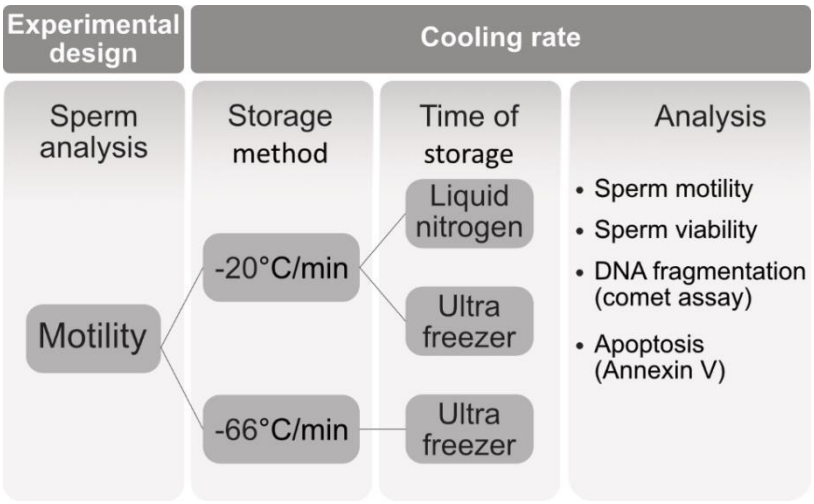

Figure 5.2 Experimental design to evaluate the effect of cooling rate (-20°C/min and -66°C/min) and storage method (LN, UF) on zebrafish cryopreserved sperm motility, viability, DNA fragmentation and apoptosis.

5.1.3.4. Sperm concentration and motility

Sperm concentration and motility were evaluated using CASA system (ISAS Integrated System for Semen Analysis, Proiser, Valencia, Spain) coupled to a phase

contrast microscope (Nikon E-200, Nikon, Tokyo, Japan) with an x10 negative phase contrast objective. The images were captured with a Basler camera A312f (BaslerAfC, Germany) and processed with CASA software. The settings of the CASA system were adapted for this species. For sperm concentration, pre-diluted sperm (individual males and pools) was diluted 1:19 in HBSS and 3 fields were sampled to determine sample concentration. For motility analysis, 0.5 µl of sperm was placed on a Mackler chamber and immediately activated with 5 µl of filtered (0.20 µm) and sterilized system water at 28°C. Each pool was measured twice. Sperm motility was characterized at 10 s post-activation for each pool and treatment according to TM (%), PM (%), VCL (µm/s), VSL (µm/s) and LIN (%). Only sperm samples with VCL>10 µm/s were considerate motile.

5.1.3.5. Membrane integrity

Sperm membrane integrity was assessed through flow cytometry using SYBR 14 (Invitrogen, Spain) and propidium iodide (PI) (Sigma Aldrich, Spain) labeling. SYBR 14 is a permeant nucleic acid stain that crosses the plasma membrane and PI is a membrane impermeable dye that label cells with the disrupted membrane. Cells with disrupted membrane are labeled in red from PI and viable cells are labeled in green from SYBR 14 (Daly and Tiersch, 2012). SYBR 14 was prepared to dilute 5 µl of stock solution in 120 µl of sterilized and filtered HBSS and PI was used undiluted. The pre-diluted sperm samples were re-diluted (1:200) in HBSS and each stain was added in a final concentration of 6.7 nM of SYBR 14 and 3.3 M of PI. Analysis was performed after 5 min of incubation in the dark at room temperature (21 to 25±1°C), in a flow cytometer (BD FACSCalibur™,BD Biosciences, Spain) adjusted for the detection of SYBR 14 through a 530 nm bandpass filter (FL1) and PI was detected with a 670 nm long pass filter (FL3). Flow cytometer settings were previously adjusted using a positive (100% dead cells) and a negative control (fresh sperm). For negative control spermatozoa were exposed to cycles of freezing-thawing (Cabrita et al., 2005). A total of 5000–10,000 events were counted for each sample.

5.1.3.6. Cell apoptosis

The Muse™ Annexin V & Dead Cell Assay (Thermo Fisher Scientific, Spain) analysis quantifies live cells, early and late apoptosis, and necrotic/dead cells by

other mechanisms. The pre-diluted sperm samples were re-diluted (1:200) in HBSS with 1% of Bovine Serum Albumin (BSA) and the labeling was conducted according to manufacturer's specifications. Samples were acquired in a flow cytometer equipped with a 488 nm laser for excitation a 530/30 BP filter and a 690/50 nm BP filter for fluorescence emission. A total of 5000–10,000 events were counted for each sample. For the annexin V apoptosis tests, the total events were collected as the relation of forward scatter (FSC; cell size characterization) and side scatter (SSC; cell granularity) plots. The gating (R1) of the sperm population was used to exclude non-sperm events and it was based on the FSC and SSC profile of zebrafish fresh sperm (Figure 5.3 A) The annexin V component has a high affinity to the phosphatidylserine in the outer leaflet of the membrane in apoptotic cells and 7-AAD (7-amino-actinomycin D) is a dead cell marker. Early apoptotic cells are labeled with annexin V, late-stage apoptotic and dead cells have both 7-AAD and annexin V labeling, necrotic/dead cells by other mechanism are labeled with 7-AAD and non-apoptotic cells (viable) are not labeled. This allows the detection of 4 subpopulations corresponding to viable cells (lower left, LL), cells in early apoptosis (lower right, LR), cells in late apoptosis (upper right, UR) and necrotic/dead cells by other mechanism (upper left, UL) (Figure 5.3 B). Flow cytometer settings were previously adjusted using a positive (100% dead cells) and a negative control (fresh sperm) where controls were incubated with each dye (annexin V or 7-AAD), separately and in combination.

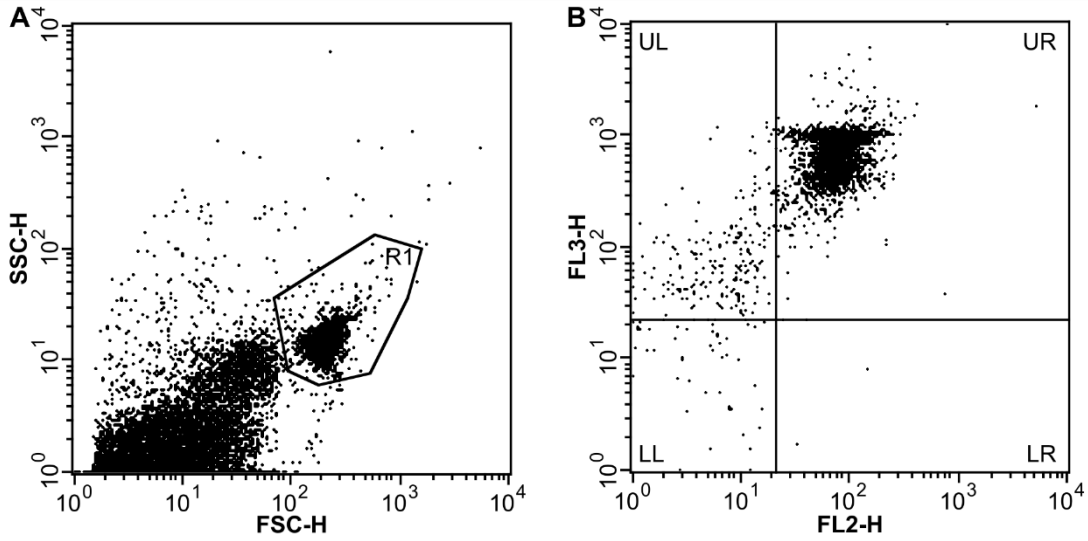

Figure 5.3 Flow cytometry analysis of annexin V of post-thaw zebrafish sperm. A) Sperm cell population gating (R1) B) sperm cell subpopulations corresponding to viable cells (lower left, LL), a cell in early apoptosis (lower right, LR), cells in late apoptosis (upper right, UR) and necrotic/dead cells by other mechanism (upper left, UL).

5.1.3.7. DNA fragmentation

DNA integrity was evaluated through Comet assay adapted from Reinardy (Reinardy et al., 2013b) with slight modifications. After sample thawing, 3 µl of sperm was diluted in 60 µl of low melting point agarose (0.5%), distributed into pre-coated slides with 0.5% of agarose (dried overnight) and covered with a coverslip 15 min at 4°C. A positive control (2 µl sperm + 2 µl 100 µM H_2O_2, incubated for 20 min at 4°C) was set up to induce DNA fragmentation. The coverslip was removed, and the slides were incubated in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1% Triton X-100) for 1 h at 4°C. Subsequently, the samples were placed in an alkaline electrophoresis solution (300 mM NaOH and 1 mM EDTA, pH 13), 20 min followed by 20 min of electrophoresis (25 V, 280–300 mA). The slides were washed twice with a neutralization solution (0.4 M Tris– HCl, pH 7.5) for 5 min and fixed in ethanol for 15 min. For slide observation, DNA was labeled with 10 µl of PI (1 mg/ml) and slides observed at ×600 in a fluorescence microscope (Olympus IX 81, Olympus, Japan) with blue excitation 450–480 nm. Images were captured and recorded with a digital camera (F-view, Olympus, Japan) and processed with the CellF image software (Olympus, Japan). At least 100 cells per slide were scored and then analyzed using Kinetic Imaging Komet 5.5 software (Andor Technology Ltd.,

United Kingdom). The DNA fragmentation was expressed in terms of DNA in tail (%) since it relates the amount and size of the DNA fragments.

5.1.3.8. In vitro fertilization

Females used for *in vitro* fertilization were maintained in a breeding tank separated from males for 16 h previously to the experiments. Females were anesthetized with MS-222 rinsed with sterile PBS (pH 7.4) and placed in a 35 mm Petri dish. To collect the oocytes, an abdominal massage was carefully performed and if the clutch had good quality characteristics (Bobe and Labbé, 2010; Carmichael et al., 2009), 100 µl of AquaBoost®OvaCoat (Cryogenetics,USA) was immediately added to avoid oocyte dehydration and prolong oocyte fertilization ability up to 30 min. Only good quality clutches (n=99) with 100–200 oocytes were selected to test all treatments. For each sperm sample (fresh or thawed), *in vitro* fertilizations were performed immediately and simultaneously for all oocyte clutches (3–6). The AquaBoost® OvaCoat was removed with a pipette before fertilization and 1×10^6 spermatozoa (Hagedorn and Carter, 2011) of either fresh or post-thaw samples were added to the oocytes ($0.5\text{-}1\times10^4$ spermatozoa/oocyte) and immediately activated with 360 µl of sterilized and filtered (20 µm) system water at 28°C. After 5 min, 5 ml of system water was added to the Petri dish. The embryos were maintained in an incubator at 28°C with the same photoperiod as in the zebrafish facilities (14 L: 10 D). The fertilization rate was measured 3 h post-fertilization (3 hpf), at the morula stage. All the dead embryos were removed, and the viable embryos transferred to 100 mm Petri dishes. Survival and hatching rates were calculated at 24 hpf and 72 hpf, respectively, according to the initial number of oocytes of each clutch. For each treatment and sampling point, each sperm pool was used to fertilize 3 to 6 clutches of oocytes. A total of 99 fertilizations were performed, where at least 13 fertilizations were done per treatment.

5.1.3.9. Data analysis

IBM SPSS Statistics 25.0 software was used for statistical analysis. Data were expressed as means±SD and normalized by logarithmic, or arcsine transformation

when results were expressed as percentages. To check the robustness of obtained data within our sample dimension (n=3), a hierarchical cluster analysis was applied to the data obtained in experiment 1. The Ward's method (Ward, 1963) was applied since this methodology is appropriate for small samples. To apply Ward's method, the squared Euclidean distance was fixed computationally. This methodology is a mechanism of agglomerative hierarchical clustering procedure to classify pools, according to a multivariate perspective. All the variables were considered in this statistical treatment adjusted according to sperm motility, viability and *in vitro* fertilization measures. This analysis is represented through a dendogram (Supplementary data 5.1), labeled by pools of sperm, which resulted in a mixture per clustering group. When we consider a rescale distance cluster combined inferior to 5, we can observe 5 clusters of sperm pools with both storage methods. The cluster representation allows the observation of sperm pools groups formation without differentiation between LN and UF storage methods. This fact reensured the lack of differentiation per storage treatments, according to the considered variables (motility, viability and *in vitro* fertilization) and therefore one-way and two-way ANOVA was performed to compare storage time and treatments and t-test to compare fresh samples and post-thaw samples.

The data of the cooling rate experiment (Experiment 2, n=7) was subjected to one-way ANOVA. Statistica differences between treatments were detected by post hoc Student-Newman-Keuls (SNK) multiple comparison tests ($P<0.05$).

5.1.4. Results

5.1.4.1. Experiment 1 — effect of zebrafish sperm storage throughout time in a ultrafreezer (−150°C) and in liquid nitrogen

The pools of fresh sperm yielded an average of 55% of total motility (at 10 s post-activation). As expected, the percentage of total motility, VCL, and VSL (Figure 5.4 A–C) of cryopreserved sperm were significantly lower when compared to fresh sperm. However, linearity was not significantly different between fresh and cryopreserved samples (Figure 5.4 D). Most importantly, there were no significant differences between treatments throughout storage time in all motility descriptors

(Figure 5.1.4). In agreement with this information, we observed that membrane viability was not significantly different between samples stored in liquid nitrogen or ultrafreezer at each sampling point (Figure 5.5).

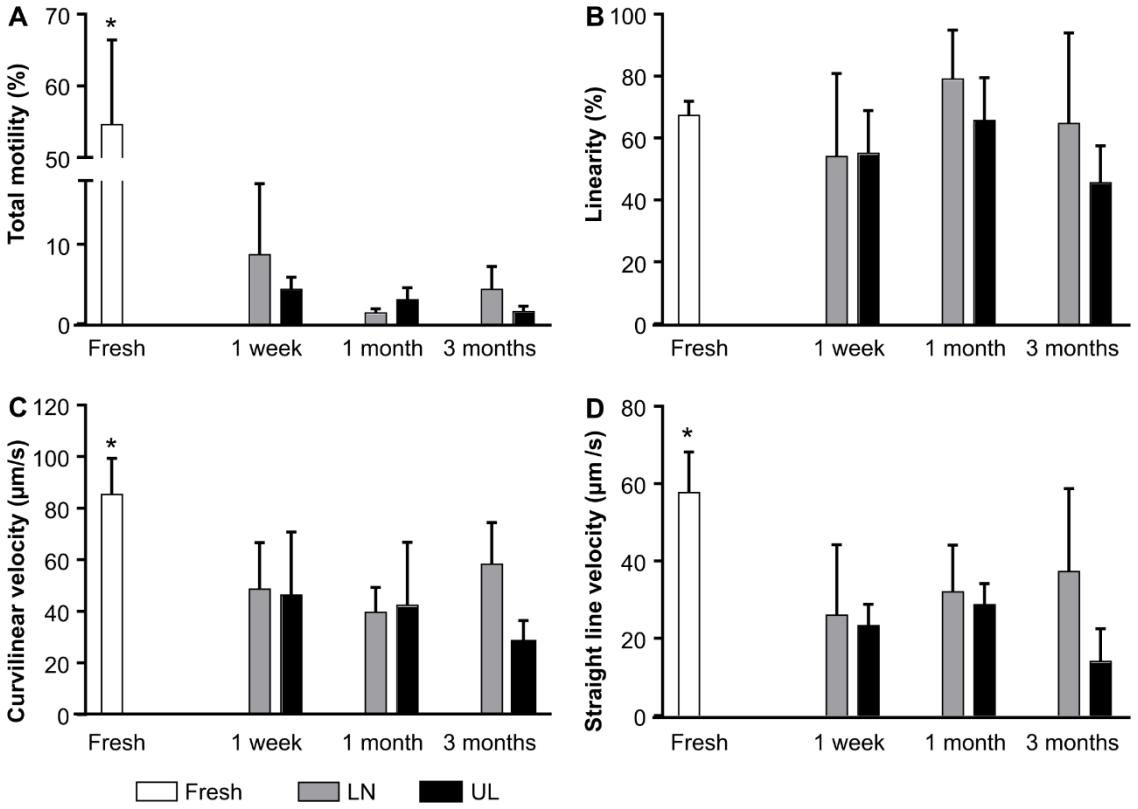

Figure 5.4 Motility of zebrafish sperm (n=3 pools) cryopreserved at -20°C/min and stored in liquid nitrogen (LN) or in ultrafreezer (UF). The samples were thawed 1 week, 1 month and 3 months after storage and characterized in terms of A) TM, B) VCL, C) VSL and D) LIN. Data is expressed as means±SD. Statistical differences (t-test, $P<0.05$) between fresh and cryopreserved sperm are represented with asterisc.

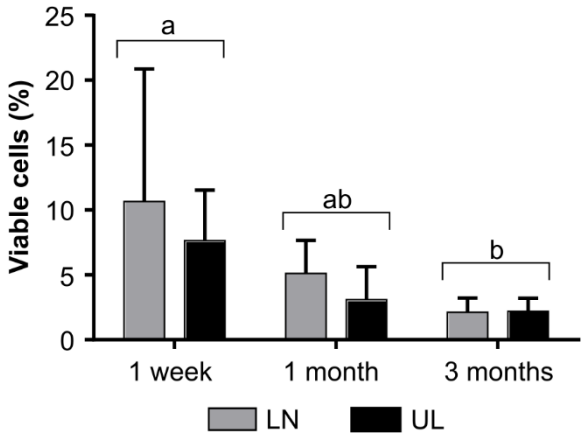

Figure 5.5 Plasma membrane integrity of zebrafish sperm (n=3 pools). Samples were cryopreserved with a -20°C/min and stored in liquid nitrogen (LN) or in a ultrafreezer (UF) and thawed 1 week, 1

month and 3 months after storage. Data is expressed as means±SD. Statistical differences within periods of storage (two-way ANOVA with post hoc SNK, P<0.05) are represented with different letters. No differences were found between the two storage methods.

Nevertheless, we observed a decrease of membrane viability throughout time in both storage conditions, where membrane viability was significantly lower at 3 months post-storage when compared to 1 week of storage.

An average of 88% fertilization rate was obtained when using fresh sperm for *in vitro* fertilization (Figure 5.6 A) and it was not significantly different when using cryopreserved sperm. There was a decrease in the embryo survival at 24 hpf and in the hatching rate, when compared to the fertilization rate at 3 hpf. However, the hatching rate had similar values to the observed for survival at 24 hpf (Figure 5.6 B and C). There were no significant differences in the hatching rate and survival at 24 hpf between liquid nitrogen and ultrafreezer storage throughout time.

5.1.4.2. Experiment 2 — effect of a fast cooling rate (−66°C/min) on zebrafish post-thaw sperm quality

Sperm cryopreserved with a cooling rate of − 66°C/min, by placing the samples directly in an ultrafreezer, had significantly higher total motility than sperm cryopreserved with a cooling rate of −20°C/min and stored in ultrafreezer (Figure 5.7 A). Sperm cryopreserved with a −66°C/min cooling rate did not show any significant differences in total motility when compared with sperm cryopreserved with a −20°C/min rate and stored in liquid nitrogen (Figure 5.1.4.4 A).

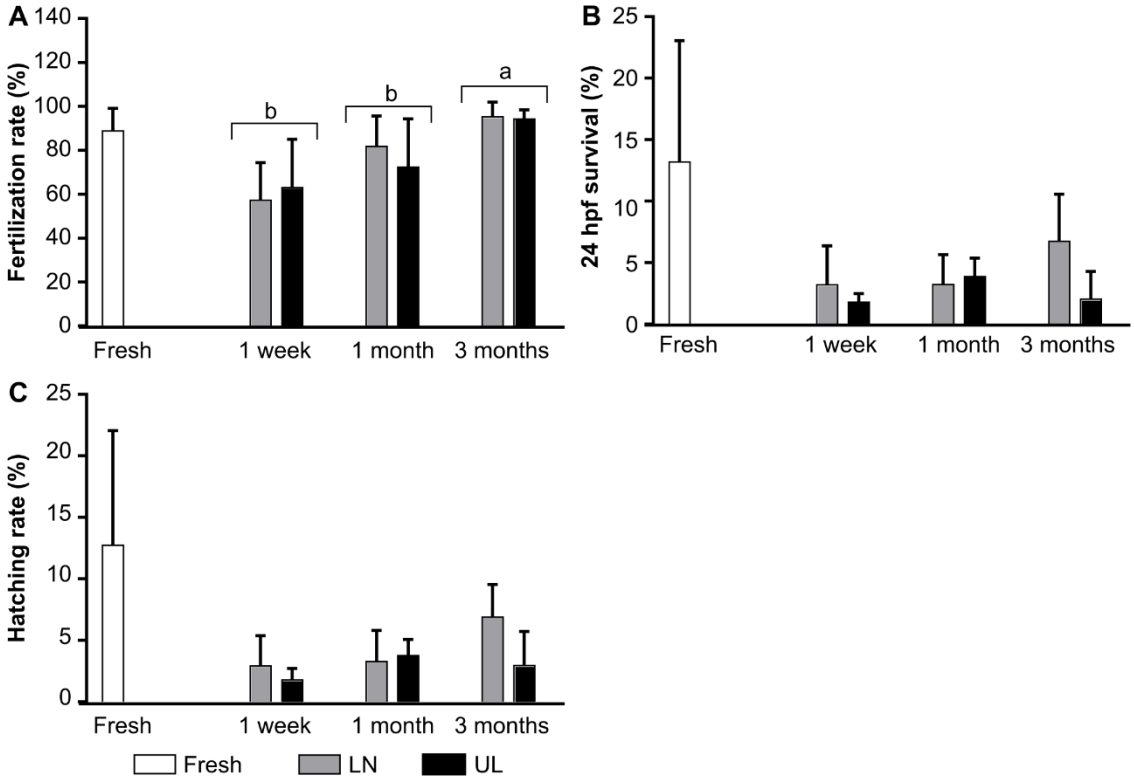

Figure 5.6 *In vitro* fertilizations performed with zebrafish sperm (n=3 pools) cryopreserved at -20°C/min and stored in liquid nitrogen or in an ultrafreezer. The samples were thawed 1 week, 1 month and 3 months after storage and the fertilization success was evaluated according to A) Fertilization rate at 3 hpf, B) Embryo survival at 24 hpf, C) Hatching rate at 72 hpf. Data are expressed as mean values±SD. Statistical differences within periods of storage (one-way ANOVA with post hoc SNK, $P<0.05$) are represented with different letters. No differences were found between the two storage methods.

There were no significant differences between treatments in terms of progressive motility (PM) and velocity (VCL and VSL) (Figure 5.7 B–D), however, there were no progressive cells in the sperm cryopreserved at −20°C/min and stored in ultrafreezer. Sperm cryopreserved with a −66°C/min cooling rate had no significant differences in linearity when compared to −20°C/min followed by liquid nitrogen or ultrafreezer storage (Figure 5.7 E).

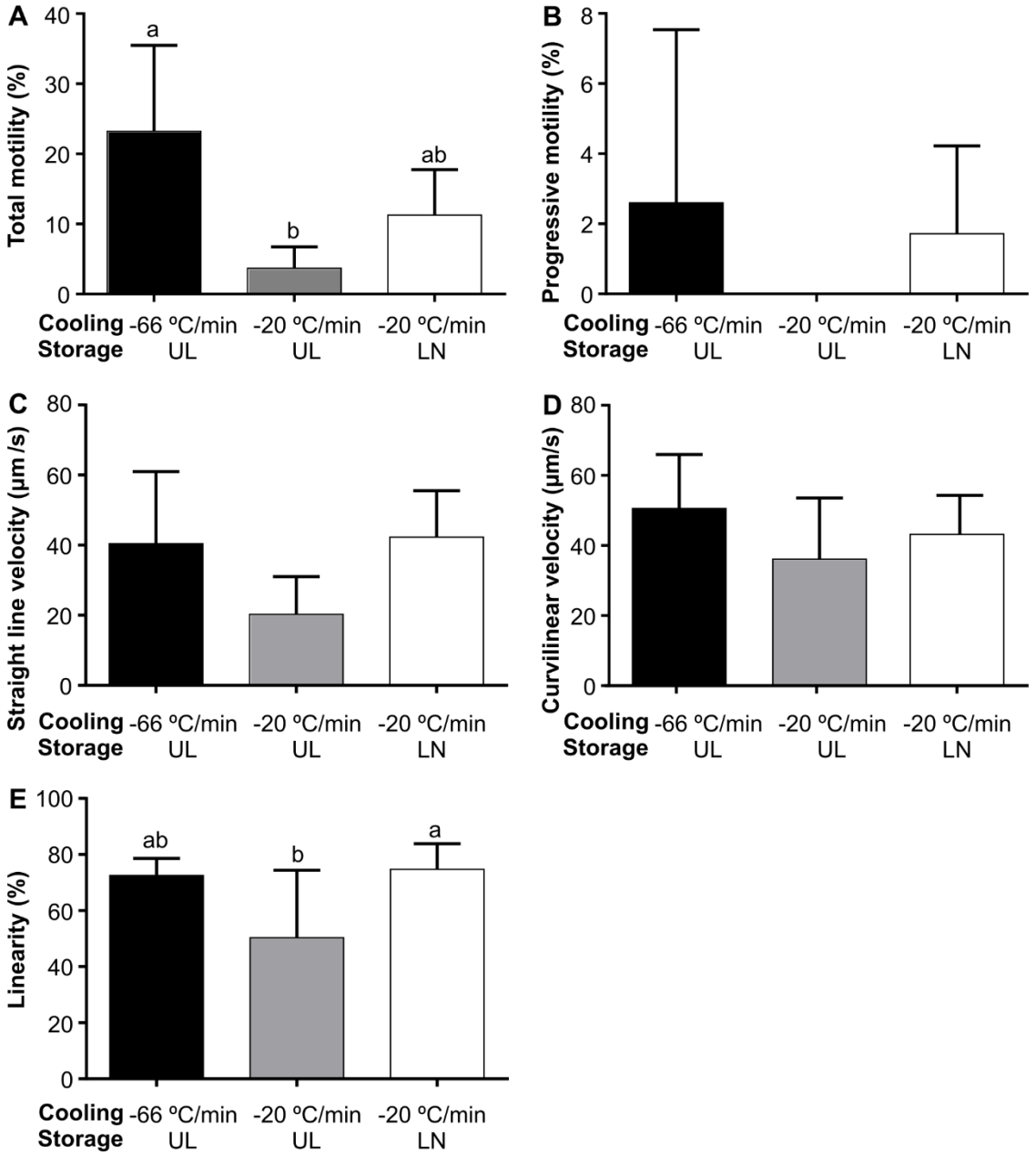

Figure 5.7 Post-thaw zebrafish sperm motility (n=7 pools) obtained from samples cryopreserved and stored directly in a ultrafreezer (-66°C/min, UF) and with a -20°C/min cooling rate with storage in ultrafreezer (-20°C/min, UF) and liquid nitrogen (-20°C/min, LN). The samples were characterized in terms of A) TM, B) PM, C) VCL, D) VSL and E) LIN. Data is expressed as mean values±SD. Statistical differences between treatments (two-way ANOVA with post hoc SNK, $P<0.05$) are represented with different letters.

The membrane viability was significantly improved in sperm with a −66°C/min cooling rate when compared to the other treatments (Figure 5.8 A). Sperm cryopreserved with a −66°C/min cooling rate showed significantly lower

DNA fragmentation when compared to the other treatment conditions (Figure 5.8 B).

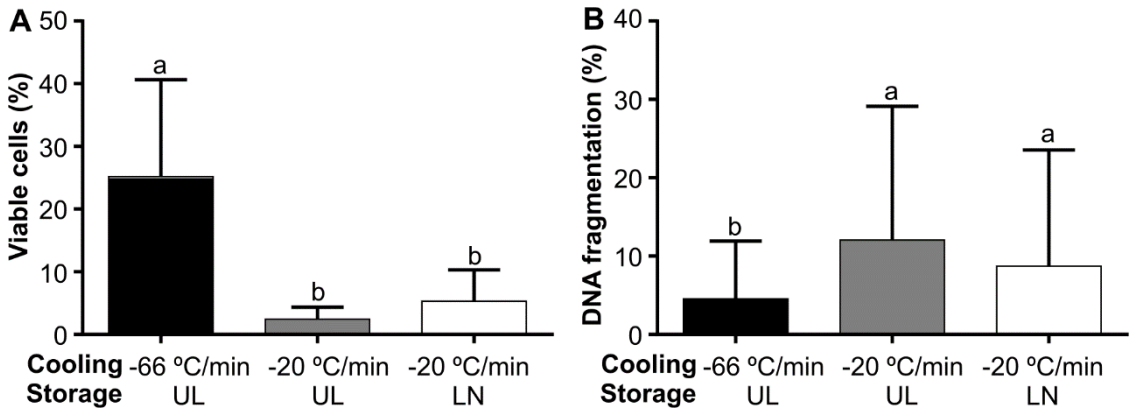

Figure 5.8 Post-thaw zebrafish sperm analysis (n=7 pools) of A) Plasma membrane integrity analyzed with flow cytometry, B) DNA fragmentation detected through comet assay. The sperm was cryopreserved directly in a ultrafreezer (-66°C/min, UF) or with a -20°C/min cooling rate with storage in ultrafreezer (-20°C/min, UF) and liquid nitrogen (-20°C/min, LN). Data is expressed as mean values±SD. Statistical differences between treatments (two-way ANOVA with post hoc SNK, $P<0.05$) are represented with different letters.

The highest DNA fragmentation was observed in the −20°C/min with ultrafreezer storage conditions. Sperm cryopreserved with a −66°C/min cooling rate had significantly higher viable cells (Figure 5.9 A) when compared to the other treatments as observed before, and significantly lower late apoptosis (Figure 5.9 B) when compared to the other treatments. However, −66°C/min cooling rate with ultrafreezer sperm storage also had a significantly higher number of necrotic/dead cells by other mechanisms (Figure 5.9 B) when compared to control method (−20°C/min cooling rate and liquid nitrogen storage). Consequently, the main cause of cells death in cryopreserved samples with −20°C/min was apoptosis, whereas in −66°C/min cryopreserved samples was another cell death cause. There were no significant differences in early apoptosis values between treatments (Figure 5.9 A).

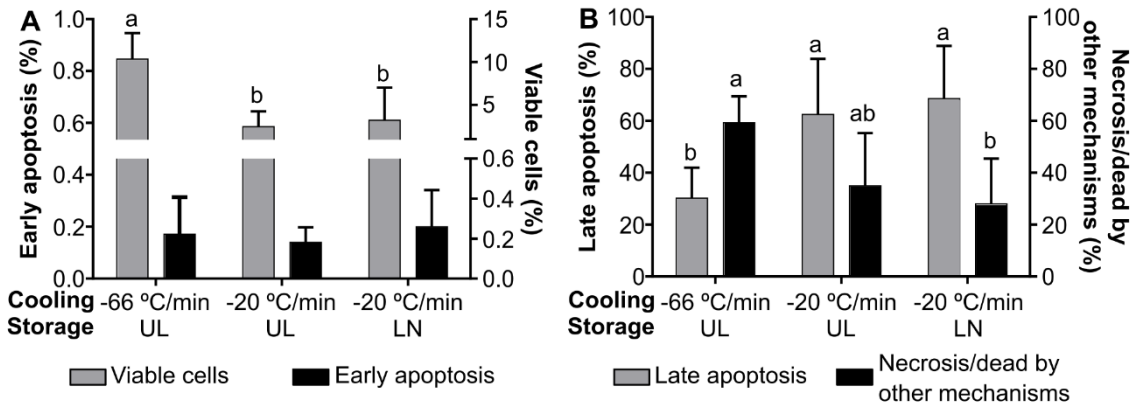

Figure 5.9 Post-thaw zebrafish sperm (n=7 pools) apoptosis detection through annexin V pathway of cryopreserved sperm directly in ultrafreezer (-66°C/min, UF) or with a -20°C/min cooling rate with storage in ultrafreezer (-20°C/min, UF) and liquid nitrogen (-20°C/min, LN). The sperm subpopulation was analyzed in terms of A) Viable cells and cells in early apoptosis, B) Cells in late apoptosis and necrotic or dead by other mechanisms. Data is expressed as mean values±SD. Statistical differences between treatments (two-way ANOVA with post hoc SNK, P<0.05) are represented with different letters.

5.1.5. Discussion

To the best of our knowledge, this is the first report of cryopreservation and storage of teleost sperm using an ultrafreezer. The ability to store cryopreserved sperm from zebrafish lines in an ultrafreezer would be extremely practical and inexpensive in these types of facilities. Consequently, it was relevant to compare the effect of sample storage throughout time in liquid nitrogen (−196°C) and in an ultrafreezer (−150°C), to ensure the viability of the storage technique. There is no other characterization of post-thaw zebrafish sperm quality using different cryostorage periods besides the present study.

Although all zebrafish sperm cryopreservation protocols have different methodologies, we selected the studies that used the CASA system to compare our data. While Yang et al. (2016) and Wang et al. (2015) started with samples of fresh sperm ranging from 80 to 95% of motility, our fresh sperm pools had an average of 55% of motility, similar to previous data published by our group (Diogo et al., 2015). This discrepancy can be explained by the fact that our motility activation is performed with system water, set at 700 µS/cm (13±3 mOsm/Kg) for a correct approximation of the fertilization microenvironment conditions to avoid

overestimations of motility, while previous studies used tap or distilled water, with lower osmolarity.

The post-thaw sperm motility registered by Wang et al (2015) after 12 h of storage was 16±3%, whereas Yang et al (2016) after 3 days of sperm storage obtained 28±15% motility. In our first experiment, we obtained 8±8% of motility after 1 week of storage. Although there were differences in methodologies and storage time since authors used different cryoprotectants, cooling rates and storage devices, it is obvious that the present study showed lower loss of motility after thawing (compared to the fresh sperm) when compared to these studies. This fact can be explained by the difference in the type of cryoprotectant used, since methanol and DMSO are known to affect negatively zebrafish sperm motility when compared to DMF (Hagedorn et al., 2012).

The analysis of membrane integrity by flow cytometry is considered a reliable method to evaluate sperm membrane viability at different conditions during storage (Figueroa et al., 2016). There were no significant differences between both storage techniques at each sampling point, which is in agreement with data reported for goat, canine and bull sperm (Álamo et al., 2005; Batista et al., 2006; Batista et al., 2009), where no significant differences were observed in motility and membrane integrity. However, there was an evident loss of membrane viability throughout storage time in both liquid nitrogen and ultrafreezer storage. Although it is generally accepted that life is on "hold" in liquid nitrogen, there are evidences of sperm quality loss over time of storage in human (Desrosiers et al., 2006) and bull sperm (Lessard et al., 2000). Lessard et al. (2000) observed a detrimental effect of storage time in the fertility marker P25b protein in bull sperm. The author hypothesized that physical vibrations at the interface between extracellular ice and plasma membrane during storage could explain P25b cryoelution. These mechanisms are still poorly understood; however, it is likely that the answer comes from the interaction of cell structures and cryosolvents. Buffers and extenders that provide different osmolarities of the cells can interact with the isotonic cytoplasm and may influence sperm viability during cryopreservation and storage (Fuller, 2004).

Although there are no universal sperm quality biomarkers, *in vitro* fertilization is considered one of the most reliable and integrative estimators of

sperm quality, since it shows the sperm ability to fertilize the oocyte (Bobe and Labbé, 2010). Yang et al. (2016) reported 62±14% of fertilization rates, which is similar to the fertilization rate (57±17%) after 1 week of storage reported in this study. Between 3 hpf and 24 hpf we observed a high degree of embryos abortion, predominantly in embryos with abnormal divisions observed initially. It has been previously described that non-motile post-thaw spermatozoa may obstruct the micropyle being forced to fertilize the eggs (Rurangwa et al., 2001), which can explain the high number of abortions and low hatching rates. It is also known that the oocyte has a mechanism that is able to repair, to some extent, spermatozoa DNA damage (Bobe and Labbé, 2010; Kopeika et al., 2004). However, in teleosts, it is reported that most embryos fertilized with damaged spermatozoa will not survive and abortion occurs between blastula and gastrula stages, where *de novo* gene expression starts to occur (Pérez-Cerezales et al., 2010). The fertilization rates can be calculated a few hours after initial cleavage, however hatching rate is a more reliable parameter, although the results take more time to be obtained (Cabrita et al., 2009). Considering the *in vitro* fertilization results achieved and the fact that hatching rates were very similar to survival at 24 hpf, we propose that the survival at 24 hpf is the most simple and accurate method to evaluate sperm fertilization ability and progeny viability produced with post-thaw zebrafish sperm.

This study validated the possibility of zebrafish sperm storage in an ultrafreezer, a method that is simpler to apply in zebrafish research facilities. However, zebrafish sperm cryopreservation would be even more simplified if it could be performed by placing samples directly in an ultrafreezer system. Therefore, in our second experiment, we tested if a fast cooling rate of −66°C/min performed directly in an ultrafreezer would benefit post-thaw sperm quality. The cooling rate is known to affect sperm survival and to interact with a medium composition (Woelders et al., 1997). Fast cooling rates can reduce the time of cell exposure to the unfavorable conditions that result from ice formation and compromise cell viability (Woelders et al., 1997). In species from zebrafish family (Cyprinidae) such as *Cyprinus carpio* (Bernáth et al., 2016) and *Perca fluviatilis* (Bernáth et al., 2015), a fast cooling rate of −56°C/min resulted in improved post-thaw sperm motility. Our results showed that −66°C/min produced improved post-thaw sperm quality in terms of total motility, membrane viability, DNA integrity and late apoptosis events

when compared to the control cooling rate of −20°C/min. Consequently, it seems that a −66°C/min cooling rate in zebrafish sperm cryopreservation reduces cryodamage risks when compared to a slower cooling rate of −20°C/min.

To perform a deeper post-thaw sperm quality characterization, we tested the effect of freezing and storage systems on zebrafish sperm DNA fragmentation and on the detection of plasma membrane phosphatidylserine externalization occurring during apoptosis mechanism. This apoptosis biomarker is a good candidate to measure damage induced by cryopreservation. Intrinsic apoptosis pathway is triggered by cell stressful factors such as radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals (Elmore, 2007). Sperm cryopreservation induces intrinsic apoptosis pathway through cold exposure or free radical production. DNA integrity is a key factor in sperm quality and progeny viability (Bobe and Labbé, 2010). The DNA fragmentation occurs late in the apoptosis process after apoptosis-inducing factors being translocated from the mitochondria to the nucleus, causing DNA fragmentation (Elmore, 2007). Annexin V is a recombinant phosphatidylserine-binding protein that binds specifically to phosphatidylserine residues. The Annexin V & Dead cell marker combination used in this study enables the quantification of different populations of cells according to the type of damage: viable cells (non-stained), apoptotic cells (early or late apoptosis) and necrotic/dead cells by another mechanism. This last subpopulation has compromised plasma membrane integrity but does not show any phosphatidylserine externalization and, therefore, cell death can be attributed to mechanisms such as ice crystal injury. This subpopulation is significantly higher in sperm cryopreserved directly in the ultrafreezer (59.27±10%), probably due to the rapid cooling which difficult water movement through the cell, allowing ice crystal formation that can cause plasma membrane disruption. However, this treatment also revealed the lowest values of late apoptosis and lower DNA fragmentation when compared to the other treatments. There are no references in the literature on zebrafish post-thaw sperm quality analysis in terms of apoptosis tests through annexin V and DNA damage. However, Reinardy et al (Reinardy et al., 2013b) obtained 9–12% of DNA damage in fresh zebrafish sperm, which is very similar to the values determined in our study using cryopreserved sperm (12.37% DNAt for −66°C/min in ultrafreezer). Although slightly different values were obtained in cell viability determined by IP/SYBR 14

and the annexin-V dead kit, both methodologies are in agreement, sustaining that a faster cooling rate of −66°C/min is more appropriate for zebrafish sperm when compared to −20°C/min cooling rate.

These results show that sperm cells cryopreserved directly in an ultrafreezer present a decrease in cell apoptosis and DNA fragmentation and that the main cause of cell death in this treatment have occurred through other mechanisms, such as the ones previously suggested.

In conclusion, our study demonstrates that ultrafreezers are a viable alternative for zebrafish sperm storage. Furthermore, a fast cooling rate of −66°C/min performed directly in an ultrafreezer improved post-thaw zebrafish sperm quality. This study optimized the cooling rate of zebrafish sperm cryopreservation, which is an important contribution to support future methodological improvements. This methodology facilitates the cryopreservation process without the need for access to expensive programmable biofreezers and can be easily applied in zebrafish facilities, reducing the global costs of cryopreservation.

5.2. CRYOPROTECTANTS SYNERGY IMPROVE ZEBRAFISH SPERM CRYOPRESERVATION AND OFFSPRING SKELETOGENESIS

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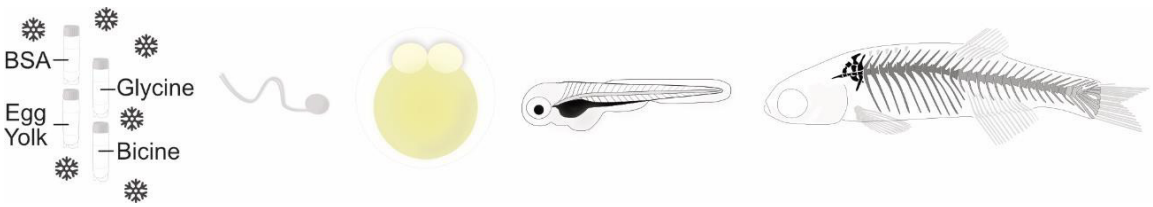

[Cryobiology, submitted]

5.2.1. Abstract

The synergy obtained by the combination of cryoprotectants is a successful strategy that can be beneficial on the optimization of zebrafish sperm cryopreservation. Recently, a protocol was established for this species using an electric ultrafreezer (-150°C) performing cooling rate (-66°C/min) and storage within one step. The ultimate objective of sperm cryopreservation is to generate healthy offspring. Therefore, the objective of this study was to select the most adequate cryoprotectant combination, for the previously established protocol, that generate high quality offspring with normal skeletogenesis. Among the permeating cryoprotectant concentrations studied 12.5% and 15% of N,N-dimethylformamide (DMF) yielded high post-thaw sperm quality and hatching rates. For these two concentrations, the presence of bovine serum albumin (10 mg/ml), egg yolk (10%), glycine (30 mM) and bicine (50 mM) was evaluated for post-thaw sperm motility, viability, *in vitro* fertilization success and offspring skeletal development (30 days post fertilization). Higher concentration of permeating cryoprotectant (15%) decreased the incidence of deformed arches and severe skeletal malformations, which suggests higher capacity to protect the cell against cold stress and DNA damage. Bicine and egg yolk were the non-permeating cryoprotectants with higher post-thaw quality. The use of these compounds results in a reduction in vertebral fusions, compressions and severity of skeletal malformations in the offspring. Therefore, 15% of DMF with 50 mM bicine was shown to be beneficial for the quality of zebrafish offspring sired by cryopreserved sperm with -66°C/min freezing rate. To the best of our knowledge, this is the first report on skeletal development of the offspring sired by cryopreserved sperm performed with different extender compositions in zebrafish.

5.2.2. Introduction

Zebrafish is the second most used model organism with increasing interest by the scientific community in the past decade. Consequently, new mutant and transgenic lines are developed continuously in laboratories across the world, posing challenges in terms of space and management that cryopreservation can solve. Until today, zebrafish sperm cryopreservation lacks standardization, yielding variable

post-thaw sperm quality and *in vitro* fertilization success (Asturiano et al., 2017; Robles et al., 2009; Yang and Tiersch, 2009). Recently, our laboratory developed the first cryopreservation protocol in a teleost species using an electric ultrafreezer (-150°C). This protocol does not require liquid nitrogen or dry ice, samples are placed directly on the electric ultrafreezer where the freezing rate (-66°C/min) and storage occur in one single step (Diogo et al., 2018). The use of ultrafreezers for sperm cryopreservation and storage allows the reduction of the global costs of cryopreservation and simplify the procedure. Therefore, it is a valuable alternative cryopreservation method for zebrafish facilities management. Following the establishment of this protocol, the present work aims to optimize the extender by modulating the permeating and non-permeating cryoprotectants composition.

A cryoprotectant agent is a solute that when present in the cells medium, allow higher post-thaw recoveries than if it is not present (Karow, 1969). Cryoprotectants are categorized as permeating and non-permeating, according to their ability to penetrate cellular membranes (Devismita and Kumar, 2015; Elliott et al., 2017). In cryobiology, it has become clear that distinct cryoprotectant classes can efficiently protect cells against freezing injuries through multiple mechanisms, many of which are still poorly understood (Elliott et al., 2017; Martínez-Páramo et al., 2017). The combination of permeating and non-permeating cryoprotectants is considered a successful strategy (Elliott et al., 2017) widely used among sperm cryopreservation protocols of teleost species (Cabrita et al., 2010).

The protocol developed in our facilities for zebrafish sperm cryopreservation (Diogo et al., 2018) comprises an extender with 10% of N,N-dimethylformamide (DMF) in Hank´s balanced salt solution (HBSS) for a -66°C/min freezing rate. This method improved post-thaw sperm DNA integrity, plasma membrane viability and late apoptosis (Diogo et al., 2018). The permeating cryoprotectant concentration was previously selected for slower cooling rates performed in dry ice (Asturiano et al., 2015; Diogo et al., 2018). Considering that cell biophysical properties vary with temperature (Elliott et al., 2017), it was essential to investigate the most appropriate concentration of DMF for a -66°C/min freezing rate, to improve the previously established protocol. In other cyprinid species similarly fast cooling rates improved post-thaw sperm quality (Bernáth et al., 2015; 2016). There are structural,

morphological and biophysical similarities observed between spermatozoa of zebrafish and other cyprinid species (Zhang et al., 2014). These facts suggest that methodological improvements for cryopreservation in cyprinid species may benefit zebrafish sperm post-thaw quality. In cyprinids, extenders commonly contain bicine and glycine (Cabrita et al., 2010; Glogowski et al., 2001; Yavaş et al., 2014), therefore it was pertinent to investigate the effect of these compounds in zebrafish sperm cryopreservation. Additionally, Bovine Serum Albumin (BSA) and Egg Yolk (EY) were selected as non-permeating cryoprotectants due to their extensive use in cryopreservation of sperm from several species, with beneficial post-thaw outcomes (Cabrita et al., 2010; Pérez-Cerezales et al., 2009; Riesco et al., 2017).

Sperm fertilizing ability is considered the most effective quality analysis to validate the effectiveness of a sperm cryopreservation protocol (Cabrita et al., 2010; Gallego and Asturiano, 2018; Rurangwa et al., 1998). However, the quality of the offspring generated by cryopreserved sperm beyond hatching rate have been poorly addressed (Dziewulska et al., 2011; Labbé et al., 2001; Miskolczi et al., 2005; Pérez-Cerezales et al., 2010; Yang et al., 2012; Young et al., 2009), particularly the incidence of malformations (Miskolczi et al., 2005; Young et al., 2009) which were only studied immediately after hatching. Since skeletal development and incidence of malformations is a well-established fish quality evaluation system (Boglione et al., 2013a; b), it is a useful tool for the characterization of offspring quality sired by cryopreserved sperm.

The description of skeletal malformations generates complex data sets with high biological variability, being therefore difficult to analyze in depth through traditional statistical methods. Machine learning is a method focused on the development of algorithms that are particularly useful for data mining. These algorithms are able to automatically learn to recognize complex patterns based on data. Classification or decision trees are machine learning methods that can provide guidelines for decision making (Breiman et al., 1984). Decision trees are non-parametric models that use algorithms to split data sets into increasingly homogeneous subsets, representing class membership through hierarchal distribution. Therefore, classification trees are considered a “knowledge discovery” technique (Cios et al., 2007), which have been considered a powerful tool for the

optimization of cryopreservation technologies (Ramón et al., 2012; Sambu, 2015), although it is still poorly explored. This modeling technique is flexible enough to handle complex problems with multiple interacting elements, yielding a straightforward interpretation (Cios et al., 2007). Consequently, it is an ideal method to explore the effects of cryoprotectant combinations during zebrafish sperm cryopreservation on the resulting offspring skeletogenesis.

The objective of this study was to select the optimal combination of permeating and non-permeating cryoprotectants for zebrafish sperm cryopreservation, performed with an electric ultrafreezer (-66°C/min freezing rate). For that purpose, the effect of permeating cryoprotectant (DMF) concentration on post-thaw sperm quality and *in vitro* fertilization was investigated. Additionally, the interactions between the combinations of two concentrations of the permeating cryoprotectant (12.5% and 15% DMF) and the addition of non-permeating cryoprotectants (10 mg/ml of BSA, 10% of EY, 30 mM glycine and 50 mM of bicine) were evaluated. Finally, the skeletal malformations of the offspring sired by sperm cryopreserved with different extender compositions were studied for the first time, to select the protocol which generated offspring with the higher skeletal quality.

5.2.3. Methods

5.2.3.1. Fish rearing

Adult AB zebrafish males and females were selected according to the age selection criteria previously established in our laboratory (6-8 months old) (Diogo et al., 2019). Zebrafish with similar size were maintained separated according to sex into 3.5 L tanks (n=15) to improve fecundity, egg viability and early larvae survival (Kurtzman et al., 2010). The fish were maintained in a water recirculation system (ZebTEC® Tecniplast, Italy). The fish room had a controlled photoperiod with a 14:10 h light/dark cycle, an independent air conditioning system (26±1°C) and an air extraction system to guarantee the air renewal in the room, maintaining the humidity close to 60%. The water rearing system was partially replaced (10%) daily and the water system maintained at 28.5±0.5°C, 700±50 µS and pH 7.5±0.1. The fish

were fed *ad libitum* twice a day with *Artemia* nauplii (AF480, INVE, Belgium) and ZEBRAFEED® diet (Sparos Lda, Portugal). Food consumption was visually controlled, and the remains removed daily. All animal manipulations were performed in compliance with the Guidelines of the European Union Council (86/609/EU) and transposed to the Portuguese law for the use of laboratory animals on research by "Decreto Lei n° 129/92 de 06 de Julho, Portaria n° 1005/92 de 23 de Outubro", and according to the European parliament council directive´s for protection of animals used for scientific research (2010/63/EU). All animal protocols were performed under a "Coordinator-researcher" license from the Direção-Geral de Veterinária, Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, Lisbon, Portugal, under the "Decreto Lei n°113/2013 de 7 de Agosto" relative to the protection of animals used for scientific research.

5.2.3.2. Sperm collection

On the day prior to the sperm collection, males (n=4) and females (n=4) were placed in 1 L breeding tanks in 1:1 sex-ratio (Tecniplast, Buguggiate, Italy) and maintained separated while sharing the same water for 16 h (Diogo et al., 2018). This method allows the exposure to the pheromones of the mating partners, which promotes the synchronization of mating behavior and oocyte release (Gerlach, 2006; Hurk and Lambert, 1983; van den Hurk et al., 1987). Sperm collection was performed, within 1 h after the beginning of the light phase of the photoperiod. Males were properly anesthetized with 0.168 mg/ml tricaine sulfonate solution (MS-222, Sigma-Aldrich, Madrid, Spain) according to Westerfield (Westerfield, 2007). When the gill movement decreased, the males were rinsed with Phosphate Buffered Saline (PBS) solution and carefully cleaned with paper towels. For sperm collection, an abdominal massage was performed and the sperm collected using a glass capillary tube attached to a mouth piece. Immediately after collection, sperm was diluted into 10 µl of sterilized and filtered (0.20 µm) Hank´s Balanced Salt Solution (HBSS) at 300 mOsm/Kg (NaCl 8.0 g, KCl 0.4 g, $CaCl_2$ x $2H_2O$ 0.16 g, $MgSO_4$ x $7H_2O$ 0.2 g, Na_2HPO_4 0.06 g, KH_2PO_4 0.06 g, $NaHCO_3$ 0.35 g, $C_6H_{12}O_6$ 1.0 g in 1000 ml of milli-Q water, pH 7.5) (Hagedorn et al., 2012; Jing et al., 2009a).

5.2.3.3. *Experiment 1 – Permeating cryoprotectant: DMF concentration*

An experiment was conducted to evaluate the adequate N-N dimethylformamide (DMF) concentration necessary to protect spermatozoa from cold damage using a -66°C/min freezing rate in an electric MDF-C2156VAN ultra-low temperature freezer (Sanyo, Demark). To perform sperm pools (n=6 pools) for this experiment, we selected sperm samples from males with total motility over 50% (at 10 s post activation) and cell concentration over 3 x 10^7 cells/ml. Each sperm pool contained sperm from 10 males. A cooling rate of -66°C/min was applied placing the samples directly in an ultrafreezer system (-150°C) as previously described (Diogo et al., 2018). Sperm was cryopreserved with 5%, 7.5%, 10%, 12.5% and 15% of DMF in HBSS in a final volume of 10 µl and stored in 2 ml cryovials (VWR® Low-Temperature Freezer Vials). After 5 days of storage in the ultrafreezer system, thawing was performed in a 40°C water bath during 8 s. Sperm quality was evaluated through sperm motility, membrane integrity and *in vitro* fertilization success (Figure 5.10).

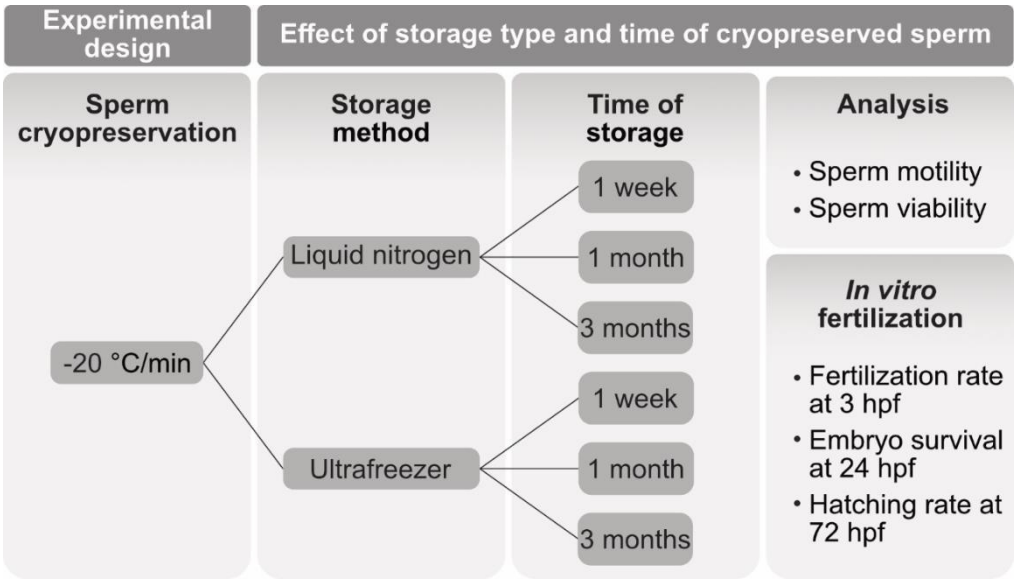

Figure 5.10 Experimental design to evaluate the effect of the storage type (LN, UF) of cryopreserved zebrafish sperm throughout storage time on sperm motility, viability and *in vitro* fertilization success.

5.2.3.4. *Experiment 2 – Non-permeating cryoprotectants: BSA, egg yolk, glycine and bicine*

According to the results obtained in experiment 1, 12.5% and 15% DMF were selected to evaluate the effect of non-permeating cryoprotectants on the extender used for zebrafish sperm cryopreservation. For each DMF concentration, a control (Ctrl) without non-permeating cryoprotectant was used and it was evaluated the effect of 10 mg/ml BSA (BSA), 10% egg yolk (EY), 30 mM glycine (Gly) and 50 mM of bicine (Bici) on the extender. The concentrations of non-permeating cryoprotectants were chosen according to the commonly used in other successful sperm cryopreservation protocols for teleost species (He and Woods III, 2003; Martínez-Páramo et al., 2013; Matthews et al., 2018; Riesco et al., 2017). To characterize post-thaw sperm quality (n=5 pools, each pool containing sperm of 16 males), the evaluation of sperm motility, plasma membrane viability and *in vitro* fertilization success was performed (Figure 5.11).

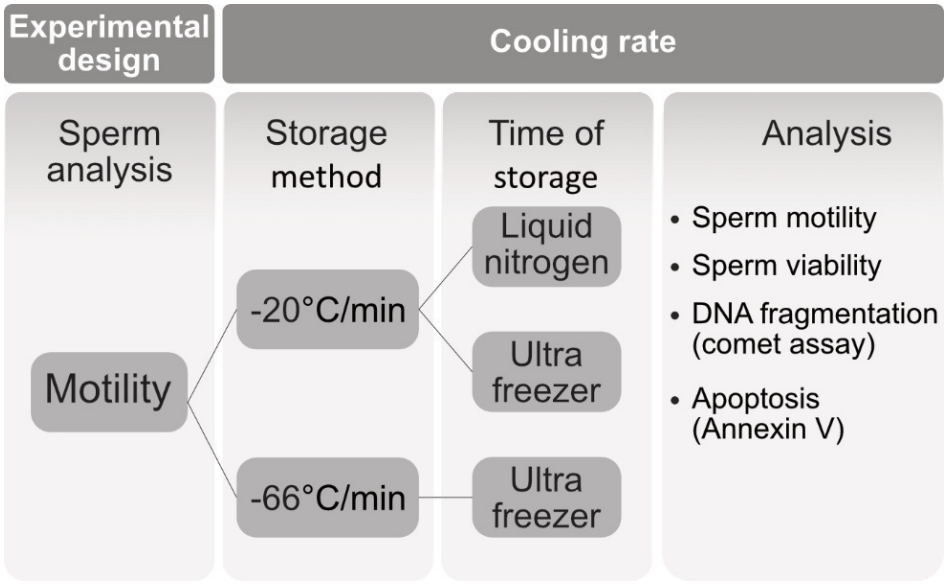

Figure 5.11 Experimental design to evaluate the effect of cooling rate (-20°C/min and -66°C/min) and storage method (LN, UF) on zebrafish cryopreserved sperm motility, viability, DNA fragmentation and apoptosis.

5.2.3.5. Sperm concentration and motility analysis

Sperm concentration and motility were evaluated using computer assisted sperm analysis (CASA) system (ISAS Integrated System for Semen Analysis, Proiser, Valencia, Spain) coupled to a phase contrast microscope (Nikon E-200, Nikon, Tokyo, Japan) with a ×10 negative phase contrast objective. The images were captured with ISAS 782C camera (Proisier, Spain) and processed with CASA software. The settings of the CASA system were adapted previously for this species namely 25 frames/s, connectivity 14, 1 to 90 mm for head area and only sperm samples with VCL > 10 μm/s were considerate motile. For sperm concentration, a dilution (1:19) was performed with HBSS and 3 fields were sampled to determine cell concentration. For motility analysis 0.5 μl of fresh sperm or 1.5 μl of cryopreserved sperm was placed on a Mackler chamber and immediately activated with 5 μl of filtered (0.20 μm) and sterilized system water at 28°C. Sperm motility was characterized at 10 s post-activation according to total motility (TM; %), progressive motility (PM; %), curvilinear velocity (VCL; μm/s), straight-line velocity (VSL; μm/s) and linearity (LIN; %).

5.2.3.6. Membrane integrity

Sperm membrane integrity was assessed through flow cytometry using SYBR 14 (Invitrogen, Spain) and Propidium Iodide (PI) (Sigma Aldrich, Spain) labeling, according to previously established methodology (Diogo et al., 2018). SYBR 14 is a permeant nucleic acid stain that crosses the plasma membrane and PI is a membrane impermeable dye that label cells nucleic acids when the plasma membrane is compromised. Consequently, spermatozoa with compromised plasma membrane are labeled in red from PI and viable cells are labeled in green by SYBR 14 (Daly and Tiersch, 2012). SYBR 14 was prepared diluting 5 μl of stock solution in 120 μl of sterilized and filtered HBSS and PI was used undiluted. The pre-diluted sperm samples were re-diluted (1:300) in HBSS and each stain was added in a final concentration of 6.7 nM of SYBR 14 and 3 ng/ml of PI. Sperm was incubated for 5 min in the dark at room temperature. The analysis was performed in a flow cytometer (BD FACSCalibur™, BD Biosciences, Spain) adjusted for the detection of SYBR 14 through a 530 nm bandpass filter (FL1) and PI was detected with a 670 nm

long pass fia lter (FL3). Flow cytometer settings were previously adjusted using a positive (100% dead cells) and a negative control (fresh sperm). As negative control, spermatozoa were exposed to successive cycles of freezing thawing (Cabrita et al., 2005). A total of 5000-10000 events were counted for each sample.

5.2.3.7. In vitro fertilization

Females used for *in vitro* fertilization were maintained in a breeding tank separated from males for 16 h previously to the experiments. Females were anesthetized with MS-222, as described above. When the gill movement decreased, the females were rinsed with sterile PBS (pH 7.4) and placed in a 35 mm Petri dish. An abdominal massage was carefully performed to collect the oocytes, avoiding any mechanical contact. If the clutch of oocytes had good quality characteristics (Bobe and Labbé, 2010; Carmichael et al., 2009), *in vitro* fertilization was performed within 1 min after collection. Only good quality clutches were selected both for experiment 1 (n=20) and experiment 2 (n=24). The sperm contained in one cryovial was used for each fertilization. Therefore, a total of 1.5 - 2 x 10^6 spermatozoa was added to the oocytes (100-200) of either fresh or thawed samples. Sperm motility activation was immediately performed with 360 µl of sterilized and filtered (0.2 µm) system water at 28°C. After 5 min, 5 ml of system water was added to the Petri dish containing the eggs. The embryos were maintained in an incubator at 28°C with the same photoperiod as in the zebrafish facilities (14L:10D). All the dead embryos were removed, and the viable embryos transferred to 100 mm Petri dishes 3 - 4 hours post fertilization (hpf). Survival and hatching rates were calculated at 24 hpf and 72 hpf, respectively according to the initial number of oocytes of each clutch (approximately 100-200). For each treatment, each sperm pool was used to fertilize 2-5 clutches of eggs.

5.2.3.8. Skeletal development analysis of the offspring obtained from both experiences

The evaluation of skeletal malformations in the offspring generated by cryopreserved sperm in both experiments was performed at 30 days post

fertilization (dpf). The fish were anesthetized with a lethal dose of MS-222 (300 mg/ml) (Sigma-Aldrich, Saint Louis, MO) (Matthews and Varga, 2012) and fixed in a 4% buffered paraformaldehyde solution at 4°C for 24 h. Larvae were further washed with PBS, pH 7.4 and stored in 75% ethanol at room temperature (Gavaia et al., 2000). A modified method of whole-mount acid-free double staining was performed using alcian blue 8GX (Sigma-Aldrich, Saint Louis, MO) for cartilage and alizarin red S (Sigma-Aldrich, Saint Louis, MO) for bone (Walker and Kimmel, 2007a). Briefly, samples were stained in alcian blue 8GX for 1.5 h and passed through a decreasing series of ethanol concentrations (96 to 25%) and hydrated with distilled water before being stained with alizarin red S in a potassium hydroxide solution 0.5% overnight. The samples were cleared with a 0.5% KOH solution and stored in a solution of 90% glycerol (Merk Millipore, Billerica, MA) at room temperature. The detection of skeletal anomalies was performed following the nomenclature by Bird and Mabee (Bird and Mabee, 2003). Briefly, the description of skeleton malformations was performed for each skeleton structure namely arches (neural and haemal) and centra (vertebrae) distributed within each region of the axial skeleton (Weberian apparatus, precaudal vertebrae, caudal vertebrae and caudal fin vertebrae). The severe malformations considered were lordosis, kyphosis and scoliosis. The occurrence of fusions, compressions, abnormal arches, extra arches, opened arches and deformed centra were evaluated and images acquired with a stereomicroscope SteREO Lumar.V12 (Zeiss, Germany).

5.2.3.9. Data analysis

IBM SPSS Statistics 25.0 software was used for statistical analysis. Data were expressed as means±SD (Standard Deviation) and normalized by logarithmic, or arcsine transformation when results were expressed as percentages. In experiment 1 to evaluate the significance of the permeating cryoprotectant concentration effect on post-thaw sperm quality and *in vitro* fertilization success, a one-way ANOVA multiple comparison tests (Student–Newman–Keuls, $P<0.05$) was performed. In experiment 2, significant differences between fresh and cryopreserved sperm were detected through independent samples t-test ($P<0.05$) and differences between permeating cryoprotectant concentration and non-permeating cryoprotectants

were detected through a two-way ANOVA, with Student–Newman–Keuls post hoc to evaluate differences between non-permeating cryoprotectants and independent samples t-test to evaluate the effect of cryoprotectant concentration ($P<0.05$).

The incidence of malformations, their severity, distribution and load per fish obtained by each treatment was investigated through Pearson´s Chi-square analysis ($P<0.05$).

For a deeper comprehension of the relationship between cryoprotectant composition during zebrafish sperm cryopreservation and the onset of skeletal malformations on the resulting offspring, a machine learning technique was performed, complementing the traditional statistical analysis. Since the variables are potentially correlated with each other a decision tree was applied through the algorithm CART (classification and regression), that uses GINI index splicing criteria (a measure of statistical dispersion). These tree models classify cases into groups or predict values of a dependent variable (criterion), based on values or categories of the independent variables (predictors). The criterion used was the malformed individuals in relation to the following predictors: permeating cryoprotector concentration, non-permeating cryoprotector, treatment (a combination of cryoprotectants), vertebral compression, fusions, additional arches, opened arches, deformed arches, deformed centra and a number of a load of deformations. A maximum tree depth of 5 levels was specified with a minimum number of classes in the initial and terminal nodes set at 20 and 10 respectively. Variables were not considered if a regression tree could not be generated.

5.2.4. Results

5.2.4.1. Permeating cryoprotectant: DMF concentration

The effect of different permeating cryoprotectant concentrations on sperm quality and *in vitro* fertilization success displayed a normal curve behavior on the analysis of total motility, progressive motility, embryo survival at 24 hpf and hatching rate (Figure 5.12). These parameters were more representative of the

effect of permeating cryoprotectant concentration when compared to sperm velocities and linearity.

Post-thaw sperm quality was significantly lower than fresh sperm in terms of total motility, progressive motility and plasma membrane viability (Figure 5.12 A, B and F). Extender containing 12.5% and 15% of DMF showed significantly higher post-thaw sperm total motility and plasma membrane viability (Figure 5.12 B and F). Additionally, these treatments showed no significant differences when compared to fresh sperm curvilinear and straight-line velocity (Figure 5.12 C and D). Linearity was affected in cryopreserved sperm with 5 and 10% of DMF when compared to fresh sperm and sperm cryopreserved with 12.5 and 15% of DMF (Figure 5.12 E).

The use of 5% of DMF yielded significantly lower embryo survival when compared to fresh sperm and the other cryopreserved treatments (Figure 5.12 G). The hatching rate was significantly higher in fresh sperm and sperm cryopreserved with 10% and 12.5% of DMF when compared to 5%, however, both concentrations showed no significant differences when compared to 7.5 and 15% of DMF (Figure 5.12 H). The treatment that yielded lower post-thaw sperm quality was 5% of DMF.

Considering the overall sperm quality and *in vitro* fertilization analysis, 12.5% and 15% of DMF were selected to investigate the effect of non-permeating cryoprotectants on post-thaw sperm quality and offspring skeletal development.

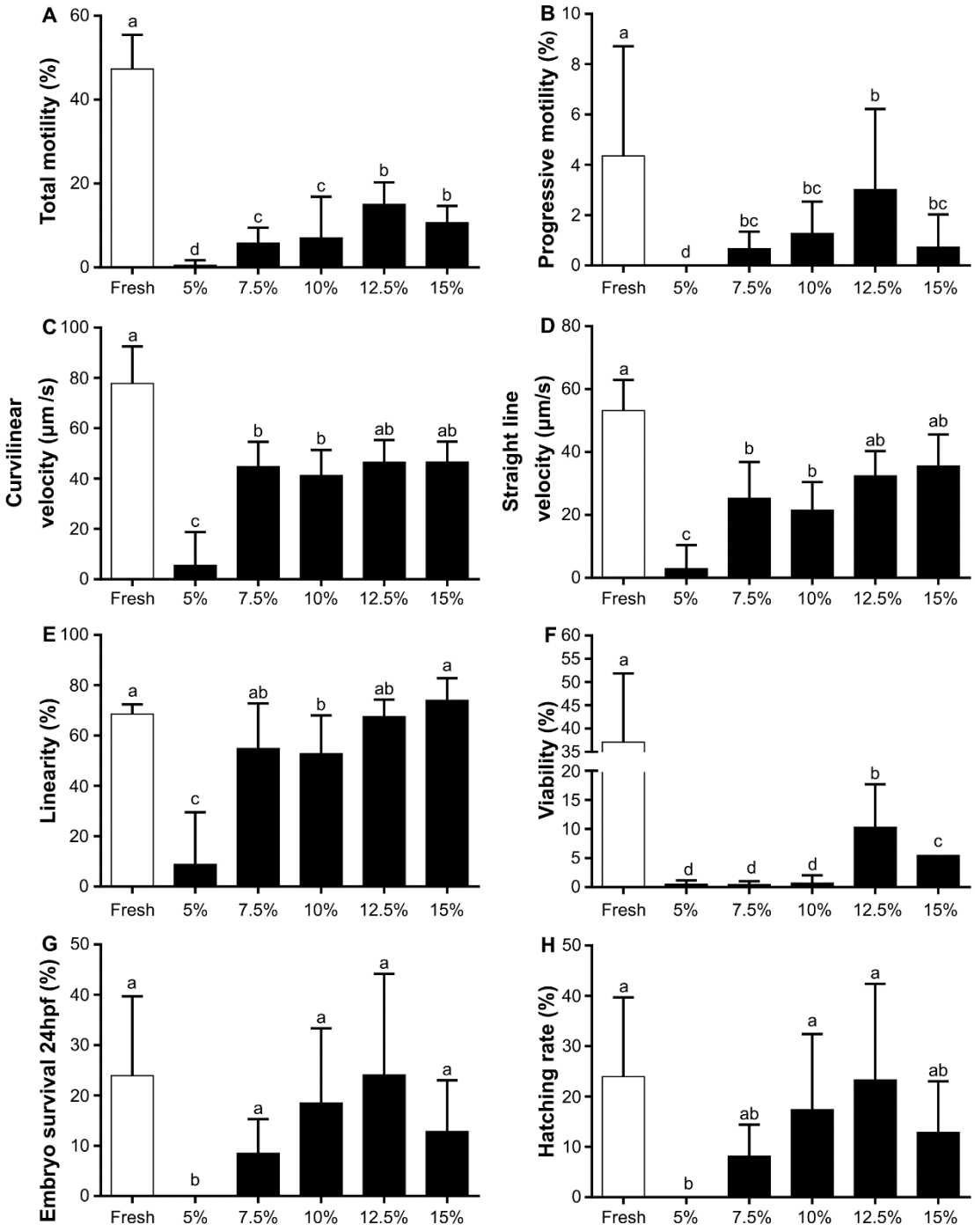

Figure 5.12 Effect of different concentrations of permeating cryoprotectant (DMF) on zebrafish sperm (n=6 pools containing sperm of 10 males) A) TM, B) PM, C) VCL, D) VSL, E) LIN and F) viability of the plasma membrane. For the same pools of sperm, the success of *in vitro* fertilizations performed with fresh (n=4) and cryopreserved sperm with 5% (n=5), 7.5% (n=4), 10% (n=5), 12.5% (n=5) and 15% (n=4) was evaluated through G) embryo survival 24 hpf and H) hatching rate at 72 hpf. The values plotted in white (fresh sperm) and black (cryopreserved sperm) bars represent means±SD. Different letters on the bars indicate significant differences (one-way ANOVA, post hoc SNK $P<0.05$).

5.2.4.2. Non-Permeating cryoprotectants: BSA, egg yolk, glycine and bicine

The post-thaw sperm quality and *in vitro* fertilization parameters analyzed did not show significant interactions (two-way ANOVA, $P<0.05$), except for total motility (Table 5.1). Consequently, the effect of permeating cryoprotectant concentration and non-permeating cryoprotectants can be studied independently, except in total motility. Progressive motility, velocities and linearity were significantly dependent on the presence of non-permeating cryoprotectants, but not on the permeating cryoprotectant concentration. Plasma membrane viability was significantly dependent on both permeating cryoprotectant concentration and non-permeating cryoprotectants addition (Table 5.1).

Fresh sperm had significantly higher total and progressive motility, velocities, linearity and plasma membrane viability when compared to cryopreserved sperm. However, the *in vitro* fertilization parameters were not significantly different from cryopreserved sperm (Figure 5.13). The use of BSA had a negative effect on post-thaw sperm quality, especially in sperm total motility, progressive motility and plasma membrane viability (Figure 5.13).

The use of egg yolk as a non-permeating cryoprotectant in the extender with 15% DMF improved significantly sperm total motility when compared to the other treatments, however, it was not significantly different from control (Figure 5.13 A).

The addition of bicine on the extender composition significantly improved progressive motility when compared to BSA, but it was not different from to the other treatments (Figure 5.13 B). Both velocities were significantly improved in control treatment when compared to BSA, however, control was not significantly different to the egg yolk and glycine treatment (Figure 5.13 C and D). Spermatozoa linearity movement was significantly higher in control treatment when compared to BSA, but not to the other non-permeating cryoprotectants (Fig Figure 5.13 E).

The plasma membrane viability of control and bicine treatment was significantly higher than BSA, but there were no differences when compared to the egg yolk and glycine treatment (Fig Figure 5.13 F).

Although there were no statistical differences in vitro fertilization parameters, it is interesting to observe that sperm cryopreserved with 12.5% of DMF with bicine and sperm cryopreserved with 15% of DMF with egg yolk, showed the highest embryo survival and hatching rates (Figure 5.13 G and H).

Table 5.1 Post-thaw zebrafish sperm quality analysis related to the effect of permeating cryoprotectant concentration, non-permeating cryoprotectants and their interactions in post-thaw zebrafish sperm.

Two-way ANOVA (P value<0.05)	Permeating	Non-permeating	Permeating*non-permeating
TM	0.010*	0.003*	0.047*
PM	0.284	0.032*	0.230
VCL	0.114	0.023*	0.118
VSL	0.082	0.008*	0.085
LIN	0.076	0.005*	0.062
Viability	0.050	0.013*	0.145
Embryo survival	0.925	0.105	0.367
Hatching rate	0.406	0.289	0.723

Significant differences (two-way ANOVA (SNK, P<0.05)) are represented with an asterisk.

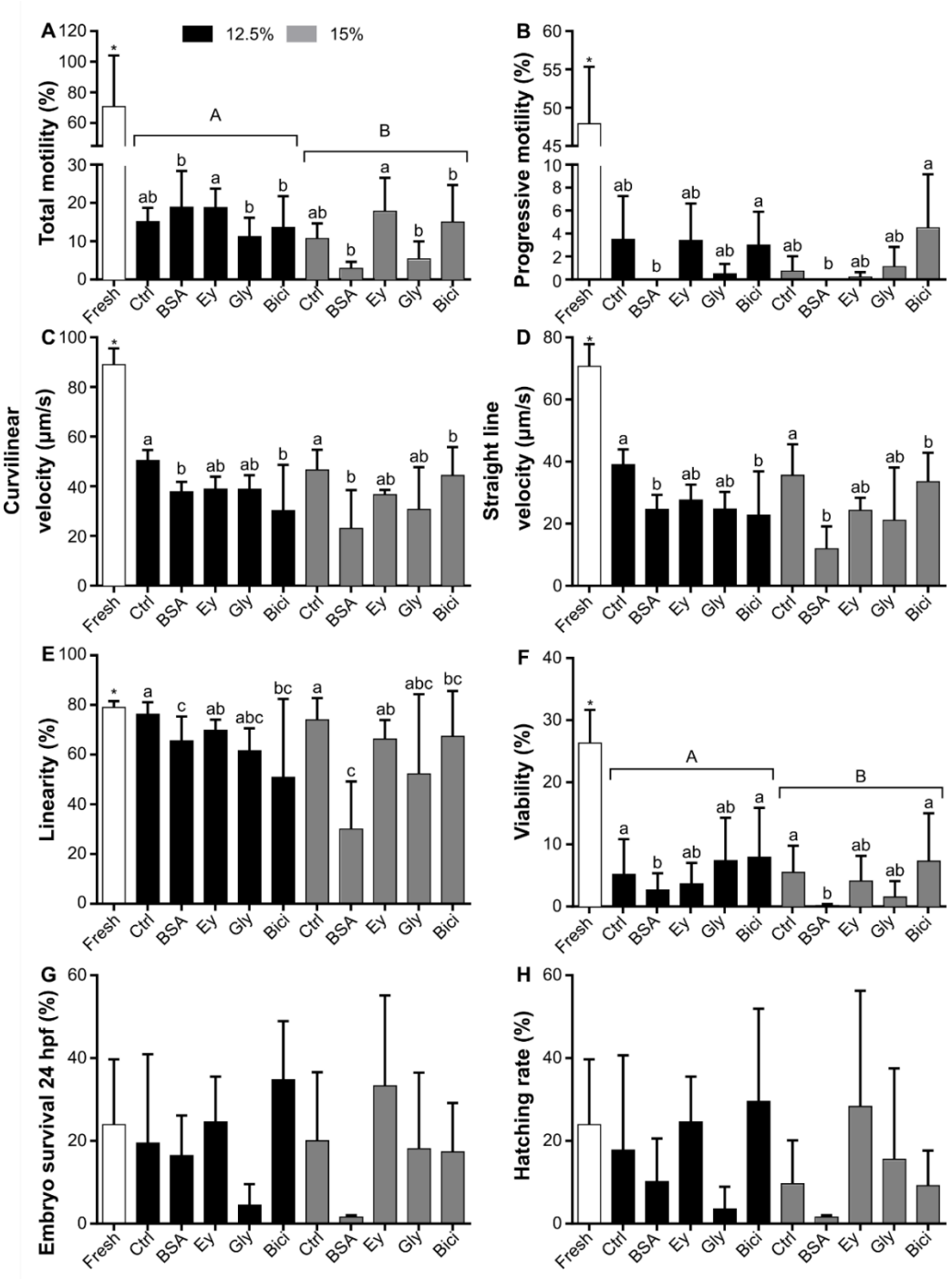

Figure 5.13 Zebrafish sperm (n=5 pools containg sperm of 16 males) cryopreserved with 12.5% (black bars) and 15% (grey bars) of DMF without non-permeating cryoprotectant (Ctrl) and with 10 mg/ml of BSA (BSA), 10% egg yolk (EY), 30 mM glycine (Gly) and 50 mM of bicine (Bici). Sperm quality was evaluated according to A) TM, B) PM, C) VCL, D) VSL, E) LIN and F) viability of the plasma membrane. For the same sperm pools *in vitro* fertilizations were performed and their success was measured through G) embryo survival 24 hpf and H) hatching rate at 72 hpf. White bars represent fresh sperm, black bars represent sperm cryopreserved with 12.5% of DMF and grey bars represent sperm cryopreserved with 15% of DMF. Values plotted represent means±SD, an asterisk indicates significant differences between fresh and cryopreserved sperm (independent samples t-test, $P<0.05$). Uppercase letters represent significant differences between permeating cryoprotectant concentration and lowercase letters significant differences between non-permeating cryoprotectants (two-way ANOVA, post hoc SNK $P<0.05$).

5.2.4.3. Axial Skeleton malformations

The characterization of severe skeletal malformations was more conclusive in terms of differences between permeating cryoprotectant percentage, in comparison to the total percentage of skeletal malformations incidence on the offspring sired by cryopreserved sperm (Figure 5.14 A and B). The percentage of deformities observed between zebrafish sired by cryopreserved sperm is highly dependent of the cryoprotectants composition used in the extender (Figure 5. A). However, sperm cryopreserved with 15% of DMF generated a significant reduction of the incidence of severe skeletal malformations on the offspring comparing with fresh sperm, except in control treatment (Figure 5.14 B). Severe skeletal malformations of zebrafish sired by cryopreserved sperm, namely lordosis, scoliosis and kyphosis, were reduced when non-permeating cryoprotectants were added to an extender with 15% of DMF. The BSA treatment resulted in very low survival and no skeletal analysis was performed. The axial skeleton malformations in zebrafish were mainly focused on the caudal and caudal fin vertebrae (Figure 5.15 A-C). Offspring generated by cryopreserved sperm display predominantly a load of 2 anomalies on the axial skeleton, although not significant (Figure 5.15 D and E). These anomalies were located on the transition between caudal vertebrae and caudal fin vertebrae (vertebrae 27-30) (Figure 5.15). In figure 5.16 are represented some of the most common skeletal malformations observed. In this figure is represented a fusion in precaudal vertebrae (Figure 5.16 A), abnormal vertebral bodies with ectopic calcifications (Fig 5B) and a fish with absent hypural connection to the urostyle (Figure 5.16 C). Additionally, in this figure is represented a fish with malformed secondary haemal arch on vertebrae number 29 with demineralization of hypural (Figure 5.16 D), an individual with abnormal neural arches, with ectopic calcification on parhypural and demineralization in hypurals (Fig 5E) and a fish with severe scoliosis (Figure 5.16 F).

To explore the potential relationships between cryoprotectant composition and the offspring skeletogenesis, the complete description of skeletal abnormalities was applied to a decision tree through the CART method (Figure 5.17). Considering if the fish were malformed or displayed a normal skeletal development (dependent variable), the severity of skeletal malformations was the factor that discriminates

treatments the most, followed by the incidence of abnormalities on the arches (Figure 5.17).

The decision tree allows to observe that the use of 15% of permeating cryoprotectant (DMF) on cryopreserved treatments reduces the onset of deformed arches on the offspring produced with cryopreserved sperm (Figure 5.17). The use of non-permeating cryoprotectants was discriminated through the incidence of fusions, and on a subsequent tree node, vertebral compression on the offspring where two non-permeating cryoprotectant groups were formed. The group formed by fresh sperm, BSA and glycine show lower number of normal individuals (53.8%) and the group formed by control, egg yolk and bicine treatments (72.9%) show a significantly higher number of normal individuals.

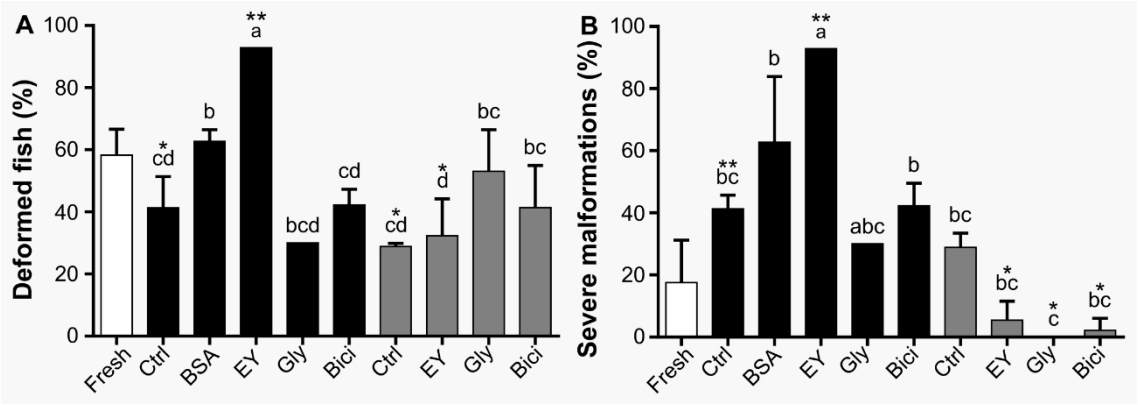

Figure 5.14 Offspring axial skeleton malformations (30 dpf) analysis through alcinan blue alizarin red staining in terms of A) malformed fish, B) severe skeletal malformations. White bars represent fresh sperm (43 fish resulting from 3 sperm pools). Black bars represent zebrafish that resulted from *in vitro* fertilization with cryopreserved sperm with 12.5% of DMF without non-permeating cryoprotectant (Ctrl, 233 fish that resulted from 8 sperm pools) and with 10 mg/ml of BSA (BSA, 37 fish that resulted from of 2 sperm pools), 10% egg yolk (EY, 14 fish that resulted from of 1 sperm pool), 30 mM glycine (Gly, 10 fish that resulted from 2 sperm pools) and 50 mM of bicine (Bicil, 90 fish that resulted from 4 sperm pools). Grey bars represent zebrafish that resulted from *in vitro* fertilization with cryopreserved sperm with 15% of DMF without non-permeating cryoprotectant (Ctrl, 59 fish that resulted from 3 sperm pools) and with 10% egg yolk (EY, 168 fish that resulted from of 7 sperm pools), 30 mM glycine (Gly, 37 fish that resulted from 2 sperm pools) and 50 mM of bicine (Bici, 73 fish that resulted from 3 sperm pools). Values plotted represent means±SD.

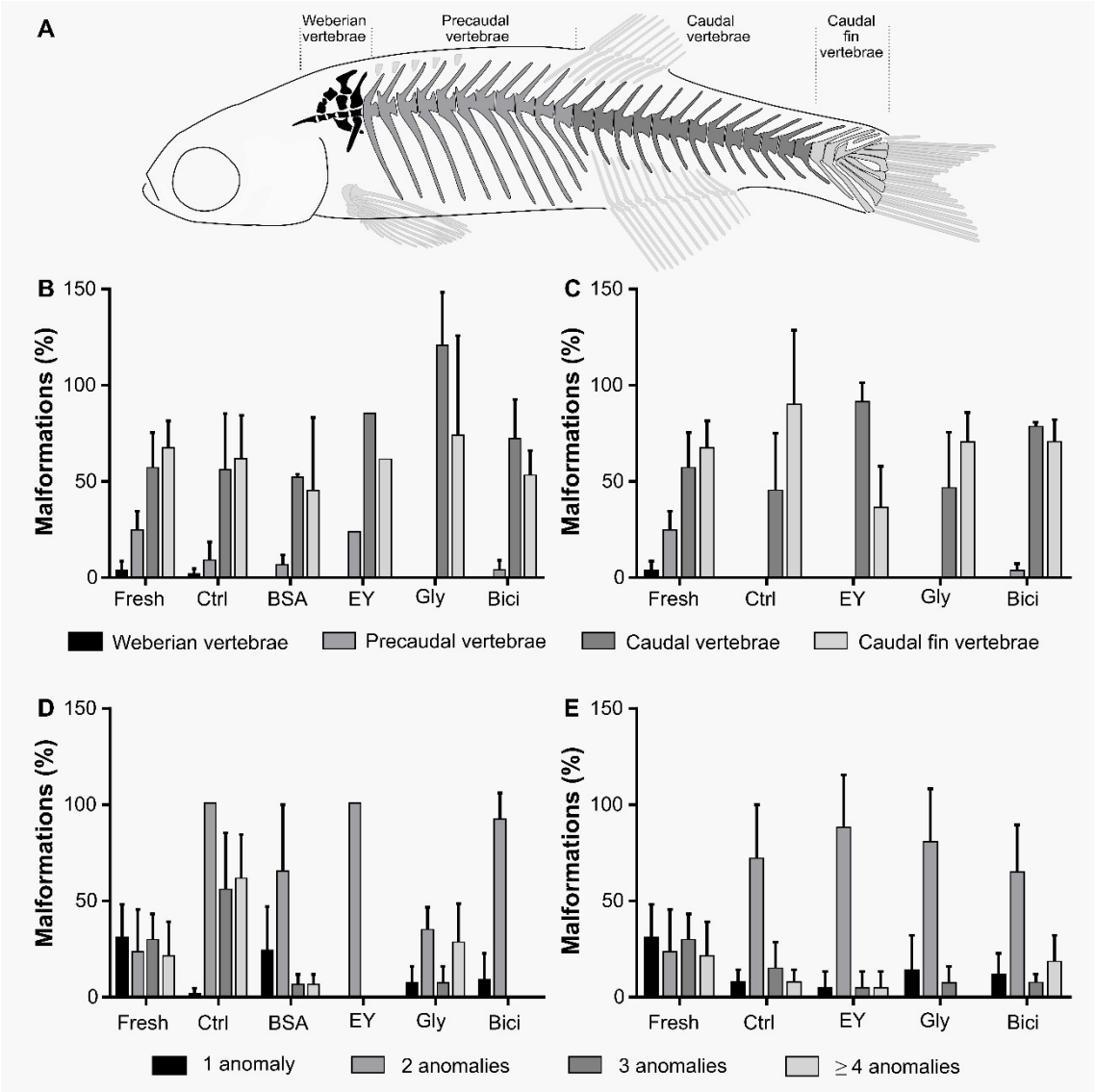

Figure 5.15 Offspring axial skeleton malformations (30 dpf) analysis through alcinan blue alizarin red staining. Upper graphics represent the distribution of malformations through zebrafish skeleton of the offspring generated from sperm cryopreserved with A) 12.5% of DMF and B) 15% of DMF. Lower graphics represent the charge of malformations on the offspring generated from sperm cryopreserved with C) 12.5% of DMF and D) 15% of DMF. For each permeating cryoprotectant concentration was tested a control without non-permeating cryoprotectant (Ctrl, 233 fish that resulted from 8 sperm pools) and the addition of 10 mg/ml of BSA (BSA, 37 fish that resulted from of 2 sperm pools), 10% egg yolk (EY, 14 fish that resulted from of 1 sperm pool), 30 mM glycine (Gly, 10 fish that resulted from 2 sperm pools) and 50 mM of bicine (Bici, 90 fish that resulted from 4 sperm pools). Values plotted represent mean±SD (Standard Deviation) and different shades of grey represent zebrafish skeleton location (A and B) or number of anomalies (C and D).

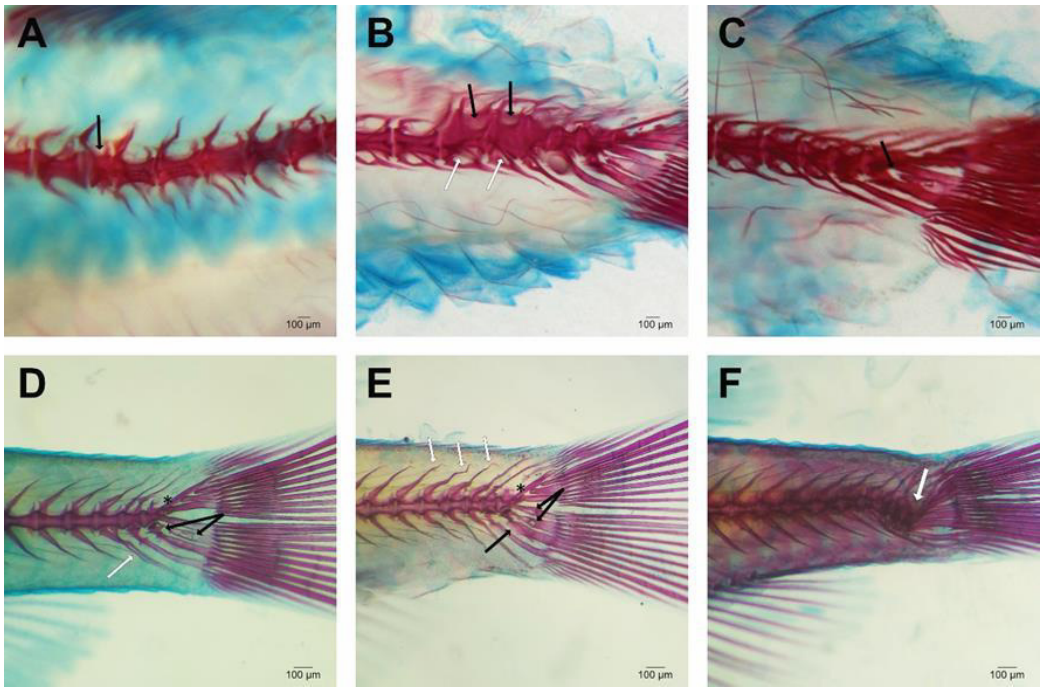

Figure 5.16 Representation of the most abundant axial skeleton malformations (30 dpf) of zebrafish sired by cryopreserved sperm A) Fusion in precaudal vertebra associated to compressive forces (black arrow), B) enlarge vertebral bodies (white arrows) with ectopic calcifications (black arrows), C) absence of hypural 1 connection to the urostyle (black arrow), D) secondary haemal arch on vertebrae No. 29 (white arrow); demineralized hypural 1 (black arrow); broken neural arch on urostyle (asterisk), E) abnormal neural arches (white arrows); ectopic calcification on parhypural (black arrow); demineralized regions in hypural 1 and 2 (black arrows); broken neural arch on urostyle (asterisk) F) Severe scoliosis associated to compressive forces (white arrow).

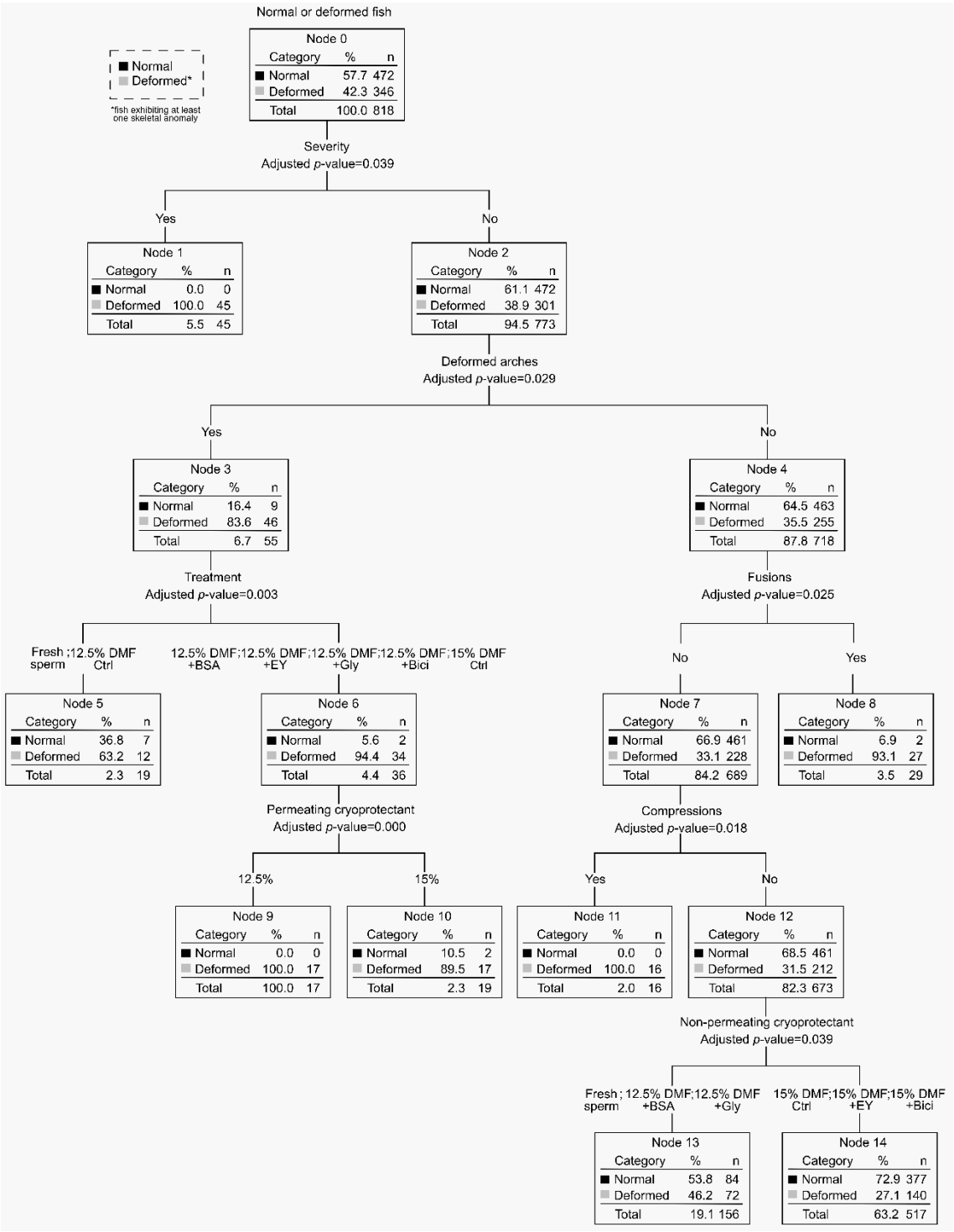

Figure 5.17 Decision tree obtained through the CART method with high significance for the malformed zebrafish sired by cryopreserved sperm with different extender compositions. The skeletal malformations analysis details the occurrence of compressions, fusions, opened arches, deformed arches, deformed centra (vertebrae). The zebrafish analyzed were obtained from sperm cryopreserved with 12.5% and 15% of DMF without non-permeating cryoprotectant (Ctrl, 233 fish that resulted from 8 sperm pools) or with the addition of 10 mg/ml of BSA (BSA, 37 fish that resulted from of 2 sperm pools), 10% egg yolk (EY, 14 fish that resulted from of 1 sperm pool), 30 mM glycine (Gly, 10 fish that resulted from 2 sperm pools) and 50 mM of bicine (Bici, 90 fish that resulted from 4 sperm pools). Statistical significance is represented in each tree node when ramification stops no significant differences are observed within the group.

5.2.5. Discussion

The ultimate objective of assisted reproduction techniques such as sperm cryopreservation is not only to accomplish oocyte fertilization but most importantly to obtain viable and healthy offspring. Spermatozoa are more than carriers of genomic information, they have a crucial role on the genetic control of the first embryonic events after fertilization (Carrell, 2011; Herráez et al., 2017; Labbé et al., 2017; Ward, 2010; Wu et al., 2011). However, the spermatozoa ability to repair DNA damage is absent (Pérez-Cerezales et al., 2010; Smith et al., 2013) and depend on oocyte DNA repair machinery to perform its genomic repair, onto some extent (Fernández-Díez et al., 2015). Cryopreservation can produce oxidative stress and increase sperm DNA damage (Cabrita et al., 2011b; Diogo et al., 2018; Martínez-Páramo et al., 2017). Fertilization with high DNA damaged spermatozoa is an important factor that leads to abortion (Ciereszko et al., 2005; Pérez-Cerezales et al., 2010). Beyond spermatozoa lethal DNA damage, there are sub-lethal effects, such as longer telomeres on offspring (Pérez-Cerezales et al., 2011), abnormal juvenile weight and cortisol response to stress (Hayes et al., 2005), malformations at hatching (Miskolczi et al., 2005; Young et al., 2009) and haploidy (Miskolczi et al., 2005) that affect progeny quality.

Cells response to the freezing process depend on the cell biophysical characteristics, which are species-specific and change in a nonlinear mode with temperature (Devismita and Kumar, 2015). Data show total post-thaw sperm motility and viability values within the previously reported in zebrafish (Diogo et al., 2018; Wang et al., 2015; Yang et al., 2016). There are few reports of zebrafish hatching rates obtained with cryopreserved sperm (Diogo et al., 2018; Harvey et al., 1982b). Hatched larvae obtained by *in vitro* fertilization with fresh sperm in our work yielded an average of 23%, whereas in Diogo et al. (2018) an average of 12% was reported. Consequently, the assisted reproduction methods may impact negatively the hatching rates of zebrafish as in mammalian species (Ramos-Ibeas et al., 2019).

Permeating cryoprotectants are among the most relevant players for cryopreservation success, they permeate the sperm plasma membrane and increase total intracellular solute concentration (Elliott et al., 2017; Martínez-Páramo et al.,

2017). Consequently, water leaves the cells through the osmotic gradient, avoiding the formation of intracellular ice crystals, which are lethal to the cell (Mazur, 1963;1984). The disadvantage of permeating cryoprotectants is their toxicity and therefore, to accomplish a feasible cryopreservation protocol, a compromise between low toxicity and complete cellular penetration must be attended (Asturiano et al., 2017; Elliott et al., 2017; Martínez-Páramo et al., 2017; Mazur, 1984). In our work, the post-thaw total and progressive sperm movement as well as *in vitro* fertilization parameters show a normal curve behavior, which represents the balance between cryoprotectant toxicity and cellular protection against cold damage. Data showed that low variations on permeating cryoprotectant concentrations impacts post-thaw sperm quality. The extender containing 5% of DMF was deleterious to sperm in all sperm quality parameters, especially in 24 hpf embryo survival and hatching rates. This result suggests that 5% of DMF is not enough to protect zebrafish spermatozoa from the cryopreservation process. Post-thaw sperm total motility and membrane viability were improved by 12.5% and 15% of DMF. These DMF concentrations produced hatching rates similar to fresh sperm and the highest of the cryopreserved treatments. Therefore, these DMF concentrations were used to study the interaction of permeating with non-permeating cryoprotectants.

Non-permeating cryoprotectants such as sugars and amino acids are able to establish interactions with membrane lipidic bilayers (Carpenter and Crowe, 1988), protecting the cells during the freezing process and improving post-thaw results (Cabrita et al., 2011b; Martínez-Páramo et al., 2013). The addition of BSA and Gly to the extender yielded lower progressive motility. Permeating and non-permeating cryoprotectants in our study showed significant interaction in total motility, where egg yolk and bicine treatment with 15% of DMF maintain higher total motility compared to the other treatments. This synergy between DMF concentration and non-permeating cryoprotectants suggest that DMF toxic effects in higher concentrations are balanced through the presence of egg yolk and bicine. This cryoprotectants combination protects therefore the plasma membrane components responsible for the triggering of zebrafish sperm motility. It is interesting to observe that the additives used in the extender composition reduce the sperm velocities and linearity when compared to the control. However, lower sperm velocity does not

result in lower 24 hpf embryo survival and hatching rates. Egg yolk yielded high post-thaw sperm quality and hatching rates, which might be explained by its high viscosity that protects the cell during cryopreservation (Morris et al., 2006) and the increase of the flagellar beating frequency in viscoelastic fluids (Lauga, 2007). Viscosity stabilizes the fertilization microenvironment, which is important in teleosts external fertilization (Lahnsteiner, 2002), particularly in species that yield low sperm volumes such as Senegalese sole (Diogo et al., 2010; Riesco et al., 2017) and zebrafish. The main disadvantages of egg yolk are difficult standardization and high susceptibility to contamination (Aires et al., 2003).

Bicine is an amino acid [N,N-Bis(2-hydroxyethyl)glycine] with high buffer capacity and recommended for biological research at low temperatures (Good et al., 1966). Bicine is commonly used in fresh water species extender composition (Cabrita et al., 2010) and was recently used in a zebrafish sperm cryopreservation protocol (Matthews et al., 2018). However, its isolated effect on post-thaw sperm quality required deeper comprehension. The use of 15% of DMF significantly reduced plasma membrane viability when compared to 12.5% of DMF. However, extenders containing 15% of DMF with egg yolk and bicine showed a reduction of skeletal malformations severity on the resulting offspring.

Sub-optimal cryopreservation protocols are known to produce genetic and epigenetic alterations with negative consequences on offspring biological performance and phenotype, affecting thus their health and lifespan (Pérez-Cerezales et al., 2011). Traditionally, skeletal malformations are associated with nutritional factors, however, the perturbation of genes responsible for the ossification is known to be responsible for abnormal skeletogenesis (Forero et al., 2018). Sperm cryopreservation in trout was associated to the alteration of genes involved in the regulation of embryo early development, particularly symmetry, axis, segmentation, gastrulation, organogenesis and tissues differentiation (Fernández-Díez et al., 2015), which are associated to skeletal development. Our results indicate that the severe skeletal malformations of the offspring sired by cryopreserved sperm provide relevant information on the effectiveness of the cryopreservation protocol, that would be otherwise disregarded. The skeletal malformations incidence on zebrafish sired by fresh sperm through *in vitro*

fertilization within the normal range for this species in natural spawns (Diogo et al., 2015; Martins et al., 2018). In our work, severe skeletal malformations that change fish external body shape such as lordosis, scoliosis and kyphosis were significantly higher with 12.5% of DMF when compared to 15% DMF. This fact suggests that 15% of DMF can protect the cell against residual intracellular ice crystals formation, cold damage or cellular stress that lead to genomic alternations and consequently to abnormal skeletal development. On early embryo, three embryonic layers are formed through extensive cellular rearrangements namely ectoderm, mesoderm and endoderm (Berendsen and Olsen, 2015). Each one of these embryonic layers will originate different body structures. Zebrafish vertebrae derive from notochord (Fleming et al., 2004) while arches derive from somite cellular line (Berendsen and Olsen, 2015). The regions most affected by skeletal anomalies in our study were caudal and precaudal fin vertebrae, which is in agreement with the typology of skeletal malformations reported in previous works in zebrafish (Bensimon-Brito et al., 2010; Diogo et al., 2015; Martins et al., 2018). Control, egg yolk and bicine treatments reduced the onset of deformed arches, vertebral fusions and compressions on the offspring. Therefore, sub-optimal extender composition in cryopreservation may cause a perturbation of the early embryo genome and structures relevant for ossification, disturbing thus zebrafish normal skeletal development. The detailed analysis of skeletal malformations generates complex data sets with inherent high biological variability, being therefore difficult to analyze through traditional statistical methods. Using decision trees, a machine learning modeling technique, it was possible to observe that using 15% of DMF reduces deformations on the arches. This fact suggests that 15% of DMF was able to protect spermatozoa genes involved on embryo somitogenesis. Our work evidences that the extenders composition that yields consistently improved sperm and offspring quality is 15% of DMF with bicine or egg yolk. However, considering egg yolk sanitary risks and standardization difficulties, we suggest that 15% of DMF with 50 mM of bicine is the adequate cryoprotectant composition of the extender for the previously established cryopreservation protocol (Diogo et al., 2018).

To the best of our knowledge, this is the first report on the skeletal malformations description of the offspring sired by cryopreserved sperm with different extender compositions in zebrafish. Our work shows that sub-lethal

damage of spermatozoa resulting from under-optimized cryopreservation protocols can increase the incidence of skeletal malformations in zebrafish offspring. Therefore, offspring skeletal development evaluation is a valuable tool for the selection of efficient cryopreservation protocols. The extender optimization performed by the present study represents an important improvement of zebrafish sperm cryopreservation through electric ultrafreezers.

CHAPTER 6. APPLICATION OF SPERM QUALITY ANALYSIS AND CRYOPRESERVATION METHODOLOGIES IN A TRANSGENIC ZEBRAFISH LINE

PREAMBLE

The major consequence of the lack of sperm cryopreservation standardization is the fact that the results achieved by these methodologies are unreliable, with low reproducibility. Therefore, the security of the highly valuable genotypes developed in laboratories and reference centers can be put at risk. Cryopreservation is a particularly valuable method for zebrafish lines that require high genomic stability and therefore are highly inbred. There are zebrafish transgenic and mutant lines more susceptible than wild type lines and with reproductive constrains. However, reliable sperm quality analysis of zebrafish strains is scarce.

Type I diabetes is a human pathology with increasing incidence worldwide that require further investigation since the cause is unknown. The development of a diabetes type I zebrafish model is highly relevant for this research. The *Tg(ins:nfsb-mCherry)* is a zebrafish line with *Escherichia coli* nitroredutase gene inserted in its genome, that upon the exposure to a pro-drug (Metronidazole) promotes the ablation of insulin-expressing β cells of the pancreas. Zebrafish has fast generations and cellular regeneration ability, and therefore the diabetic condition is transient until full β cells regeneration, which is useful for this pathology investigation. Similarly, to humans, zebrafish under type I diabetes transient state show negative consequences on sperm motility, plasma membrane viability and DNA integrity. Additionally, sperm obtained from *Tg(ins:nfsb-mCherry) males under diabetic conditions shows a higher quantity of insulin (insa) and glucose carrier (slc2a2) transcripts.* Since diabetes has relevant negative outcomes in the male reproductive system, this study aimed to understand if zebrafish is a useful model to support the investigation of the mechanisms by which this disease affects sperm quality and potentially its transgenerational effect.

6.1. TYPE I DIABETES IN ZEBRAFISH REDUCES SPERM QUALITY AND INCREASES INSULIN AND GLUCOSE TRANSPORTER IN SPERMATOZOA

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[Disease Models & Mechanisms, in preparation]

6.1.1 Abstract

Type I diabetes is a human pathology with increasing incidence however, its causes are still largely unknown. Diabetes promotes detrimental effects on reproduction such as lower sperm motility and DNA integrity. Hence, investigating the underlying mechanisms of this metabolic disturbance in reproduction and transgenerational consequences is of utmost importance. Zebrafish is a useful model considering its high homology to human genes, fast generation turnover and regeneration ability. Therefore, we proposed to investigate the putative effects of diabetes in sperm quality, particularly in sperm motility, the viability of plasma membrane, DNA integrity and in the expression of *insulin a* (*insa*), *insulin receptor a* (*insra*) and *glucose transporter 2* (*slc2a2*) transcripts in spermatozoa. The *Tg(ins:nfsb-mCherry) transgenic zebrafish line was used as model for type I diabetes. Diabetic Tg(ins:nfsb-mCherry) males showed a significantly higher quantity of transcripts of insa and slc2a2 compared to control. Sperm obtained from these diabetic fish showed significantly lower sperm motility, plasma membrane viability and DNA integrity when compared to the non-diabetic controls. Considering the pathology onset in early age progressing with time, sperm cryopreservation can be a useful tool to safeguard the possibility of in vitro fertilization. Upon sperm cryopreservation, sperm freezability from diabetic males was reduced, which could be a consequence of their initial poor sperm quality, highlighting the necessity of sample selection in cryopreservation of diabetic patients. Our data show similar detrimental effects of type I diabetes in spermatozoa at the cellular and molecular level and validates the zebrafish model for type I diabetes research in germ cells.*

6.1.2 Introduction

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism. This metabolic unbalance results from defects in insulin secretion and/or action (Alberti KG, 1998) (Alberti). It is estimated that 382 million people suffer from diabetes with 8.2% of prevalence (Guariguata et al., 2014). The projection the for human population suffering from diabetes in 2035 is expected to increase up to 592 million people (Guariguata et al., 2014). Type I diabetes is a chronic autoimmune disease

characterized by the loss of insulin-producing β cells in the pancreas, leading to insulin deficiency. Type II diabetes is the acquired insulin resistance, which can occur in combination with reduced insulin secretion (Butler et al., 2003). Both types of diabetes show a growing incidence among the human population. The cause of type II diabetes is known to be primarily life style factors and genetic predisposition (Kahn et al., 2006; Risérus et al., 2009). However, the cause of type I diabetes is still to be fully understood (Xia et al., 2019).

Considering the alarming increase of diabetes incidence and the fact that environmental factors affect both maternal and paternal germ cells, there is a great need for intergenerational and transgenerational studies of type I diabetes (Ding et al., 2015) on offspring inheritance and reproductive function. For this purpose, a model species with fast generation turnover is necessary. Zebrafish is a promising candidate for this type of research since it is a small teleost with fast generations, the genome fully sequenced and presents 74% homology with human genes (Howe et al., 2013). Additionally, there are established transgenic zebrafish models for type I diabetes (Bergemann et al., 2018; Pisharath et al., 2007). The Tg*(ins:nfsb*-mCherry) type I diabetes model was developed by Pisharat et al (Pisharath et al., 2007) with a Tübingen AB background to investigate pancreatic β cells regeneration. The earliest known marker of β cells in zebrafish embryo is the preproinsulin (*ins*) gene, its promoter is expressed in the nascent endocrine pancreas (Huang et al., 2001). This zebrafish line expresses *nfsb* gene and produces nitroreductase (NTR) enzyme, which converts prodrugs such as metronidazole (Met; Sigma-Aldrich, Spain) to cytotoxins, resulting in cell apoptosis (Pisharath et al., 2007). Consequently, the Tg*(ins:nfsb*-mCherry) line in the presence of Met will convert this prodrug into cytotoxins through the NTR enzyme, therefore ablating the pancreatic β cells and losing the mCherry fluorescence on the pancreas. The fact that this species has high regeneration ability, therefore being able to regenerate the ablated pancreatic β cells (Carvalho et al., 2017), makes it a particularly useful model for the investigation of type I diabetes.

Among other complications, diabetes causes disturbances in the male reproductive system, since glucose metabolism is an important event not only in spermatogenesis (Ding et al., 2015) but also in mature spermatozoa metabolism

(Dias et al., 2014; Urner and Sakkas, 2005). When critical developmental points of spermatogenesis are affected, environmentally-induced epigenetic modifications may become permanent in the germ line epigenome with potential consequences on subsequent generations (Anway et al., 2005; Ding et al., 2015). Numerous studies were performed both in humans and murine models, confirming the deleterious effects of diabetes on reproduction and gametes quality (Ding et al., 2015; López-Escobar et al., 2015). In males, these deleterious consequences were particularly evident in sperm quality parameters such as DNA fragmentation, chromatin quality, sperm motility and seminal plasma composition (Agbaje et al., 2008). These deleterious effects on reproduction are observed both in type I and type II diabetes (Ding et al., 2015), and even in pre-diabetic and obesity conditions (Palmer et al., 2012; Rato et al., 2013).

In human male populations, the onset of type I diabetes occurs typically in children and adolescents and the disease progresses with age towards several andrological and reproductive complications (La Vignera et al., 2009; Xia et al., 2019). Sperm cryopreservation is a valuable resource to support assisted reproduction (Cabrita et al., 2008; Diogo et al., 2018; Morris et al., 1999)*. Therefore, sperm cryopreservation can be a valuable tool to safeguard the possibility of in vitro fertilization later in life (Ranganathan et al., 2002). The zebrafish type I diabetes model is a useful tool to understand if sperm freezability is affected by the diabetic condition.*

Spermatozoa need the energy to acquire and maintain motility in order to reach the oocyte. This process requires the consumption of adenosine triphosphate (ATP). The metabolic pathways for energy production in spermatozoa are anaerobic glycolysis, mitochondrial oxidative phosphorylation and the pentose phosphate pathway (Bucci et al., 2013). Sperm uses primarily sugars such as glucose, mannose and fructose as energy fuels for ATP production (Bucci et al., 2013). These sugars are incorporated passively through lipid bilayers in a slow and inefficient manner and therefore require carriers. Hexoses (sugars) are transported into sperm actively through sodium-dependent glucose transporters, or passively through glucose transporters (GLUTs) (Scheepers et al., 2004). GLUTs, currently known as solute carrier family 2 (SLC2A), are essential during the passive glucose transport through

the blood-testes barrier during spermiogenesis. In addition, GLUTs are present in mature spermatozoa, which require carriers to incorporate energetic resources as mentioned previously. GLUT 2 is expressed at a very high level in pancreatic β cells and is a high-affinity glucose transporter. This protein is located on acrosomal and end pieces of the tail of human spermatozoa (Bucci et al., 2013). The GLUTs are markers of sperm quality, useful for both clinical and commercial purposes (Bucci et al., 2013).

Unexpectedly, it was demonstrated that mammalian spermatozoa have stores of insulin (Aquila et al., 2005; Carpino et al., 2010; Kim and Moley, 2007). Moreover, spermatozoa are known to secrete this hormone in a short autocrine loop to recruit glucose as energetic subtract (Andò and Aquila, 2005). This recruitment of glucose through insulin secretion is performed according to their metabolic needs or alterations in the systemic energy homeostasis (Andò and Aquila, 2005). The presence of insulin in teleosts sperm is still to be investigated. However, if similar to mammals, the role of insulin can be relevant not only in somatic but also in germ cells. Therefore, it is pertinent to understand the alterations of the transcripts of insulin and insulin receptor and glucose carrier under transient diabetic conditions.

The objective of this study was to investigate the sperm quality in a type I zebrafish transgenic model under transient diabetic condition. The evaluation of target genes relevant for diabetes research such as insulin a (*insa*), insulin receptor a (*inra*) and glucose carrier 2 (*slc2a2*) were also studied in zebrafish sperm for a deeper understanding of the usefulness of this type I diabetes model for the study of reproductive complications.

6.1.3 Methods

1.1.1.1. Fish husbandry

Adult AB zebrafish males and *Tg(ins:nfsb-mCherry)* (8-12 months old) were selected according to similar size and maintained in 3.5 l tanks with 15 fish each. The wild type AB line was provided by Max Planck Institute for Heart and Lung Research (Bad Nauheim, Germany) and maintained at the Centre of Marine Sciences

(CCMAR, Portugal). The reporter line *Tg(ins:nfsb-mCherry) was kindly given by the Laboratory of Molecular Biology and Genetic Engineering (GIGA Research, Liege, Belgium). The Tg(ins:nfsb-mCherry) was previously screened and selected according to the presence of cell signalling on the pancreas during larval stage.* Both fish lines were reared in a ZebTEC® (Tecniplast, Italy) recirculation system with 980 l of water, as previously described in Diogo et al. (Diogo et al., 2018). The water system was maintained at 28.2±0.5°C, 700±75 µS and pH 7.5±0.2. The fish were fed twice a day with *Artemia* nauplii (AF480, INVE, Belgium) and ZEBRAFEED® diet (Sparos Lda, Portugal) *ad libitum*. The Tg*(ins:nfsb*-mCherry) type I diabetes model was developed by Pisharat et al (Pisharath et al., 2007) with a Tübingen AB background to investigate pancreatic β cells regeneration. The earliest known marker of β cells in zebrafish embryo is the preproinsulin (*ins*) gene, its promoter is expressed in the nascent endocrine pancreas (Huang et al., 2001).

All animal manipulations were performed in compliance with the Guidelines of the European Union Council (86/609/EU) and transposed to the Portuguese law for the use of laboratory animals on research by "Decreto Lei n° 129/92 de 06 de Julho, Portaria n° 1005/92 de 23 de Outubro", and according to the European parliament council directive´s for protection of animals used for scientific research (2010/63/EU). All animal protocols were performed under a "Coordinator-researcher" license from the Direção-Geral de Veterinária, Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, Lisbon, Portugal, under the "Decreto Lei n°113/2013 de 7 de Agosto" relative to the protection of animals used for scientific research. All the fish sampling procedures were performed by licensed researchers.

1.1.1.1. Induction of diabetes on Tg(ins:nfsb-mCherry) zebrafish model

The Tg(*ins:nfsb*-mCherry) zebrafish line has inserted the *nfsB* gene of *Escherichia coli* and the florescent protein mCherry downstream to the promoter region of the *insa* gene in the Tg*(ins:nfsb*-mCherry) line.

The diabetes induction was performed with metronidazole (Met; Sigma-Aldrich, Spain) resulting in ablation of β cells in *Tg(ins:nfsb-mCherry) line*. Met was dissolved in 0.5 ml/l of dimethyl sulfoxide (DMSO) and diluted in system water in a

final concentration of 10 mM by vigorous agitation. This concentration was selected since in zebrafish it does not produce germ cells ablation below 48 h of exposure to the drug (Dranow et al., 2013) and have no significant effect on sperm parameters in several species (Foote, 2002). For the control, the same conditions of water and fish housing were used with the exception of Met exposure. Males from both treatments were incubated at 28ºC in the dark for 24 h in glass tanks (14 fish/l of water). After incubation, the males were returned to clean system water tanks. Zebrafish have regeneration ability, therefore the transient diabetic conditions reach a maximum β cells ablation 3 days after the exposure to the drug and fully regenerate pancreatic cells 14 days after Met exposure (Moss et al., 2009). Additionally, zebrafish males require 6 days to complete spermatogenesis (Leal et al., 2009). Due to the previously mentioned factors, a second induction was performed 7 days after the first Met exposure, as described above, to ensure that all males have the full spermatogenic cycle exposed to diabetic (or control) conditions.

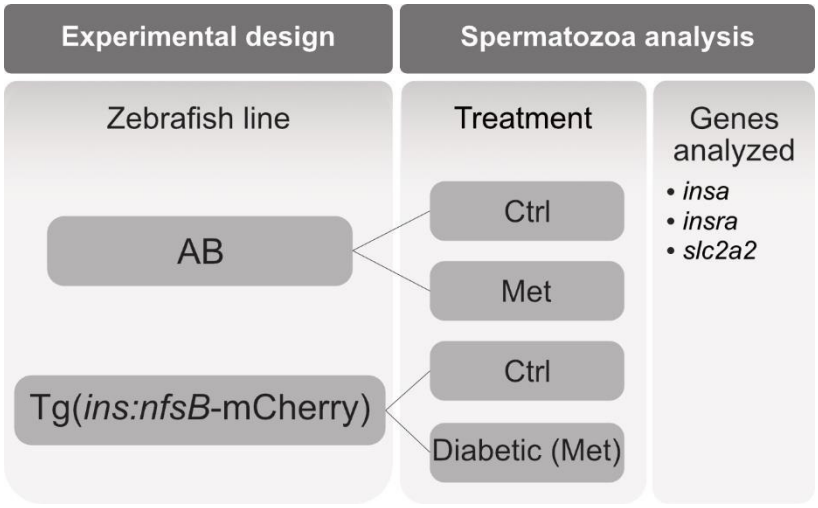


Figure 6.1 Experimental design to evaluate the effect of a transient type I diabetes state on zebrafish Ins2 line of fresh and post-thaw sperm motility, viability and DNA fragmentation.

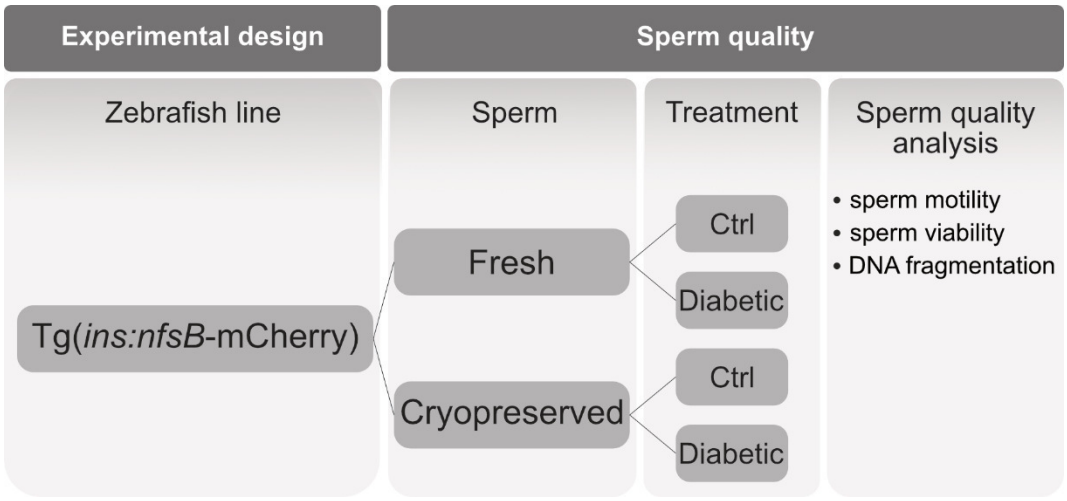


Figure 6.2 Experimental design to evaluate the effect of a transient type I diabetes state on zebrafish Ins2 line on zebrafish sperm RNA transcripts.

6.1.3.1 Experimental design

Adult AB and *Tg(ins:nfsb-mCherry)* zebrafish males (8-12 month) were selected and separated into 2 groups the control and the diabetic treatment respectively. *A second group of males was used as control where males were only exposed to the vehicle in the system water. The sperm and blood were collected three days after the second exposure to Met, when the maximum β cells ablation occurs (Moss et al., 2009) for further analysis.*

To understand the impact of the diabetic conditions on zebrafish spermatozoa at the molecular level, a set of target transcripts was investigated with high relevance for this metabolic disorder*. In fresh sperm, the levels of insa, inra and slc2a2 transcripts was evaluated in both zebrafish lines in Met treated and untreated males.*

6.1.3.2 Sperm collection

Zebrafish were euthanized by hypothermal shock in ice and water slurry at 2±2ºC (monitored with a probe). The ice was removed from the slurry to avoid the direct contact of fish skin with the ice. This method avoids the known interference of anesthetics with blood glucose analysis (Eames et al., 2010) and allows a fast euthanasia respecting fish welfare (Matthews and Varga, 2012; Wilson et al., 2009).

In less than 3 min after the beginning of the tank manipulation, the fish were properly euthanized, the males were rinsed in phosphate buffered saline (PBS) solution and cleaned with a paper towel to avoid sperm motility activation. Sperm was collected immediately by an abdominal massage using a glass capillary tube connected to a mouthpiece. Sperm was immediately diluted with 10 µL of sterilized and filtered (0.20 µm) Hank's Balanced salt solution (HBSS) at 300 mOsm/kg (Jing et al., 2009) to prevent motility activation, in accordance with previous studies (Hagedorn and Carter, 2011). After sperm collection, the samples were maintained at 4ºC in the dark until quality analysis was performed (between 1 and 2 h after collection).

6.1.3.3 Blood glucose determination and pancreas fluorescence observation

To confirm the diabetic conditions of the zebrafish Tg(*ins:nfsb*-mCherry) males, the blood glucose levels were evaluated. In zebrafish, blood glucose rises 3 min after the exposure to stress (Eames et al., 2010).To avoid blood glucose analysis biases related to stress, 3 days prior to blood collection males were separated into glass tanks (2 l of water) with 2 males of the same treatment. Therefore, on the sampling day, males of each tank could be collected and euthanized within 3 min, without blood glucose increase due to stress. Moreover, prior to sampling, males were fasted for 24 h to avoid differential blood glucose fluctuations related to food consumption. The tail of the fish was excised and the blood of the peduncle was immediately measured with a blood glucose monitoring system (Glucocard™ G+meter, Arkray Europe B.V., Netherlands) according to the manufacturer's instructions. The use of hand-held glucose meters designed for use by human diabetics yields valid results with zebrafish blood (Eames et al., 2010). Glucose analysis was performed in AB males from control (n = 7) and Met treated fish (n = 24), and in Tg*(ins:nfsb*-mCherry) from control (n = 8) and Met treatment (n = 21). Glucose levels were also evaluated in zebrafish AB males randomly collected from their housing tanks under stress-free conditions (n = 7) to control the fish handling effect.

The observation of fluorescence in the pancreas of *Tg(ins:nfsb-mCherry) males were immediately performed after the blood glucose measurements. In adult fish, the observation of fluorescence in the pancreas is impaired due to the high muscular density surrounding the tissues. Therefore, each fish was dissected and the fluorescence observed under a MZ 7.5 fluorescence stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a green light filter (λex =530–560 nm and λem =580 nm) coupled to a black and white F-View II camera (Olympus, Hamburg, Germany), controlled by the Cell^F v2.7 software (Olympus Soft Imaging).*

6.1.3.4 RNA extraction and complementary DNA synthesis

Total RNA was extracted from sperm pools of AB (n = 4) and *Tg(ins:nfsb-mCherry)* (n = 7) untreated *males (control) and met treated* (n = 4 and n = 7 respectively). Each pool contained sperm from 5 males. The sperm sample of each male (1-2 µl) was collected and immediately diluted in 10 µl of Phosphate buffer (PBS) and added to 400 µl of NZYol Reagent (NZYTech, Portugal) according to the manufacturer's specifications. To improve the efficiency of the RNA extraction 0.5 µl of glycogen (Thermo fisher scientific, US) was added to the sample according to the manufacturer's specifications. The concentration and purity of the total RNA samples were evaluated using NanoDrop 1000 (Thermo Fisher Scientific, USA). The integrity of the obtained RNA was assessed through Experion RNA analysis (Biorad, USA). Complementary DNA (cDNA) was synthesized from 500 ng of the total RNA using M-MLV reverse transcriptase kit (Thermo Fisher Scientific, USA) with an oligo (dT) primer rate groups following the manufacturer protocol. Reverse transcription conditions were 37ºC for 1 h, 70ºC for 15 min and samples were stored at -20ºC until further analysis.

6.1.3.5 Quantitative real-time polymerase chain reaction (qPCR)

Relevant genes affected by diabetes and hyperglycemia were selected for analysis in zebrafish spermatozoa namely *insa*, *insra* and *slc2a2* according to previous studies (Ahmed Alfar et al., 2017; Im et al., 2005; Michel et al., 2016;

Schoeller et al., 2012). A DNAse treatment was performed with RQ1 RNase-Free DNase product (Promega, USA) to remove genomic DNA contamination.

The qPCR primers were designed using Perl Primer software (open-source PCR primer design). The nucleotide sequences of the primers are described in Table 6.1. The quantitative real-time PCR (qPCR) conditions were optimized for the different primers (Table 6.1). The amplification was monitored and analyzed by the intercalation of the fluorescent dye, SYBR Green, to double-stranded DNA. Reaction mixtures (20 µl of total volume) contained template cDNA (100 ng cDNA), SYBR Green PCR Master Mix (10 µl) and 10 µM of forward and reverse primer (0.8 µl). The qPCR reaction was initiated with a pre-incubation phase of 30 s at 95°C followed by 50 cycles of 95ºC of denaturation for 10 s and the temperature for primer extension (60ºC) (Table 6.1) for 20 s. To check the specificity of qPCR amplifications, dissociation curve analysis was also included: 1 cycle of 95ºC for 15 s, 60ºC for 1 min, followed by slow ramping of the temperature to 95ºC and finally 95ºC for 15 s. qPCR was carried out in a StepOnePlus™ System (Applied Biosystems, USA) according to the guidelines provided. StepOnePlus™ Systems software v.2.0 was used to calculate threshold cycle values (Ct). The *ef1α* was used as the endogenous reference gene to correct for the differences in reverse transcription efficiency and template quantity (McCurley and Callard, 2008). The mRNA levels were calculated as fold expression relative to the reference group, for each zebrafish line the met treated males were compared to untreated males. Each sample (n = 4 to 7 pools of sperm) was analyzed in technical replicates and the results were expressed according to the method described by Bustin (Bustin et al., 2009). Relative changes in gene expression were quantified using the $2^{-\Delta\Delta Ct}$ methods (Livak and Schmittgen, 2001).

Table 6.1 Forward and reverse primers used for target genes analysis through real-time PCR.

Target gene	Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Annealingt emperature (°C)
ef1a	elongation factor 1alpha	AGCCCCTCCTGGCTTTCACCC	TGGGACGAAGGCAACACTGGC	60
insa	preproinsulin	CATTCCTCGCCTCTGCTTC	TGCCTGGGTTAGTGCTTACA	60
insra	insulin receptor a	TCTACAGCGAGGAAAACAAGC	AGAGATAAGATGCGTCCGTTTT	60
Slc2a2	solute carrier family 2 member 2	GCCATAACAGCAGGACTACT	GATGACAGACCACAGTACAATCC	60

6.1.3.6 Sperm cryopreservation and thawing

For sperm cryopreservation, sperm samples from individual *Tg(ins:nfsb-mCherry)* males were re-diluted in HBSS (1:2) containing 10% of DMF. Sperm was cryopreserved in a final volume of 10 µl in each cryovial with a controlled freezing rate of -10ºC/min through a programmable biofreezer (Assymptote EF600M, Grant, UK). The cryopreserved samples were then plunged into liquid nitrogen and stored in the cryobank. The samples were thawed in a 33ºC bath for 8 s and immediately analyzed for motility, cell viability and DNA integrity.

6.1.3.7 Sperm plasma membrane viability analysis

Sperm membrane viability of fresh and cryopreserved samples was assessed through flow cytometry using SYBR 14 (Invitrogen, Spain) and propidium iodide (PI) (Sigma Aldrich, Spain) labeling. The plasma membrane viability of spermatozoa was evaluated in untreated Tg*(ins:nfsb*-mCherry) males (n = 5) and males treated with Met (n = 6). SYBR 14 is a permeant nucleic acid dye that permeates the cellular plasma membrane and PI is a membrane impermeable dye, therefore PI only labeled cells with the disrupted membrane. Consequently, cells with disrupted membranes are labeled in red from PI and viable cells are labeled in green from SYBR 14 (Daly and Tiersch, 2012). The SYBR 14 used was diluted with 5 µl of stock solution added to 120 µl of sterilized and filtered HBSS, while PI was used undiluted. The pre-diluted sperm samples were re-diluted (1:300) in HBSS and each stain was added for a final concentration of 6.7 nM of SYBR 14 and 3 ng/ml of PI. The samples were

incubated for 5 min in the dark at room temperature (21 to 25±1°C). The flow cytometer (BD FACSCalibur™, Biosciences, Spain) settings were adjusted for the detection of SYBR 14 through a 530 nm bandpass filter (FL1) and PI was detected with a 670 nm long pass filter (FL3). Prior to the beginning of the experiments, the settings were adjusted for zebrafish sperm analysis using a positive (100% dead cells) and a negative control (fresh sperm). For negative control, spermatozoa were exposed to cycles of freezing-thawing (Cabrita et al., 2005). A total of 5000– 10,000 events were counted for each sample.

6.1.3.8 Sperm motility analysis

Sperm motility analysis was evaluated in fresh and cryopreserved sperm obtained from untreated Tg*(ins:nfsb*-mCherry) (n = 5) and Met treated males (n = 8 and n = 10 respectively). Sperm motility was evaluated using computer-assisted sperm analysis (CASA) system (ISAS Integrated System for Semen Analysis, Proiser, Valencia, Spain) coupled to a phase contrast microscope (Nikon E-200, Nikon, Tokyo, Japan) with a x10 negative phase contrast objective. The images were captured with a Basler camera ISAS 782C camera (Proisier, Spain) and processed with CASA software. The settings of CASA system were adapted for this species with 25 frames/s, connectivity 14, 1 to 90 mm for head area and only sperm samples with VCL > 10 μm/s were considered motile. Motility analysis was performed by placing 1 μl of pre-diluted sperm in a Mackler chamber and immediately activate its motility with 5 μl of filtered (0.20 μm) and sterilized system water set at 28 °C and 700 μS/cm. Sperm motility was characterized during 1 min each 10 s post activation according to total motility (TM; %), progressive motility (PM; %), curvilinear velocity (VCL; μm/s), straight-line velocity (VSL; μm/s) and linearity (LIN; %).

6.1.3.9 DNA integrity evaluation through comet assay

DNA integrity was evaluated in fresh and cryopreserved sperm obtained from untreated Tg*(ins:nfsb*-mCherry) males (n = 4 and n = 5 respectively) and Met treated males (n = 8 and n = 6 respectively). The DNA fragmentation was evaluated through Comet assay methodology adapted from Reinardy et al. (2013), with some

modifications, as previously described (Diogo et al., 2018). The fresh prediluted sperm (1 µl) or thawed sperm (3 µl) were diluted in 60 µl of low melting point agarose (0.5% in PBS). The samples diluted in low melting point agarose were distributed into pre-coated slides with 0.5% of agarose in PBS (dried overnight) and covered with a coverslip 15 min at 4°C. For positive control, 2 µl of pre-diluted sperm was incubated with 2 µl of 100 µM H_2O_2 20 min at 4°C to ina duce DNA fragmentation. The coverslip was removed, and the slides were incubated in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1% Triton X-100) for 1 h at 4°C. Afterward, the slides were placed in an alkaline electrophoresis solution (300 mM NaOH and 1 mM EDTA, pH 13) for 20 min to unwind the DNA. The electrophoresis was performed during 20 min set at 25 V and 280–300 mA. The slides were washed twice with neutralization solution (0.4 M Tris– HCl, pH 7.5) for 5 min and fixed in ethanol during 15 min. For sample visualization, the DNA present in each slide was labelled with 10 µl of PI (1 mg/ml) and immediately observed at ×600 in a fluorescence microscope (Olympus IX 81, Olympus, Japan) with blue excitation (450–480 nm). Images were captured and recorded with a digital camera (F-view, Olympus, Japan) and processed with the Cell^F image software (Olympus, Japan). At least 100 cells per slide were scored and further analysed using Kinetic Imaging Komet 5.5 software (Andor Technology Ltd., United Kingdom). DNA fragmentation was expressed in terms of DNA in tail (%).

6.1.3.10 Data analysis

IBM SPSS Statistics 25.0 software was used for statistical analysis. Data were expressed as mean ± SD (Standard Deviation) and normalized by logarithmic, or arcsine transformation when results were expressed as percentages. Blood glucose analysis was evaluated by one-way ANOVA with post hoc Tukey ($P<0.05$). The genomic transcripts present in spermatozoa obtained from qPCR were evaluated through one-way ANOVA with post hoc Student–Newman–Keuls (SNK) ($P<0.05$). Plasma membrane viability results were compared with independent samples t-test ($P<0.05$). A repeated measures ANOVA was applied for sperm motility analysis and each time post activation was evaluated with independent samples t-test for fresh and cryopreserved sperm ($P<0.05$).

To evaluate if sperm freezability was affected by the diabetic condition, fresh and cryopreserved sperm from untreated and Met treated Tg*(ins:nfsb*-mCherry) males were evaluated according to sperm motility and DNA fragmentation parameters. Viability of the sperm plasma membrane was not used since the preliminary data analysis revealed that it was not useful in the comparison between fresh and cryopreserved samples in the present work. Due to the high number of variables related to sperm quality (4 DNA fragmentation parameters + 5 motility parameters x 6 post activation times = 34 sperm quality variables) measured for each sample, their degree of redundancy was investigated. Consequently, a Principal Component Analysis (PCA) was used to assess the possibility to aggregate all variables into a small number of components, without significant loss of information.

To check the sperm freezability of untreated (fresh n = 5; cryopreserved n = 4) and Met treated (fresh n = 7; cryopreserved n = 9) Tg*(ins:nfsb*-mCherry) males, a hierarchical cluster analysis was applied to the scores PCA components. The Ward's method (Ward, 1963) was applied since this methodology allows to form hierarchical groups of mutually exclusive subsets, where each member of the group maximally similar in relation to their inherent characteristics (i.e. sperm motility and DNA fragmentation). To apply Ward's method, the squared Euclidean distance was fixed computationally. This methodology is a mechanism of agglomerative hierarchical clustering procedure to classify the homogeneity of samples, according to a multivariate perspective. All the variables were considered according to treatment (control and diabetic) and sperm (fresh and cryopreserved) sample. This analysis is represented through a dendrogram.

6.1.4 Results

To evaluate the effectiveness of induction of diabetic and control conditions in Tg*(ins:nfsb*-mCherry) males, two methods were used, namely blood glucose analysis and observation of the pancreas under a fluorescence stereomicroscope. For a comprehensive analysis of glucose conditions of the fish, a set of controls were used namely AB males with and without exposure to Met and males without

manipulation (collected directly from the ZebTec housing tanks). The AB and Tg*(ins:nfsb*-mCherry) males from control showed no significant differences in blood glucose levels when compared to stress-free AB males, however, they were also not significantly different from Met treatments. Both AB and Tg*(ins:nfsb*-mCherry) lines showed significantly higher blood glucose levels when compared to stress-free AB males (Figure 6.1 A).

Immediately after the dissection of the Tg*(ins:nfsb*-mCherry) males the fluorescence of β-cells in the pancreas was observed. The loss of fluorescence in the pancreas of Tg*(ins:nfsb*-mCherry) males exposed to Met when compared to the control allowed the confirmation of the pancreatic β-cells ablation and onset of diabetes (Figure 6.1 C and D).

In spermatozoa, the transcripts of *insa* and glucose carrier *slc2a2* were significantly upregulated in Met treated Tg*(ins:nfsb*-mCherry) males when compared to untreated Tg*(ins:nfsb*-mCherry) males, and to AB males with and without Met exposure (Figure 6.2 A and B). The *insra* transcripts were present in significantly higher quantities in sperm from Met treated Tg*(ins:nfsb*-mCherry) males when compared to AB males exposed to Met, however, it was not significantly different to sperm from both untreated zebrafish lines (Figure 6.2 C).

In fresh sperm, the plasma membrane viability of Tg*(ins:nfsb*-mCherry) males under diabetic conditions was significantly reduced when compared to untreated males (Figure 6.3). After cryopreservation, the Tg*(ins:nfsb*-mCherry) sperm plasma membrane viability was compromised with no significant differences between both treatments (Figure 6.3).

For sperm motility analysis, initially a repeated measures ANOVA was used to investigate the effect of the treatments (control and diabetic) and sperm cryopreservation through post activation time on sperm motility parameters and their interactions (Table 6.2). Both treatment and cryopreservation factors showed significant differences in sperm motility parameters without interactions between these factors (Supplementary data 6.1). To investigate thoroughly the effects of each treatment an independent samples t-test was used to study the differences at each time post activation on fresh and cryopreserved sperm (Supplementary data 6.1).

The sperm total motility was significantly reduced in males from Tg*(ins:nfsb*-mCherry) under diabetic conditions when compared to the control (Figure 6.4 A, Table 6.2) in the last seconds of the spermatozoa lifespan both in fresh and cryopreserved sperm (Supplementary data 6.1). Progressive motility was significantly lower in sperm from Met treated males when compared to untreated males. Sperm from males with transient diabetes showed no post-thaw sperm progressive motility (Figure 6.4 B). The Tg*(ins:nfsb*-mCherry) males under diabetic conditions showed significantly reduced sperm velocities and linearity when compared to the control (Figure 6.4 C-E) after 30 s post activation until the end of their lifespan. This result was observed both in fresh and cryopreserved sperm (Supplementary data 6.1).

The DNA fragmentation was, as expected, significantly increased due to cryopreservation procedure both in sperm from Tg*(ins:nfsb*-mCherry) untreated males and in diabetic conditions (Figure 6.5). The Tg*(ins:nfsb*-mCherry) males under diabetic conditions produced significantly higher spermatozoa DNA fragmentation when compared to the control (Figure 6.5).

The sperm quality components obtained by PCA (i.e., component PC1, PC2, PC3 and PC4) consisted of a weighted median of the motility and DNA integrity measurements (after standardization). The scores of the components obtained with PCA were used for further cluster analysis since these components retain a total of 94.1% of the accumulated variance. With the exception of PM at 40 and 50 s post activation, all sperm motility parameters (TM, PM, VCL, VSL and LIN) displayed a high degree of positive correlation among them, being aggregated into a single latent variable (PC1), which retains 74.3% of the observed variation. All DNA fragmentation parameters were completely differentiated from motility parameters, being positively correlated among each other and aggregated into a variable (PC2) retaining 11.5% of the observed variation. The components PC3 and PC4 explained 5.3% and 3% of the observed variance respectively. The components obtained by PCA data correlation of sperm motility and DNA fragmentation of fresh and cryopreserved sperm from diabetic and control treatment of Tg*(ins:nfsb*-mCherry), were used to investigate the levels of association among samples and therefore sperm freezability through a hierarchical cluster analysis (Figure 6.6).

Data were aggregated into 3 clusters where one cluster contain samples of fresh sperm both from untreated and Met treated males. The other two clusters are predominantly composed by cryopreserved sperm. One cluster is composed mainly of cryopreserved samples of the control treatment (except one sample of fresh sperm of control). However, in this cluster, there is one sample of cryopreserved sperm of Met treated males, that is more distant and with lower homogeneity compared to the other samples. The last cluster is composed predominantly of cryopreserved samples from Met treated males (Figure 6.6). Overall, in fresh sperm, both treatments are homogeneously mixed, however, after cryopreservation sperm from transient diabetic conditions are separated from the control treatment. Consequently, sperm from Met treated males has reduced freezability when compared to the control.

The mechanism of action of diabetic conditions in Tg*(ins:nfsb*-mCherry) zebrafish spermiogenesis and sperm quality is systematized in Figure 6.7.

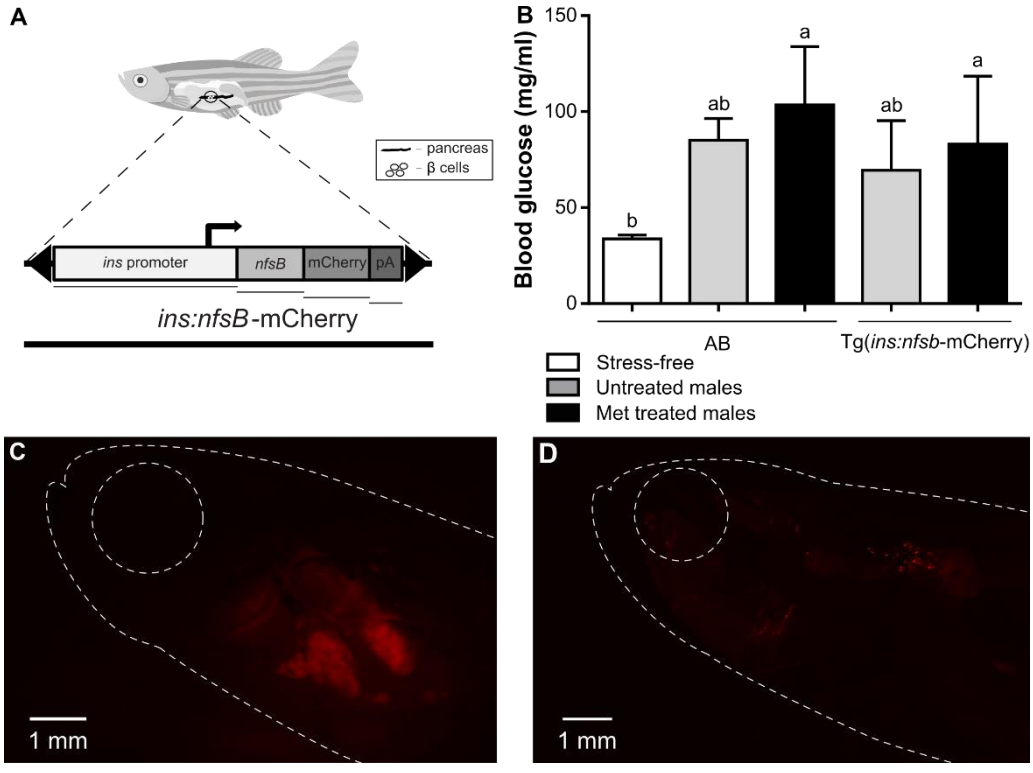


Figure 6.3 Confirmation of pancreatic β cells ablation and onset of diabetes in zebrafish males. A) graphic representation of the construct inserted in Tg(*ins:nfsb*-mCherry) genome; B) blood glucose analysis of stress-free AB males (n=7), AB males in control (=7) and Met treatment (n=24), and Tg(*ins:nfsb*-mCherry) in control (n=8) and Met treatment (n=21); C) observation of pancreas fluorescence in Tg(*ins:nfsb*-mCherry) control males after dissection; D) observation of pancreas lack of fluorescence in Tg(*ins:nfsb*-mCherry) diabetic males. The values plotted represent means±SD. Different letters represent statistical differences (one-way ANOVA, post hoc Tukey, P<0.05).

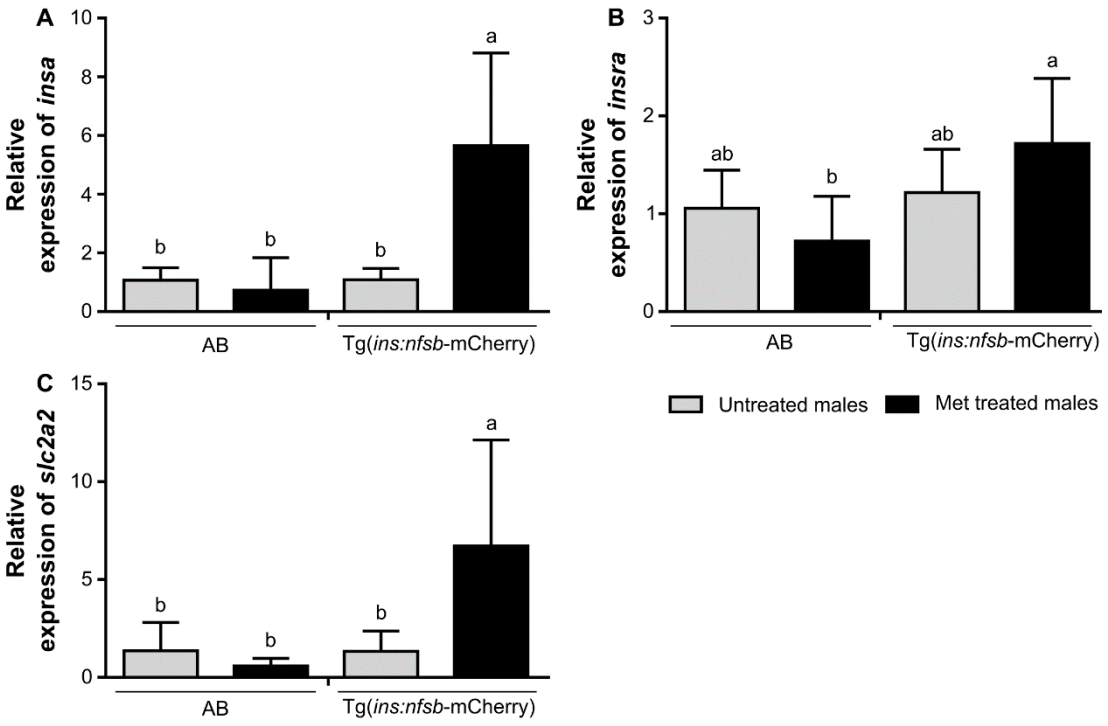


Figure 6.4 Genes relative expression to *ef1α* in zebrafish sperm pools using $2^{-\Delta\Delta Ct}$ method: A) *insa* in spermatozoa pools of AB males in control (n=4) and Met treatment (n=4), and Tg*(ins:nfsb*-mCherry) in control (n=5) and Met treatment (n=7); B) *insra* in spermatozoa pools of AB males in control (n=4) and Met treatment (n=4), and Tg*(ins:nfsb*-mCherry) in control (n=7) and Met treatment (n=7); C) *slc2a2* in spermatozoa pools of AB males in control (n=4) and Met treatment (n=4), and Tg*(ins:nfsb*-mCherry) in control (n=5) and Met treatment (n=6). The values plotted represent means±SD. Different letters represent statistical differences (one-way ANOVA, post hoc SNK, P<0.05).

Table 6.2 Statistical analysis of sperm motility parameters (data for 1 minute, each 10 s post activation) of Tg(*ins:nfsb*-mCherry) zebrafish line under control and diabetic conditions and their interactions (P values).

Repeated measures ANOVA	TM (%)	PM (%)	VCL (μm/s)	VSL (μm/s)	LIN (%)
Treatment (control/diabetic)	0.017*	0.050*	0.023*	0.018*	0.006*
Sperm (fresh/cryopreserved)	<0.001*	<0.001*	0.045*	0.005*	0.005*
Treatment x sperm	0.530	0.165	0.717	0.943	0.999

Significant differences (repeated measures ANOVA, P<0.05) are represented with asterisk.

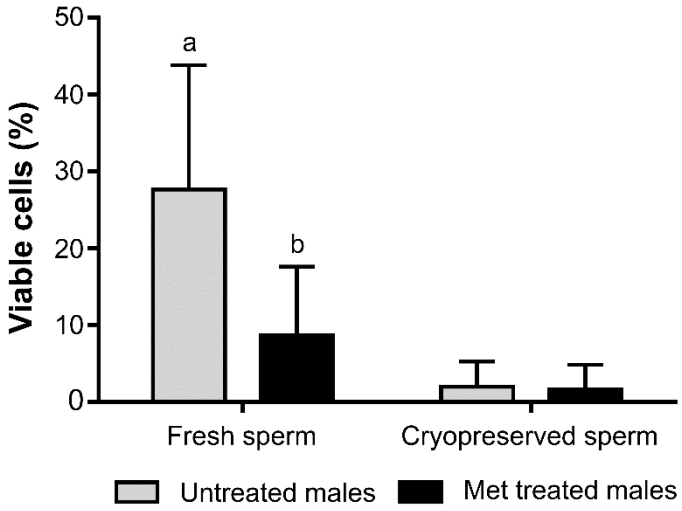


Figure 6.5 Spermatozoa plasma membrane viability of fresh and cryopreserved sperm from untreated Tg(*ins:nfsb*-mCherry) males (n=5) and met treated males (n=6). The values plotted represent means±SD. Asterisk represent statistical differences (independent samples t-test, $P<0.05$).

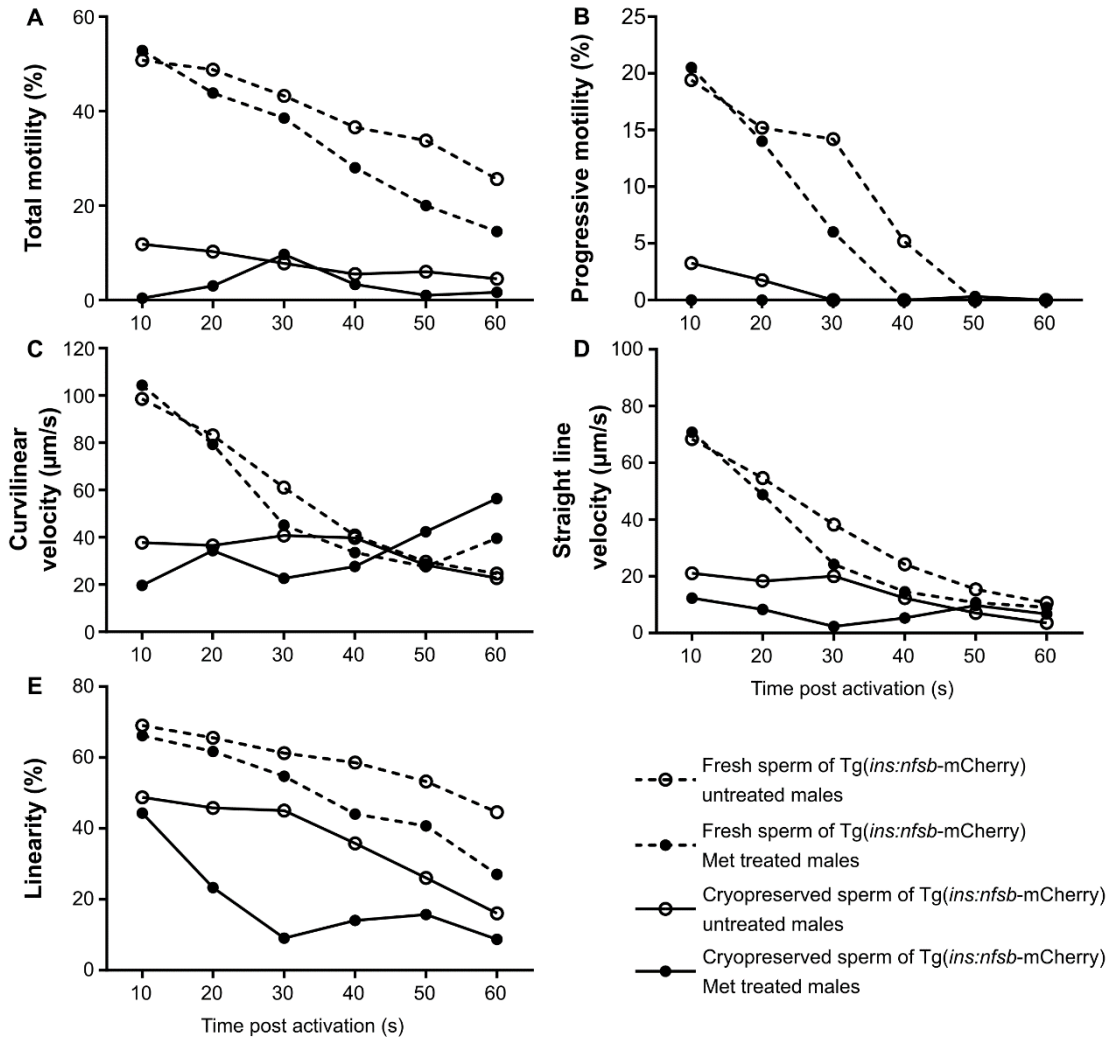


Figure 6.6 Sperm motility parameters of fresh sperm obtained from untreated Tg(*ins:nfsb*-mCherry) males (n=5) and metronidazole (Met) treated males (n=8), and cryopreserved sperm from untreated (n=5) and Met treated males (n=10). Sperm was activated, and motility parameters were recorded every 10 s for 1 minute in terms of: A) total motility (%); B) progressive motility (%); C) curvilinear velocity (µm/s); D) straight line velocity (µm/s) and E) Linearity (%). The values plotted represent means. Dashed line represents fresh sperm and continuous line represent cryopreserved sperm. Sperm from untreated males is represented with white circle and sperm from Met treated males with dark circle.

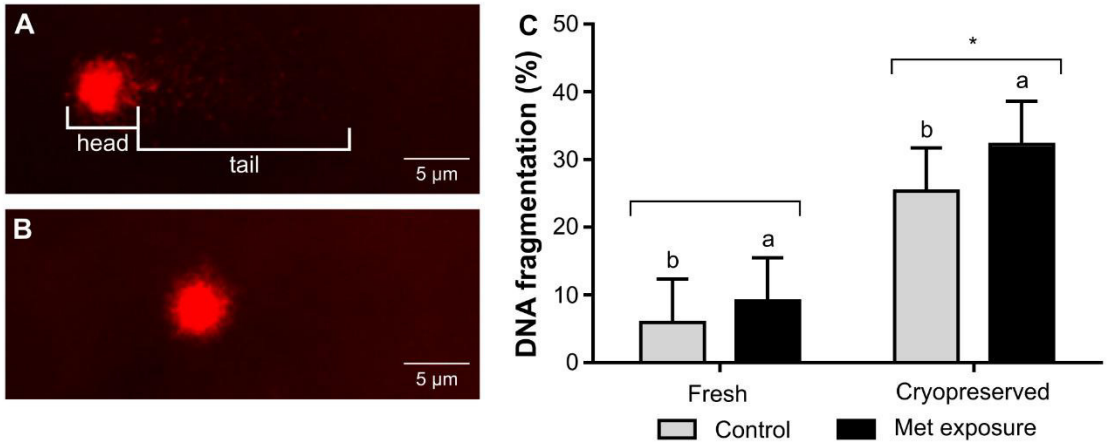


Figure 6.7 DNA integrity in zebrafish sperm: A) comet with high DNA fragmentation; B) comet with low DNA fragmentation; C) DNA fragmentation of fresh sperm obtained from untreated Tg(*ins:nfsb*-mCherry) males (n=4) and metronidazole (Met) treated males (n=8), and cryopreserved sperm from untreated males (n=5) and Met treated males (n=6). The values plotted represent means±SD. Different letters represent statistical differences between treatments and asterisk represent statistical differences between fresh and cryopreserved sperm (independent samples t-test, P<0.05).

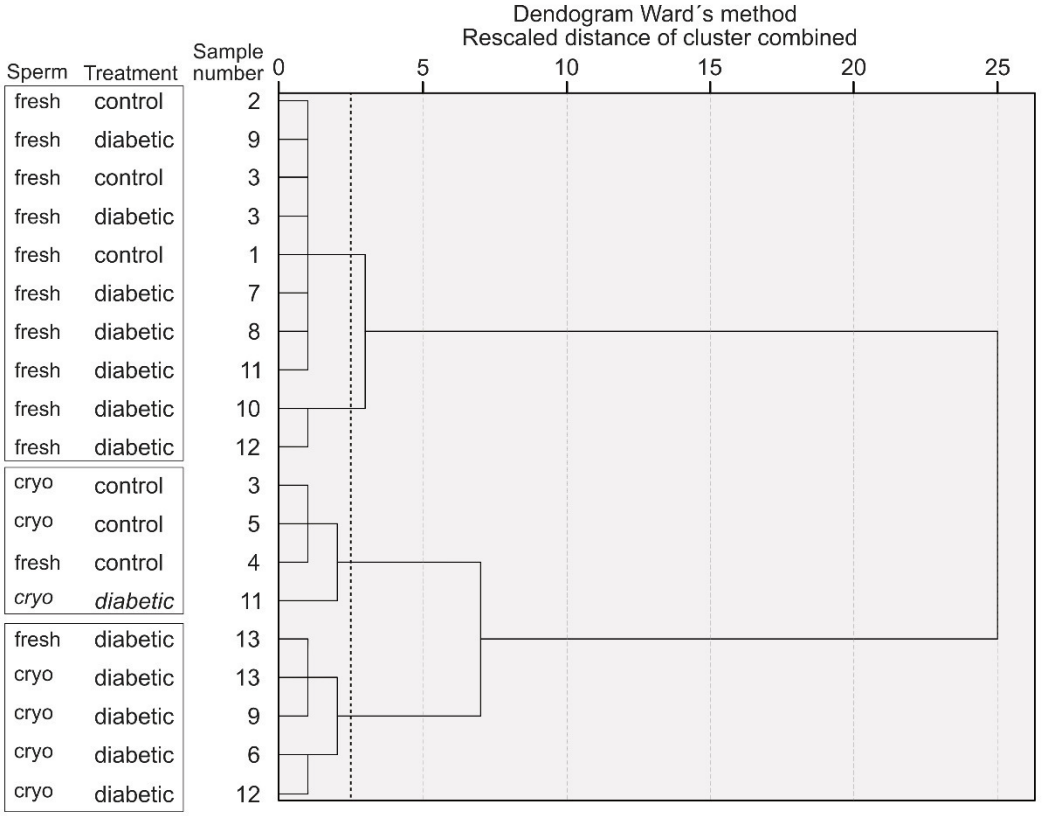


Figure 6.8 Dendogram of Ward´s hierarchical cluster analysis for fresh and cryopreserved sperm (of principal components resulting from motility and DNA fragmentation data) of zebrafish sperm cryopreserved with a -10°C/min cooling rate. Rectangles discriminate clusters of samples within fresh and cryopreserved sperm from untreated and Met treated Tg*(ins:nfsb*-mCherry) males.

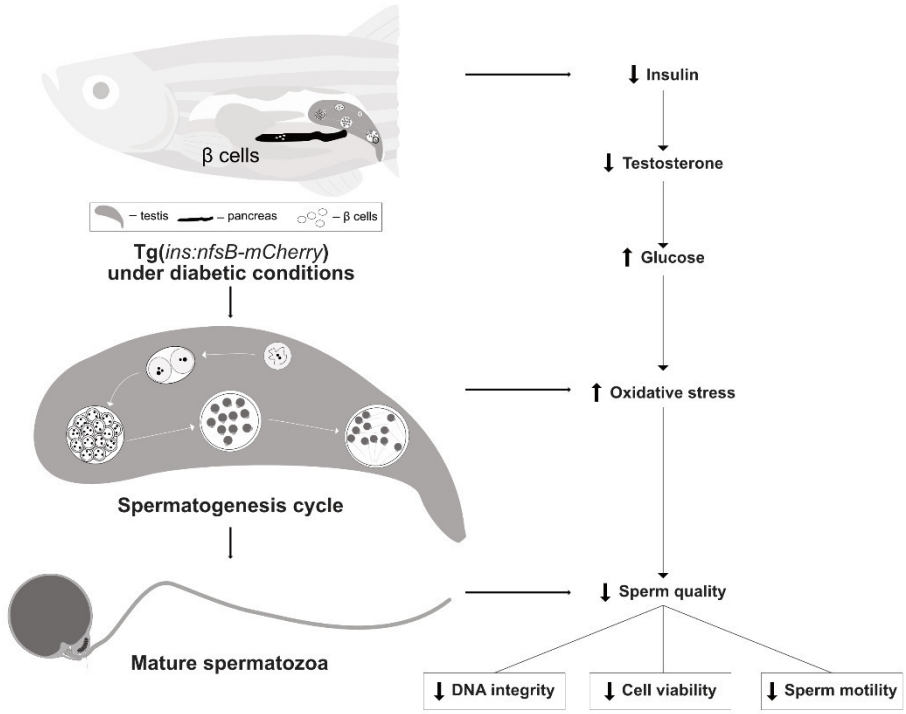


Figure 6.9 Hypothetic model of putative effect of diabetes in sperm quality.

6.1.5 Discussion

Among the most important complications related to diabetes mellitus is the impairment of male reproduction, affecting both spermatogenesis and mature sperm metabolism (Dias et al., 2014). This disease modulates spermatozoa substrate consumption and/or production due to altered glycolytic behavior, with a deregulation of the glucose uptake and metabolism (Dias et al., 2014). Zebrafish is a strong candidate to model prominent human pathologies such as diabetes mellitus. These species has been considered a useful vertebrate model for research in reproduction (Hoo et al., 2016) and metabolic diseases such as diabetes (Patton and Tobin, 2019). Therefore, zebrafish is a suitable model for the investigation of diabetes effect in the male germ line.

The onset of diabetes promotes impaired insulin secretion and high systemic glucose levels. In our study, we observed that Tg*(ins:nfsb*-mCherry) zebrafish with impaired pancreatic activity produced sperm with an increase in the number of transcripts of *insa*, and *slca2a2*. This event can be a consequence of cellular transcription during spermatogenesis exposed to high glucose levels. The onset of

diabetes promotes impaired insulin secretion and high systemic glucose levels (Carvalho et al., 2017; Moss et al., 2009; Pisharath et al., 2007). Our data demonstrate that Tg*(ins:nfsb*-mCherry) zebrafish sperm showed higher levels of *slc2a2* transcripts relative to control, which suggests that glucose uptake was favored. A zebrafish mutant model lacking a functional leptin receptor has a similar glucose homeostasis phenotype, shows reduced reproductive competence and upregulation of *insa* and *slc2a2* transcripts in the liver, where systemic glucose metabolism occurs (Michel et al., 2016). As in liver, spermatozoa have high glucose metabolism. Similarly, to this study higher levels of *insa* and *slc2a2* transcripts along with low sperm quality occurs under transient diabetic conditions. Therefore, our results suggest that during zebrafish spermatogenesis the lack of systemic insulin secretion promotes an increase in *insa* and *slc2a2* in spermatozoa. In our study, *insra* transcripts in sperm from males under transient diabetes conditions were significantly higher than sperm from Met treated WT males, indicating a response to the experimental diabetes induction. The observed upregulation of insulin-related genes can be a consequence of cellular transcription during spermatogenesis under diabetic conditions.

In type I diabetes male patients, spermatogenesis disruption and germ cells apoptosis is observed. Upon the activation of the apoptosis cascade, the cell experiences a series of cellular degradation events, including DNA fragmentation, that leads ultimate to cell death (Elmore, 2007; Martinvalet et al., 2005). In patients with type I diabetes, changes in the expression of the genes involved in DNA repair and replication were correlated with the increase in sperm DNA fragmentation (Agbaje et al., 2008). In our work, it was possible to observe that sperm from zebrafish under diabetic conditions show significantly lower sperm motility, plasma membrane viability and DNA integrity when compared to sperm from untreated males. This data suggests that the ablation of the pancreatic β cells, which have been shown to impair insulin secretion (Moss et al., 2009; Pisharath et al., 2007), produced germ cells apoptosis leading to reduced sperm quality and DNA integrity. The spermatozoa plasma membrane viability of diabetic and untreated males was compromised after cryopreservation. This result shows high susceptibility of Tg*(ins:nfsb*-mCherry) zebrafish line to cryopreservation and methodological

improvements in the protocol could be adopted to improve post-thaw viability, as in previous works (Diogo et al., 2018).

In zebrafish, stored ATP is considered the basis for motility soon after initiation of motility. However, prolonged motility relies on oxidative phosphorylation and *de novo* ATP synthesis (Ingermann et al., 2011). Our results show that sperm collected from diabetic conditions, both in fresh and cryopreserved sperm, show significant lower motility parameters when compared sperm from untreated males. There is a significant decrease in sperm motility parameters such as VCL, VSL and LIN, particularly after 30 s post activation in sperm from *Tg(ins:nfsb-mCherry)* males under diabetic conditions. This result suggests that under diabetic conditions, the ATP stores allow sperm normal motility in the first seconds of motility. In the lasts seconds of motility when *de novo* ATP synthesis through oxidative phosphorylation occurs (Ingermann et al., 2011) motility is impaired which suggests mitochondria deregulation as observed in sperm from human patients (Agbaje et al., 2007). The high levels of glucose uptake can promote inhibition of glycolysis pathway, which can lead to mitochondrial activity impairment, promoting a decrease in sperm motility (Agbaje et al., 2007; Ding et al., 2015).

Factors affecting the plasma membrane composition and fluidity (Cabrita et al., 2008), sperm subpopulations structure (Flores et al., 2009), intrinsic male variability (Pérez-Patiño et al., 2019; Roca et al., 2006) and differences in the abundance of proteins relevant for sperm function (Dietrich and Ciereszko, 2018; Pérez-Patiño et al., 2019) are associated to sperm freezability. However, sperm freezability predictors are not universally manifested in sperm quality traits across species prior to cryopreservation (Roca et al., 2006). Recently, good freezability in carp (Cyprinus carpio) sperm were related to high concentrations of proteins responsible for the maintenance of flagella structure, membrane fluidity, sperm motility and energy production, which can be markers of spermatozoa full maturation (Dietrich and Ciereszko, 2018). The freezability was reduced in sperm from diabetic treatment. We hypothesize that this result could be caused either by their lower initial sperm quality, due to apoptotic events caused by systemic metabolic malfunction, or failure in sperm full maturation due to abnormal glucose transport

during spermatogenesis. This data suggest that additional care should be taken in sperm quality analysis and sample selection of diabetic patients for cryopreservation purposes.

We propose a hypothetic model for the mechanism of action of pancreatic cells impairment on sperm quality in *Tg(ins:nfsb-mCherry)* zebrafish (Figure 6.7) as a consequence of high systemic glucose levels which can affect affects sperm microenvironmental conditions. The pancreatic β cell ablation promotes impaired insulin secretion in the pancreas, hyperglycemia, high ROS production and oxidative stress, low antioxidant defenses, and high apoptosis (Alfar et al., 2017; Delgadillo-Silva et al., 2019). With low insulin secretion in the pancreas and hyperglycemia, it is expected upregulation of *ins* transcripts in germ cells (Aquila et al., 2005; Michel et al., 2016). Moreover, with the high glucose present in the organism, it is expected that germ cells in the testes increase the transcripts for glucose carriers which favors an increase in intracellular glucose levels, which can potentially promote cell stress. Consequently, in zebrafish under transient diabetic conditions it is expected that during spermatogenesis, germ cells in the testes are exposed to oxidative stress, mitochondrial dysfunction and high apoptosis environment that can lead to DNA fragmentation, loss of cell viability. Moreover, under these conditions, spermatozoa have deficient differentiation and maturation and therefore upon sperm cryopreservation shows lower sperm freezability. If spermatozoa mitochondria are affected by oxidative stress events, after the exhaustion of ATP stores, its *de novo* synthesis through oxidative phosphorylation is impaired, affecting sperm motility at the end of their lifespan, therefore reducing sperm fertilizing ability.

The results of this work reveal that under diabetic conditions detrimental effects are observed on zebrafish sperm viability, motility and DNA fragmentation similar to human patients. Therefore, this species is a suitable model to assay reproductive dysfunctions. The use of zebrafish in diabetes research respects the 3R´s rule, since this species regenerate the pancreatic β cells and the diabetic condition can be induced repeatedly on the same individuals. The present work reveals compelling evidence that zebrafish is a suitable model to investigate the effects of type 1 diabetes mellitus on male reproductive function.

CHAPTER 7. GENERAL DISCUSSION

General Discussion

The methodological standardization of zebrafish sperm cryopreservation is essential to overcome the high variability of post-thaw sperm quality and *in vitro* fertilization success. The improvement of this methodology is highly relevant for research centres worldwide with facilities dedicated to the use zebrafish as a model for research areas such as biomedicine, toxicology, pharmacology and developmental biology, among many others. There is an urgent need for an improved and consistent cryopreservation method to support the management of zebrafish lines, since the lack of space for the exponential generation of new zebrafish lines is one of the main constrains in zebrafish facilities. This thesis provided an integrative approach to tackle this issue, with a multidisciplinary research strategy.

7.1. Identification of specific constraints in zebrafish male donors affecting reproductive performance

Successful cryopreservation depends on high quality sperm, which is ensured by having high quality breeders. Consequently, broodstock selection and management is a priority to improve sperm cryopreservation. Broodstock nutrition is considered one of the most important factors affecting reproduction and progeny quality (Beirão et al., 2015a; Izquierdo et al., 2001). Diet has a preponderant effect on gamete quality, particularly phospholipid and fatty acid contents (Meinelt et al., 1999; Nowosad et al., 2017), that are one of the main constituentes of plasma membranes. Nutrition is one of the earliest factors affecting gametogenesis, and therefore the quality of gametes. Zebrafish are fed with highly different diets and feeding protocols in research centres worldwide, which might be a contributing factor for reported reproduction variability (Martins et al., 2018). In chapter 3 of the present thesis, we investigated how the supplementation of the phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) included in purified diets affected zebrafish reproductive performance, gamete quality and incidence of skeletal malformations in the progeny (Diogo et al., 2015). Both phospholipids are

particularly relevant for teleosts, considering that spermatozoa membranes and egg yolk contain high quantities of PC and PE (Drokin, 1993b; Rønnestad et al., 1995). The inclusion of these phospholipids may be particularly important in zebrafish spermatozoa, which have lower viability than other species, since these cells need to maintain the integrity of plasma membrane when exposed to the fertilizing media, suffering an hypoosmotic stress. It has been seen in other species (Senegalese sole) that plasma membrane can be modulated through diet promoting an increase in the resistance to osmotic stress under fertilization conditions (Beirão et al., 2015a). The experimental data obtained showed that fish fed with PC supplemented diet presented good sperm quality parameters and the highest hatching rate. However, this diet also revealed deleterious effects on zebrafish larvae skeletal development. Broodstock fed with diets supplemented with PE promoted good quality sperm without compromising the offspring skeletal development. This work provided evidences on the relevance of dietary phospholipids on sperm quality and supported the development of a standardized diet, which is essential for the reduction of variability on the reproductive performance among facilities.

Sperm quality is defined by the ability to successfully fertilize an egg, which is dependent on factors such as spermiation period, favourable environmental conditions for activation of sperm motility, parental aging and sperm output frequency (Alavi and Kazemi, 2006; Cabrita et al., 2011a; Migaud et al., 2013; Rurangwa et al., 2004). Therefore, the establishment of optimal age and sperm collection frequency is a relevant resource for zebrafish broodstock management and supports zebrafish sperm cryopreservation. The non-lethal method for sperm collection is more adequate for zebrafish management purposes and respects the 3 R's rule. Consequently, sperm can be collected repeatedly from the same individual through a non-invasive method (reuse), the same fish can be used for other experiments after an appropriate resting time (recycle), and therefore a lower number of fish can be used for experimental purposes (reduce). In zebrafish, the comparison between lethal and non-lethal sperm collection methods was already investigated (Daly and Tiersch, 2012; Jing et al., 2009a; Morris et al., 2003). The non-lethal technique has become more used in research due to its benefits (Diogo et al., 2015; 2018; Draper and Moens, 2009; Hagedorn and Carter, 2011; Hagedorn et al.,

2012; Harvey et al., 1982b; Matthews et al., 2018; Panigrahi et al., 2018; Park et al., 2012; Silva et al., 2019) when compared to the lethal method (Bai et al., 2013; Hagedorn et al., 2009; Yang et al., 2007; Yang et al., 2016).

In chapter 4 the minimum required time for males to rest between sperm collections was investigated, in order to ensure optimal sperm quality recovery. With this study it was possible to establish a minimum of 14 days of rest between non-invasive samplings to the same individual. Moreover, it was determined that zebrafish between 6 to 8 months of age presented a higher sperm quality in comparison to older fish (12-14 months), and therefore, younger males should be selected for cryopreservation purposes, decreasing the labour-intensive process of sample selection (Diogo et al., 2019).

7.2. Optimization of zebrafish sperm cryopreservation methodologies

Under the objectives of the present thesis, a new sperm cryopreservation and storage method is presented in chapter 5.1. This new procedure uses an ultrafreezer and was established for the first time in a teleost species (Diogo et al., 2018). This methodological innovation is highly relevant for zebrafish facilities management, since it decreases the overall costs of cryopreservation. The extender solution was improved according to cryobiological principles and, to the best of our knowledge, we reported for the first time the incidence of skeletal malformations in zebrafish offspring sired by cryopreserved sperm.

The hypothesis of an alternative storage method in an ultrafreezer was validated through the comparative assessment of cryopreservation of pooled sperm in a programmable biofreezer and subsequent storage in either liquid nitrogen or in a ultrafreezer. To evaluate the storage efficiency, samples were thawed at different time points. Storage in an ultrafreezer was not significantly different to liquid nitrogen storage in terms of sperm motility, viability and hatching rates. Ultrafreezer storage was beneficial in relation to liquid nitrogen since it has higher storage capacity, easier sample manipulation and, unlike liquid nitrogen storage, it does not require periodic reposition, making the global costs of cryopreservation lower (Batista et al., 2009). Considering these results, we aimed to understand if it

was possible to simplify even more the protocol and to improve it using a fast-freezing rate, placing the samples directly in the ultrafreezer (-66ºC/min). This was a particularly relevant hypothesis, since the fastest freezing rate reported for zebrafish sperm was -25ºC/min (Bai et al., 2013; Wang et al., 2015).

As previously discussed, zebrafish spermatozoa are similar to sperm from other cyprinids (Zhang et al., 2014). Spermatozoa structure depends on the cell water content; as such, spermatozoa can have different cryoprotectant permeability. Therefore, spermatozoa structure is an important cryobiological feature contributing to the species-specific cell biophysical properties that affect the freezing process and post-thaw survival (Hagedorn et al., 2009). Since in species such as *Cyprinus carpio* (Bernáth et al., 2016) and *Perca fluviatilis* (Bernáth et al., 2015) a fast cooling rate of −56°C/min improved post-thaw sperm motility, the hypothesis of a fast freezing rate to improve zebrafish post-thaw sperm quality was evaluated. The post-thaw sperm quality was improved as shown by the evaluation of sperm viability, DNA fragmentation and late apoptosis in relation to the conventional cryopreservation methodology. Our study demonstrated that ultrafreezers are a viable alternative for zebrafish sperm storage and a fast cooling rate of −66°C/min performed directly in an ultrafreezer improve post-thaw zebrafish sperm quality. The optimization of the cooling rate for zebrafish sperm cryopreservation, represents an important contribution to support future methodological improvements. This methodology facilitates the cryopreservation process without the need of expensive programmable biofreezers and can be easily applied in zebrafish facilities, reducing the global costs of cryopreservation.

Considering the relevant findings described previously, in chapter 5.2 we performed a refinement of the extender composition for the previously established protocol using an electric ultrafreezer, according to cryobiological principles. Therefore, the optimal concentration of permeating cryoprotectants was evaluated with narrow ranges, because low differences in cryoprotectant concentrations are known to have a high impact on the freezing process and on post-thaw sperm quality (Anchordoguy et al., 1987; Judycka et al., 2018). Non-permeating cryoprotectants such as sugars and amino acids are able to establish interactions with membrane lipidic bilayers (Carpenter and Crowe, 1988), protecting the cells during the freezing

process and improving post-thaw results (Cabrita et al., 2011b; Martínez-Páramo et al., 2013).

Our results showed that concentrations of 12.5 to 15% of N-N dimethylformamide (DMF) were able to protect spermatozoa during the cryopreservation process yielding high total and progressive motility, plasma membrane viability and hatching rates. Egg yolk yielded high post-thaw sperm quality and hatching rates, which might be explained by its high viscosity that protects the cell during cryopreservation (Morris et al., 2006). Viscosity stabilizes the fertilization microenvironment, which is important in teleosts external fertilization (Lahnsteiner, 2002), particularly in species that yield low sperm volume such as Senegalense sole (Diogo et al., 2010; Riesco et al., 2017) and zebrafish. The main disadvantages of egg yolk are the difficult standardization probably due to the variation of biochemical composition depending of the source, the high susceptibility to contamination by pathogenic agents (Aires et al., 2003) and the fact that is a animal-derived product that presents sanitary risks due to possible contamination due to the introduction of exotic diseases via semen media containing egg yolk (Gavin-Plagne et al., 2018; 2019). Additionally, egg yolk provides aromatic amino acids and therefore contributes to the production of reactive oxygen species by dead spermatozoa to the detriment of live spermatozoa (Shannon and Curson, 1982; Vishwanath and Shannon, 2000).

Bicine is an amino acid [N,N-Bis(2-hydroxyethyl)glycine] with high buffer capacity and recommended for biological research at low temperatures (Good et al., 1966). Bicine is commonly used in fresh water species extender composition (Cabrita et al., 2010) and was recently used in a zebrafish sperm cryopreservation protocol (Matthews et al., 2018). However, its isolated effect on post-thaw sperm quality required deeper comprehension. We observed that bicine yields high post-thaw sperm quality and *in vitro* fertilization success without the disadvantages of egg yolk mentioned previously.

The incidence of severe malformations, namely lordosis, scoliosis and kyphosis and the occurrence of deformed arches were the main effects observed within skeletal malformations in the offspring sired by cryopreserved sperm. The skeletal malformations incidence on control treatment of zebrafish obtained with

fresh sperm are within the normal range for this species in natural spawns (Diogo et al., 2015; Martins et al., 2018). It was observed higher incidence of skeletal malformations in caudal and precaudal fin vertebrae in zebrafish sired with cryopreserved sperm. The extender composition affects differently the occourence of severe malformations (lordosis, kyphosis and escoliosis) and deformed arches of the offspring. These results suggest that different types and concentrations of cryoprotectants, and their combinations, are differently able to protect cells against deleterious effects on genes involved in developmental processes that will affect skeletal formation. This characterization allowed to observe effects of the extender composition that could be otherwise disregarded. Therefore, our study evidences that the characterization of the skeletal development of offspring sired by cryopreserved sperm is a useful tool for evaluation of the quality of the gametes and can be used on the optimization of sperm cryopreservation protocols.

7.3. Zebrafish sperm quality assessment methodologies

One of the most striking issues among zebrafish studies related to sperm cryopreservation is the considerably high number of studies relying only on sperm motility estimations (Hagedorn et al., 2012; Harvey et al., 1982a; Morris et al., 2003; Yang et al., 2007; 2016) rather than quantitative analysis through computer-assisted sperm analysis (CASA) system (Bai et al., 2013; Diogo et al., 2015; 2018; Matthews et al., 2018; Wang et al., 2015). As previously mentioned, CASA systems allow for a precise quantification and qualification of motility parameters. Therefore, the high number of studies relying on motility estimation rather than quantification with CASA system consists in an important source of variability among studies and on post-thaw sperm quality analysis. Zebrafish are reared in captivity under controlled environmental conditions.

Water osmolarity is the most important factor controlling zebrafish sperm motility activation (Dadras et al., 2017; Jing et al., 2009a). In the recirculation systems water is maintained at 28ºC, and ions present in the water are controlled through addition of salts and a water conductivity probe controlling the automatic adjustment. Therefore, the settings used for water temperature and conductivity,

will be the system water conditions present in the fertilization microenvironment of natural spawns that can affect the fertilization success. However, the research community does not have this parameter standardized and the water conductivity is highly variable among facilities (≥400 up to 1600 μS/cm). This fact, by itself, could affect sperm motility activation and characteristics. Surprisingly, throughout the literature the activation of sperm motility for quantification procedures is performed with distilled water, or using several buffers diluted in distilled water, in different proportions with few exceptions (Caetano Da Silva et al., 2019; Diogo et al., 2015, 2018).

Spermatozoa motility depends on the energy released with ATP hydrolysis to produce flagellum beating (Alavi and Cosson, 2005). Water temperature affects motility characteristics (Dadras et al., 2017) and dynein motors of flagellum (Cosson et al., 2008a). In comon carp spermatozoa sperm motility is longer at 20°C than at 26-30°C (Billard and Cosson, 1992), decreasing sperm motility duration at lower temperatures (Billard et al., 1995). No differences were found in sperm motility duration of brown trout spermatozoa between 2-28ºC, however Atlantic salmon decrease sperm motility duration with higher temperature (Vladiĉ and Jätrvi, 1997). Therefore, the optimal temperature for sperm motility activation is species specific and determined by each species ecological adaptations (Dadras et al., 2017). In zebrafish sperm motility activation is routinely performed without temperature control. We demonstrated that water temperature affects zebrafish sperm metabolism, and showed that, for zebrafish, 28°C improves sperm motility throughout sperm lifespan.

Osmolarity is one of the major factors contributing to sperm motility activation through cell signaling cascade (Morisawa et al., 1983). Osmolarity os the activation medium is the main responsible for zebrafish motility activation (Ingermann et al., 2011). Osmolarity is correlated with water conductivity as observed in our study. It was observed that distilled water in zebrafish sperm motility activation improve sperm longevity in relation to 0.3% of NaCl and HBSS 170 mOsm/Kg. In our study, distilled water yields higher motility values in relation to the water conductivity set up in the systems, which results in sperm analysis biases in relation to spermatozoa behaviour under natural spawning conditions.

The results of our work suggest that this species is adapted to low water conductivity conditions, hence 700 µS/cm is the most suitable conductivity to set up in the recirculation systems. This work constitutes a powerful tool for the standardization of the sperm quality analysis as well as hatchery management.

Standardization or harmonization of methodologies implies the normalization, according to an established common consensus, to reduce variability and ensure consistent results (Torres and Tiersch, 2018). Therefore, standardization of procedures is essential to improve experimental reliability and replicability (Hagedorn et al., 2018; Lawrence, 2016; Martínez-Páramo et al., 2017; Torres and Tiersch, 2018; Torres et al., 2017; Varga et al., 2018). Although the standardized procedures might not be useful or applicable in all experimental designs, they would allow comparison between studies and facilitate knowledge improvements in a faster and more reliable manner. The lack of standardization of zebrafish sperm cryopreservation has been routinely considered the most important reason behind the low post-fertilization success (Hagedorn et al., 2018; Torres et al., 2017; Varga et al., 2018). We consider that this is an important source of biases, but not the only responsible for low embryo survival. Throughout the several experiments performed in the present thesis, several *in vitro* fertilization trials were performed where fertilization with fresh sperm yielded similarly low hatching rates. Therefore, the methodologies used during *in vitro* fertilization, such as sperm pipetting and oocyte manipulation may be relevant stressors for zebrafish *in vitro* fertilization, as observed in assisted reproduction techniques applied to other species such as human, bovine and mouse (Ramos-Ibeas et al., 2019).

In addition to the improvement of sperm motility activation and analysis through CASA system, DNA fragmentation analysis through comet assay, flow cytometry evaluations of plasma membrane viability and cell apoptosis, proved to be valuable tools for sperm quality analysis and sample selection. One of the most relevant issues in sperm zebrafish studies is the few methods employed in the characterization of sample quality. The use of sperm samples with inconsistent quality is an important source of variability in this species. The gathered analysis of sperm plasma membrane viability and motility, particularly progressive movement, are robust, practical to apply and predictive of hatching rates. One of the main

constraints in this species is the very low sperm volume available. Therefore, flow cytometric analysis using dyes to characterize cellular constituents are the most appropriate methods, since with low sample volume a robust analysis is obtained rapidly. DNA fragmentation analysis is an important but complex analysis relevant for protocol optimization purposes. To the best of our knowledge, this is the first study where an analysis of spermatozoa apoptosis was conducted in zebrafish.

In vitro fertilization is considered the most accurate way to observe spermatozoa fertilizing ability (Rurangwa et al., 2004) and the final objective of assisted reproductive techniques. There are few reports in zebrafish sperm cryopreservation performing *in vitro* fertilization with post-thaw sperm. Among these studies, the characterization of hatching rates was only reported twice and fertilization rates at 3 hpf consist the main measure of success of *in vitro* fertilization (Harvey et al., 1982b). However, at 3 hpf an embryo sired with spermatozoa containing DNA damage is still being repaired. The maternal machinery responsible for repairing paternal DNA damage only stops its action at 8 hpf (Fernández-Díez et al., 2018). Therefore, fertilization rates only describe the entry of sperm in the oocyte, disregarding possible damage that this cell may contain, and consequently embryo putative abortion. In chapter 5.1 it is possible to observe high abortion rates between 3 and 24 hpf in embryos sired with cryopreserved sperm. In agreement with our work, Fernández-Diez et al. (2018) observed high embryo abortion due to paternal genotoxicity between 8 and 24 hpf. Considering the *in vitro* fertilization results achieved in our work and the fact that hatching rates were very similar to survival at 24 hpf, we propose that the survival at 24 hpf is the most simple and accurate method to evaluate sperm fertilization ability and progeny viability produced with post-thaw zebrafish sperm. In this work we observed a high discrepancy between fertilization and hatching rate and concluded that fertilization rate is a highly biased quality assessment in zebrafish. Although fertilization rates are often used as representation of *in vitro* fertilization success, zebrafish embryo survival at 24 hpf is the earliest and most accurate measure to predict embryo viability.

7.4. Application of sperm cryopreservation to relevant animal models

Zebrafish natural populations have a wide range of genotype and phenotypes variations associated to their ecology and evolution (Whiteley et al., 2011). However, this species was domesticated and established in research centres worldwide. The domestication of the species introduced genetic variations modulating also parameters such as behaviour and reproduction (Holden and Brown, 2018; Whiteley et al., 2011; Wilson et al., 2014), especially in highly inbred lines (Balik-Meisner et al., 2018). Moreover, zebrafish lines produced with gene editing tools have additional genomic alterations that can have an impact in this species biological performance (D'Agati et al., 2017; Lawrence, 2016). The characterization of gametes quality and reproductive success is poorly investigated in most of zebrafish lines, and it is highly important in the establishment of reproductive programs, assisted reproduction and sperm cryopreservation methodologies. The last objective of this thesis was the application of sperm cryopreservation in relevant zebrafish lines.

The application of zebrafish sperm cryopreservation protocols is particularly relevant for valuable mutant and transgenic lines, due to their high value and in some cases difficulty in breed naturally. However, all the twelve zebrafish sperm cryopreservation studies rely uniquely on wild-type AB line (Diogo et al., 2018; Draper and Moens, 2009; Hagedorn et al., 2012; Harvey et al., 1982a; Matthews et al., 2018; Morris et al., 2003; Silva et al., 2019; Wang et al., 2015; Yang et al., 2007; 2016) and/or uncharacterized wild type zebrafish (Bai et al., 2013; Yang et al., 2016); and one is a general protocol description (Carmichael et al., 2009). In our work, sperm quality of mutant (e.g. *casper*) and transgenic lines (e.g Tg(*runx2:*eGFP) and Tg(*ins:*nfsb-mCherry)) were evaluated, and shown to present considerable differences in relation to wild-type lines. The *casper* mutant line is known to have reproductive constraints (Lawrence, 2016) and here we demonstrated that sperm motility and metabolism is impaired in comparison to AB wild type males. In opposition, the Tg(*runx2*:eGFP) males showed significantly higher sperm motility and plasma membrane viability, although they require more time to rest between samplings to recover sperm quality (Diogo et al., 2019).

The trangenic line Tg(*ins:*nfsb-mCherry) was used in chapter 6 to study the effect of type I diabetes in zebrafish reproductive performance. This model has a high biomedical relevance since could be used to compare the effects of this desease in humans. The hyperglycemia present in the organism during spermatogenesis under diabetic conditions can cause an abnormal glucose intake on the germ cells in the testes. High cellular glucose is known to promote oxidative stress events, which are detrimental to the cell and can lead to apoptosis and autophagy (La Sala et al., 2015; Ma et al., 2013; Zhang et al., 2009). Moreover, the high levels of glucose uptake in the cell can promote an inhibition of glycolysis pathway, leading to mitochondrial activity impairment and decreasing sperm motility (Agbaje et al., 2007). Our work revealed that similarly to human patients, zebrafish under transient diabetic conditions have detrimental effects on sperm quality, namely in sperm motility, plasma membrane viability and DNA integrity. The decrease in all these quality parameters in the zebrafish males under transient diabetic conditions when compared with the same males without treatment suggest that oxidative stress could be the primary damage affecting several functionalities in the cell. Although in the present study, ROS (reactive oxygen species) were not determine, and therefore could be other causes behind these effects, its has been shown in several species including zebrafish that high production of free radicals (e.g. H_2O_2, O^-) reduces spermatozoa functionality (Hagedorn et al., 2012; Sanocka and Kurpisz, 2004). The use of molecular tools allowed to observe that sperm from zebrafish under diabetic conditions have higher levels of transcripts related to this pathology such as *insulin a* (*insa*) and *glucose carrier 2* (*slc2a2*). The same findings were observed by previous studies in mouse models (Alfar et al., 2017; Im et al., 2005; Michel et al., 2016; Schoeller et al., 2012). This means that during gametogenesis this transgenic line under diabetic conditions suffers an overexpression of these transcripts, which will pass to spermatozoa as remanants. Other effects has been shown during spermatogenesis of diabetes patients such as lower testicular volume, azoospermia, altered epidimary volume and altered thickness of seminal vesicles (La Vignera et al., 2009). The diabetic conditions during spermatogenesis are also known to imprint the paternal genome and epigenetic marks that will be inherited by the offspring (Ding et al., 2015).

With our work we validate zebrafish as a useful model for the investigation of type I diabetes in male germline. Considering the pathology onset in early age and progressing with time, sperm cryopreservation is a useful tool to safeguard the possibility of *in vitro* fertilization. Upon sperm cryopreservation, Tg(*ins:*nfsb-mCherry) under diabetic conditions showed lower sperm freezability which can be a consequence of their lower initial sperm quality. Therefore, the selection of sperm samples from type I diabetes patients with the highest quality possible (early age) is essential to improve the post-thaw sperm quality.

Sperm cryopreservation protocols are particularly relevant for vulnerable zebrafish lines, particularly when they have reproductive constraints. Therefore, Tg(*ins:*nfsb-mCherry) zebrafish model for type I diabetes is a valuable model for the investigation of male reproductive dysfunctions associated to this pathology.

Main conclusions and perspectives

This thesis allowed the identification of specific constraints in zebrafish assisted reproduction. The methodologies established during this thesis are particularly relevant for small and intermediate zebrafish facilities since we established practical and cost-effective procedures. Overall, the present doctoral thesis allowed the establishment of relevant guidelines for zebrafish sperm cryopreservation and *in vitro* fertilization.

One of the major objectives of the present thesis was to detect specific constraints of zebrafish male donors that affect reproductive performance, which we summarize as: 1) very low sperm volume, 2) high uncertainty of sperm volume, biasing values of sperm concentration yielded by each male, 3) high male to male variability, 4) low number of males with high quality, which is variable between populations and facilities, 5) high variability between sperm quality of model zebrafish strains, 6) high variability on sperm motility activation for its analysis and *in vitro* fertilization purposes, and 7) highly variable fertilization media composition, osmolarity and volume used during *in vitro* fertilization.

There are several major sources of variability among sperm cryopreservation methodologies such as extender composition, freezing and thawing rates. However through this study it was possible to detect the following constraints were detected: 1) reduced quantitative selection of sperm samples prior cryopreservation, 2) biases produced by sperm activation with distilled water with uncontrolled environmental temperature, 3) use of new cryopreservation methodologies and solutions without previous investigation on post-thaw quality by themselves in comparison with an adequate control, 4) use of inadequate vials for small/medium zebrafish research centres, since a single French straw requires a high number of males with high quality sperm that will be used only for one fertilization, as opposed to cryovials.

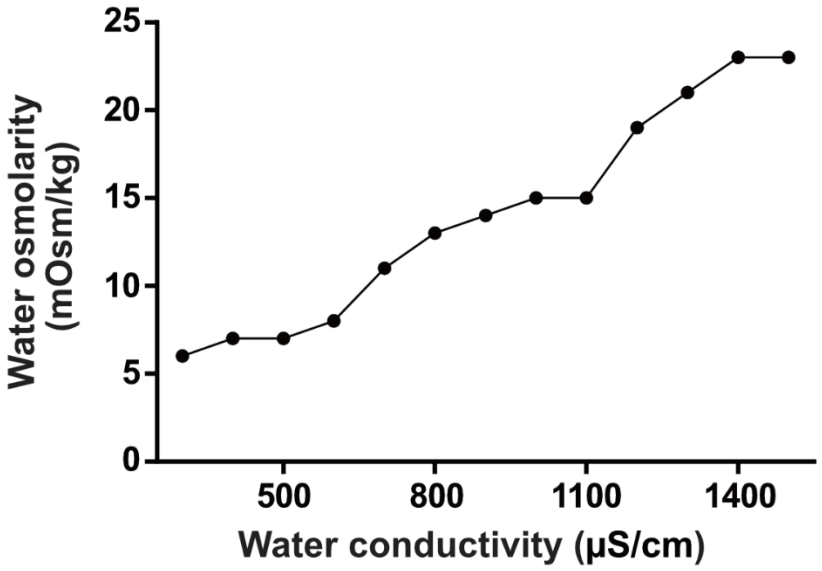
With the gathered knowledge obtained by the present thesis we provide robust guidelines for sperm quality analysis as well as male donor management and selection guidelines, as follows: 1) sperm motility analysis and activation for *in vitro* fertilization should be performed with solutions at 28°C, 2) the sperm motility

should be activated with system water with low water conductivity (we propose 700 µS/cm), 3) the diet should be optimized for the species and standardized among facilities, 4) males from 6 to 8 months old should be selected for sperm collection, 5) males require at least 14 days of rest between non-invasive sperm collections, 6) sperm quality of each zebrafish line should be studied prior to cryopreservation protocols, 7) ultrafreezer is a viable alternative to liquid nitrogen storage, 8) a fast freezing rate of -66°C/min is beneficial for zebrafish post-thaw sperm quality, 9) embryo survival at 24 hpf is the most accurate measure to predict embryo viability.

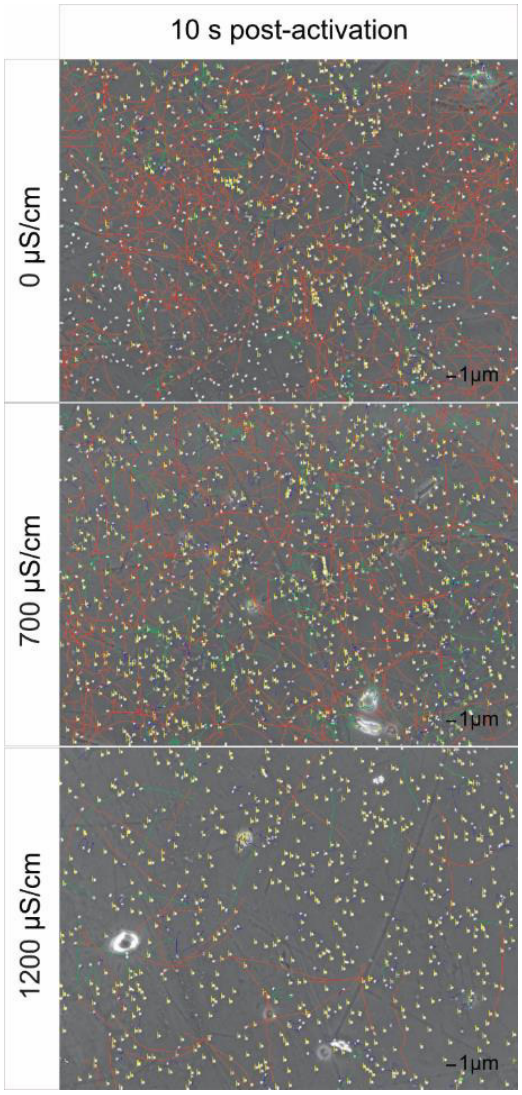
In future studies special attention should be payed to this species hierarchical structure and dominance-subordinate relationships to understand the impact of male selection criteria for cryopreservation on the offspring. Additionally, the *in vitro* fertilization methodologies would require refinements regarding gametes collection and manipulation, particularly oocyte handling.

CHAPTER 8. APPENDIX

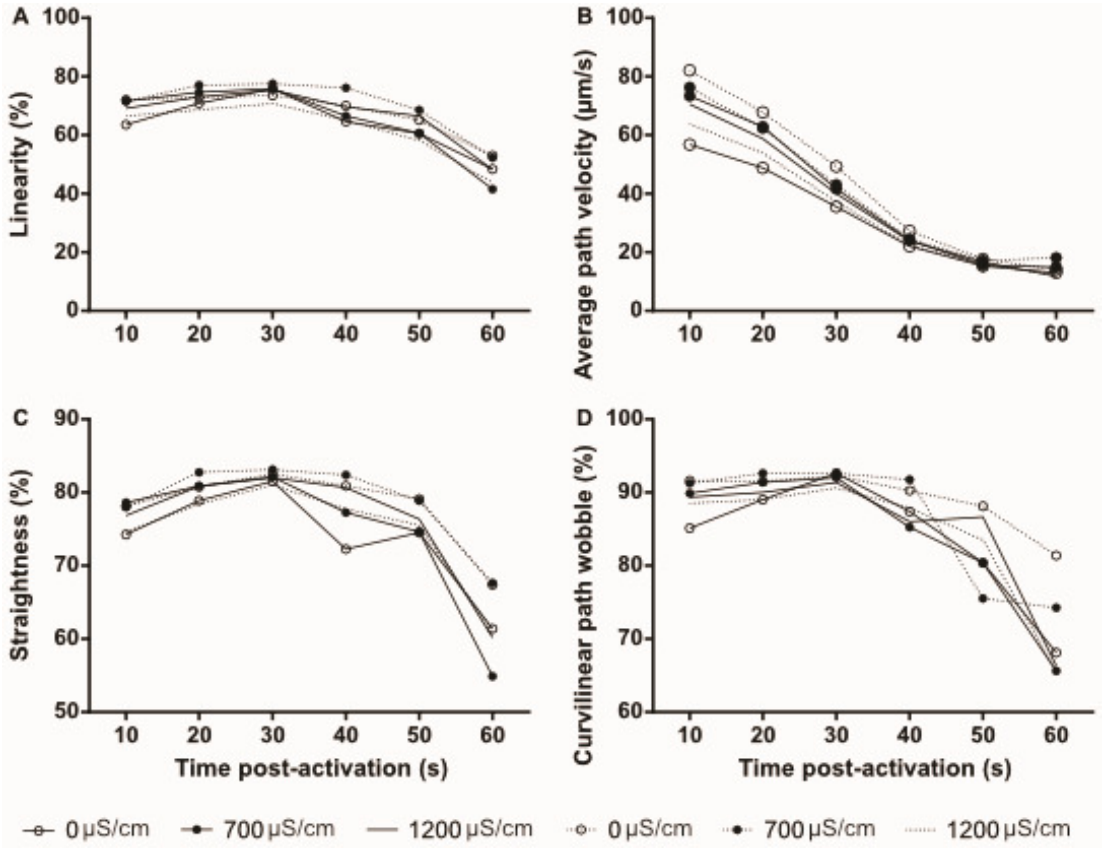
SUPLEMENTARY DATA CHAPTER 2



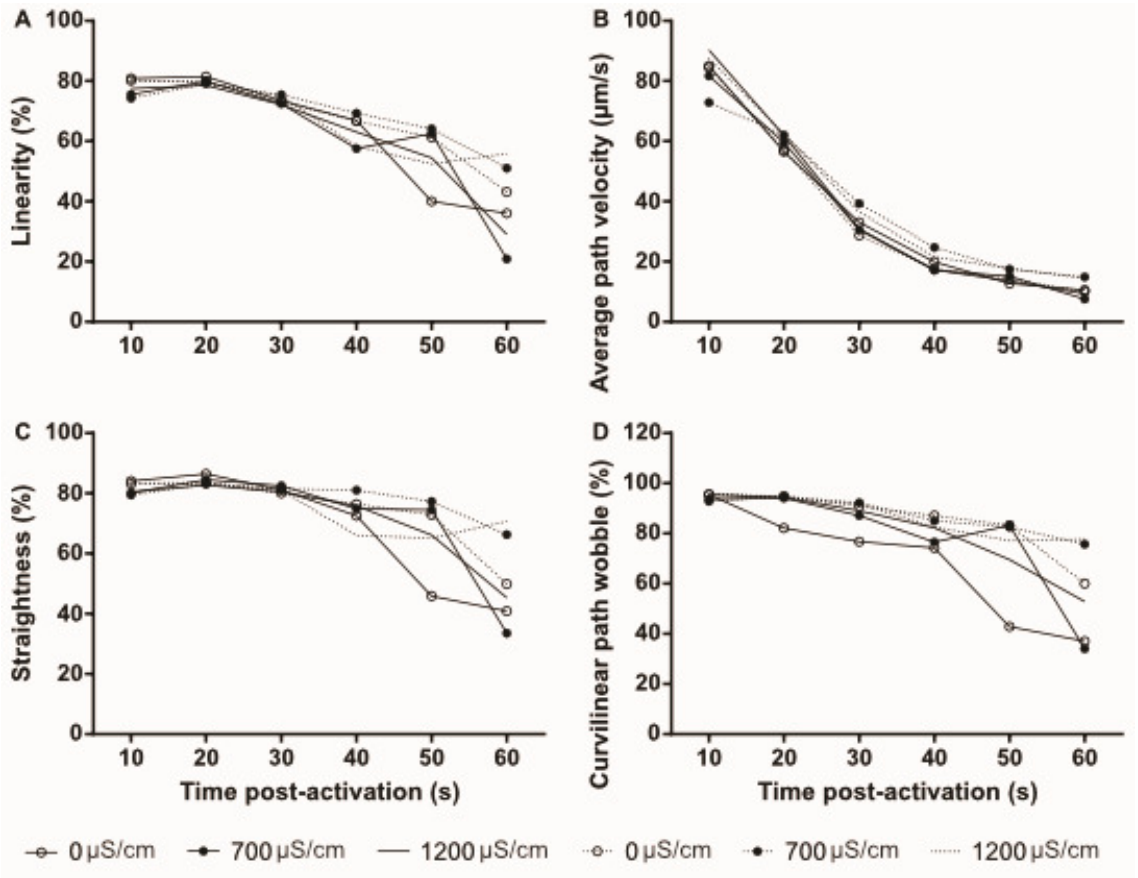
Supplementary Figure 2.1- Relation between system water conductivity and osmolarity. The water conductivities plotted are the most used in zebrafish rearing.



Supplementary Figure-2.2 Zebrafish AB line sperm motility activation (with the same pool) using CASA system with different water conductivities: A) 0 µS/cm, B) 700 µS/cm and C) 1200 µS/cm. The lines with different colors represent different spermatozoa velocities. Red lines represent fast spermatozoa (10-45 µm/s), green lines represent medium velocities (45-100 µm/s) and blue lines represent slow spermatozoa (<150 µm/s). Yellow crosses represent immotile spermatozoa (>10 µm/s).



Supplementary Figure 2.3 Effect of water temperature (20°C and 28°C) and water conductivity (0, 700 and 1200 µS/cm) on sperm motility parameters of zebrafish AB line (n=8). Sperm was activated, and motility parameters were recorded each 10 s for 1 minute in terms of: A) linearity (%), B) average path velocity (µms/s), C) straightness (%) and D) curvilinear path wobble (%). The values plotted represent means, continuous line represent 20°C and dashed line represent 28°C. The values plotted represent mean, continuous line represent 20°C and dashed line represent 28°C of the activation medium. Activation medium with 0 µS/cm is represented with white circle, 700 µS/cm with a dark circle and 1200 µS/cm without symbol.



Supplementary Figure 2.4 Effect of water temperature (20°C and 28°C) and water conductivity (0, 700 and 1200 µS/cm) on sperm motility parameters of zebrafish *casper* line (n=8). Sperm was activated, and motility parameters were recorded each 10 s for 1 minute in terms of: A) linearity (%), B) average path velocity (µm/s), C) straightness (%) and D) curvilinear path wobble (%). The values plotted represent means, continuous line represent 20°C and dashed line represent 28°C. The values plotted represent mean, continuous line represent 20°C and dashed line represent 28°C of the activation medium. Activation medium with 0 µS/cm is represented with white circle, 700 µS/cm with a dark circle and 1200 µS/cm without symbol.

SUPLEMENTARY DATA CHAPTER 4

Supplementary table 4.1 Sperm motility parameters from CASA system of zebrafish AB line at 10 s post-activation

Sperm motility	VCL (µm/s)	VSL (µm/s)	TM (%)	PM (%)	LIN (%)
Age (months)[a]					
6 months	84.4 ± 16.1	53.0 ± 24.5	22.6 ± 12.4	17.0 ± 9.4	59.6 ± 19.5
8 months	70.2 ± 8.7	56.6 ± 12.3	27.8 ± 25.9	12.0 ± 9.4	80.2 ± 11.9
12 months	71.3 ± 34.8	50.6 ± 28.7	21.3 ± 14.2	8.7 ± 10.6	62.2 ± 24.2
14 months	65.5 ± 14.5	39.8 ± 12.4	34.0 ± 18.6	7.3 ± 3.0	58.3 ± 8.3
Frequency (days)[b]					
0	84.4 ± 16.1	53.0 ± 24.5	22.6 ± 12.4	17.0 ± 9.4	59.6 ± 19.5
2	71.3 ± 15.0	42.4 ± 16.0	23.3 ± 16.0	8.7 ± 8.2	58.3 ± 14.2
7	85.9 ± 18.7	57.8 ± 20.0	31.3 ± 21.7	11.3 ± 9.2	65.8 ± 9.8
14	59.4 ± 32.3	40.4 ± 23.6	32.6 ± 17.5	8.2 ± 6.0	67.6 ± 35.2

[a] In baseline (0) sperm collection frequency

[b] Males with 6 months of age

Supplementary table 4.2 Sperm motility parameters from CASA system of zebrafish Tg(*runx2:eGFP*) line at 10 s post-activation.

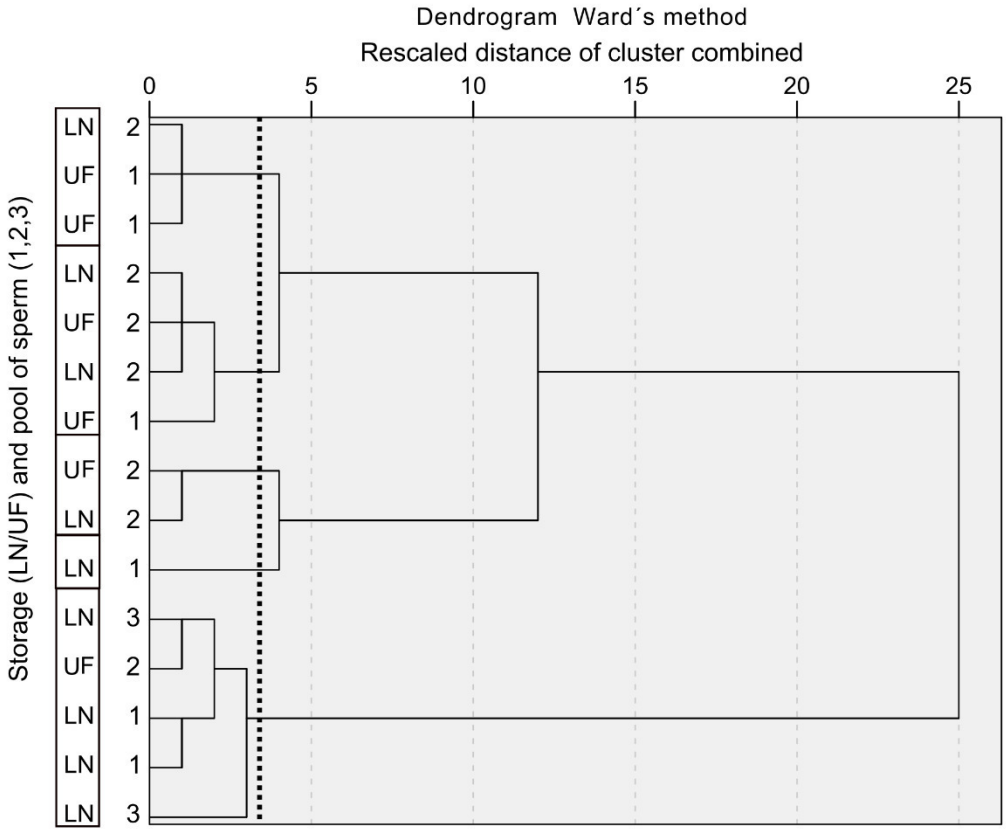
Sperm motility	VCL (µm/s)	VSL (µm/s)	TM (%)	PM (%)	LIN (%)
Age (months)[a]					
6	105.9 ± 22.3	73.9 ± 19.8	58.9 ± 2 1.6	25.0 ± 13.6	68.6 ± 4.8
8	96.6 ± 20.0	63.1 ± 16.7	53.7 ± 30.3	21.2 ± 16.5	64.2 ± 5.9
12	69.0 ± 16.0	51.8 ± 15.0	36.3 ± 25.2	18.5 ± 14.9	74.3 ± 8.1
14	54.3 ± 26.5	40.5 ± 20.3	23.8 ± 22.3	9.5 ± 11.1	65.0 ± 26.6
Frequency (days)[b]					
0	105.9 ± 22.3	73.9 ± 19.8	58.9 ± 21.6	25.0 ± 13.6	68.6 ± 4.8
2	78.4 ± 4.6	56.0 ± 9.0	32.8 ± 21.7	14.0 ± 10.4	71.0 ± 1.2
7	103.2 ± 31.3	71.0 ± 24.5	52.2 ± 27.8	22.8 ± 14.1	68.0 ± 4.4
14	86.2 ± 5.4	61.8 ± 7.5	50.2 ± 7.9	20.0 ± 5.0	71.2 ± 4.9

[a] In baseline (0) sperm collection frequency

[b] Males with 6 months of age

SUPLEMENTARY DATA CHAPTER 5.1

Supplementary Figure 5.1. Dendogram of cluster analysis through Ward´s method with an evenly distribution of liquid nitrogen (LN) and ultrafreezer (UF) storage methods.



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