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Special thanks to Bruno Rebocho for the motivation and constant vitality in all the work progress. For our little being who was about to meet us, but meanwhile decided to give us an early visit…never give up and always follow your dreams, whatever they may be, little Raquel.

Toutes les grandes personnes ont d'abord été des enfants.
(Mais peu d'entre elles s'en souviennent.)

Antoine de Saint-Exupéry *in* **LE PETIT PRINCE**

Resumo

À medida que novas linhas novas geneticamente modificadas vão sendo geradas, aumenta o desafio de manter este vasto número de peixes-zebra. Desta forma, a criopreservação de esperma tem sido considerada uma ótima opção para o armazenamento a longo prazo de material genético reduzindo assim custos inerentes à sua manutenção.

Os métodos tradicionais de criopreservação e vitrificação são os mais utilizados para criopreservar células. A vitrificação apresenta inúmeras vantagens sobre o método tradicional, entre as quais a não formação de cristais de gelo através de elevadas taxas de arrefecimento. Desta forma, a concentração de agentes crioprotetores utilizados pode ser menor, diminuindo o seu efeito tóxico nas células.

A falta de padronização e resultados coesos em estudos anteriores foram as principais razões para o desenvolvimento de um método simples e consistente na Plataforma de Peixes da Fundação Champalimaud. Desta forma, o objetivo principal desta tese foi o desenvolvimento de um protocolo fácil, económico e coerente, designado por congelamento ultra-rápido. Este método otimizado para a criopreservação de esperma de peixe-zebra terá um impacto muito importante na comunidade científica.
De 23 protocolos testados (n = 201), foram escolhidos para criopreservação de esperma de peixe-zebra os que apresentaram melhores resultados tendo em conta a percentagem de recuperação de linhas, a taxa média de fertilização, a taxa média de sobrevivência das larvas e a taxa média de malformações. Desta forma, foram selecionados dois protocolos com combinações de *extender* e crioprotector distintas.

Em relação aos métodos complementares utilizados na otimização do protocolo de criopreservação, a estimulação hormonal das fêmeas com 17α,20β-DHP traduziu-se claramente numa melhoria na qualidade e quantidade dos oócitos, sendo um passo muito importante na otimização deste protocolo; a quantificação da concentração de esperma é útil apenas com amostras translúcidas, no entanto indicou que a concentração de esperma não pode ser correlacionada com a taxa de fertilização; o protocolo otimizado de FIV (fertilização *in vitro*) é, no momento, um serviço prestado pela Plataforma de Peixes da Fundação Champalimaud.

Paralelamente à otimização da criopreservação de esperma, foi otimizado um protocolo de transgénese usando o sistema de transposão Tol2 com microinjeção *in vitro* de

oócitos seguido pela técnica de FIV otimizada anteriormente. Com este método, a percentagem de fundadores transgénicos de uma linha transgénica estável foi de 66.67%, muito superior à observada em microinjeção no estádio de zigoto. Foi ainda realizada uma análise da viabilidade do esperma em peixes alimentados com duas dietas comerciais (Skretting Gemma® e SparosZebrafeed®) tendo como conclusão que ambas as dietas são semelhantes.

Palavras-chave: criopreservação; fertilização *in vitro*; esperma; peixe-zebra.

Summary

As new genetically modified lines are being generated the challenge of maintaining this vast number of zebrafish increases. Therefore, cryopreservation of sperm has been considered a good option for the long-term storage of genetic material, thus reducing costs inherent to its maintenance.

The traditional cryopreservation method and vitrification are the most used methods to cryopreserve cells. Vitrification has many primary advantages and benefits over the other methods, such as no ice crystal formation through increased cooling rates. This way, the concentration of cryoprotectant agents used can be less, decreasing its toxic effect in the cells.

The lack of standardization and coherent expectable results in previous studies were the major causes for the development of a simple and consistent method at the Champalimaud Fish Platform. This way, as the main goal of this thesis, an easy, cheap and reliable protocol procedure was developed and optimized, designated by ultra-fast freezing. This optimized method for zebrafish sperm cryopreservation will have a very important impact in the zebrafish community.
The best protocols of 23 tested (n=201) were chosen for zebrafish sperm cryopreservation according to the percentage of line recovery, mean fertilization rate, mean larvae survival rate, and mean malformation rate. This way, two protocols with different combinations of extender and cryoprotectant were selected.

Regarding the complementary methods for the ultra-fast freezing method, female stimulation with 17α,20β-DHP was clearly an improvement in the oocyte quality and quantity and a very important step in the optimization of this protocol; quantification of sperm concentration is useful only with transparent samples but indicated that sperm concentration can´t be correlated with the fertilization rate; IVF (*in vitro* fertilization) optimized protocol is at the moment a state-of-the-art service in the Champalimaud Fish Facility.

In parallel with sperm cryopreservation optimization, a transgenic protocol was optimized using the Tol2 transposon system with oocyte *in vitro* microinjection followed by the optimized IVF technique previously optimized. With this method the percentage of germline transgenic founders was 66.67%, a higher percentage than the one observed in

one-cell stage microinjection. It was also performed a sperm viability analysis using fish fed with two dietary regimens currently commercialized (Skretting Gemma® and SparosZebrafeed®), concluding that both feedings are equal.

Keywords: cryopreservation; *in vitro* fertilization; sperm; zebrafish.

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List of abbreviations, acronyms and symbols

17α,20β-DHP - 17α,20β-Dihydroxy-4-pregnen-3-one
BSMIS - Buffered sperm motility inhibiting solution
CPA - Cryo Preservation Agent
DMA - N,N-dimethylacetamide
DMF - N,N-Dimethylformamide
dpf – Days post fertilization
E3 - Embryo medium
FIV - Fertilização *in vitro*
FTIR - Fourier transform infrared spectroscopy
GOI - Gene of interest
HBSS300 - Hanks' balanced salt solution at an osmolality of 300 mOsmol/kg
hpf – Hours post fertilization
IVF – *In vitro* fertilization
LN2 – Liquid nitrogen
Lp - Water permeability
N2- Nitrogen
PGCs - Primordial germ cells
RFP - Red fluorescent protein
RT – Room temperature
SG – Spermatogonia
SOP – Standard operations procedure
UAS - Upstream Activating Sequence
Vb - Osmotically inactive component

I. Introduction

Sperm cryopreservation is a technique involving many steps including sample collection, sperm extension, cryoprotectant selection, cooling, storage, thawing, and viability detection (Tiersch 2000). Its success can be assessed by *in vitro* fertilization and production of live offspring. Protocols for sperm cryopreservation can vary due to species-specific differences in sperm size, shape, and biochemical characteristics. In sperm cryopreservation, Cryo Preservation Agents (CPAs) are additives necessary for protection against freezing damage due to intracellular ice crystal formation and excessive dehydration (Yang et al. 2007).

Using genome manipulation techniques, biomedical research has been creating thousands of new mutant strains of mice and zebrafish, the two main vertebrate animal models. For mice, the number of strains is so big that it has become impossible in terms of cost and space to maintain more than a fraction as breeding colonies. Consequently, an increasing number and proportion of strains are being maintained by cryopreservation of their germplasm (Mazur et al. 2008). Zebrafish is by now the second most used animal model in biomedical research and the increasingly fast generation of new transgenic and mutant fish lines in recent years (Clark et al. 2011, Howe et al. 2013, Varshney et al. 2013, Ata et al. 2016) urges for simple and efficient cryopreservation programs. The development of more effective, reproducible, easier and cheaper methods of sperm cryopreservation not only guarantees safe preservation of the genotypes but also addresses the inevitable space limitation to maintain live strains in fish facilities thus limiting research.

With recent improvements in the methodology, cryopreservation of spermatozoa in zebrafish is quickly becoming the favoured method for archiving animal lines, leading to much less ‘‘front-end’’ work required for safely storage. One disadvantage of sperm cryopreservation is that only one haploid gamete is preserved. However, the gamete cryopreservation remains the most used technique for the archiving and shipping of valuable animal models (Du et al. 2010).

There are two main cryopreservation techniques used for sperm conservation, the slow equilibrium freezing cryopreservation (traditional method) and vitrification. The most commonly used cryopreservation method relies in sperm being slowly frozen and then stored in liquid nitrogen. However, as in humans and rodents, this technique has drawbacks, including loss of motility and vitality, and membrane damage. For zebrafish, published

protocols are ponderous, with multiple steps rendering them error-prone. More recent studies have demonstrated that the use of French straws can improve this method (Yang et al. 2009), however a programmable freezer is very expensive which can be a limitation to many zebrafish facilities. Vitrification on the other hand involves suspending cells in sufficiently high concentrations of mixtures of CPAs that, in combination with sufficiently high cooling and warming rates, prevent the cells and the surrounding medium from undergoing ice formation during cooling or warming (Mazur et al. 2008). Vitrification is a freezing method with several advantages over the traditional cryopreservation method, including a significant increase in sperm motility in humans and rodents (Kasai & Mukaida 2004). In the latest tests in zebrafish, vitrification consisted of freezing primordial germ cells, however this method had very low success rates and entailed quite laborious steps. Simultaneously, there were suggestions that cryopreservation of these cells can have negative consequences in gametogenesis due to hypermethylation (Riesco & Robles 2013).

II. Aims

Due to the inherent problems associated to the slow equilibrium freezing in cryopreservation (traditional method) and vitrification, it is thus becoming urgent to have a better, easier and functional method for zebrafish sperm cryopreservation. To achieve this goal it was decided to develop an ultra-fast freezing method. This is an intermediate freezing method that, to the best of our knowledge, had not been adopted yet in zebrafish. This new method for zebrafish sperm cryopreservation was developed based on previous murine and zebrafish protocols. Therefore, the main goal of this project was to develop and optimize a new sperm cryopreservation protocol that would be easily reproducible not requiring excessive training nor specific skills, not too laborious and as cheap as possible making this procedure accessible for all the zebrafish community. More importantly, we wanted a protocol as more independent as possible from individual sperm quality in order to have more balanced success rates.

To assess the success of the procedure, fertilization rate was used as a quality check. In order to have a reliable fertilization rate, the *in vitro* fertilization technique itself was optimized using several approaches.

III. Literature review

1. Gamete production

In contrast to other vertebrate groups, reproduction in fishes exhibits great diversity and many original features. Reproductive strategies are as diverse as the adaptations to numerous aquatic environments that are found in fishes. This diversity may concern sexuality, spawning, and parental behavior, sensitivity to environmental factors, and specific features of gametogenesis (Jalabert, 2005 in Bone & Moore 2008). Knowledge of fish reproduction and life history is important. The prospects of breeding fish requires an understanding of reproductive physiology, breeding behavior, and genetics (Purdom, 1993 in Bone & Moore 2008). Because fish have evolved to live in diverse environments, there are substantial differences in fish morphofunctional characteristics. Fish have had to develop adaptation mechanisms to survive in diverse environmental conditions, and as a result spermatozoa from fish species demonstrate significant differences in their reactions to cryopreservation protocols. For example, there is a striking difference in post-thaw survival of reproductive cells of marine and freshwater species. Sperm of marine species were successfully cryopreserved and reported (Blaxter 1953 in Stoss 1983 and in Agarwal 2011, Tsai & Lin 2012) soon after the discovery of the first cryoprotectant, whereas the cryopreservation of freshwater fish gametes was more challenging and took longer to achieve (Graybill & Horton 1969, Moczarski 1977, Stein & Bayrle 1978 in Stoss 1983, Tsai & Lin 2012).

The fish male yields several hundreds of billions of spermatozoa per year per kg of body weight, or more than $100x10^6$/g of testis per day, which is 10 times higher than production recorded in mammals. Sperm concentration, also very high, is between 10 and $40x10^9$ spermatozoa/ml of sperm in trout and pike, $7x10^9$ in coregones, $14x10^9$ in carp, and $10x10^9$ and $30x10^9$ in the perch. However, only a part of these spermatozoa can be collected in some species during the reproductive period, the rest remain in the testis where they are gradually reabsorbed. Two original features characterize the spermatozoa physiology of most of the studied species: immotility in the genital tract and extremely short lifespan after motility is triggered. Immotility can be due to the presence of a specific ion in the seminal fluid but other factors, such as elevation of osmotic pressure or sucrose may also inhibit motility (Bellard 1988 in Alavi & Cosson 2006). Female fecundity is generally high, depending on the species and the mode of reproduction. Fecundity or egg size in the same species may also depend on the time of reproduction, which, in turn, depends on seasonal differences in food availability for the parents (Bagenal 1971 in Demartini & Sikkel 2006). After ovulation,

the fertilization ability of the ova remaining in the female genital tract declines more or less rapidly (varying from hours to weeks), depending on the species (Bellard 1988 in Alavi & Cosson 2006).

2. Biophysics of zebrafish sperm

Cell cryopreservation cannot be improved without proper basic physiological knowledge. Successful cryopreservation of germplasm must consider intrinsic biophysical properties (e.g., water and cryoprotectant permeability, osmotic tolerance limits, intracellular ice nucleation) to maximize survival (Rall 1993 in Hagedorn et al. 2009). It is important to understand and avoid the mechanisms by which sperm is damaged or destroyed during cryopreservation.

Zebrafish sperm have small, round heads and a smaller midpiece (Figure III.1) that together are approximated as a prolate spheroid with an average major and minor axes of the combined head and mid-piece of 2.2± <0.1 µm (SEM) and 1.9 µm ± <0.1 m, respectively, an average tail length of 27.6 ± 0.5 µm with an average tail thickness of 0.4 ± <0.1 µm in diameter (Hagedorn et al. 2009).

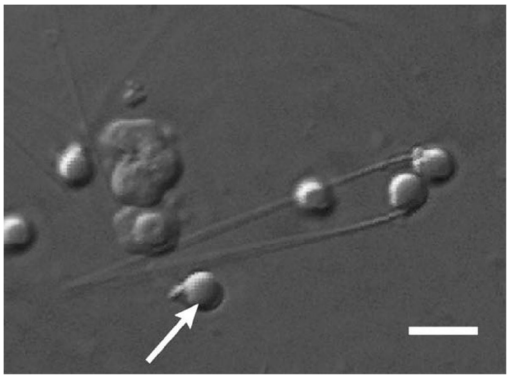

Figure III.1: Zebrafish sperm morphology. Zebrafish sperm display a prolate head and mid-piece (arrow), and a tail ~30 µm in length. Bar = 4 µm (*in* Hagedorn et al. 2009).

Sperm volume measured in the Coulter electronic particle counter is 12.1 μm^3. The optimal osmotic range for these cells is from approximately 200 to 600 mOsm/kg (Hagedorn et al. 2009).

Zebrafish sperm have a low osmotically-inactive component (Vb) (determines how much osmotically-active water is in the cell) compared to that of sperm from most mammals. Vb of zebrafish is 0.37 compared to 0.61 for mouse, for example. Water permeability (Lp) for zebrafish sperm is low, approximately 30-fold lower than in mammalian sperm, as might be

predicted for a cell that must function in a hypotonic environment (e.g., fresh water) when fertilizing an egg (Hagedorn et al. 2009).

3. Cryopreservation

The preservation of biological material in a stable state is a fundamental requirement in biological/medical science, agriculture, and biotechnology. It has enabled standardization of experimental work over time, has secured lifesaving banks of cells and tissue ready for transplantation and transfusion at the time of need, and has assured the survival of critical germ plasm in support of programs for the conservation of species. Cryopreservation is one of the widely accepted and preferred techniques for achieving long-term storage, and has been applied to an increasingly diverse range of biological materials. (Day & Stacey 2007). Advantage of cryopreserving the fish semen is well established. It is not only a useful management tool, it offers several benefits such as stock protection due to outbreak of diseases, natural disaster, or over exploitation. Other application of cryopreservation includes stable supply of sperm for optimal utilization in hatchery production, and easy stock transportation among hatcheries, with stocks being maintained more economically and effectively, and for laboratory experiments providing experimental material for advanced studies such as gene transfer (Agarwal 2011).

Although the basis for many methodologies is common, many laboratories lack expertise in applying correct preservation and storage procedures and many apply out-dated or inappropriate protocols for storing samples or cultures (Day & Stacey 2007).

Cryopreservation is the use of very low temperatures to preserve structurally intact living cells and tissues (Agarwal 2011, Pegg 2007). This technique allows virtually indefinite storage of biological material without deterioration over a time scale of at least several thousands of years (Agarwal 2011, Mazur 1985 in Hiemstra et al. 2005), but probably much longer. Important progress in cryobiology was achieved in the second half of the previous century (Hiemstra et al. 2005).

In cryopreservation, cells are suspended in a suitable solution, cooled, stored in liquid nitrogen, warmed to room temperature, and returned to a physiological solution. During each step of this process, cells are at risk for various types of damage. The primary injury is that caused by the formation of intracellular ice during cooling and warming (Kasai & Mukaida 2004). The biological effects of cooling are dominated by the freezing of water, which results in the concentration of the solutes that are dissolved in the remaining liquid phase. Rival theories of freezing injury have envisaged either that ice crystals pierce or tease apart the cells, destroying them by direct mechanical action, or that damage is from secondary effects via changes in the composition of the liquid phase. Cryoprotectants, simply

by increasing the total concentration of all solutes in the system, reduce the amount of ice formed at any given temperature, but to be biologically acceptable they must be able to penetrate into the cells and have low toxicity. Both damaging mechanisms are important, their relative contributions depending on the cell type, cooling rate, and warming rate (Pegg 2007).

Whether freezing is permitted (conventional cryopreservation) or prevented (vitrification), the cryoprotectant has to gain access to all parts of the system. However, there are numerous barriers to the free diffusion of solutes (e.g., membranes), and these can result in transient, and sometimes equilibrium, changes in compartment volumes, which can be damaging. Hence, the processes of diffusion and osmosis have important effects during the introduction of cryoprotectants, the removal of cryoprotectants, the freezing process, and during thawing (Pegg 2007).

It was not until 1948 that a general method was discovered allowing for the freezing of many types of animal cells with subsequent restoration of structure and function (Agarwal 2011). In 1949, Polge et al. published their landmark paper in which they showed that the inclusion of 10–20% of glycerol enabled the spermatozoa of the cock to survive prolonged freezing at –80°C. Regarding fish, several different approaches were initially tested including storage of fish sperm in medium saturated with different gases (Holtz et al 1976), preservation of sperm at temperatures above zero (Ginzburg 1968), as well as in the frozen state (Blaxter 1953) and drying (Zell 1978). However, to date, low-temperature preservation has proven to be the most effective approach, with the first successful cryopreservation of fish sperm being reported by Blaxter in 1953 (Agarwal 2011).

3.1. Embryo, oocyte and primordial germ cell cryopreservation

Teleost primordial germ cells (PGCs), as the embryonic precursors of gametes, have tremendous importance in the fields of developmental biology and aquaculture. They are an optimal cell type to be cryopreserved because they conserve both paternal and maternal genomes. Moreover, recent studies have demonstrated the competence and suitability of these cells for surrogate production. The implementation of these technologies provides precise control over many relevant reproductive aspects, for example, PGCs or spermatogonias (SGs) xenotransplantation that could offer a solution for the management of species with reproductive failures, or for those species with long maturation periods (Robles et al. 2017).

In zebrafish, PGCs have been cultured and marked by using a transgenic line that expresses red fluorescent protein (RFP) under the PGC-specific vasa promoter (optimizing

the culture conditions by counting the number of fluorescent cells) (Fan et al. 2008). The possibility of generating PGCs *in vitro* would represent a powerful tool in biotechnological research and aquaculture. The number of PGCs is limited in embryos and cell proliferation is difficult to achieve once they are cultured *in vitro*. Generation of these cells *in vitro* would provide the means to increase the number of cells per embryo which could be important for germplasm banking purposes, it would be a source of cells for surrogate production and it would increase the possibility to genetically manipulate embryonic cells (easier to transfer than PGCs) in teleosts (Robles et al. 2017).

Both embryo and oocyte cryopreservation have not been successful in fish yet. Most of the cryopreservation protocols have been developed for sperm, which disregards the female genome (Robles et al. 2017). Teleost oocytes and embryos have intrinsic biophysical properties that make their cryopreservation difficult. To minimize cryodamage and maximize survival rates, water exchange and cryoprotectant influx have to be studied and tested, taking into account that both these factors are influenced by membrane permeability, osmotic tolerance limits, surface-volume ratio, and yolk amount. (Hagedorn et al 1997). All previous attempts to cryopreserve fish embryos have been unsuccessful so far. The analysis of the permeability parameters of the zebrafish embryo predicted that a major site for lethal cryodamage would occur within the yolk compartment (Robles et al. 2017). Presumably, without sufficient cryoprotectant entering the yolk, damaging ice-crystals will form (Hagedorn et al 1997 in Robles et al. 2017). Therefore, protocols for fish embryo vitrification with removal of some yolk have been studied (Higaki et al. 2013). Regarding oocytes, after cryopreservation, these cells require post-thaw *in vitro* maturation and fertilization, thus a functional protocol for germ cell survival would not necessarily guarantee successful production of zygotes. Therefore, cryopreservation of PGCs and spermotogonia represent an important tool in gene banking until fish embryo cryopreservation is successfully achieved (Robles et al. 2017).

Besides the cryoprotectant exposure time (Higaki et al. 2009), combination of external and internal CPAs at lower doses (Robles et al. 2007), microencapsulation with dissociated cells (Kasai & Mukaida 2004), or incorporation of antifreeze protein effects, other strategies, such as yolk removal (Higaki et al. 2013) have been used to examine the effects of this partial removal and cryoprotectant mixtures on the viability and the differentiation ability of cryopreserved zebrafish PGCs. All of these studies have provided important advancements for PGC cryobanking and have established a basis for future improvements.

3.2. Sperm cryopreservation

The low-temperature preservation method has been applied widely and has become not only a routine tool in aquaculture for fish hybridization and selective breeding, but also an important tool in programs of biodiversity and preservation of endangered species. Gamete banks of rare or almost extinct species were created (Harvey et al. 1998) with the objective of protecting endangered species. The technique has also found applications in research programs for maintaining laboratory animals and sperm of more than 200 species of fish have been successfully cryopreserved (Agarwal 2011, Blesbois & Labbé 2003 in Hiemstra et al. 2005, Rana & Gilmour 1996). However, despite the extensive number of studies that have been undertaken there is still ambiguity (great variability and poor reproducibility) in the data reported in the literature, primarily because of lack/poor standardization of methodology and data analysis. Zebrafish sperm cryopreservation protocols are far from optimized and further improvement is necessary.

Because oocyte or embryo cryopreservation has not yet been successful in zebrafish (Guan et al. 2010, Lin et al. 2009, Robles et al. 2017), sperm freezing is currently the best option for genetic resource banking. Although there are many protocols available for low-temperature storage of sperm of freshwater fish (Agarwal 2011, Kopeika & Novikov 1983, Tiersch & Mazik 2003) there is much work still to be done to improve this technology.
Most of the events associated with freezing are a result of the osmotic properties of cells. The cellular damage during the freezing process is all due to the osmotic shock, intracellular ice formation, increased intracellular concentration of solutes and solution effects (Agarwal 2011). In general, approximately 40–90% of spermatozoa from freshwater species are usually damaged after cryopreservation, whereas only 10–20% of spermatozoa are damaged in marine species (Tiersch & Mazik 2003). Post-thaw survival of fish sperm is strongly predetermined by their sensitivity to osmotic changes in extracellular media (Tiersch & Mazik 2003), leading to a generally low (0–30%) average post-thaw motility (Morris et al. 2003, Yang et al. 2007). Decrystallization is pointed out as the main cause for cryodamage, and rewarming is the critical step for post-thaw survival (Mohammad et al. 1997, Medrano et al. 2002).

To develop reliable protocols of cryopreservation for fish spermatozoa, individual fish and species-specific properties must be taken into consideration (Agarwal 2011). A cryopreservation protocol needs to have several optimized steps such as gamete collection, stimulation of maturation (used in specific cases), gamete storage and equilibration, freezing, storage in liquid nitrogen, thawing, and fertilization. Due to the multiple steps and their

interactions, errors at each step can accumulate and lead to considerable losses of viable cells. Thus, careful attention should be given to the numerous details at each step, and care should be taken to reduce or eliminate sources of uncontrolled variation (Leibo, 2000).

3.2.1. Cryopreservation solutions

The solutions used for sperm cryopreservation include:

(1) Extender to storage the gametes and retain the functional capability and fertilizing ability of sperm by controlling pH, osmolality, ion concentration, and in some cases, the supply of energy (Stoss & Holtz 1981 in Yang & Tiersch 2009). The choice of appropriate extender depends on the species. The osmolality of the extender solution is one of the most important factors in preparation of an appropriate extender (Kopeika et al. 2007). Specifically, for zebrafish the most common extenders are Ginsburg Ringer's fish solution with skim milk powder, Hanks' balanced salt solution (HBSS300) and buffered sperm motility inhibiting solution (BSMIS) and all of them generally function well to retain fertility of post-thaw sperm (Harvey et al. 1982, Morris et al. 2003, Draper et al. 2004, Yang et al. 2007).

(2) Cryoprotectant solution. The absence of an ideal cryoprotectant, makes selection of a common single cryoprotectant difficult for different species. However, the optimal cryoprotectant can be determined empirically. The addition of cryoprotectants interacts with the membranes to make them more flexible and thus reduces damage due to solution effects. Thus, the basic principle of cryopreservation is to cause cell dehydration and eventually concentrate the cytosol with minimum injury so that ice crystallization in the cytosol is minimized during cooling in liquid nitrogen (Agarwal 2011). The concentration of cryoprotectant usually varies in the range between 5 and 12% (v/v) (Kopeika et al. 2007). Better cell protection can be achieved by employing higher concentrations, but this has to be balanced with toxicity effects of the cryoprotectant (Yang & Tiersch 2009). The addition of non-penetrating agents, such as sucrose, is generally considered to be beneficial. However, direct mixing of fish sperm with cryoprotectants inevitably leads to the death of all cells (Scott & Baynes 1980 in Gwo et al. 2009). The level of dilution of the cryoprotectant medium is equally important and it is species sensitive (Agarwal 2011, Lahnsteiner 2000). The most commonly used cryoprotectants for fish sperm cryopreservation are permeating ones, such as dimethyl sulfoxide (DMSO), ethylene glycol, methanol, ethanol, glycerol, and N,N-dimethylacetamide (DMA) and non-permeating ones, such as egg yolk, milk, and proteins (Kopeika et al. 2007, Yang & Tiersch 2009). For zebrafish, the toxicity of DMSO, N,N-dimethyl acetamide, methanol, and glycerol at concentrations of 5, 10, and 15% have been evaluated with sperm cells. Glycerol was the most toxic, and was eliminated for sperm cryopreservation. The other three chemicals have been used for sperm cryopreservation,

and analysis of post-thaw motility have shown that methanol at a concentration of 8% was the best choice (Yang et al. 2007). This was also the choice in two earlier studies (Harvey et al. 1982, Draper et al. 2004). In addition, DMA (10%) was used as a cryoprotectant for zebrafish sperm (Morris et al 2003), but the fertilization level after thawing (9–14%) was lower than that observed (28–51%) when methanol was used (Harvey et al. 1982, Draper et al. 2004, Yang et al. 2007). Cryoprotectant permeabilities are in the range expected for most sperm (~10-4 cm/min). Sperm suffers changes in cell volume caused by dimethylsulfoxide, however 10% methanol and 10% N,N-dimethylformamide do not cause any changes in cell volume as they enter and exit the cell (Hagedorn et al. 2009).

Hagedorn et al. 2009 analysed sperm membranes with Fourier transform infrared spectroscopy (FTIR), which is an established tool for biophysical characterization of cell membranes (Crowe et al. 1989) extremely sensitive to changes in lipid conformational order, and allows for measurement of membrane fluidity and lipid organization in intact cell membranes. The FTIR data suggest that freezing zebrafish sperm without cryoprotectant causes membrane damage and large-scale lipid reorganization.

Cold shock damage has been directly linked to lipid phase transitions that cause the sperm membrane to become transiently leaky, thereby compromising membrane integrity (Agca et al. 2005, Arav et al. 2000, Drobnis et al. 1993 in Hagedorn et al. 2009). Ice formation and changes in osmotic pressure are the major causes of spermatozoa damage during cryopreservation, and the ability of sperm plasma membrane to resist structural damage during cryopreservation may be related to the type of fatty acids in the spermatozoa plasma membrane and the strength of the bonds between membrane components (Agarwal 2011) causing irreversible phase separation (clustering) and rearrangement of membrane components in sperm (DeLeew et al 1990, Hotl & North 1984 in Hagedorn et al. 2009). During chilling, the key is to minimize the number and cooperativity, or sharpness, of lipid phase transitions, thus keeping the membrane fluid and structurally intact (Hagedorn et al. 2009).

3.2.2. Freezing

The freezing step can be achieved using different methods:

(1) Freezing in vapour-phase liquid nitrogen, which implies placing vials or straws above the liquid nitrogen horizontally on a rack at a predetermined position. The position of the sample and the time of exposure at that position depend on the sample volume, type of container, and temperature at that position;

(2) Freezing in alcohol baths. Similar results can be obtained by freezing sperm in cold baths that are capable of maintaining a set temperature;

(3) Freezing in dry-ice using Falcon type tubes as a support placed deep in dry ice;
(4) Controlled-rate cooling using programmable freezers.

Freezing in liquid nitrogen vapour or in dry ice are more practical methods compared to a controlled-rate freezer and are also the closest easily achieved approximation to an exponential cooling regime (Agarwal 2011, Harvey et al. 1982, Kopeika et al. 2007).

Cooling rate is a crucial factor in sperm cryopreservation because it affects the osmotic and pH balance of intracellular and extracellular solutions during freezing. Theoretically, with an excessively slow cooling rate, osmotic equilibrium is maintained, and much of the freezable water leaves the cell resulting in excessive dehydration; with an excessively fast cooling rate, little or no freezable water leaves the cell, and thus large intracellular crystals can form, causing damage to the cell. Ideally, a balanced situation allows survival when the cooling rate is fast enough to minimize the time of exposure to concentrated solutions and yet is slow enough to minimize the amount of intracellular ice formation. Optimum cooling rates vary with different cryoprotectants and the physiology of sperm cells from different species (Yang & Tiersch 2009).

The packaging of samples for freezing and storage is also important to standardize the cooling rate, and to assure proper sample identification. Currently, several different kinds of containers have been used, such as plastic cryovials, glass tubes and ampules, and plastic straws. The different materials and shapes of these containers result in different heat transfer properties during freezing and thawing. Even for the same style of container, differences can exist with products from different manufacturers, which can result in variation of cooling or thawing rates (Yang & Tiersch 2009).

Currently, reported sperm cryopreservation protocols on zebrafish include:

(1) Freezing in glass capillary tubes on dry ice using methanol and powdered skim milk as cryoprotectants (Harvey et al. 1982) and various adaptations of this method (Westerfield 1995, Ransom & Zon 1999, Brand et al. 2002, Draper et al. 2004) (Agarwal 2011). Morris et al. 2003 were unable to reproduce the results reported by Harvey et al. 1982 and its updated protocols;
(2) Freezing in 1.5 mL cryotubes on dry ice using N,N-dimethylacetamide as cryoprotectant (Morris et al. 2003, Berghmans et al. 2004) or methanol (Draper & Moens 2009);
(3) Freezing in 0.25 mL French straws with a programmable freezer using methanol as cryoprotectant (Yang et al. 2007). Bai et al. 2013 were unable to repeat the success with

methanol when samples were frozen in 0.25 mL French straws with a programmable freezer. DMA was found to be worse than methanol in the straw freezing method (Yang et al. 2007).
(4) Cryomicroscopy which allows real time observation of the entire freezing and thawing process, tracking throughout all temperature regions events such as cell motility, membrane integrity, and ice formation status. Cryomicroscopy yields a two-step freezing protocol that employs a faster cooling rate of 25°C/min initially from 4 to 30°C, and then a slower cooling rate of 5°C/min from 30 to 80°C before plunging into liquid nitrogen for permanent storage. For freezing, the equipment is a controlled-rate freezer and sperm is suspended in 8% DMSO in 0.25 ml French straws (Bai et al. 2013). Bai et al. 2013 tested the efficiency of this method through sperm motility observation.

3.2.3. Thawing and activation

By the time sperm is thawed and ready to be used for fertilization, it has gone through a series of stresses. Therefore, special care has to be taken during handling of sperm after thawing and pure water should not be used as an activator for cryopreserved-thawed sperm during fertilization (it affects functional activity of weak sperm cells post-thaw). Improved activation will be attained in activation media that have higher osmolality than pure water. However, the increase in osmolality in the activating medium has to be within the range that is safe for the eggs (Kopeika et al. 2007). In zebrafish, once activated by hypotonic osmolality, sperm have a short burst of motility (30 s to 5 min) (Yang et al. 2007).

Theoretically, the process of thawing is the reverse of freezing, and thus the damage that can occur during cooling can also occur during warming, primarily through formation of ice crystallization between −40 and 0°C (Leung 1991, Til et al. 2016). Therefore, it is usually desirable to rapidly thaw cryopreserved samples to minimize the period of crystal propagation (termed “recrystallization”) (Yang & Tiersch 2009). Studies on optimization of the thawing regime have demonstrated that the best thawing regime for 1–2 mL vials is using a water bath between 33 and 40°C (Kopeika et al. 2007, Draper & Moens 2009).

3.2.4. *In vitro* fertilization

In vitro fertilization (the collection of spermatozoa and ova and their mixing together in various media that keep spermatozoa motile) is commonly carried out in several freshwater species, such as salmonids, cyprinids and acipenserids. The eggs of most teleosts are fertilized externally, which means that after passing through the micropyle, the spermatozoon penetrates the cytoplasm. Traditionally, fresh water (or sea water for marine species) is used as the medium in which the male and female gametes are mixed. However,

fresh water is not a very favourable medium because hypotonic shock causes the sperm structure to deteriorate in several minutes and the egg is activated quickly. These problems can be avoided by using as media various saline solutions of different composition, depending on the species. These media prevent sperm deterioration, prolong slightly the duration of motility or limit it, and prevent or defer the cortical reaction. The length of gamete survival is an important factor to consider in carrying out artificial reproduction (Agarwal 2011, Bellard 1988 in Alavi & Cosson 2006).

For zebrafish, artificial fertilization protocols have been established with fresh sperm, and can be directly modified to provide fertilization analysis of cryopreserved sperm (Westerfield 1995 in Yang & Tiersch 2009). Eggs can be collected by squeezing of females, held in isotonic buffer to retain fertility, and then be mixed with a sperm suspension for fertilization. After mixing of sperm and eggs, fresh water needs to be added to activate gametes for fertilization. Fertilization and hatching are determined by assessing the percentage of developing embryos or hatched fry (Yang & Tiersch 2009).

IV. Methods

1. Zebrafish and husbandry procedures

Several strains of zebrafish that are widely used in biomedical research: wild-type AB and TU, the Nacre (*mitfa*-/-) mutant and several transgenic lines were used. Housing and husbandry of all animals were performed according to Martins et al. 2016. Fish were housed in 3.5L tanks at a maximum density of 10 fish per liter or housed in 3.5L tanks with a divider dividing males and females at a maximum density of 4 fish per liter (depending on the purpose). Fish did not stay divided for more than two consecutive weeks.

The feeding regimen implemented consisted of feeding the fish 3 times per day (one time with live *Artemia nauplius* and two times with powder Skretting® Gemma Micro 500).

2. Techniques directly involved in sperm cryopreservation

2.1. *In vitro* fertilization

Spawning trials are necessary to test the fertility of cryopreserved sperm. This process includes a series of steps: egg collection, holding of eggs prior to fertilization, thawing of cryopreserved sperm, mixing of the sperm and eggs, gamete activation, fertilization confirmation, hatching of fertilized eggs, and offspring harvest (Yang & Tiersch 2009).

Large numbers of synchronously developing embryos can be obtained by i*n vitro* fertilization (IVF). IVF can be performed when experiments depend on synchronized embryos, when natural mating doesn´t occur or for line recovery of a cryopreserved sperm sample. Fertilization with cryopreserved sperm requires slightly more time and equipment that fertilizion using fresh sperm.

Gametes were collected from breeding adults by gentle pressure and stored in commercial solutions (Aquaboost® Ovacoat for oocytes and Aquaboost® Spermcoat for sperm) in order to retain fertility (figure IV.1). These commercial solutions can maintain sperm inactive for 24 hours on ice and oocytes for 30 minutes at room temperature (CUG 11/13 2015), allowing for the collection of several or pooled samples and the time optimization of the procedure.

Briefly, after collection, the gametes were mixed together in a petri dish, fish water was added to the egg-sperm mixture and fertilization took place very rapidly in 20 to 60 seconds (figure IV.1). After 1 minute, the sperm is no longer active. Embryos were then placed in the

incubator at 28°C with a photoperiod of 14h:10h/light:dark. Between 15 and 24hpf the success of fertilization was checked by observing on the stereoscope the development of the embryos (figures IV.2 and IV.3, appendix A).

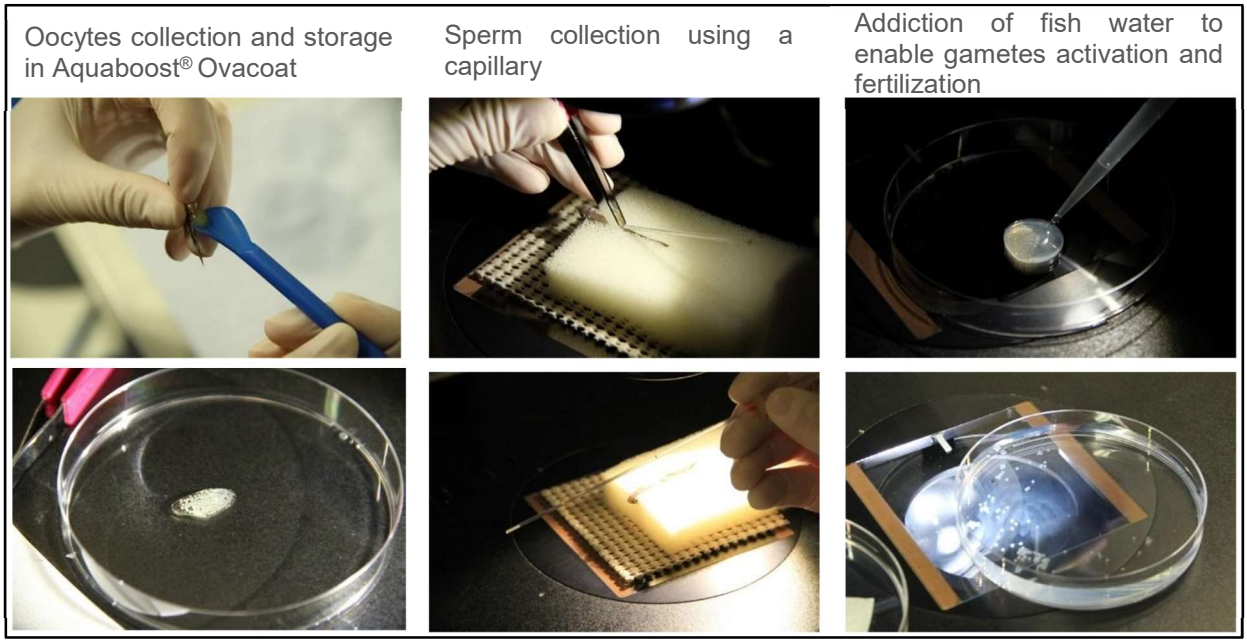

Figure IV.1: Zebrafish *in vitro* fertilization main steps. Procedure currently in use at the Champalimaud Fish Facility.

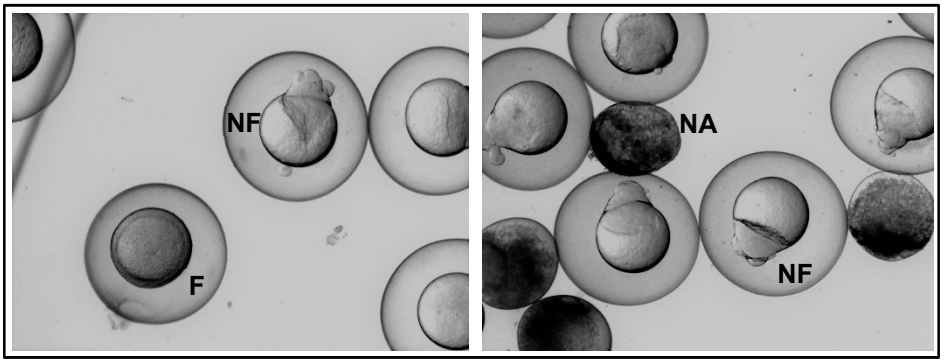

Figure IV.2: Zebrafish embryo development at 6hpf, 80x magnification. F – fertilized embryo; NF – egg not fertilized; NA – egg not activated.

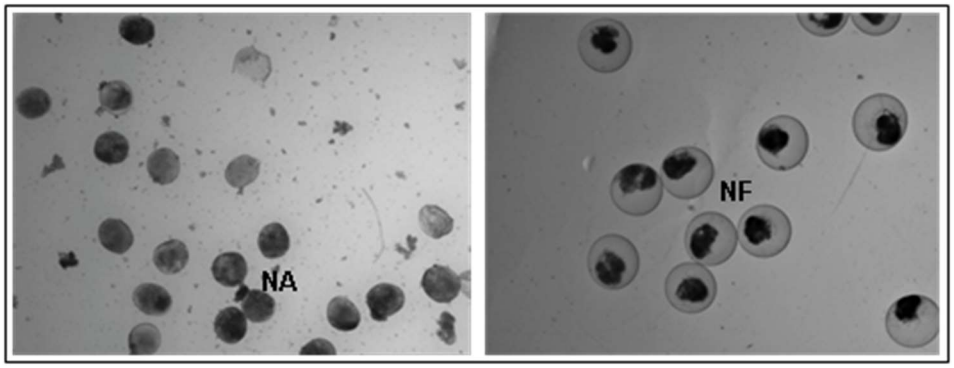

Figure IV.3: Zebrafish embryo development at 24hpf, 40x magnification. NF – eggs not fertilized; NA – eggs not activated.

The Champalimaud IVF protocol was optimized based on several established protocols (ZFIN, ZIRC, UCL and Cryogenetics). The standard operations procedure (SOP) is described in appendix B.

For the IVF tests, good breeders were selected. Four month-old females and males were incrossed at least three times in a weekly basis and housed in pairs in 1.1L tanks. The couples that produced, during the three mating episodes, at least 50 to 200 fertilized embryos were considered good breeders and transferred to a 3.5L tank with a divider dividing males and females at a maximum density of 4 fish per liter.

Males were squeezed every two weeks, females were squeezed every two/three weeks. Fish were used in a rotating system as being separated for too long reduces their productivity and can trigger inflammation caused by egg accumulation.

IVF was performed in the first three hours after the room lights turn on. Alternatively, when female hormonal stimulation was used, IVF was performed approximately 6-7 hours after the room lights turn on.

2.2. Female hormonal stimulation (adapted from Tokumoto et al. 2009).

Female egg quality is an important factor in successful IVF but it can sometimes be a challenge to obtain good usable eggs. Not all females are fecund but 1/3 of squeezed females can have good quality eggs (whereas males will give sperm >90% of the times) (Pegg 2007).

After the first trials, *in vitro* fertilization started to be hampered by the quantity and quality of oocytes. In a first attempt to understand how many females were needed to guarantee enough oocytes to perform IVF and to reduce costs and human resources, the percentage of high quality oocyte clutches (yellow oocytes, with no white debris indicative of degradation, dry and sticking together) within two wild type strains was determined for four months. For this study, only data from wildtype AB and TU females was collected.

In order to trigger zebrafish oocyte maturation and ovulation, to make sure the female population has higher quality clutches, the natural teleost maturation-inducing hormone, 17alpha,20beta-dihydroxy-4-pregnen-3-one (17α,20β-DHP) was used as a tool for artificially inducing ovulation in zebrafish (adapted from Tokumoto et al. 2009). 100nM 17α,20β-DHP was administered directly to the water where zebrafish were housed. This direct administration allows the steroid hormone to penetrate the fish body, causing an effect upon oocyte maturation. Besides being an enhancer of good egg clutches, this technique allows IVF procedures to have an extra daily working window of 2-3 hours, as fertilizable

oocytes can be obtained up to 4-5 hours upon addition of 17α,20β-DHP. The respective SOP is described in appendix C.

2.3. Sperm ultra-fast freezing

For testing and optimization of cryopreservation protocols, sperm from four to eleven month-old males was collected using a glass capillary attached to a mouthpiece and flat forceps. Sperm was collected in the first three hours after the room light turns on (Engeszer et al. 2007 in Lawrence 2012). Sperm was then added to an extender that was previously stored in a cryovial on ice, and freezing medium comprised of a mixture of extender and cryoprotectant was immediately added. Forthwith, the cryovial without cap was transferred into the upper rack on the Styrofoam box for fifteen minutes. After this period the cryovial was capped and transferred into liquid nitrogen (figures IV.4 and IV.5).

Figure IV.4: Ultra-fast freezing set up.

Figure IV.5: Zebrafish sperm ultra-fast freezing main steps currently in use at the Champalimaud Fish Facility. 1 - Extender storage on ice; 2 - Styrofoam box with LN2; 3 – Sperm collection; 4 – Sperm addition to extender; 5 – Freezing medium addition; 6 – Cryovial transfer without cap; 15 minutes in N2 vapour; 7 – Capping of cryovial and transfer to LN2; 8 – Storage in N2 chamber at ~-180°C.

The container used in the cryopreservation procedure was not, as in classical protocols, an expensive metal container. A Styrofoam box filled with 5000 to 6500cm^3 of liquid nitrogen was used as the cryopreservation main set up. The ultra-fast freezing set up was comprised of the Styrofoam box and two centrifuge tube racks - one placed at the bottom of the box completely immersed with liquid nitrogen and the other one for cryovials placed on the top of the other rack. In general, the total volume of solutions and sperm cryopreserved was 23µL per cryovial. The respective SOP is described in appendix D.

All tested cryopreservation solutions (extender and cryoprotectant) are schematized in figure IV.6. The freezing medium was a mixture of extender and cryoprotectant.

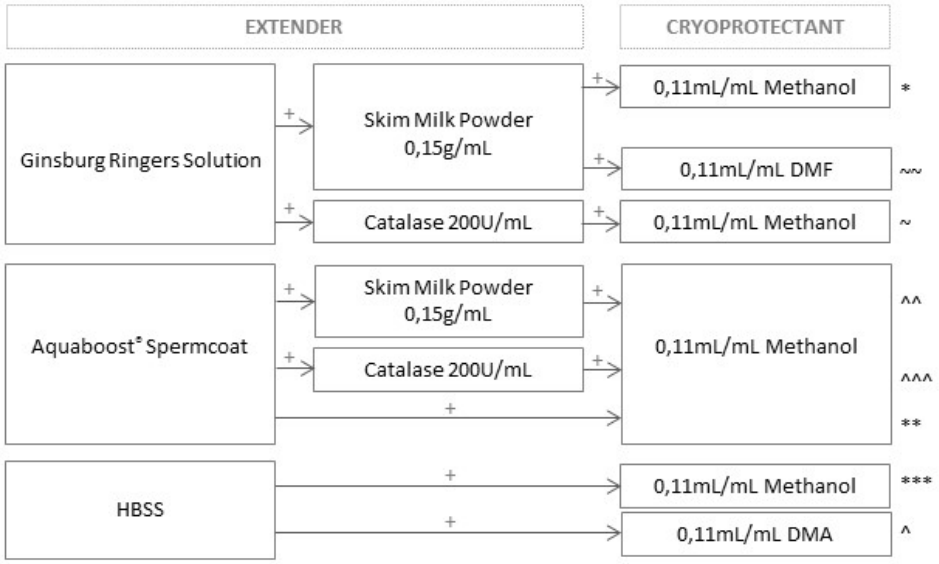

Figure IV.6: Tested combinations of extenders and cryoprotectants used in the sperm ultra-fast freezing tests. Symbols *, **, ***, ^, ^^, ^^^, ~ and ~~ identify each combination.

2.4. Sperm thawing and reconstitution

Examination of sperm viability generally includes evaluation of morphology, membrane integrity, motility, ability to bind oocytes, and fertilization. Motility is the most widely used assay, but fertilization is considered to be the most informative (Yang & Tiersch 2009). Fertilization rate was achieved by performing *in vitro* fertilization and was determined by assessing the percentage of developing embryos or hatched fries at 24hpf. Survival and malformations rates (defined as larvae viability rate) were evaluated between 6 and 7dpf.

A water bath was set at 33°C in order to pre-heat the extender and to use this temperature to thaw the sperm samples. Meanwhile, oocytes were collected from selected

females into petri dishes and stabilized in the extender Aquaboost® Ovacoat in order to prevent their activation.

Each cryovial was removed from liquid nitrogen, the cap opened, the liquid nitrogen tipped out and quickly immersed ~1/2 way into 33°C water bath for 15 seconds. 70µL of pre-heated extender was immediately added and mixed by gently pipetting up and down 2-3 times. A 200µL pipette tip was used with the tip cut off to prevent spermatozoa damage (figure IV.7). Before adding the sperm to the oocytes, Aquaboost® Ovacoat had to be gently removed from the oocytes to ensure proper contact between the two gametes. Immediately, 750µL of fish water was added and incubated 5 minutes at room temperature. After incubation, the petri dishes were filled with fish water and transferred to an incubator at 28°C with a photoperiod of 14h:10h/light:dark. The respective SOP is described in appendix D.

Figure IV.7: Sperm thawing main steps. 1. Cryovial removal from N2; 2 – Immersion of ~1/2 cryovial into a 33°C water bath; 3 – Addition of pre-heated extender and mix.

2.5. Determination of sperm concentration

A sperm suspension for analysis was obtained sampling 2 to 3µL of sperm with extender. Sperm concentration was estimated with a microspectrophotometer (NanoDrop®, Thermo Scientific, Wilmington, DE). The protocol for microspectrophotometry analysis was previously established by Tan et al. 2010. The standard equation used was:

$$Y = (3x10^{8}) X - 3x10^{7}$$

with “X” being defined as the absorbance measured at 400nm. Briefly, 1µL sample of sperm suspension was loaded onto the lower pedestal of the NanoDrop, and absorbance was measured at 400 nm.

3. Technological procedures performed in parallel with sperm cryopreservation optimization

Despite sperm cryopreservation being the main goal of this study, other zebrafish procedures that needed optimization of the IVF technique were performed, showing the general importance of this technique. Therefore, those methods and results were included as part of this project.

3.1. *In vitro* oocyte injection

This subchapter is part of an on-going collaboration for the development of new transgenic procedures between the Fish Facility and CR researchers, in this particular case, the Orger Lab. A newly developed method involving reproduction techniques, namely IVF, was tested.

3.1.1. Microinjection

In vitro oocyte injection to improve transgenesis was performed based on the protocol described by Xie et al. 2016. Low efficiencies of genome editing and germline transmission result in time-intensive and laborious screening work, thus the optimization of strategies to minimize screening is crucial.

The standard method of introducing foreign genomic material into zebrafish is by microinjecting it in fish embryos immediately after fertilization, at one-cell stage. This new method consists on injecting oocytes instead of embryos (figure IV.8) and incubating them in a specific oocyte storage medium to significantly improve efficiencies of genome editing and germline transmission. According to Xie et al. 2016, micro-injecting zebrafish oocytes substantially improved genome editing efficiency, especially for sgRNAs with low targeting efficiency, providing an efficient alternative to decrease the time frame of generating heritable mutants in zebrafish by using the CRISPR/Cas9 system.

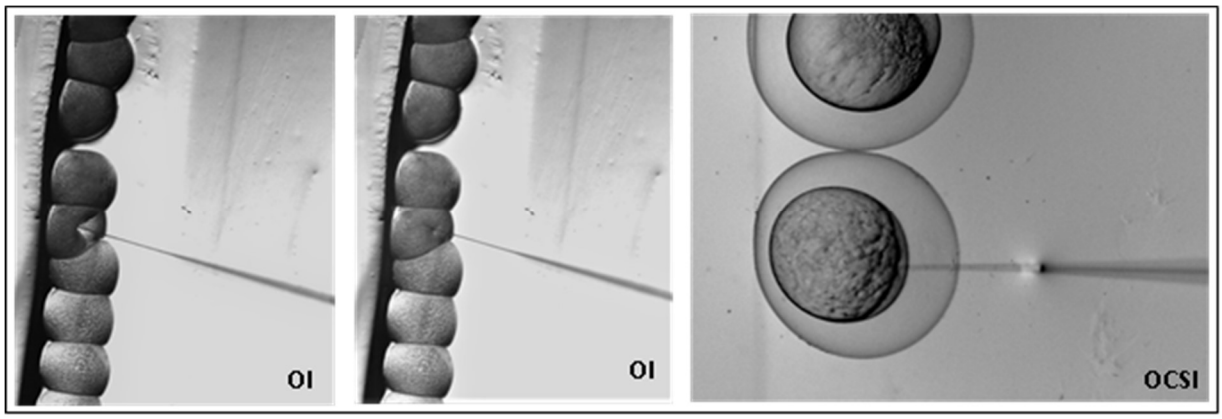

Figure IV.8: Comparison between oocyte injection (OI), 20x magnification, and one-cell stage injection (OCSI), 60x magnification.

The method developed in this study had slight differences to the original published method (Xie et al. 2016), namely the type of construct injected (the Tol2 system was used to generate random insertions of a transgene as opposed of using the CRISPR/Cas9 system to generate gene-specific mutants) and some variations in the storage medium. Usually, the agarose plates used for embryo injections are prepared with E3 medium but for oocyte injection this this would cause the oocytes to be activated immediately which is not

compatible with the procedure. Therefore, the medium in which the agarose is melted needs to be an extender, in this case Ginsburg Ringers Solution (also used in the sperm cryopreservation process described in subchapter 2.3.) or 90% Leibovitz's L-15 medium with L-glutamine and 0.5mg mL^{-1} bovine serum albumin, pH 9.0 (Xie et al. 2016).

The constructs injected - HuC:GFF 10xUAS:GCaMP6sEF05 and HuC:GCaMP6sEF05 - were chosen by the Orger Lab.

The SOP is described in appendix F. Injection was done in a TU zebrafish strain with Nacre (*mitfa*+/-) background. At 24hpf plates were cleaned off of dead embryos and the medium was replaced with fresh E3.

3.1.2. Transient expression

At 48hpf all injected larvae were screened for pan-neuronal transgenic transient expression on a PentaFluor-equipped V8 stereoscope (Zeiss) using a blue filter with a spectrum range from 400–460nm.

Positive transgenic larvae (stringently selected: enough labelled cells and expected panneuronal expression pattern for the HuC/elav3 promoter) were raised according to the conditions published by Martins et al. 2016.

3.1.3. Screening for stable expression

Fish were raised until adulthood (~3 months or when sexual maturity was observed). Fish were then individually crossed with the driver line *Isl3:Gal4*$^{(+/+)}$ and the offspring was screened on a PentaFluor-equipped V8 stereoscope (Zeiss) using a blue filter with a spectrum range from 400–460nm. Animals with positive progeny were kept. From the positive progeny (F0, founders), stable transgenic lines (F1) were established.

3.1.4. Establishment of stable transgenic lines

F1 stable lines were generated crossing each Tg(10x*UAS:GCaMP6sEF05*) F0 (founder) fish with the driver line *HuC:GFF*. At 48-72hpf the expression pattern was checked (strong fluorescence in the brain tectum) and the positive larvae raised.

3.2. Sperm viability analysis taking into account two dietary regimens

This subchapter is part of a parallel study performed by the Champalimaud Fish Facility in which the impact of different feeding regimens on zebrafish survival, growth and reproductive performance was studied. Two feeding regimens were created using combinations of two commercial dry feeds - Skretting® Gemma Micro and Sparos®

Zebrafeed - and one live feed (rotifers). Results from this study were submitted for publication.

3.2.1. Sperm viability

To evaluate the influence of the dietary regimen on reproduction, both embryo development and sperm viability were studied.

In order to have more information to analyse, fertilization rate assays were performed. Thus fertilization was achieved by performing *in vitro* fertilization and was determined by assessing the percentage of developing embryos or hatched fries 24hpf. Oocytes from 5-10 month-old wild type fish (AB and TU strains) were collected by female squeezing. Clutches from each female were divided in two petri dishes and held in an extender Aquaboost® Ovacoat to retain fertility. In order to calculate the fertilization rate depending on the diet on which each male had been fed, sperm from two individual regimens (dietary groups 2 and 5; Table IV.1) was mixed to the half clutch for *in vitro* fertilization. After mixing, fresh fish water was added to activate gametes in order for fertilization to occur. Males were squeezed during four months every two weeks (6-10 month-old fish). Survival and malformation rates (defined as larvae viability rate) were evaluated between 6 and 7dpf.

3.2.2. Statistical analysis

All data was analysed with the IBM SPSS Statistics software (v. 23, IBM Corp., Chicago, IL). As assumptions were not verified (normality and homoscedasticity), the Wilcoxon-Mann-Whitney test was used followed by pairwise comparison. Results were considered statistically significant for *p-values* <0.05.

3.2.3. Feeding Regimens

Skretting® Gemma®Micro 150, 300 or 500 was provided to animals with <30 dpf, 30-90 dpf, and >90 dpf, respectively. Similarly, Sparos® Zebrafeed® 200-400 and 400-600 was used to feed fish with ages 30-90 dpf, and >90 dpf, respectively. Regardless of the dietary regimen, all fish were fed 4x/day between 8 dpf and 60 dpf, 3x/day from 60 to 90 dpf and 2x/day from 90 dpf onwards. On weekends and holidays, they were fed 1x/day with the dry feed of the corresponding experimental group. All tanks were given a similar volume of rotifer solution and a similar amount of dry feed. The density of each tank was readjusted at 30 dpf, to ensure uniformity and reduce the influence of density on the results. Two experimental dietary groups were designed using different combinations of dry feeds (Skretting® Gemma® Micro or Sparos® Zebrafeed®) and a live feed (type “L” saltwater rotifers) (table IV.1).

Table IV.1: Feeding regimens for dietary groups 2 and 5

Dietary Group	6 - 30dpf		30 - 60dpf		60dpf - end of study
	Live Feeding	**Dry Feeding**	**Live Feeding**	**Dry Feeding**	**Dry Feeding**
2	2x Rotifers	2x Gemma Micro 150	1x Rotifers	2x Gemma Micro 300	2x Gemma Micro 500
5	2x Rotifers	2x Gemma Micro 150	1x Rotifers	2x Gemma Micro 300	2x Zebrafeed 400-600

V. Results

1. Techniques directly involved in sperm cryopreservation

1.1. *In vitro* fertilization (IVF)

The first IVF test was performed using two strains of wild type zebrafish, AB and TU, one mutant strain, Nacre (*mitfa -/-*), and one transgenic strain, Tg(*Isl3:Gal4*). AB, TU and Nacre fish had been previously selected as good breeders and were four to six months old. The transgenic fish were nine months old and were randomly selected. Before using the gametes for IVF, a quality assessment was done. Only yellow, with no white debris indicative of degradation, dry and sticking together oocytes and bright white sperm were used.

The mean fertilization rate at 24hpf was 69.58±22.42%, 70.72±27.30% and 38.66±8.10% for wild type, Nacre and *Isl3:Gal4*, respectively (figure V.1).

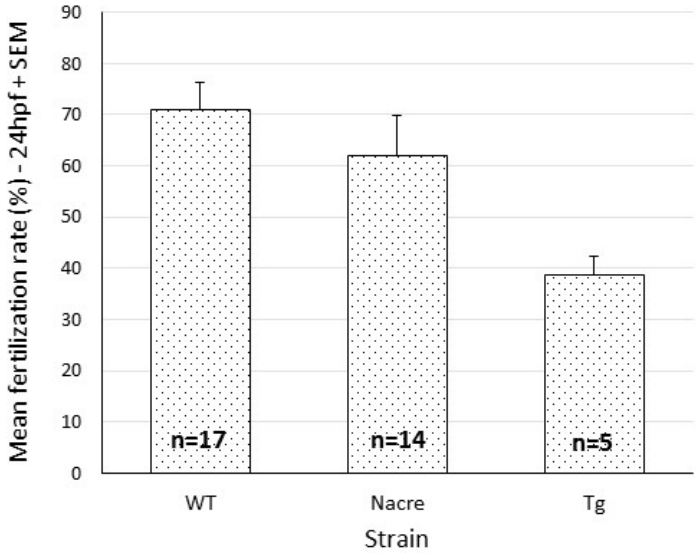

Figure V.1: Mean fertilization rate ± standard error at 24hpf using the IVF technique, according to the strain. x̄(WT)=69.58±22.42%, n=17; x̄(Nacre)=70.72±27.30%, n=14; x̄(Tg)=38.66±8.10%, n=5. WT is a pool of AB and TU lines.

Eight IVF trials were performed using oocytes in phase I and II of maturation and with some percentage of white debris or using sperm that had low motility (<50% of spermatozoa without motility observed after activating the gametes with fish water). All the experiments resulted in no fertilized embryos (data not shown), further emphasizing that samples with these characteristics should never be used. Indispensable characteristics for success in IVF are oocytes with a transparent to yellow colour, a firm and rounded chorion and clutches should form an aggregation of cells within a transparent fluid. Sperm should be opaque white and present at least with 80% of motility.

Some of the fish lines existing at the Champalimaud Fish Facility lost their natural mating behaviour due to either inbreeding through many generations (a requirement for the particular type of behavioural research for which they are used) or to aging. Therefore, in these circumstances, IVF has to be performed to maintain the line. One extra step in the initial optimized IVF procedure was added, in which the natural teleost maturation-inducing hormone (17α,20β-DHP) was used. Figure V.2 demonstrates that IVFs performed with oocytes collected from females stimulated with the hormone 17α,20β-DHP have higher fertilization rates.

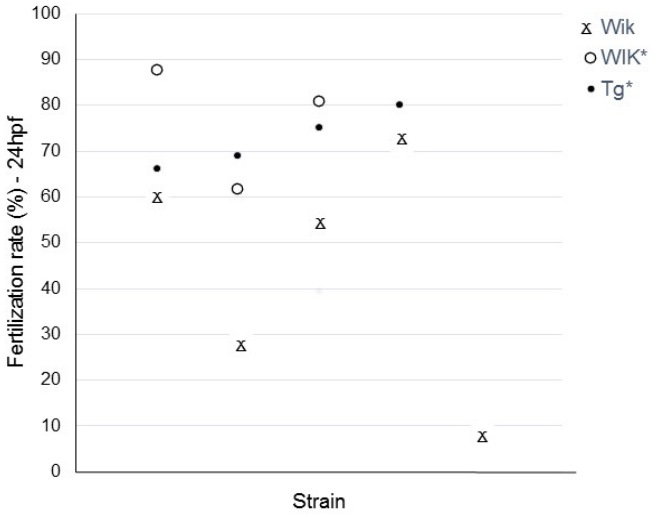

Figure V.2: Percentages of fertilization success for every IVF performed due to loss of natural mating behaviour. Wik lines lost the natural mating behaviour due to inbreeding; Tg line (HuC:GFF UAS:mCherry) lost the natural mating behaviour due to fish aging. * refers to IVFs performed with oocytes from females stimulated with the hormone 17α,20β-DHP.

1.2. Female hormonal stimulation

In order to understand how many females would be sufficient to have always enough oocytes to perform IVF, a study with a total number of 473 females was performed. The mean of females with good clutches (yellow, with no white debris indicative of degradation, dry and sticking together oocytes) after squeezing was 33.23±27.51% and 19.50±24.29% for AB and TU, respectively (figure V.3A). Similar data from females stimulated with 17α,20β-DHP, shows a much higher number of females producing good quality clutches, 66.34±32.53% and 50.82±26.54% for AB and TU, respectively (figure V.3B).

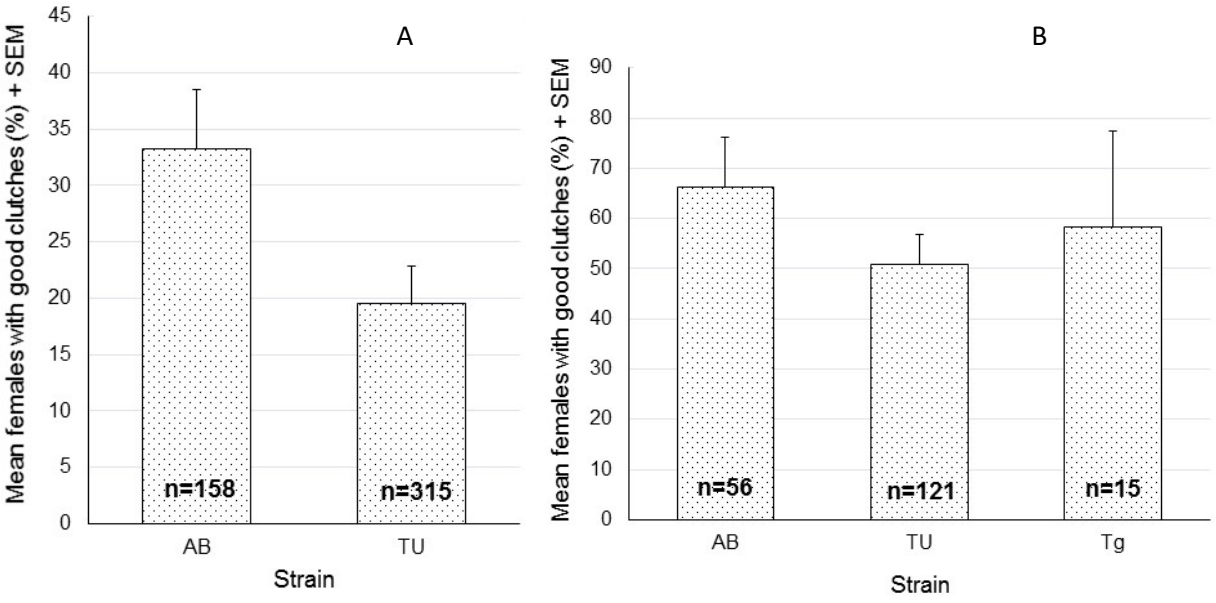

Figure V.3: Mean ± standard error of females providing good quality clutches; A - According to the wild type strain without hormonal stimulation: x̄(AB)= 33.23±27.51%, n=158; x̄(TU)=19.50±24.29%, n=315. B – According to the strain after stimulation with 17α,20β-DHP: x̄(AB)= 66.34±32.53%, n=56; x̄(TU)=, 50.82±26.54%, n=121; x̄(Tg)=58.33±38.19%, n=15. Tg is a pool of several transgenic lines (*Isl3:Gal4; 10xUAS:GCaMP6SEF05; HuC:GFF UAS:mCherry*).

1.3. Sperm ultra-fast freezing

As the choice of the extender and/or the CPA is very important, different extenders and cryoprotectants previously mentioned in literature were tested.

In order to overcome the high sperm variability, a collection of sperm from different males in the same cryovial was performed. Seconds after the sperm was collected and before its freezing, cells of some samples were observed in the stereoscope showing that sperm stored in different extenders is activated within different timeframes and in some extenders quicker than expected.

A total of 201 samples with sperm mean concentration of $4{,}82x10^6 \pm 3{,}34x10^6$ cells/µL were cryopreserved. 23 protocols were tested, which differed in: type and volume of extender (and if it was stored frozen or not), type and volume of freezing medium (percentage of cryoprotectant), sperm collected from several numbers of males, quantity of vials cryopreserved per sample, type of vials used in the cryopreservation process and exposure time to nitrogen vapour (appendix E). For each protocol the fertilization rate with thawed sperm was determined at 24hpf (figure V.4). Data showed a high variability in fertilization rates even within the same protocol. Of the 23 tested protocols, two can be highlighted as having 100% of line recovery (protocols 11 and 21 - Ginsburg Ringers Solution + Skim Milk Powder + Methanol) and two protocols had the higher fertilization rates (protocol 1 - Ginsburg Ringers Solution + Skim Milk Powder + Methanol; and protocol 17 –

Spermcoat + Skim Milk Powder + Methanol) (figure V.4). Three protocols were chosen as the best ones to analyse embryo survival and viability (protocols 1, 11 and 17).

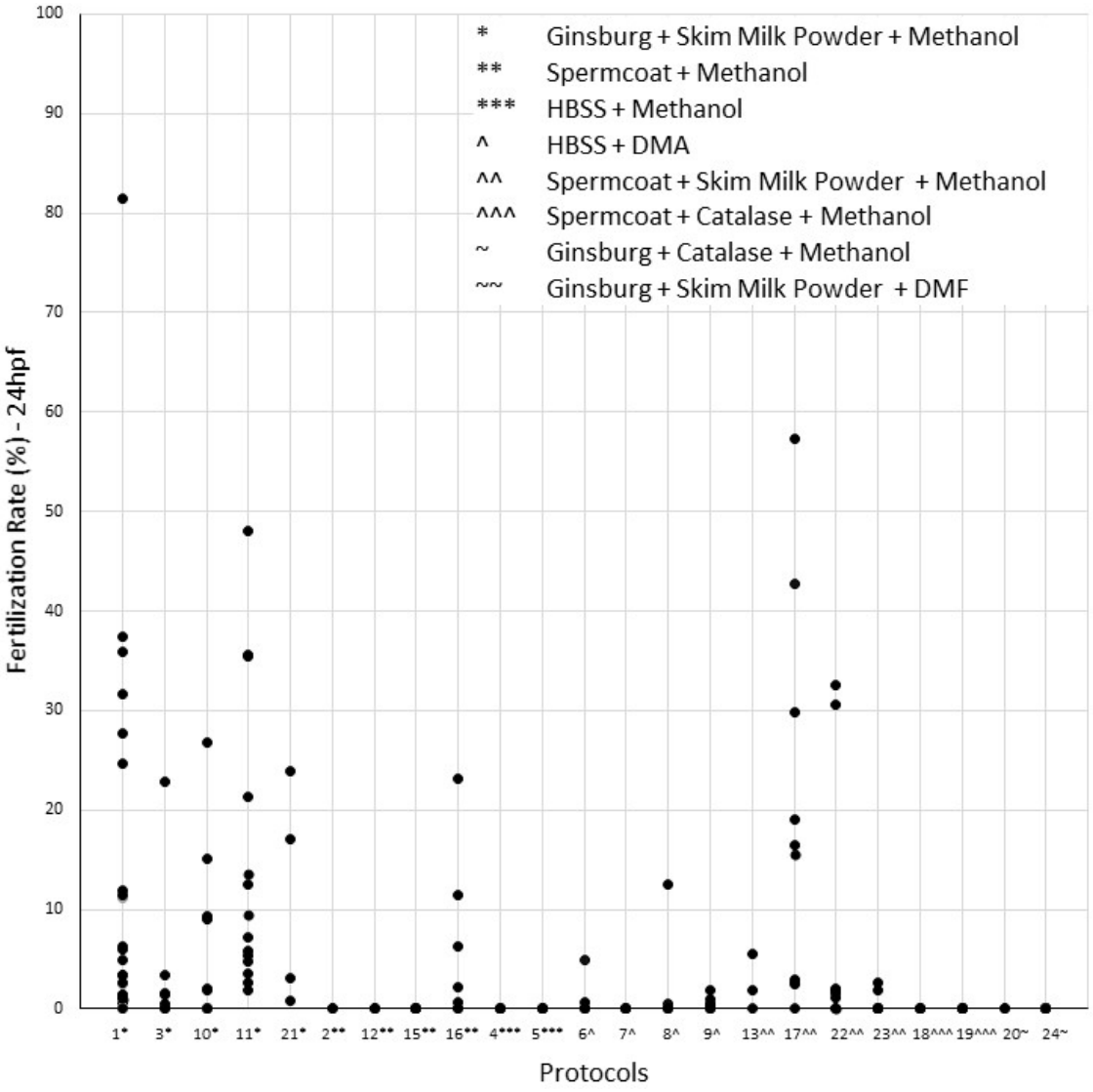

Figure V.4: Fertilization rate at 24hpf for 23 protocols tested. All samples cryopreserved by ultra-fast freezing (n = 201). Protocols organized by combination of extender and freezing medium (*, **, ***, ^, ^^, ^^^, ~and ~~).

In the analysis of the mean fertilization rate was observed that protocol 1, despite having high fertilization rates per sample, had the lowest mean fertilization rate (9.37±16.55%) as compared to protocol 11 and 17 (13.90±14.09% and 17.91±17.65%, respectively) (figure V.5, appendix E).

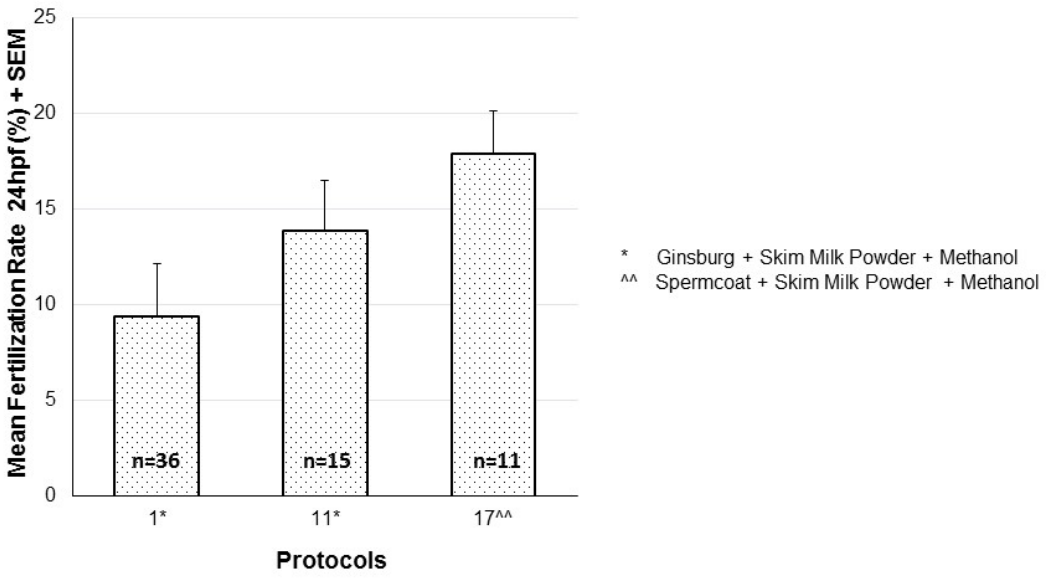

Figure V.5: Mean fertilization rate ± standard error at 24hpf for the three best protocols for ultra-fast freezing. $\bar{x}$(Protocol 1*)= 9.37±16.55%, n=36; $\bar{x}$(Protocol 11*)=13.90±14.09%, n=15; $\bar{x}$(Protocol 17^^)=17.91±17.65%, n=11. Protocols organized by combination of extender and freezing medium (*, ^^).

Therefore, protocol 11 and 17 were selected. In protocol 11, the extender was comprised of 0,15g/mL skim milk powder solubilized in Ginsburg Ringers Solution and the freezing medium was comprised of 0,15g/mL skim milk powder solubilized in Ginsburg Ringers Solution with 0,11mL/mL methanol. To perform protocol 11, sperm from one male was collected and added to 6,6µL of extender one ice to which was immediately added 40µL of freezing medium. The final sperm solution was divided in two cryovials from Nunc (CryoTube internal thread 1mL, Thermo Scientific) and transferred to nitrogen vapour for 15 minutes. Sperm was ultra-fast frozen in 8,7% cryoprotectant. In protocol 17 the extender was comprised of 0,15g/mL skim milk powder solubilized in Aquaboost® Spermcoat and the freezing medium was comprised of 0,15g/mL skim milk powder solubilized in Aquaboost® Spermcoat with 0,11mL/mL methanol. To perform protocol 17, sperm from one male was collected and added to 6µL of extender in a cryovial from Nunc (CryoTube internal thread 1mL, Thermo Scientific) stored on ice, to which was immediately added 20µL of freezing medium and transferred to nitrogen vapour for 15 minutes. Sperm was ultra-fast frozen in 7,65% cryoprotectant. As shown in figures V.6 and V.7, larvae survival rate was higher and the percentage of malformations was lower using the protocol where sperm was cryopreserved with a lower percentage of cryoprotectant (protocol 17), confirming the toxic effect of cryoprotectants.

Regarding the mean larvae survival rate at 7dpf (figure V.6), results were 87.25±18.7%, 89.44±9.79%, and 96.97±4.79% for protocol 1, 11 and 17, respectively, with no significant differences between the three methods. Malformations were also evaluated at 7dpf (figure V.7), the mean larvae malformation being 2.88±9.11%, 1.06±2.08%, and 0.42±1.18% for protocol 1, 11 and 17, respectively.

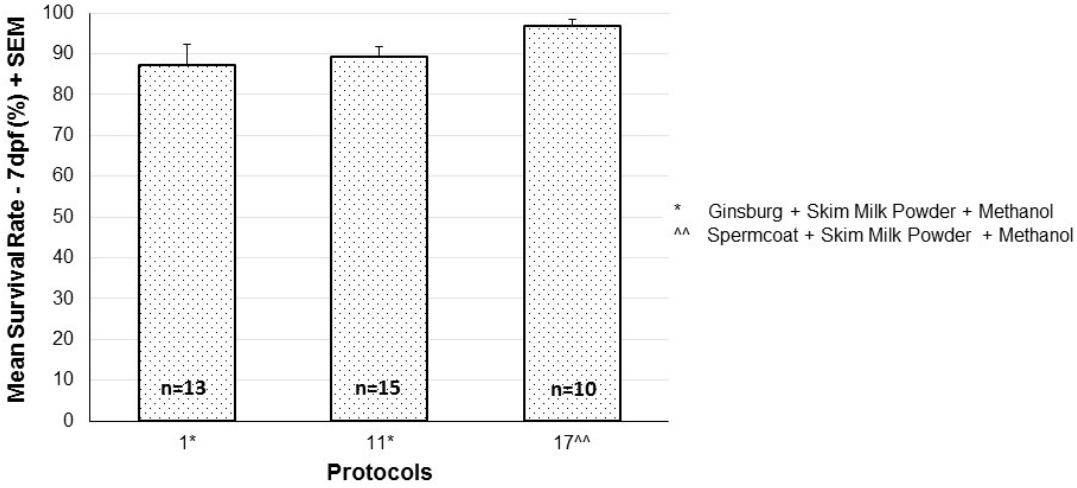

Figure V.6: Mean survival rate ± standard error of larvae at 7dpf of the three best protocols. x̄(Protocol 1*)=87.25±18.7%, n=13; x̄(Protocol 11*)=89.44±9.79%, n=15; x̄(Protocol 17^^)=96.97±4.79%, n=10. Protocols organized by combination of extender and freezing medium (*, ^^).

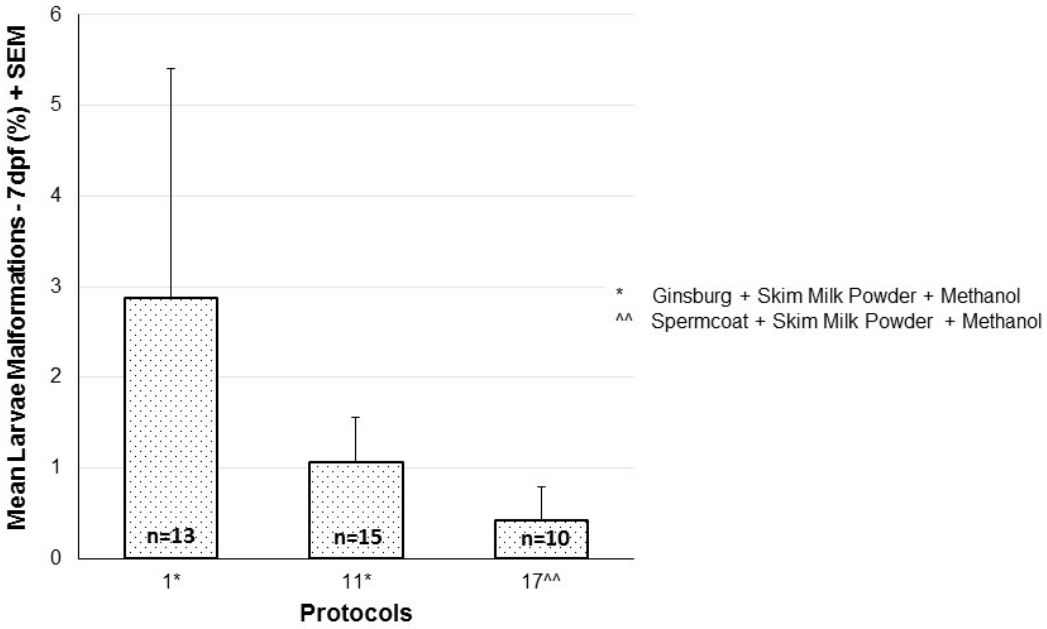

Figure V.7: Mean malformations ± standard error of larvae at 7dpf for the three best protocols. x̄(Protocol 1*)=2.88±9.11%, n=13; x̄(Protocol 11*)=1.06±2.08%, n=15; x̄(Protocol 17^^)=0.42±1.18%, n=10. Protocols organized by combination of extender and freezing medium (*, ^^).

Other extender used was Ginsburg Ringers Solution with skim milk powder combined with methanol (protocols 1, 3, 10, 11, and 21), based on procedures published previously (Draper & Moens 2009).

HBSS300 has proven to be a very difficult reagent to prepare due to its osmolality. When measured in a osmometer the osmolality was always different from 300mOsmol/kg (+/- 70mOsmol/kg) which could activate the sperm during the storage step before the addition of the freezing medium. In general, it was observed that sperm was activated very quickly (within 2-3 seconds) when using this extender. Another problem with this extender is that bicarbonate in HBSS has a low buffering capacity and is unstable over time because the release of CO_2 causes the pH to rise (Freshney 2000 in Mathews et al. 2018).

The procedures tested using this extender when combined with methanol (protocols 4, and 5) did not result in fertilized embryos. HBSS combined with DMA (protocols 6, 7, 8, and 9) resulted only in 0 to 2.17% mean fertilization rates (figure V.4, appendix E).

Figure V.8 demonstrates an attempt to establish a relation between the sperm concentration and the fertilization rate of thawed sperm that underwent cryopreservation. As observed there isn´t a direct relation between sperm concentration and fertilization using cryopreserved sperm to perform IVF.

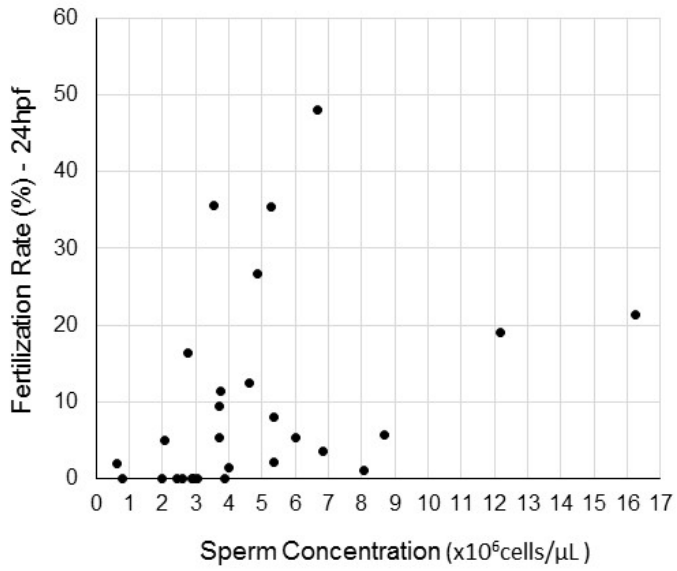

Figure V.8: Fertilization rate with thawed sperm *versus* concentration of fresh sperm (n=28). *In vitro* fertilization after thawing was performed with the same sperm sample in which the concentration was measured. Sperm concentration was estimated in Nanodrop® (absorbance measured at 400nm).

2. Technological procedures performed in parallel with sperm cryopreservation optimization

2.1. *In vitro* oocyte injection

2.1.1. Microinjection

The oocyte microinjection was performed in collaboration with A. Raquel Tomás from the Orger Lab at the Champalimaud Foundation. The parameters used in each experiment are reported in Table V.1.

Transfection, the process of introducing genetic material into a eukaryotic cell, for example by microinjection, is a way of getting nucleic acids of interest — whether plasmid DNA or various types of RNA (messenger, short interfering or micro) - into a cell without destroying it.

Table V.1: Summary of parameters tested and rates associated with survival, fluorescence expression and germline transmission for each experiment testing Tol2 system in oocytes injection.

Experiment	**A**	**B**	**C**	**D**
Construct concentrations	HuC:GFF: 12ng/µL* 10xUAS:GCaMP6sEF05: 8ng/µL	HuC:GFF: 12ng/µL* 10xUAS:GCaMP6sEF05: 8ng/µL	HuC:GCaMP6fEF05: 100ng/µL	HuC:GCaMP6fEF05: 18 and 180ng/µL
Tol2 mRNA concentration	100ng/µL	100ng/µL	1µg/µL	1µg/µL
Agarose plate - 1% agarose in:	Ginsburg Ringers Solution	Leibovitz medium with glutamine + BSA, pH 9.0	Ginsburg Ringers Solution	Leibovitz medium with glutamine + BSA, pH 9.0
# Injected oocytes	1500	1200	1200	2400
Injection + IVF strains	♂ Nacre; ♀ TU	♂ Nacre; ♀ TU and AB	♂ Nacre; ♀ TU	♂ Nacre; ♀ TU
Survival rate (%) – 24hpf	10	5.25	2	0
Primary Screen				
Larvae with positive expression – 48-72hpf (%)	33.33	15.87	0	-
Malformation rate of positive larvae (%)	40	10	-	-
Line name	10xUAS:GCaMP6sEF05	10xUAS:GcaMP6sEF05	-	-
# Fish raised	3	5	-	-
Survival rate (%) – 90dpf	33.33	60	-	-
# Fish survived – 90dpf	1	3		
Germline Transmission Screen				
# Founders	0	2		
Progeny with positive expression (%)	-	11.94 10.62	-	-

*transient expression; # number of

2.1.2. Primary screen

The Gal4/UAS transactivation system is frequently used in zebrafish to easily obtain transgene expression in a cellular population of choice, since it allows for flexible

combination of driver lines with reporter lines. The Gal4 protein binds as a dimer to short DNA sequences upstream of target genes, Upstream Activating Sequence (UAS), and recruits transcriptional machinery to adjacent promoters. Gal4 can also bind cooperatively to UAS tandem repeats, enhancing gene expression. The Gal4/UAS system allows for combination of a promoter sequence with a gene of interest (GOI), by crossing a promoter:Gal4 driver line with a UAS:GOI reporter line (figure V.9) (Halpern et al. 2008, Asakawa & Kawakami 2008). Orger Lab works with a wide range of promoter:Gal4 driver lines because the cloning of tissue-specific promoters allows labeling of specific embryonic structures in live embryos using fluorescent reporter transgenes (Kwan et al. 2007). For Orger Lab it is important to have stable lines of UAS:GOI reporter lines allowing several combinations using this Gal4/UAS system.

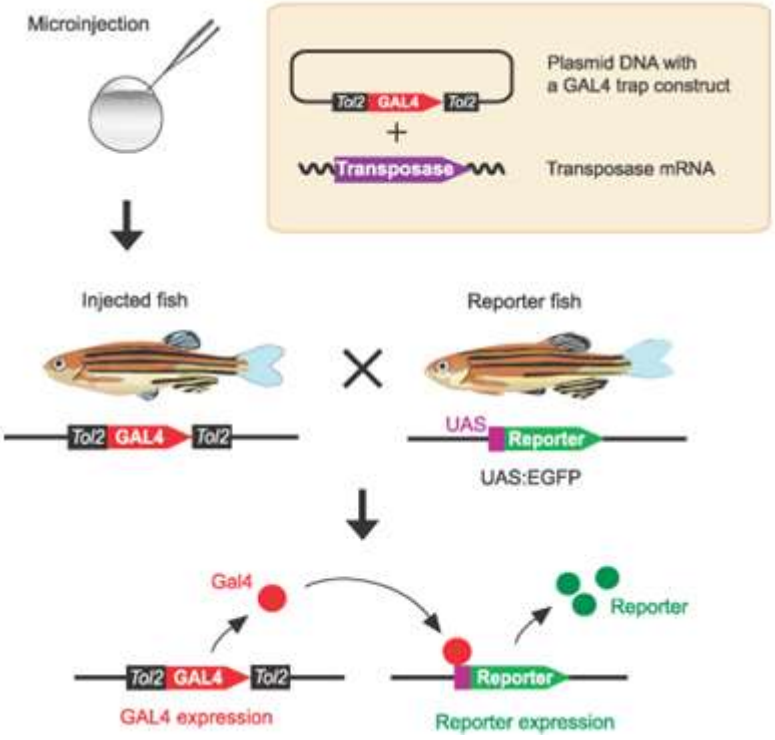

Figure V.9: Gal4/UAS transactivation system in zebrafish. A Gal4 driver line crossed with a UAS:reporter line results in double transgenic embryos expressing the reporter protein in Gal4 expressing cells. Adapted from Asakawa & Kawakami 2008.

In the first experiments (A and B) the material injected consisted of two plasmids plus the Tol2 transposase mRNA (Kawakami 2007). One plasmid had DNA for *HuC:GFF* with transient expression and the other DNA for 10x*UAS:GCaMP6sEF05* (table V.1). The *HuC:GFF* expression was transient because it did not integrate into the cell´s genome due to the lack of Tol2 transposase recognition flanking sequences in the plasmid, leading to a transiently transfected construct for a finite period of time in the cells. This system leads to

the necessity of using the Gal4/UAS system in progeny screens, in order to check for the presence of the UAS:GOI reporter line.

In the primary screen, larvae were checked for transfected cells that expressed both the *HuC:GFF* and the 10x*UAS:GCaMP6sEF05* expressions (figure V.10A). Very few embryos survived the microinjection (5.25 to 10% for experiment B and A, respectively, table V.1) due to the complexity of the method. The percentage of embryos with positive expression varied between 15.87 and 33.33% (experiment B and A, respectively) with some malformations observed (figure V.10B, table V.1) that resulted in the euthanasia of those larvae (10 and 40% of malformations for experiment B and A, respectively).

In experiments C and D the material injected consisted of Tol2 transposase mRNA (Kawakami 2007) and a plasmid DNA construct containing *HuC:GCaMP6fEF05.* The embryo survival rate was lower than in experiments A and B probably due to higher concentration of material injected compared to experiments A and B, introducing the parameter toxicity. The embryos that survived were all negative for *HuC:GCaMP6Fef05* expression (table V.1) probably due to the size of the plasmid (~11KB, against ~4.5Kb) diminishing the probability of insertion in the oocyte genome.

Figure V.10: Transient expression of *HuC:GFF 10xUAS:GCaMP6sEF05; A* - Healthy larvae, B – Deformed larvae.

2.1.3. Germline transmission

Germline transmission of the construct can be selected in the F0 generation (founders), by outcrossing the injected fish with the promoter:Gal4 driver line *Isl3:Gal4*$^{(+/+)}$. The presence of an *Isl3*-driven fluorescent pattern was checked (figure V.11). If the progeny had fluorescence in the trigerminal nerve, eye and specific brain regions it meant that the founder had a stable transfection in the germline and the next generations resulted in a stably transfected zebrafish line. A minimun of 100 embryos were screend for each fish raised as positive transgenics in the primary screen.

The only founders resulted from experiment B, with 11.94% and 10.62% of progeny with positive expression for the UAS:GOI reporter line 10x*UAS:GCaMP6sEF05* (table V.1).

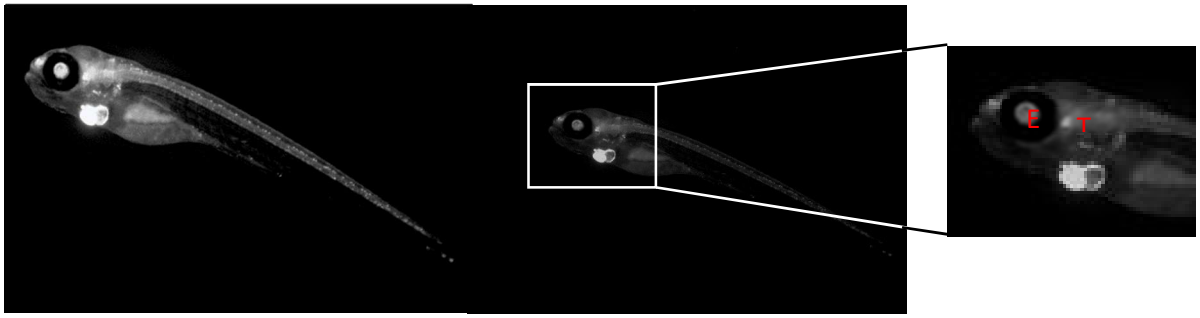

Figure V.11: Progeny (with different fluorescence intensity) from a founder *10xUAS:GCaMP6sEF05* crossed with the promoter:Gal4 driver line *Isl3:Gal4*$^{(+/+)}$. Fluorescence in trigerminal nerve (TG) and eye (E) typical of the promotor I*sl3:Gal4*.

2.2. Sperm viability analysis taking into account two dietary regimens

It has been previously reported by Diogo et al. 2015 that Zebrafeed® increments sperm quality, revealed by a higher total and progressive motility and higher velocities of the spermatozoa as compared to other commercial feedings. Dietary group 5 was created to test if the replacement of Gemma® by Zebrafeed® at 60 dpf would show any difference when compared with group 2 (fish fed with Gemma® after 60 dpf).

For the analysis of sperm viability, embryo development and viability analysis were performed. It was verified that there are no statistically significant differences between the two dietary groups (groups 2 for Gemma® and group 5 for Zebrafeed® after 60dpf) for both fertilization rate (figure V.12A) and larvae survival rate (figure V.12B).

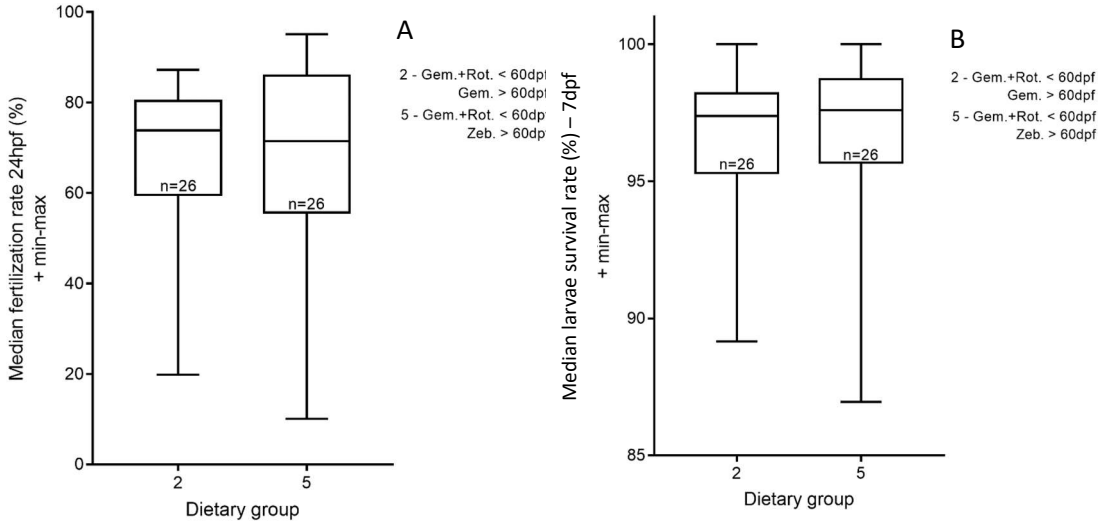

Figure V.12: Comparison of two dietary regimens (diet 2 and 5) to test the replacement of Gemma® by Zebrafeed® at 60dpf. A - Median of fertilization rate at 24hpf with minimum and maximum percentages using the IVF technique: $\tilde{x}$ (diet 2)=73.85% (19.83 to 87.27%) , n=26; $\tilde{x}$ (diet 5)=71.43% (10.06 to 95.12%) , n=26. B – Median of larvae survival rate at 7dpf with minimum and maximum percentages: $\tilde{x}$ (diet 2)=97.38% (89.15 to 100%) , n=26; $\tilde{x}$ (diet 5)=97.58% (86.96 to 100%), n=26.

VI. Discussion and conclusions

1. *In vitro* oocyte injection

A transgenic protocol based on *in vitro* oocyte microinjection was tested based on a novel technique developed and optimized by Xie et al. 2016 to improve efficiencies of CRISPR/Cas9 genome editing and germline transmission in zebrafish. The new protocol we developed focused on the Tol2 transposon system. The Tol2 system has a high insertion frequency (Kawakami 2005), when used in one-cell stage microinjection, but has low transgenesis efficiency, leading to mosaicism in transient transgenics and infrequent germline incorporation (Kwan et al. 2007). In order to overcome the low germline transmission, the transgenesis can be applied in the haploid gametes (oocytes or sperm) mediating the genetic transmission to the next generation. In zebrafish it is much easier to manipulate oocytes.

The Tol2 element, identified in medaka fish, is an autonomously-active transposon of 4.7kb encoding a transposase protein. The transposase is capable of catalysing transposition of a non-autonomous Tol2 construct, i.e., a construct that contains only the Tol2 arms (200bp and 150bp sequences) flanking a region where a DNA of interest (up to 10kb) can be cloned. Stable transposition is achieved when the Tol2 construct is co-injected with transposase mRNA: the transposase mRNA is translated and the protein catalyses the excision of the flanked DNA, which integrates into the genome. When both mRNA and protein degrade, transposase activity is lost and the insertion gets stabilized (Kawakami 2007).

From the four experiments, only experiments A and B resulted in positive fish in the primary screen (3 and 5 fish, respectively). In both experiments, the material injected consisted of two plasmids, one encoding for transient transgenesis of *HuC:GFF* and the other one for stable transgenesis *of 10xUAS)*. The difference between stable and transient transgenesis is the presence or absence of the Tol2 transposase recognition flanking sequences flanking the sequence of the gene of interest.

The absence of positive fish in the primary screen for experiments C and D could be due to the a high concentration of the injection mix (resulting in toxicity) leading to a high mortality rate or due to the large size of the insertion *HuC:GCaMP6fEF05* decreasing the probability of insertion and consequently of having positive transgenic fish.

Regarding germline transmission, it was only possible to have a F1 generation in experiment B. The number of putative positive fish raised in both experiments A and B was very low due to the high mortality observed in the first 24hpf.

The goal of the Tol2 system is to generate stable transgenic fish lines. However, the rates of germline transgenesis using injection in one-cell stage embryos are low with plasmid-based transgenesis, requiring the injection, raising, and screening of hundreds of potential founders to ensure recovery of a stable line (Kwan et al. 2007). Usually the injection of supercoiled or linear DNA yields 1-30% germline transgenic founders (Balciunas et al. 2006, Kwan et al. 2007, Stuart et al. 1988, 1990, Urasaki et al. 2006). With this method the percentage of founders was 66.67% (2 of the 5 fish considered positive in the primary screen were founders), a very high percentage due to the injection of the gene of interest being performed in gametes (oocytes in this case). For both founders, the percentage of F1 offspring expressing fluorescence (germline transmission) was around 11% which can be considered an efficient result.

The survival rate at 24hpf was very low in all experiments. After observing the cells in the stereoscope most of the oocytes didn´t undergo the activation step not allowing fertilization to occur. One possible cause is that the injection of a strange body in those cells may inhibit the development of the cavity between the shell and vitelline membrane due to the break of the natural reproduction machinery. In Xie et al. 2016, the choices of effective storage media and appropriate storage time were critical factors affecting the probability of successful oocyte storage *in vitro.* However, relatively longer storage time resulted in higher oocyte deformity rates. This is still a time point that needs to be studied, especially when using the Tol2 system.

In order to have higher survival rates at 24hpf and subsequently increase the number of founders, there are multiple steps that can still be changed, such as the number of trials, alternative setups for the oocyte injection (use of other kind of physical support instead of agarose plates to minimize damage to the oocytes when removing them from agarose), test different concentrations of plasmids, perform controls of the same construct using one-cell stage injection to determine if the mortality is due to the toxicity of the plasmid or to the technique itself, or test other incubation times.

If the mortality observed in the 24hpf could be overhaul, the number of founders would be higher and the effort required to create stable lines could be drastically reduced. There are no doubts that this method has a real potential and needs to be further optimized.

2. Sperm viability taking into account different dietary regimens

Zebrafish facilities need to make important decisions on how to invest resources among apparent competing processes such as growth and reproduction and to have always

several commercial alternatives regarding feeds and reagents. This way it is important to have an active role in testing and choosing the best feeding regimens. To evaluate the influence of the dietary regimen on reproduction, embryo development and viability, and sperm viability were studied.

Different fish populations may respond differently to dietary regimes depending on their original facility conditions. An increased resource availability can improve breeding success and egg production in females and fertilization rates in males (Newman et al. 2016). Different and higher embryo viability rates, depending on the dry feed offered to breeders, is correlated with the type of nutrients present in the diet (Rainuzzo et al. 1997 in Izquierdo et al. 2001). Experiments performed by Nowosad et al. 2017 revealed that feeding spawners with feed enriched with polyunsaturated fatty acids increased fertility, survival rate, and the post hatching size of larvae, compared to the control group. Other nutrients such as vitamins should also be present in the diet (Miller et al. 2012).

As shown in figure V.12, there were no significant differences between the two dietary feeding regimens, when analysing the fertilization rate at 24hpf and the viability of larvae with 7dpf. Using these two parameters as markers, the conclusion is that both feedings are equal and suitable for zebrafish maintenance. Besides fertilization rate and viability of larvae, other parameters that can be analysed are growth (dry weight and fork length), sexual maturity, sex ratio, and fecundity.

3. Importance of complementary procedures for sperm cryopreservation optimization

The method performed for quantification of sperm concentration using the Nanodrop was not considered a sensitive method to be applied as such when the samples measured were diluted in solutions containing skim milk powder. Several samples had a negative absorbance, maybe due to the interference of the extender added to sperm before the absorbance measurement in order to maintain the sperm inactivation. In this situation it was not possible to have a blank control and these samples were not included in the results and analysis.

The sperm concentration couldn´t be correlated with the fertilization rate (figure V.8). In samples where the concentration of sperm collected in the cryovials was very high, the volume of extender and freezing medium was not enough to retain proper storage and freezing conditions of all spermatozoa, thereby not resulting in higher fertilization rates.

Regarding female hormonal stimulation with 17α,20β-DHP, it was clearly an improvement in the oocyte quality and quantity of zebrafish (figure V.3) so, whenever possible, it is a method that can be adopted. This stimulation decreases the number of females needed to perform IVF, following the 3Rs guidelines (Reduce, Reuse, and Recycle), which are very important ethical guidelines in animal research. The 3Rs tenet is followed by scientists in the design of their animal-based research projects and by animal ethic committees and welfare bodies during their ethical review of the projects. Use of the 3Rs tenet assists in improving the welfare of animals used in science in several ways: it addresses a range of concerns about scientific animal use; it places a focus on individual animals; it adapts and responds to new information; it balances the needs of research and the needs of the animals; and it unites disparate groups with an interest in the welfare of animals used in science (European Agency Medicines 2016, Fenwick et al. 2009).

The optimized IVF procedure had very good results, as mentioned previously (figure V.1), the use of Cryogenetics reagents (Aquaboost® Ovacoat for oocytes and Aquaboost® Spermcoat for sperm) improved gamete storage time resulting in higher fertilization rates, compared with other published protocols. The procedure (appendix B) is currently in use at the Champalimaud Fish Platform providing a state-of-the-art service. The plus of having female hormonal stimulation with 17α,20β-DHP further improved fertilization rates.

4. Sperm ultra-fast freezing

From the two most used cryopreservation methods (slow freezing cryopreservation and vitrification), vitrification has many advantages over the traditional method such as no ice crystal formation (less chilling injuries) because the water content is lowered before cooling by adding high concentrations of CPAs. Increased speed of temperature conduction, which allows for a significant increase in cooling rates in this technique, prevents the risk of intracellular ice formation. This permits the use of less concentrated cryoprotectants causing less toxic effects (Isachenko et al. 2004, Kattera & Chen 2009).

The survival rates of vitrified cells are dependent on the type and concentration of the cryoprotectant (virtually all cryoprotectants are toxic), the temperature of the vitrification solution at exposure, and the type of device that is used for vitrification (Kattera & Chen 2009). Up to now, vitrification has been tested in zebrafish PGCs but with very low success rates (Higaki et al. 2013), urging for the development of other viable methods for cryopreservation of zebrafish sperm. Regarding the traditional cryopreservation method, many studies have shown that it is very difficult to replicate reported results in different

facilities and many protocols suffer from great variability and poor reproducibility (Bai et al. 2013, Mathews et al. 2018, Morris et al. 2003). Slow progress can be due partly to the lack of a proper extender to maintain the viability of spermatozoa and to the general use of toxic cryoprotectants.

The lack of standardization and coherent expectable results in previous studies (Bai et al. 2013, Mathews et al. 2018, Morris et al. 2003) were the major causes for the development of a simple and reliable method at the Champalimaud Fish Facility. This way, a new procedure was developed and optimized, designated by ultra-fast freezing since it is an intermediate method between cryopreservation and vitrification. At the end of each procedure, both cells are storaged at the same temperature (~196°C, liquid nitrogen temperature) (Agarwal 2011) but the difference between these two methods is in the cell freezing rate and in the cryoprotectant solutions. In vitrication the freezing is much faster (within seconds) than in the traditional cryopreservation (~30 minutes) and the type of cryoprotectants used allows no ice formation at all and consequently less damage. In this ultra-fast freezing process there weren´t used any specific CPAs usually used in vitrification (resulting in less steps) but the cooling rate was higher than in slow freezing cryopreservation.

In general, sperm quality had high variability within each tested protocol, when using fertilization rate as a sperm quality marker (figure IV.5), even when the sperm collection was made from the same male but in different trials. In other studies performed with teleost species, like the redside dace or the northern pike, showed also very high variation in the ability of sperm to endure freezing. It was also shown that spermatozoa with higher motility could not sustain it after thawing and high variability in sperm cryoresistance between individual males was observed (Babiak et al. 1997, Butts et al. 2013). This male-to-male variation poses a problem in the cryopreservation optimization. However, in the particular case of zebrafish there are no reports of such variation. There was variability when vials from the same sample were frozen at the same time point. This high variability can be due to several parameters.

Health status, besides animal age and holding density, is also expected to affect animal and sperm cell quality (Castranova et al. 2011, Hu et al. 2013, Murray et al. 2016, Torres et al. 2016, Watts et al. 2012). Food availability can also improve breeding success and egg production in females and fertilization rates in males (Newman et al. 2016).

Common parasites of zebrafish, such as *Pseudoloma neurophilia*, can infect nearly every tissue in the body. It has an overwhelming preference for neural tissue, particularly the nerve roots, spinal cord, and hindbrain (Sanders et al. 2014). This *Pseudoloma* infection has a very high prevalence; from 2000 to 2013 ZIRC has diagnosed five hundred and fifty-nine infected zebrafish from 86 laboratories. High numbers of Parasite clusters within brain and spinal cord structures mediating startle responses and anxiety suggests that related behaviours could be altered by neural microsporidiosis (Spagnoli et al. 2015). Reprodutive behaviour can be also altered as serotonin is involved in a wide range of reproductive functions (Prasad et al. 2015) and is mostly synthesized in the serotonergic cell bodies that are present in the hindbrain (Dahlbom et al. 2012). Some genes that are responsible for sex steroids are also highly expressed in this preferred *Pseudoloma* infected region of the brain (Pradhan & Olsson 2015). This way, health status is a very important factor to take into account when collecting sperm, as infected animals will probably have lower sperm cell quality.

As the success of cryopreservation strongly depends on the initial quality of the sperm, the period in which the sperm is collected is very important. Higher quality sperm can be obtained from matured breeders in the middle of a breeding season (Billard et al. 1977 in Kopeika et al. 2007, Bulostovich 1979) and the quality of sperm drops substantially by the end of the spawning period in trout, herring, and others species (Turdakov 1972 in Kopeika et al. 2007). In other teleost fish it is well known for many years that the sperm fertilizing ability decreases or not during the spawning period depending of the species (Bellard 1988 in Kopeika et al. 2007).

Zebrafish natural history and physiology influences mating and spawning behaviour with daily and seasonal variations in the natural environment. Zebrafish reproduction takes place primarily during the rainy months, a period of resource abundance (Talwar & Jhingran 1991 in Lawrence 2012), in the early morning and along the margins of flooded water bodies, often in shallow, still, and heavily vegetated areas (Engeszer et al. 2007 in Lawrence 2012). Zebrafish raised in captivity such as in a fish facility have the same environmental conditions during the whole year, mimicking the best conditions for the spawning and for the production of good quality gametes and stimulation and/or releasing of reproductive pheromones. On the other hand, can this controlled and invariable conditions “confuse” the intrinsic cycle regulated by the variations in the natural environment and uncontrol the reproductive behaviour? The circadian rhythmicity is the adaptation to the regular changes in the environment, defined mostly by the 24-hour period of earth’s rotation relative to the sun. Anticipating illumination, temperature, or food availability changes allows organisms to adjust all of their metabolic and behavioural processes in advance and to do everything ‘‘on time’’.

These intrinsic clocks are affected by environmental temperature, food availability, and predation risk and, in some species, may even play a more important role than light. However, biological clocks have evolved, and even in the absence of any environmental cues (like a facility with controlled physical-chemical parameters, for example) they autonomously oscillate with a circadian period (Zhdanova & Reebs 2006). It remains unknow if circadian cycle, besides depending on environmental inputs, can also be influenced by genetic factors consequence of inbreeding or low variability among generations.

To understand if there is still happening a circadian cycle in the zebrafish raised in research facilities can be a very useful study. If the circadian cycle is still present in these animals, having more information about the periods in which fish could have higher quality in gametes (independently of the constant and controlled conditions they are raised in) would be very important for the zebrafish community.

When developing a protocol for fish sperm manipulation it is very important to keep in mind its immobility in the genital tract and the extremely short lifespan after motility. In zebrafish, the spermatozoa lifespan is one-two minutes in natural medium (spawning/fish water) (Bai et al. 2013). This short period of activity time addresses two extremely important points: the speed of the operator and the efficiency of the extender. Intrinsic biophysical properties such as water and cryoprotectant permeability, osmotic tolerance limits, and intracellular ice nucleation must be considered for both extender and cryoprotectant. *In vivo* gamete survival (after the release of sperm) in controlled conditions varies among species, from one to several weeks for sperm (under oxygen and with antibiotics) (Bellard 1988 in Yang & Tiersch 2009) but for zebrafish there is only one commercial extender available - Aquaboost® Spermcoat. Procedures tested with Spermcoat in addition with skim milk powder had some of the best fertilization rates (figures V.4 and V.5) but regarding larvae survival and viability this extender combined with methanol resulted in the best combination (figures V.6 and V.7). Milk is a complex physiologic medium and its mechanism of action during cryopreservation is not well understood (Mathews et al. 2018). Lactose present in skim milk powder played a very important role in these cryopreservation protocols. Lactose is a saccharide and these molecules occur in the cells of plants, insects and fish that are exposed to freezing temperatures (Bachmann et al. 1944, Koster & Lynch 1992, Storey 1981, Yancey et al. 1982 in Hino et al. 2007). Lactose was found to possess some CPA activity and it can be significant in avoidance of chemical toxicity to cells of the diol CPAs (Fuller 2004). Saccharides limit intracellular dehydration during freezing and protect proteins against denaturation (Storey 1981a, Storey 1981b, Yancey et al. 1982 in Hino et al. 2007). Moreover, some saccharides stabilize bilayers and preserve membrane function and

structure (Rdolph & Crowe 1985 in Hino et al. 2007). Several studies indicate that casein micelles are the active components in milk that protect sperm by preventing cholesterol and lipid loss from sperm cell membranes (Bergeron & Manjunath 2006, Bergeron et al. 2007, Garcia & Graham 1987 in Mathews et al. 2018, Manjunath 2012). The addition of skim milk powder originated, in general, a minimum raise of 5% in fertilization rate success. The procedures tested without skim milk powder had only a maximum mean fertilization rate of 6.14%.

Samples comprised of pooled samples of sperm (protocols 8, 10,15, 16, and 23) didn´t show promising line recovery rates (figure V.4). Analysing all data can be concluded that there is still a lack of proper extenders to maintain the viability of spermatozoa and for now sperm cryopreservation has better results when cryopreserving one sperm sample at a time. Despite this hindrance, one key step in all procedures is to always make sure the freezing medium is well mixed in the sample without formation of bubbles.

In order to have less steps each time the procedure was performed, the freezing of some extenders was tested - any extender with skim milk powder cannot be frozen; after thawing the milk proteins sediment and do not regain the ability to solubilize. For example, the Ginsburg Ringers Solution can be frozen and the skim milk powder added after thawing.

The best protocols were chosen according to the percentage of line recovery (percentage of samples with fertilized embryos), mean fertilization rate (to analyse sperm viability), mean larvae survival rate, and mean malformation rate (for embryo survival and viability) (figures V.5, V.6 and V.7) - protocols 1 and 11 (Ginsburg Ringers Solution + Skim Milk Powder + Methanol), and 17 (Spermcoat + Skim Milk Powder + Methanol).

At the Champalimaud Fish Facility, the fertilization rate after performing the optimized *in vitro* fertilization protocol (appendix B) using fresh sperm and Spermcoat is very high (figures V.1 and V.2). These results depend of the strain but, for example, for wild-type strains the average is around 70%. Considering these results using the extender Spermcoat and the fact that sperm can be stored for 24 hours in Spermcoat (CUG 11/13, 2015), freezing samples using this extender were tested. Mainly three combinations were tested: Spermcoat with skim milk powder (protocols 13, 17, 22, and 23 with the best fertilization rates); Spermcoat without skim milk powder (protocols 2, 12, 15, and 16, without successful fertilization rates, between 0 and 6.14% mean fertilization rates) and Spermcoat with catalase (protocols 18 and 19, without successful fertilization rates, 0%) (figure V.4). The use of Spermcoat could have been a good choice for pooled samples, however the fertilization

rates weren´t as expected (0%, 6.14% and 0.38% mean fertilization rate for protocols 15, 16, and 23, respectively) maybe due to inappropriate proportion of extender in the freezing medium resulting in cell death during the freezing process.

Catalase was added as an alternative to skim milk powder in order to work as an antioxidant. This choice was based in Hagedorn et al. 2012 who reported that catalase as well as the cryoprotectant DMF did not decrease the sperm post thaw motility. However, the tested protocols in which catalase was used (protocols 18, 19, and 20) as an addition to the extender and DMF as cryoprotectant (protocol 24) did not have satisfactory fertilization rates (always 0% of fertilized embryos) (figure V.4). In the study made by Hagedorn et al. 2012, these two reagents were tested through the observation of spermatozoa motility, however in salmonids, for example, the length and intensity of spermatozoa on motility are not invariably correlated with fertilizing ability, especially when involving sperm stored *in vitro* for several days or deep frozen (Bellard 1988 in Yang & Tiersch 2009). The probable absence of correlation between these two parameters can be due to the weakened spermatozoa not having enough strength to disrupt the oocyte membrane and this lack of vitality can be the answer for the failure of the protocols using catalase and DMF. When performing *in vitro* fertilization it is necessary to use more spermatozoa per egg than with fresh sperm due to the loss of spermatozoa viability during the cryopreservation process.

It is important to emphasize that not providing the same conditions (water physical-chemical parameters, type of feeding and even infectious agents) to the models studied can deeply influence their general and natural performance. These differences in results can happen not only regarding reproductive performance but can also affect behaviour, regeneration or cancer research data.

There are no cryopreservation processes that can reproduce the fresh sperm quality, and establish stable success rates and reproducibility in sperm cryopreservation methods are the most important aspects in a protocol so there is still a wide range of factors to be deeply studied. The design of new extenders and cryoprotectants is a path that must be taken. The latest publication in zebrafish cryopreservation (Matthews et al. 2018) describes an average motility of 13%–20% and an average post-thaw fertilization rate of 16%–68% using a new hypertonic extender (E400) and a cryoprotective medium containing raffinose, skim milk, methanol, and a bicine buffer. Unfortunately, the set up and method continues to be the same laborious one, using cryovials and 15mL falcon type tubes immersed in dry ice. This study focused on sperm cell density and motility assessment to

develop the cryopreservation protocol. The use of advanced technology like the computer-assisted sperm analysis (CASA) System helps to monitor the sperm motility. Previous to Mathews et al. 2018, the validation of sperm cryopreservation methods did not include cell-density or motility measurements usually because it needs a costly equipment. The only options were to count cells with a hemocytometer and observe motility under a microscope. Although these methods are useful for establishing reference densities and to calibrate equipment such as spectrophotometers, they are relatively time consuming and, thus, impractical for routine use (Mathews et al. 2018).

The results from the ultra-fast freezing method optimized are nevertheless more than adequate for preserving genetic diversity via sperm banks and for *in vitro* fertilization. The main goal of this project was achieved: an easy, cheap and reliable protocol was obtained.

The results acquired and the optimized protocol are now being prepared for publication. It has made an impact in the zebrafish community and the Champalimaud Fish Platform will start providing cryopreservation services to the entire national community as part of the CONGENTO.

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Appendices

Appendix A: Zebrafish embryo development time lapse

Fresh or cryopreserved sperm is added to oocytes. Fertilization occurs upon gamete activation, by adding fish water.

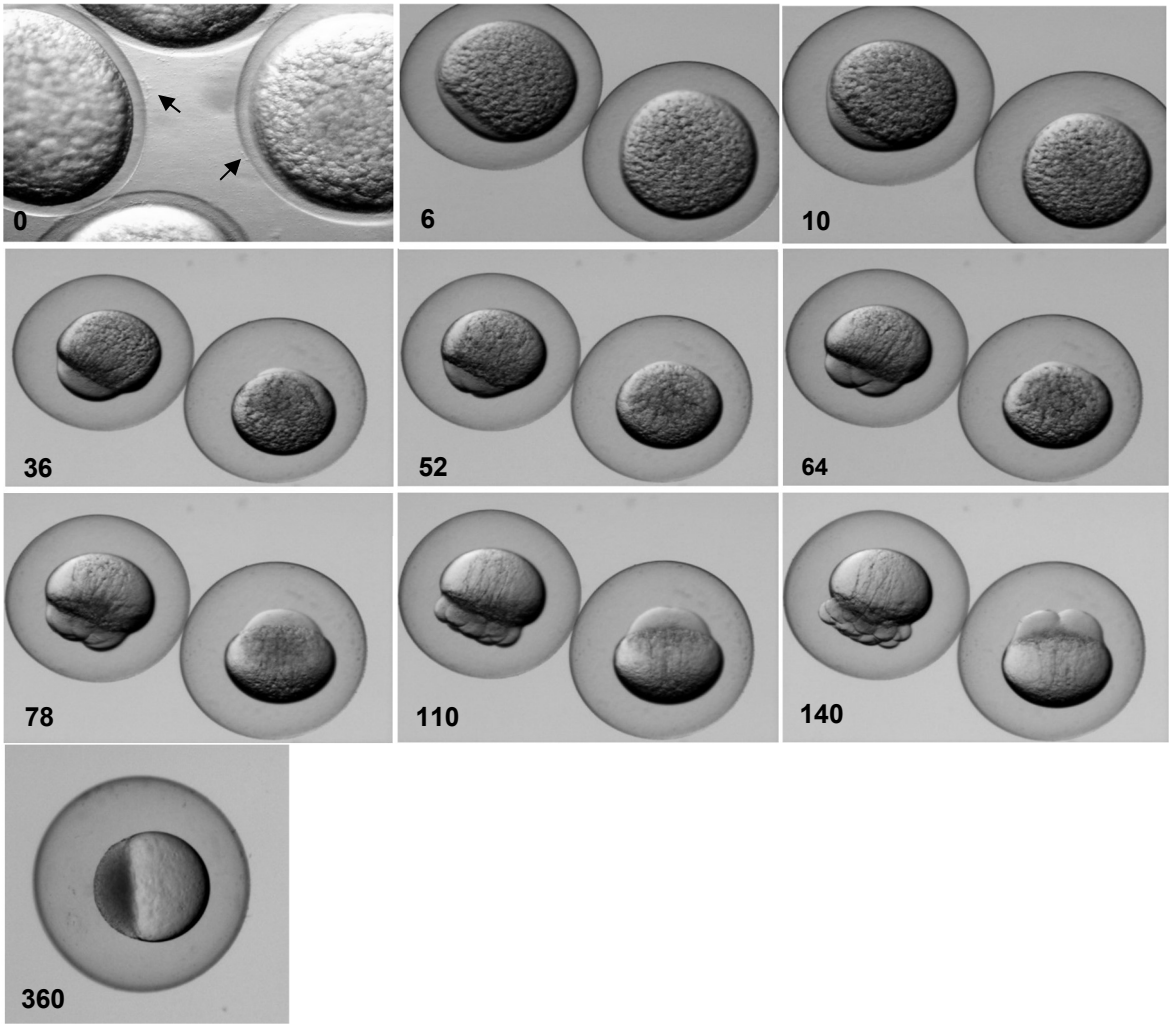

Figure 1: Embryo development after IVF, 80x magnification. Minutes post fertilization;

sperm ⟶

Appendix B: *In vitro* fertilization SOP

Zebrafish *In vitro* Fertilization

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Materials

- 0.5 mL Eppendorf tubes
- 1 L Breeding tank for anaesthesia (0,16 g/L MS-222)
- 1 L Breeding tank for recovery (system water)
- 1 L Breeding tank for rinse (PBS pH7.4)
- 500 mL Beaker with system water
- 12 cm long Capillaries
- Flat forceps
- Ice in a Styrofoam small box
- Kimwipes
- Lamp for direct illumination
- Permanent marker
- Mouth piece, capillary adaptor and hose with a syringe filter in the middle
- Nets - one to collect fish from Tricaine (MS-222) and another to collect fish from the recovery breeding tank
- 210mm Polypropylene spatula
- Paper towels
- Petri dishes
- Pipettes - P20 and P200
- Pipette tips - 20 µl and 200 µl
- Plastic spoon
- Stereoscope
- Sponge fish holder
- Timers

Solutions

- 0,16 g/L MS-222 for anaesthesia
- Fish water in beaker
- Aquaboost® Ovacoat at room temperature
- Aquaboost® Spermcoat on ice

Fish Preparation

- Keep fish at a low density (4 fish/L)
- Separate males from females in a 3,5 L tank with a divider
- Feed 3 times/day (1x artemia; 2x dry powder)
- Use fish with 5-11 months old
- Use females that have been laying eggs every 2 weeks or being squeezed every month since 4 months old
- Males have to be conditioned (separated from females in a 3,5 L tank with a divider) for a minimum of 2-4 weeks prior to sperm collection

The day before

Set up crosses using 2 L breeding tanks with divider. Place 3 females and 2 males in each tank.

Before the collection of gametes

- Attach the capillary to the hose with the mouthpiece. Introduce a pipette tip in the mouthpiece in order to replace it every time the IVF is performed.
- Place 100 µl of Aquaboost® Ovacoat in a petri dish for the eggs of each female.
- Pipette 10 µl of Aquaboost® Spermcoat to 0.5 mL Eppendorf tubes.

Sperm collection

1. Anesthetize males with 0,16 g/L MS-222 until gill movement slows down.
2. Remove one male from Tricaine and rinse the fish in PBS. Remove very quickly the male from this tank with the spoon and place it in the paper towel.
3. Dry the male by rolling gently front to back on a paper towel using the spoon. If the ventral side is still moist use a Kimwipe and gently dry by blotting. Water activates sperm so it is important to thoroughly dry the skin around the cloaca.
4. Put the male belly up in the sponge holder and place it in the scope with direct lighting.
5. Use the end of the capillary to spread the pelvic fins apart and expose the urogenital pore.

6. To extract the sperm place the capillary in the urogenital pore and gently press with the flat forceps stroking the sides of the fish. Collect sperm in capillary tube as it is expelled using suction. Avoid faeces that may be expelled with sperm. Collect 2-3 µL of sperm. Verify the quality of sperm: a dense concentration and good quality sperm will appear bright white.
7. Expel the sperm into the 0.5 mL Eppendorf tube with 10 µL of Aquaboost® Spermcoat. Store on ice (maximum 24 hours) until fertilization.
8. Recover the fish in fresh system water.

Female squeezing

1. Anesthetize females with 0,16 g/L MS-222 until gill movement slows down.
2. Remove one female from Tricaine and rinse the fish in PBS. Remove very quickly the female from this tank with the spoon and place it in the paper towel.
3. Dry female by rolling gently front to back on a paper towel using the spoon. If the ventral side is still moist use a Kimwipe and gently dry by blotting. Water activates eggs so it is important to thoroughly dry the skin around the cloaca.
4. Place the female belly up between thumb and index finger and squeeze gently to expel eggs. Fingers should be damp but not wet. Collect the eggs with the polypropylene spatula. Examine the eggs. They should be yellow, with no white debris indicative of degradation, dry and sticking together. Any egg lays with turbidity, dirt or watery eggs shouldn´t be used.
5. Immediately dip the polypropylene spatula in the Aquaboost® Ovacoat in the petri dish in order to remove the eggs from the spatula. Eggs are very delicate and should be handled with extreme care.
6. Keep eggs in Aquaboost® Ovacoat between 5 and 30 minutes.

Perform IVF

IVF should be performed up to 3 hours after the light cycle begins.

1. Gently remove excess Aquaboost® Ovacoat from eggs using a 100 µL pipette tip to ensure proper contact between sperm and eggs. Be careful not to touch the eggs.
2. Add 10 µL of sperm in Aquaboost® Spermcoat.
3. Add 200 µL of system water to activate the eggs and sperm. Swirl to mix. Incubate 2-5 minutes at room temperature.

4. Examine the sperm motility. Zebrafish sperm will loose motility 1 minute after activation.
5. Fill the dish with system water and place it for incubation (28ºC, photoperiod 14h day/10h night).

24 hours after IVF, check if fertilization was successful. Determine the success rate.

Zebrafish Female Hormonal Stimulation

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Materials

- Breeding tanks
- Pipette tips - 200 µl

Solutions

- System water
- 100nM 17α,20β-Dihydroxy-4-pregnen-3-one (Sigma P6285-5MG)

Hormone Preparation

- Dissolve β-DHP (Sigma P6285-5MG) in 100uL absolute ethanol
- Dilute this solution in 100mL of deionized water
- Aliquot this stock solution in 1,5mL Eppendorf tubes, keep them on the fridge (stable for at least 1 year)

Hormonal Stimulation

1. 1 to 2 hours after the lights turn on in the room transfer females from the system tank to a breeding tank (no need to use the grid). For each female use 100mL of system water. The females can be together with the males in the tank.
2. For each 100mL of system water add 15uL of β-DHP stock solution.
3. Incubate the females for 4-5 hours

Female squeezing

1. Anesthetize females with 0,16 g/L MS-222 until gill movement slows down.
2. Remove one female from Tricaine and rinse the fish in PBS. Remove very quickly the female from this tank with the spoon and place it in the paper towel.

3. Dry female by rolling gently front to back on a paper towel using the spoon. If the ventral side is still moist use a Kimwipe and gently dry by blotting. Water activates eggs so it is important to thoroughly dry the skin around the cloaca.
4. Place the female belly up between thumb and index finger and squeeze gently to expel eggs. Fingers should be damp but not wet. Collect the eggs with the polypropylene spatula. Examine the eggs. They should be yellow, with no white debris indicative of degradation, dry and sticking together. Any egg lays with turbidity, dirt or watery eggs shouldn´t be used.
5. Immediately dip the polypropylene spatula in the Aquaboost® Ovacoat in the petri dish in order to remove the eggs from the spatula. Eggs are very delicate and should be handled with extreme care.
6. Keep eggs in Aquaboost® Ovacoat between 5 and 30 minutes.

Zebrafish Sperm Ultra-Fast Freezing

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Controlled rate freezing processes in the presence of cryoprotectants can cryopreserve cells in suspended state for indefinite periods.

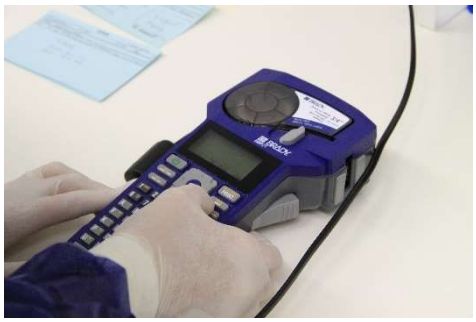
Labelling

Cryopreserved samples ID: **eg. SUF00101**

S = sperm

UF = ultra-fast freezing (method)

numeric code with 5 numbers



Labels printer

Set up

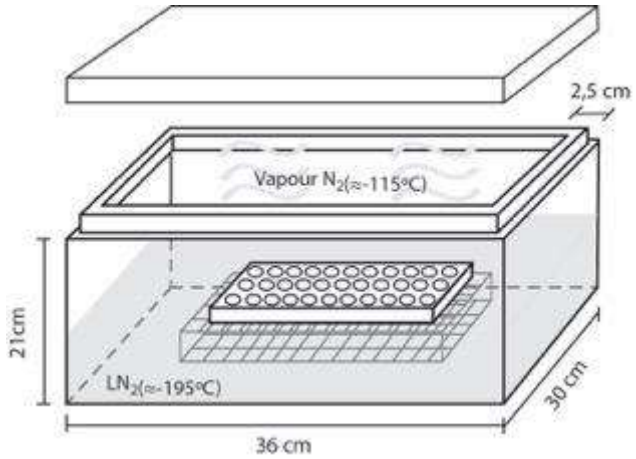
Styrofoam box (36x21x30cm or similar)

2 supports (one for 15mL Falcons - bottom, one for cryovials - top)

Fill Styrofoam box with liquid nitrogen until the bottom support is completely immersed

Close the Styrofoam box with the lid for at least 30 minutes

The sperm in cryovials is going to be frozen in the vapour phase nitrogen. The cryovials are positioned in a support above a shallow reservoir of liquid nitrogen. In this phase there is no risk of cross-contamination from liquid nitrogen, low temperatures are achieved and is a very simple and reliable method, however temperature fluctuations can occur.



PROTOCOL A (Sperm from 1 ♂ - 2 cryovials)

Solutions

Prepare volume of solutions depending on the number of samples being cryopreserved

Ginsburg Fish Ringers Solution (Make fresh and then freeze in aliquots)

In 450ml sterile ddH2O dissolve:

NaCl 3.25g

KCl 0.125g

CaCl2•2 H2O 0.175g

Then add NaHCO3 0.10g

Bring final volume to 500ml with sterile dH2O

Freezing Medium (Make fresh daily, solutions with skim milk powder can´t be frozen)

WITHOUT Methanol

Ginsburg Fish Ringers 10mL

Powdered Skim Milk 1.5g

WITH Methanol

Ginsburg Fish Ringers 9mL (room temperature)

Methanol 1ml

Powdered Skim Milk 1.5g

Sperm Freezing Protocol

1. Pipette 6.6µL of freezing medium without methanol to one cryovial and place both cryovials on ice (label both cryovials with the same ID)
2. Collect sperm and add it to the cryovial
3. Add 40µL of freezing medium with methanol, stir and pipette up/down 2-3 times
4. Immediately transfer 23.3µL to the other cryovial
5. Place the cryovials without cap into the upper support on the Styrofoam box for 15 minutes
6. Close the Styrofoam box lid every time a cryovial is placed in the support
7. After 15 minutes cap the cryovial and transfer it to the liquid nitrogen

PROTOCOL B (Sperm from 1 ♂ - 1 cryovial)

Solutions

Prepare volume of solutions depending on the number of samples being cryopreserved

Freezing Medium (Make fresh daily, solutions with skim milk powder can´t be frozen)

WITHOUT Methanol

Aquaboost® Spermcoat 10mL
Powdered Skim Milk 1.5g
WITH Methanol
Aquaboost® Spermcoat 9mL (room temperature)
Methanol 1ml
Powdered Skim Milk 1.5g

Sperm Freezing Protocol

1. Pipette 6µL of freezing medium without methanol to the cryovial and place it on ice
2. Collect sperm and add it to the cryovial
3. Add 20µL of freezing medium with methanol, stir and pipette up/down 2-3 times
4. Place the cryovial without cap into the upper support on the Styrofoam box for 15 minutes
5. Close the Styrofoam box lid every time a cryovial is placed in the support
6. After 15 minutes cap the cryovial and transfer it to the liquid nitrogen

***In Vitro* Fertilization Protocol Using Cryopreserved Sperm**

1. Set a water bath to 33°C
2. Preheat the Ginsburg solution in the water bath
3. Gently remove excess Aquaboost® Ovacoat from eggs using a 100 µL pipette tip to ensure proper contact between sperm and eggs. Be careful not to touch the eggs.
4. Remove one cryovial containing sperm sample from liquid nitrogen, open the cap, tip out liquid nitrogen and quickly immerse vial ~1/2 way into 33°C water bath for 15 seconds
5. Fill up immediately with 70µL pre-heated Ginsberg solution and mix by pipetting up and down
6. Immediately add to eggs, activate sperm and eggs by adding 750 µl fish water, swirl to mix
7. Incubate 5 minutes at room temperature.
8. Fill dish with system water and transfer to incubator (28°C; 14:10h/light:dark).
9. 15-24hpf remove dead embryos and fill the dish with E3.

Appendix E: Protocols summary regarding the main steps in each procedure tested for the ultra-fast freezing method of sperm. Mean fertilization rate and standard deviation for each protocol.

Extender + Cryoprotectant Combination (symbol associated)	*Protocol*	*Extender (mL)*	*Freezing Medium (mL)*	*# Males Squeezed*	*# Vials Cryopreserved*	*Vial capped in N2 vapour?*	*Fertilization Rate (%) mean±SD*
Ginsburg + Skim Milk Powder + Methanol (*)	1	3.3	20	1	1	NO	9,37±16,55
	3	3.3	20	1	1	YES	3,85±7,27
	10	10x5 vials	330	5	2	NO	6,49±7,75
	11	6.6	40	1	2	NO	13,90±14,09
	21	6	20	1	1	NO	11,18±8,58
Spermcoat + Methanol (**)	2	3.3	20	1	1	NO	0
	12	6.6	40	1	2	NO	0
	15	11.5	100	5	4	NO	0
	16	10x5 vials	13x5 vials	5	4	NO	6,14±742
HBSS + Methanol (***)	4	3.3	20	1	1	NO	0
	5	21.3	2	1	1	NO	0
HBSS + DMA (^)	6	3.3	20	1	1	NO	1,80±2,34
	7	3.3	20	1	1	NO	0
	8	10x5 vials	445	5	2 straws	sealed ends	2,17±4,63
	9	10	15	1	2	NO	1,37±2,33
Spermcoat + Skim Milk Powder + Methanol (^^)	13	6.6	40	1	2	NO	4,68±4,32
	17	6	20	1	1	NO	17,91±17,65
	22	3.3	20	1	1	NO	7,33±12,17
	23	6.6	40	2	2	NO	0,38±0,86
Spermcoat + Catalase + Methanol (^^^)	18	6	20	1	1	NO	0
	19	3	20	1	1	NO	0
Ginsburg + Catalase + Methanol (~)	20	3	20	1	1	NO	0
Ginsburg + Skim Milk Powder + DMF (~~)	24	3.3	20	1	1	NO	0

Injection of Tol2 constructs in Zebrafish oocytes + IVF

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Materials

- 0.5 mL Eppendorf tubes
- 1 L Breeding tank for anaesthesia (0,16 g/L MS-222)
- 1 L Breeding tank for recovery (system water)
- 1 L Breeding tank for rinse (PBS pH7.4)
- 500 mL Beaker with system water
- Capillaries for sperm collection
- Pulled 12 cm long capillaries for injection
- Flat forceps
- Ice in a Styrofoam small box
- Kimwipes
- Lamp for direct illumination
- Permanent marker
- Mouth piece, capillary adaptor and hose with a syringe filter in the middle
- Nets - one to collect fish from Tricaine (MS-222) and another to collect fish from the recovery breeding tank
- 210mm Polypropylene spatula
- Paper towels
- Petri dishes
- Pipettes - P20 and P200
- Pipette tips - 20 µl and 200 µl
- Plastic spoon
- Stereoscope
- Sponge fish holder
- Timers
- Agarose plates with rows to place eggs for injection (1% agarose in Ginsburg Ringers Solution or 90% Leibovitz's L-15 medium with L-glutamine and 0.5mg mL−1 bovine serum albumin, pH 9.0)

Solutions

- 0,16 g/L MS-222 for anaesthesia
- Fish water in beaker
- Aquaboost® Spermcoat on ice
- Oocyte storage medium (extender): 90% Leibovitz's L-15 medium with L-glutamine and 0.5mg mL−1 bovine serum albumin, pH 9.0

Gamete collection

- Collect the sperm and expel it into the 0.5 mL Eppendorf tube with 10 µL of Aquaboost® Spermcoat. Store on ice.
- Collect the oocytes and transfer them to the agarose plate, use the spatula to divide them through the slots in the agarose. Add <5µL of oocyte storage medium to each group of oocytes.

Oocyte microinjection and IVF

Use the microinjector PV 820 Pneumatic PicoPump to inject mature oocytes.

1. Align the oocytes using an autoclaved tricot needle
2. Carefully insert the capillary needle into the cell and press the injection pedal in order to inject the DNA mix
3. Add oocyte storage medium to the plate and with the help of the tricot needle carefully unstick the oocytes from the agarose
4. Incubate at room temperature (RT), in the dark, for 30 minutes
5. Transfer the oocytes to a petri dish and withdraw all the medium
6. Add the sperm and 200µL of fish water. Incubate for 5 minutes at RT.
7. Fill the petri dish with fish water and transfer it to the incubator (28°C; 14:10h/light:dark).

Appendix G: Preparation of chemicals and solutions

- 100nM 17α,20β-Dihydroxy-4-pregnen-3-one (P6285-5MG Sigma Aldrich)
- Aquaboost® Ovacoat (Cryogenetics)
- Aquaboost® Spermcoat (Cryogenetics)
- Catalase (C40-100MG Sigma Aldrich)
- Embryo medium (E3) prepared as follow: 29,38 g NaCl, 1,26 g KCl, 4,86 g CaCl2.2H2O, 8,14 g MgSO4.7H2O, 60 ml 0.01% Methylene Blue Solution (0.05 g Methylene Blue powder in 500 ml MQ water). Fill to 18L with fish water.
- Ginsburg Ringers Solution prepared as follow: in 450ml sterile deionized water dissolve NaCl 3.25 g, KCl 0.125 g, CaCl2•2 H2O 0.175 g and NaHCO3 0.10 g. Bring final volume to 500 ml with sterile deionized water (all reagents from Sigma Aldrich)
- Hanks' balanced salt solution at an osmolality of 300 mOsmol/kg abbreviated as HBSS300 prepared as follow: 0.137 M NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 1.0 mM MgSO4, 0.25 mM Na2HPO4, 0.44 mM KH2PO4, 4.2 mM NaHCO3, and 5.55 mM glucose, pH 7.2 (all reagents from Sigma Aldrich)
- Methanol (UN1230 Fisher Scientific)
- N,N-dimethylacetamide 99,55 (38840-1L-F Honeywell) abbreviated as DMA
- N,N-dimethylformamide 99.8%, Extra Dry, AcroSeal™ (ACROS Organics) abbreviated as DMF
- Skim Milk Powder (70166-500G Fluka Analytical)