# CHAPTER IV

# SPERM CRYOPRESERVATON IN TURBOT (*SCOPHTHALMUS MAXIMUS*)

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## 1. Introduction

A demersal species, turbot is distributed along the northeastern Atlantic coast, from Norway and Iceland to the Mediterranean and Black Seas. The reproductive period varies depending on the region, ranging from February to April (Mediterranean), mid-April to mid-June (Black Sea), April to August (North Sea), and May to July (North Atlantic) (Suquet et al., 2008; Cardinale et al., 2021; Baiandina et al., 2022).

In the aquaculture sector, egg quality is known to be the main indicator of fertilisation success. Likewise, sperm quality is emerging as a critical factor in artificial insemination (Lahnsteiner et al., 1998; Aydın et al., 2022). In addition to using high-quality gametes, techniques are needed to keep them alive for longer and maximize their efficiency. Among the approaches aimed at improving the breeding management of cultivated species, the preservation of gametes, especially sperm, is of great importance (Chereguini et al., 2003).

Cryopreservation of fish sperm is emerging as a potential application for fish farmers and breeders in artificial reproduction and enhancement of genetic diversity of aquatic species and is recognised as an essential technique for prolonged conservation of germplasm (Yongsheng et al., 2020; Thilak Pon Jawahar & Betsy, 2020). In this field, frozen sperm technology has enabled the long-term preservation of sperm, allowing superior germplasm to be spread over a wider area (Thilak Pon Jawahar & Betsy, 2020). In the rapidly growing aquaculture sector, the significant impact of biotechnology is particularly evident in the field of breeding. Broodstock management is carried out using cryopreservation technology, overcoming time and space constraints in genetic

improvement. (Pan et al., 2023). Specific applications have been advanced for certain freshwater fish varieties, notably trout, mullet, carp, and catfish (Lahnsteiner, 2000). Until today, significant research has also been conducted on marine species, particularly flatfish species such as turbot (Chereguini et al., 1992; Dreanno et al., 1997; Chen et al., 2004; Polat & Kurtoğlu, 2023), flounder (Chen & Tian, 2005; Liu et al., 2015), four species of sole (Pullin, 1972; Saitoh, 1996; Richardson et al., 1999) and halibut (*Hippoglossus hippoglossus, Verasper variegatus*) (Bolla et al., 1987; Zidni et al., 2020). As a result, the sperm of over 200 fish species have been successfully frozen (Chereguini et al., 2003; Cabrita et al., 2010; Thilak Pon Jawahar & Betsy, 2020).

The high market value of turbot, its efficient feed conversion ratio, easy of adaptation to aquaculture conditions, resistance to disease, high reproduction rate, and ongoing research and development efforts have made it a prominent species in flatfish farming. In addition, its tough, white meat and mild flavour make it highly prized in the culinary world. It has established itself as a preferred variety among both chefs and consumers. The turbot is a serial spawner and lays eggs maximum 10 times during the spawning season. The asynchronous egg supply process results in a prolonged spawning season, which is incompatible with intensive production (Beken, 2024). Turbot (*Scophthalmus maximus*) was last assessed for the IUCN Red List of Threatened Species in 2020. Although turbot is listed as “Least Concern”, its population trend shows a declining trend (Cardinale et al., 2021). The importance of cryopreserved sperm becomes evident at this point. However, the effective use of cryopreservation methods in turbot remains challenging due to species-specific physiological and biochemical traits.

This chapter aims to provide a comprehensive overview of sperm cryopreservation techniques specifically adapted for turbot. These techniques encompass practical protocols, critical factors influencing the success of cryopreservation, and methods for assessing sperm quality after thawing.

## 2. Biological and Technical Background

### *2.1. Reproductive Biology of Turbot*

The flatfish turbot has a benthic lifestyle, and therefore, lives mostly in shallow waters during its larval and juvenile stages, migrating to deeper waters when it reaches adulthood (Figueras et al., 2016). The turbot is a predatory species that inhabits sandy, rocky, or sandy-rocky bottoms; it is widespread in both marine and brackish waters (Danancher & García-Vázquez, 2007; Froese

and Pauly, 2025). It exhibits a mysterious body colouration, with mucous-covered skin, and undergoes a complete metamorphosis to adapt to benthic life (Amaoka et al., 2001; Figueras et al., 2016; Beken, 2020).

Turbot exhibits remarkable sexual dimorphism in growth. Females grow faster than males by transferring energy to growth, and therefore, reach sexual maturity later than males. Eight months after hatching, males begin to grow less than females and this difference in growth rate continues throughout the rest of the reproductive cycle, including sexual maturation (Imsland et al., 1997; Aydın et al., 2011). This situation has led producers to take an interest in producing entirely female populations (Martínez et al., 2014; Taboada et al., 2018). In turbot, the first genetic differences between male and female gonads become apparent at 90 dpf, and therefore the 75–90 dpf range is considered to be the initial period of sex differentiation (Robledo et al., 2015). In addition to this, histological differentiation between female and male gonads occurs at around 100 dpf (≈7 cm) (Taboada et al., 2018).

Female turbots have a high reproductive potential and lay average 7-8 batches of eggs. During the spawning season, she produces about 0.8 million oocytes per 1 kg of her total weight (2 to 10 kg) (Giragosov, 2020; Baiandina et al., 2022). For a fish obtained from wild with an average total length of 55.7 cm and an average weight of 3742 g, the total fecundity is 2,400,000 eggs and the relative fecundity is 635,000 eggs/kg, while for a fish obtained through hatchery-reared with an average total length of 54.3 cm and an average weight of 3604 g, the total fecundity is 2,300,000 eggs and a relative fecundity of 565,000 eggs/kg (Aydın et al., 2020).

### *2.2. Challenges in Sperm Cryopreservation*

Aquatic organisms have five different reproductive systems: internal fertilisation without copulation, external fertilisation, viviparous, oviparous, and parthenogenesis (Yoshida, 2020). Many fish species carry out external fertilisation (Crowe and Russell, 2009; Gallego and Asturiano, 2019; Yoshida, 2020). Depending on whether they are freshwater or saltwater species, fish spermatozoa are totally immobile in the sperm duct until becoming motile when discharged into the outside environment with a hyper- or hypo-osmotic shock (Morisawa 2008; Gallego & Asturiano, 2019). Reproduction of fish is controlled by manipulating gametes and using the best artificial insemination techniques (Chauvaud et al., 1995). Sperm quality is commonly indicated by the evaluation of the motility parameter. This information is important because sperm must

have enough energy to be fully motile in order to penetrate the egg. Therefore, any sperm characteristic that is measurable and directly related to fertilisation success can be used as a potential sperm quality biomarker. The sperm biomarkers associated with the ability to fertilise the egg in the studies conducted so far are osmolality, pH and chemical composition of seminal plasma (Alavi et al. 2004); enzymatic activity (Burness et al. 2005); ATP concentration (Dzyuba & Cosson 2014); spermatocrit and sperm density (Sørensen et al., 2013); sperm motility (Ottesen et al., 2009; Gallego et al., 2013); or sperm morphology and ultrastructure (Ciereszko et al., 2015). Cryopreservation of turbot sperm enables the production of high-quality offspring, gamete synchronisation, preserves sperm from superior individuals, and provides material for gene banks (Aydın et al., 2020; Polat & Kurtoğlu, 2023).

Cryopreservation provides many advantages such as preservation of genetic diversity of endangered species and valuable wild stocks, creation of genetic banks of specimens threatened with extinction or with favourable traits obtained through genetic manipulation, eliminating the need for continuous breeding for artificial reproduction under farm conditions, creation of hybrids of geographically distant species, solution to the maturation delay between males and females seen in captivity, reducing transport costs and breeding stocks, and reducing the risk of disease transmission (Chereguini et al., 2003; Abed-Elmdoust et al., 2021). The advancement of cryopreservation technologies has caused significant changes in reproductive biotechnology of animals (Figueroa et al., 2019). However, these methods are related to cellular and molecular damage influencing mitochondrial function and spermatozoa quality, and limited studies in fish have been conducted (Figueroa et al., 2016a, 2016b; Magnotti et al., 2018; Figueroa et al., 2019). The DNA integrity, cell metabolism and mobility of cryodamaged spermatozoa are adversely affected, resulting in impaired fertilisation and embryo development (Xin et al., 2020). In addition, the adverse effects of cryopreservation are listed as significant changes in plasma membrane structure, mitochondrial membrane potential ($\Delta\Psi m$), nDNA and mtDNA fragmentation, enzyme inactivation, production of free radicals such as reactive oxygen and nitrogen species (ROS and RNS), ATP concentration and intracellular calcium homeostasis $(Ca^{+2})_i$. Cryopreserved spermatozoa show significant decreases in motility, fertilisation rate, mitochondrial membrane potential and $Ca^{+2}{}_i$. (Figueroa et al., 2018, 2019).

Commonly seen genomic damage after cryopreservation is DNA fragmentation accompanied by lipid peroxidation caused by ROS (Ciereszko

et al., 2020). The addition of antioxidants to extenders reduces the level of cryoinjury (Ciereszko et al., 2020; Polat & Kurtoğlu, 2023).

## 3. Cryopreservation Techniques

### *3.1. Sperm Collection and Preparation*

One of the essential parts of cryopreservation is sperm collection because, based on the species, it allows for the pre-selection of high-quality material to be used. It is essential to use methods that aim to minimise contamination of samples; however, such approaches may limit the volume of sperm obtained from some individuals. Gametes are produced naturally, following hormone administration or by photoperiod treatments. In general, males do not need hormone administration because they produce sperm. The optimum dose for turbot males is 500 IU HCG and 7 mg WSPG/kg bw per liveweight if a high volume of sperm is required. Females are not like males, they need hormone treatment. Maturing females with oocytes larger than 0.4 mm are administered 100 µg pellets of LHRHa/kg per body weight. Although gamete collection may seem simple at first glance, it is often a delicate procedure that requires careful attention, which can adversely affect the quality of the gametes (Gallego & Asturiano, 2019).

Firstly, the urinary bladder is discharged by light pressure on the abdomen of the fish to prevent sperm contamination with urine. Sperm is collected from ripe males by manually stripping it into a 1 mL sterile syringe after carefully cleaning the genital area to prevent accidental contact with water, urine, feces and/or other activating substances that could activate the sperm and stored at 4°C until use (Drenno et al., 1997; Chen et al., 2004; Cabrita et al., 2022) (Figure 1). Sperm samples with high initial motility (min >70%, preferably >90% after 10 seconds of activation) are used for cryopreservation (Drenno et al., 1997). In comparison to other species, turbot produce sperm at a low rate. Wild-caught fish produce an average of 2.3±1.2 mL of sperm during the breeding season (Baiandina et al., 2022), while hatchery-origin individuals produce an average of 4.05±0.30 mL of sperm in the Black Sea region (Polat et al., 2014). Stripped wild Black Sea turbot males are characterised by low and variable spermatozoa density (0.2 $\times 10^9$ to 11 $\times 10^9$ $ml^{-1}$). The average height of 8-year-old Black Sea turbot males originating from hatcheries was 56.20 ± 1.05 cm, and their average weight was 3.25 ± 0.17 g. The average spermatozoid consantration was determined to be $2.2 \pm 0.1 \times 10^9$ $mL^{-1}$ (Polat et al., 2014).

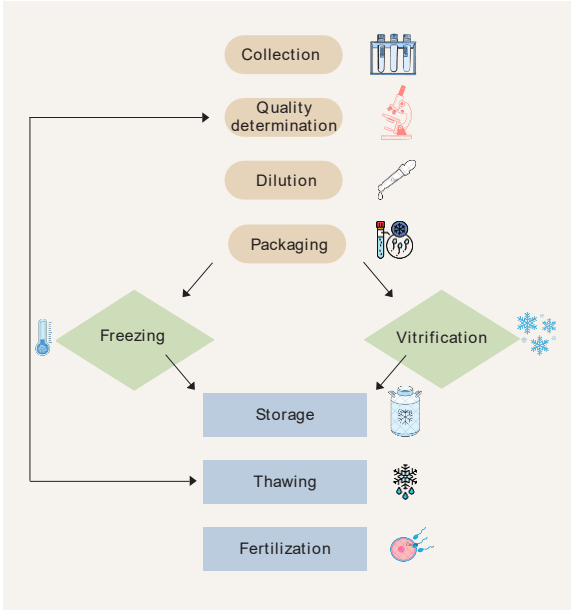


**Figure 1.** Procedures for the cryopreservation of fish sperm (modified from Horváth and Urbányi 2020)

### *3.2. Cryoprotectants and Extenders*

Cryoprotectants are used to preserve cells from the detrimental impacts of freezing and thawing. By binding to water, they lower the solution's freezing point, thereby preventing ice crystals from forming and possibly stabilising cell membranes. (Horváth and Urbányi 2020). Cryoprotectants are classified into two types: non-penetrating and penetrating, according to their action external or internal to cells. Sugars or polymers form external cryoprotectants and have the function of maintaining the osmolarity of the surrounding solution, protecting cells from osmotic stress, lowering the freezing temperature of the solution and providing spermatozoa with nutrients (Lahnsteiner et al., 1993). Glycerol was used as the first internal cryoprotectant. During these periods, numerous chemicals with cryoprotective properties were identified (Blaxter, 1953). However, few cryoprotectants are used for fish sperm cryopreservation. These are dimethylsulfoxide (DMSO), methanol (MeOH), dimethylacetamide (DMA), ethylene glycol (EG), propylene glycol (PG) and methyl glycol (Mounib 1978; Legendre and Billard 1980; Kurokura et al., 1984; Harvey et al., 1982; Lahnsteiner et al., 1996; Horváth et al. 2003; McNiven et al., 1993; Horváth and Urbányi 2000; Morris et al. 2003; Jähnichen et al., 1999; Conget

et al., 1996; Viveiros et al., 2012, 2014; Horváth and Urbányi 2020). In addition to cryoprotectants, they have demonsrated that the freezing rate also has a profound effect on cryopreservation success and that a two-stage slow freezing process with a slow initial cooling rate of 0.5-1 °C/minute is suitable for almost all organisms and cell types. However, vitrification, which has been used as a rapid freezing technique in recent years, also gives very successful results in the cryopreservation of alternative cell sources. (Arslan 2023).

Sperm is diluted in a solution called cryomedia before freezing. Extender and cryoprotectant are the two main components that make up cryomedyum. Extenders are composed of salt, sugar, and other chemicals and are responsible for immobilising sperm so that it can move after being thawed (Glenn III et al., 2011; Horváth & Urbányi, 2020). Besides, it contains chemicals that minimise the formation of cryoinjury in the cell by ice crystals that occur between cells during the freezing and thawing stages of cryopreservation (Thilak Pon Jawahar & Betsy, 2020). The success of cryopreservation therefore depends on preventing intracellular ice formation, controlling the increasing solute concentration by removing water from the medium, and managing the interplay between these two conditions (Thilak Pon Jawahar & Betsy, 2020). The extender contents used for turbot sperm cryopreservation are listed in Table 1.

**Table 1**. Extender composition for turbot sperm

| Compsition | D15 | MPRS | TS-2 | MMM | ASL1 | ASL2 | MRM | RM |
|---|---|---|---|---|---|---|---|---|
| NaCl (mM) | 136.75 | 60.35 | - | - | 70 | 70 | 74 | 150 |
| NaHCO3 (mM) | - | 3.0 | - | - | | 25 | 1.59 | 7 |
| KCl (mM) | 6.71 | 5.23 | - | - | 1.5 | 1.5 | 26.8 | 2.5 |
| $CaCl_2$ (mM) | - | - | - | - | 2.7 | 2.7 | 1.8 | 3.5 |
| $MgCl_2$ (mM) | - | - | - | - | 6.1 | 6.1 | - | 1 |
| D-Glucose (mM) | 83.33 | 55.55 | - | - | - | - | - | 2.8 |
| Sucrose (Mm) | - | - | 110 | 125 | - | - | - | - |
| $KHCO_3$ (Mm) | - | - | 100 | 100 | - | - | - | - |
| Reduced gluthation (Mm) | - | 1.8 | - | 6.5 | - | - | - | - |
| HEPES (mM) | - | - | - | - | - | - | - | 10 |
| BSA (mg/ml) | - | 1.13 | - | 10 | 10 | 10 | 10 | - |
| Tris–Cl (mM) | - | 1.13 | 10 | - | - | - | - | - |
| pH | 6.5 | 6.68 | 8.20 | 7.8 | 8.2 | 8.0 | 8.0 | 7.4 |
| Osmotic pressure (mosM/kg) | 363 | 202 | 335 | 310 | 200 | 200 | 200 | |
| DMSO | 10 % | 10 % | 10 % | 10 % | 10 % | 10 % | 10 % | 10 % |
| Dilution ratio (sperm: extender) | 1:3 | 1:3 | 1:3 | 1:2 | 1:2 | 1:2 | 1:2 | 1:9 |
| Source | Legendre and Billard, 1980 Chereguini et al., 2003 Chen et al., 2004 | Chen et al., 2004 Yao et al., 2000 | Chen et al., 2004 | Drenno et al., 1997 Suquet et al., 1998 | Drenno et al., 1997 | Drenno et al., 1997 | Drenno et al., 1997 | Polat and Kurtoğlu, 2023 |

D-15: Developed from Chinese carps cryopreservation medium (Chen et al., 1992)
MPRS: modified plaice Ringer solution
TS-2: Modified Mounib (Mounib, 1978)
MMM: Modified Mounib Medium
ASL: Artificial Seminal Liquid
MRM: Modified Ringer Medium
RM: Ringer Medium

### *3.3. Freezing and Thawing Protocols*

Freezing protocols have been developed for species specific around the world. Numerous studies have focused on species that live in temperate climates and reproduce seasonally, such as salmon and carp, which have been studied most extensively due to their high commercial value (Martínez-Páramo et al., 2016). Sperm packed in straws or cryovials can be frozen in LN (liquid nitrogen) vapour under controlled or uncontrolled circumstances. Uncontrolled

cooling is performed by placing a floating tray oo liquid nitrogen that has been inserted into a Styrofoam box. A controlled-rate freezer consists of a freezing chamber and a computer. The cooling speed can be adjusted by the user through a program installed on the computer (Diogo et al., 2018; Horváth & Urbányi, 2020).

The freezing process involves the Styrofoam box is filled with liquid nitrogen, and a floating rack is placed inside so that it is 5 cm above the nitrogen vapour. Sperm is first stored in straws with volumes of 0.25 and 0.5 mL or in cryovials with volumes of 1.2 and 5 mL for varying periods depending on the volume, and then directly plunged in liquid nitrogen (Horváth & Urbányi, 2020). Cryovials are gradually cooled in liquid nitrogen vapour. In this context, they are first equilibrated for 10 minutes approximately 6 cm above the surface of the LN (at around -180 °C), then placed on the surface of the LN and left for another 5 min. In the final stage, cryovials are immersed directly into liquid nitrogen to complete the freezing process (Chen et a., 2004). Turbot sperm extender ratios of 1:1, 1:2, 1:3, 1:4 and 1:9 were applied in different ways (Drenno et al., 1997; Chereguini et al., 2003; Chen et a., 2004; Suquet et al., 2008; Beken et al., 2014; Polat & Kurtoğlu, 2023).

Straws are filled with semen and equilibrated 6.5 cm above the surface of LN, and then involved in the LN (Drenno et al., 1997; Polat & Kurtoğlu, 2023). Straws are thawed by placing them in a water bath set at 40 °C for 7 sec. After the straws have been dissolved, their ends are cut off and they are transferred to Eppendorf tubes for further analysis and fertilisation trials (Chereguini et al., 2003).

## 4. Evaluation of Cryopreserved Sperm

### *4.1. Assessment of Sperm Viability and Motility*

CASA is a technique used worldwide that allows a large number of spermatozoa to be analysed quickly and highly objectively, leading to a set of kinetic parameters (Boryshpolets et al., 2018). In recent years, CASA has become the standard procedure in fish sperm cryopreservation research to determine sperm quality (Beirão et al. 2011; Bernáth et al., 2016; Judycka et al., 2018; Riesco et al., 2017; Horváth & Urbányi, 2020). Among sperm quality indicators, sperm density is measured by the number of spermatozoa per milliliter of sperm using various haemocytometers (Thoma, Improved Neubauer, Neubauer, Funchs-Rosenthal etc.). The membrane integrity of sperm cells, often referred to as viability, serves to characterise the quality of sperm (Horváth & Urbányi,

2020). CASA systems consist of a phase contrast microscope, a camera and software that allows the video sequences to be analysed (WHO, 2010). The accuracy of these expensive systems relies on the analyse configuration and the sperm dilution rate (Horváth & Urbányi, 2020). In addition to providing better measurement of motility parameters, this software yields more accurate results as it can generate various variables related to sperm velocity, movement patterns and trajectory linearity and track each spermatozoon (motile or immobile) (Robles et al., 2009).

As an alternative method, a CASA software package that functionsas an add-on to the United States National Institutes of Health (NIH) ImageJ software is publicly accessible and free of charge, allowing all research laboratories to access CASA software and creating standardisation opportunities. Although the IMAGEJ plug-in is publicly available, for a large number of video sequences, it is not as efficient as other CASA software (e.g., ISAS, Hamilton Thorne), as the data processing time after video acquisition is significant (Purchase & Earle, 2012).

To determine the sperm quality of the turbot under a microscope, 1 µL of sperm is pipetted into a Macler chamber and activated by adding 10 µL of seawater with a salinity of 18 ppt as the activation medium (Chauvaud et al., 1995; Beken unpublished).

### *4.2. Fertilization Success and Hatchability*

In fertilisation studies conducted with cryopreserved turbot sperm to date, sperm-egg ratios of 1000:1, 2000:1, 3000:1, 5000:1, 6000:1, 10000:1 and 20000:1 have been used (Drenno et al., 1997; Suquet et al., 1998; Chen et al., 2004; Polat & Kurtoğlu, 2023). Sperm samples are immediately added to the eggs and activated with seawater. Five minutes after activation, the eggs are rinsed with incubation water and than incubated in a temperature-controlled incubator or incubation tanks, if large volumes are used. In fertilisation experiments with frozen turbot semen, Ringer's solution and modified Mounib diluent were used as diluents as well as different sperm: extender ratios (1:2, 1:3) and no difference was found between the results obtained. Fertilisation rate is calculated by counting at least 100 eggs in each group by determining the number of eggs that have reached the cell stage in 2-4 division within the total number of eggs (Chereguini et al., 2001). In the study, fertilisation rates were obtained between <69.2% and >90.4%. (Chereguini et al., 2003).

In another study, Modified mounib solution was used at a ratio of 1:2 (sperm: extender) and fertilisation rates were obtained between <50%

<70%. Cryopreservation of turbot sperm was carried out with two different sized containers (0.5 ml straws and 2 ml cryotubes) with no significantly different fertilisation rates observed. Fresh and cryopreserved sperm samples collected from forty-nine turbot did not show any significance difference in fertilisation and hatching rates (Chereguini et al., 2003; Suquet et al., 2008). Cryopreserved sperm shows similar fertilisation and hatching rates to fresh sperm (Chen et al., 2004).

## 5. Conclusion

Turbot has very low sperm volume and spermatozoa concentration, and therefore the application of sperm preservation techniques for artificial reproduction is inevitable (Cabrita et al., 2008). Cryopreserved sperm samples can provide high-quality male genetic information ready for hatcheries, intensive larval production, selective breeding programmes or research needs (Boryshpolets et al., 2020). However, it is necessary to continue studies aimed at optimising cryopreservation processes by taking into account species-specific biological characteristics, evaluating sperm quality using objective criteria, and increasing the fertilisation capacity of frozen sperm. Conservation programmes need large populations to ensure biodiversity. On the other hand, aquatic organisms are threatened and their numbers are declining (Bozkurt, 2023).

Genetic resources, which are of vital importance for future generations, are declining day by day due to factors such as excessive and uncontrolled hunting, habitat destruction and global climate change. In this context, aquatic organisms have not been sufficiently protected and many of them have disappeared or are in danger of extinction (Bozkurt, 2023). Although many protocols can obtain viable spermatozoa after thawing in aquatic species, the economic applications of cryopreservation are still very limited (Beken, 2023). Furthermore, the dissemination of cryobanking practices plays a strategic role in preserving the genetic diversity of the species and supporting sustainable breeding programmes for future generations. In conclusion, it is of great importance to further strengthen the research on turbot sperm cryopreservation with multidisciplinary approaches.

Research on turbot sperm cryopreservation has made significant progress; however numerous topics remain to be investigated in light of new technologies and applications. Future studies should focus on optimising species-specific cryoprotectants and their concentrations, developing sperm extender ingredients and standardising freeze-thaw protocols. Besides, it would be helpful to follow the long-term effects of fertilisation capacity, which is a good indicator of

cryopreservation success, on embryo development and offspring quality. The use of advanced imaging techniques, biomarker analyses, and artificial intelligence-assisted monitoring systems should be encouraged to assess sperm quality after cryopreservation objectively. However, the expansion of cryobanking practices for the conservation of genetic diversity is a strategic approach in terms of sustainable breeding and genetic breeding programmes.

**References**

Abed-Elmdoust, A., Uysal, O., Rahimi, R., & Farahmand, Y. (2021). Antifreeze proteins are robust cryoprotectants for sperm cryopreservation in fishes: A systematic review and meta-analysis. *Aquaculture*, *534*, 736250.

Alavi SMH, Cosson J, Karami M, Abdolhay H, Amiri BM (2004) Chemical composition and osmolality of seminal fluid of *Acipenser persicus*; their physiological relationship with sperm motility. Aquaculture Research 35: 1238–1243.

Amaoka, K., Yoseda, K., Şahin, T., Üstündağ, C. ve Çiftçi, Y., 2001, Flatfishes (Order Pleuronectiformes) Found in Black Sea and Its Adjacent Waters. Special publication No.1, Central FisheriesResearch Institute, Ministry of Agriculture and Rural Affairs, Trabzon, Turkey, 27 pp.

Arslan T., 2023. Cryopreservation of alternative cell sources for cryobanking of aquatic species. Bozkurt Y, editor. Modern Reproductive Biotechnologies in the Conservation of Aquatic Genetic Resources. 1st ed. Ankara: Türkiye Klinikleri. p.18-30.

Aydın, I., Polat, H., & Sahin, T. (2020). Reproductive performance of wild and hatchery-reared black sea turbot, *Psetta maxima*, in the southern black sea coast. *Turkish Journal of Fisheries and Aquatic Sciences*, 20(5), 351–357. https://doi.org/10.4194/1303-2712-v20_5_03

Aydin, I., Sahin, T., Kolotoglu, L., & Özongun, M. (2011). The effect of sexual dimorphism on growth of the black sea turbot, Psetta maxima. *Journal of FisheriesSciences. com*, *5*(1), 47.

Aydin, İ., Öztürk, R. Ç., Polat, H., Beken, A. T., Terzi, Y., Özel, O. T., ... & Küçük, E. (2022). Sperm characteristics of wild-caught and hatchery-reared turbot, Scophthalmus maximus, originated from the Black Sea. *Journal of Applied Ichthyology*, *38*(1), 73-83.

Baiandina, I., Giragosov, V., & Khanaychenko, A. (2022). Male reproductive potential in the Black Sea turbot (Scophthalmus maximus) spawning populations. *Fisheries Research*, *253*, 106367.

Beirão J, Cabrita E, Pérez-Cerezales S, Martínez-Páramo S, Herráez MP (2011) Effect of cryo- preservation on fish sperm subpopulations. Cryobiology 62:22–31. https://doi.org/10.1016/j. cryobiol.2010.11.005

Beken AT., 2023. Biobanking of aquatic species. Bozkurt Y, editor. Modern Reproductive Bio- technologies in the Conservation of Aquatic Genetic Resources. 1st ed. Ankara: Türkiye Klinikleri; p.12-9.

Beken, A.T., 2020. *In Vivo* and *In Vitro* Evolution of the Effects of Cypermethrin on Turbot (*Scophthalmus maximus*, Linnaeus, 1758) Spermatozoa. Ege University Institute of Natural Sciences, Doctoral Thesis 170p.

Beken, A.T., 2024. Yassı Balık Yetiştiriciliğine Bakış: Kalkan. *Skretting Insight Su Ürünleri Dergisi* 1, 32-35s.

Beken, A.T., Polat, H., Aydın, ˙I., 2014. Cryopreservation of Turbot (*Psetta maxima*) Sperm with Different Cooling Protocols. In: Aydin, I. (Ed.), FABA 2014: International Symposium on Fisheries and Aquatic Sciences. CFRI press, Trabzon, Turkey, p. 339. https://doi.org/10.13140/RG.2.2.24023.93607, 580 pp.

Bernáth G, Bokor Z, Żarski D, Várkonyi L, Hegyi Á, Urbányi B, Radóczi Ifj J, Horváth Á (2016) Commercial-scale out-of-season cryopreservation of Eurasian perch (*Perca fluviatilis*) sperm and its application for fertilization. Anim Reprod Sci 170:170–177. https://doi.org/10.1016/j. anireprosci.2016.05.005

Blaxter JHS (1953) Sperm storage and cross-fertilization of spring and autumn spawning herring. Nature 172:1189–1190

Bolla S., Holmefjord I. & RefstieT. (1987) Cryogenic preservation of Atlantic halibut sperm. Aquaculture 65, 371-374.

Boryshpolets, S., Kholodnyy, V., Cosson, J., & Dzyuba, B. (2018). Fish sperm motility analysis: the central role of the flagellum. *Reproduction, Fertility and Development*, *30*(6), 833-841.

Boryshpolets, S., Kholodnyy, V., Cosson, J., & Dzyuba, B. (2020). Fish sperm quality evaluation after cryopreservation. *Cryopreservation of fish gametes*, 117-133.

Bozkurt Y., 2023. Conservation strategies for the aquatic genetic resources: Opportunities and challenges. Bozkurt Y, editor. Modern Repro- ductive Biotechnologies in the Conservation of Aquatic Genetic Resources. 1st ed. Ankara: Türkiye Klinikleri. p.1-5.

Burness G, Moyes CD, Montgomerie R (2005) Motility, ATP levels and metabolic enzyme activity of sperm from bluegill (*Lepomis macrochirus*). Comparative Biochemistry and Physiology – A Molecular and Integrative Physiology 140: 11–17

Cabrita, E., Horváth, Á., Marinović, Z., & Asturiano, J. F. (2022). Technologies and strategies for ex situ conservation of aquatic organisms: the role of cryopreservation in long-term management. In *Cellular and Molecular Approaches in Fish Biology* (pp. 1-48). Academic Press.

Cabrita, E., Robles, V. & Herráez, P., eds. 2008. *Methods in reproductive aquaculture: marine and freshwater species.* Boca Raton, Florida, CRC Press.

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., & Herráez, M. P. (2010). Cryopreservation of fish sperm: applications and perspectives. *Journal of Applied Ichthyology*, *26*(5), 623-635.

Cardinale, M., Chanet, B., Martínez Portela, P., Munroe, T.A., Nimmegeers, S., Shlyakhov, V., Turan, C. & Vansteenbrugge, L. 2021. *Scophthalmus maximus*. *The IUCN Red List of Threatened Species* 2021: e.T198731A144939322. https://dx.doi.org/10.2305/IUCN.UK.2021- 2.RLTS.T198731A144939322.en

Chauvaud, L., Cosson, J., Suquet, M. *et al.* Sperm motility in turbot, *Scophthalmus marimus*: initiation of movement and changes with time of swimming characteristics. *Environ Biol Fish* **43**, 341–349 (1995). https://doi.org/10.1007/BF00001167

Chen, S. L., & Tian, Y. S. (2005). Cryopreservation of flounder (Paralichthys olivaceus) embryos by vitrification. *Theriogenology*, *63*(4), 1207-1219.

Chen, S. L., Ji, X. S., Yu, G. C., Tian, Y. S., & Sha, Z. X. (2004). Cryopreservation of sperm from turbot (*Scophthalmus maximus*) and application to large-scale fertilization. *Aquaculture*, *236*(1-4), 547-556.

Chen, S.-L., Liu, X.-T., Lu, D.-C., Zhang, L.-Z., Fu, C.-J., Fang, J.-P., 1992. Cryopreservation of spermatozoa of silver carp, common carp, blunt snout bream and grass carp. Acta Zool. Sin. 38, 413–424.

Chereguini O., Fernandez Pato C. & Rasines I. (1992) Adaptacion de la tecnica de crioconservacion de esperma de rodaballo (*Scophthalmus maximus*) y besugo (Pagellus bogaraveo). InformeTecnico Instituto Espanìol de Oceanografia 117, 1-13.

Chereguini, O., I. Garíca De La Banda, Inmaculada Rasines, and A. Fernandez. "Larval growth of turbot, Scophthalmus maximus (L.) produced with fresh and cryopreserved sperm." *Aquaculture Research* 32, no. 2 (2001): 133-143.

Chereguini, O., García de la Banda, I., Herrera, M., Martinez, C., & De la Hera, M. (2003). Cryopreservation of turbot Scophthalmus maximus (L.) sperm: fertilization and hatching rates. *Aquaculture Research*, *34*(9), 739-747.

Ciereszko A, Hatef A, K_ri_sann J, Dzyuba B, Boryshpolets S, Rodina M et al. (2015) Sperm morphology, physiology, motility, and cryopreservation in percidae. In: Kestemont P, Dabrowski K, Summerfelt RC (eds) Biology and Culture of Percid Fishes: Principles and Practices, pp. 163–191. Springer, Netherlands, Dordrecht.

Ciereszko, A., Judycka, S., Nynca, J., Słowińska, M., & Dietrich, M. A. (2020). Factors influencing milt quality in fishes and its usefulness to cryopreservation. *Cryopreservation of fish gametes*, 25-67.

Conget P, Fernández M, Herrera G, Minguell JJ (1996) Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) spermatozoa using programmable freezing. Aquaculture 143:319–329

Cosson, J., Groison, A. L., Suquet, M., Fauvel, C., Dreanno, C., & Billard, R. (2008). Studying sperm motility in marine fish: an overview on the state of the art. *Journal of Applied Ichthyology*, *24*(4), 460-486

Crowe TP, Russell R (2009) Functional and Taxonomic Perspectives of Marine Biodiversity. In: Wahl M (ed) Marine Hard Bottom Communities. In: Marine Hard Bottom Communities: Patterns, Dynamics, Diversity, and Change, pp. 375–390. Springer, Berlin Heidelberg, Berlin, Heidelberg.

Danancher, D. and García-Vázquez, E., 2007, Turbot-Scophthalmus maximus. Genetic impact of aquaculture activities on native populations. Genimpact-Evaluation of genetic impact of aquaculture activities on native populations, 55- 61 p. (http://genimpact.imr.no/) (Eylül 2019)

Ding, F., Lall, S. P., Li, J., Lei, J., Rommens, M., & Milley, J. E. (2011). Cryopreservation of sperm from Atlantic halibut (*Hippoglossus hippoglossus*, L.) for commercial application. *Cryobiology*, *63*(1), 56-60.

Diogo P, Martins G, Quinzico I, Nogueira R, Gavaia PJ, Cabrita E (2018) Electric ultrafreezer (-150 °C) as an alternative for zebrafish sperm cryopreservation and storage. Fish Physiol Biochem 44(6):1443–1455. https://doi.org/10.1007/s10695-018-0500-6

Dreanno, C., Suquet, M., Quemener, L., Cosson, J., Fierville, F., Normant, Y., & Billard, R. (1997). Cryopreservation of turbot (*Scophthalmus maximus*) spermatozoa. *Theriogenology*, *48*(4), 589-603

Dzyuba V, Cosson J (2014) Motility of fish spermatozoa: from external signaling to flagella response. Reproductive Biology 14: 165–175.

Fauvel C, Savoye O, Dreanno C, Cosson J, Suquet M (1999) Characteristics of sperm of cap- tive seabass in relation to its fertilization potential. J Fish Biol 54:356–369. https://doi. org/10.1111/j.1095-8649.1999.tb00835.x

Figueras, A., Robledo, D., Corvelo, A., Hermida, M., Pereiro, P., Rubiolo, J.A., Gómez-Garrido, J., Carreté, L., Bello, X., Gut, M., Gut, I.G., Marcet-Houben, M., Forn-Cuní1, G., Galán, B., García, J.L., Abal-Fabeiro, J.L., Pardo, B.G., Taboada, X., Fernández, C., Vlasova, A., Hermoso-Pulido, A., Guigó, R., Álvarez-Dios, J.A., Gómez-Tato, A., Viñas, A., Maside, X., Gabaldón, T., Novoa1, B., Bouza, C., Alioto, T., Martínez, P., 2016, Whole genome sequencing of turbot (*Scophthalmus maximus*; Pleuronectiformes): a fish adapted to demersal life. DNA Research, 23(3), 181-192 p. DOI: doi: 10.1093/dnares/dsw007

Figueroa, E., Lee-Estévez, M., Valdebenito, I., Farías, J.G., Romero, J., 2018. Potential biomarkers of DNA quality in cryopreserved fish sperm: impact on gene expression and embryonic development. Rev. Aquac. 1–10. https://doi.org/10.1111/raq.12323.

Figueroa, E., Lee-Estevez, M., Valdebenito, I., Watanabe, I., Oliveira, R. P. D. S., Romero, J., ... & Farías, J. G. (2019). Effects of cryopreservation on mitochondrial function and sperm quality in fish. *Aquaculture*, *511*, 634190.

Figueroa, E., Valdebenito, I., Farias, J.G., 2016a. Technologies used in the study of sperm function in cryopreserved fish spermatozoa. Aquac. Res. 47, 1691–1705.

Figueroa, E., Valdebenito, I., Merino, O., Ubilla, A., Risopatron, J., Farias, J.G., 2016b. Cryopreservation of Atlantic salmon *Salmo salar* sperm: effects on sperm physiology. J. Fish Biol. 89, 1537–1550.

Froese, R. and Pauly, D., 2025, FishBase. *Psetta maxima* (Linnaeus, 1758). Accessed through: World Register of Marine Species at: http://www.marinespecies.org/aphia.php?p=taxdetails&id=154473 (Access date: 08.06.2025).

Gallego V, P_erez L, Asturiano JF, Yoshida M (2013) Relation- ship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (*Takifugu niphobles*). Aquaculture 416–417: 238–243.

Gallego, V., & Asturiano, J. F. (2019). Fish sperm motility assessment as a tool for aquaculture research: a historical approach. *Reviews in Aquaculture*, *11*(3), 697-724.

Giragosov, V.E., 2020. Forming and realization of fecundity in the Black Sea Turbot *Scophthalmus maeoticus* (Scophthalmidae) at the South-Western Shelf of the Crimean Peninsula. J. Ichthyol. 60 (4), 608–617. https://doi.org/10.1134/s0032945220040050.

Glenn DW III, Lang RP, Tiersch TR (2011) Evaluation of extenders for refrigerated storage of koi carp and goldfish sperm. In: Tiersch TR, Green CC

(eds) Cryopreservation in aquatic species, 2nd edn. World Aquaculture Society, Baton Rouge, pp 107–124

Harvey B, Kelley RN, Ashwood-Smith MJ (1982) Cryopreservation of zebra fish spermatozoa using methanol. Can J Zool 60:1867–1870

Horváth Á, Miskolczi E, Urbányi B (2003) Cryopreservation of common carp sperm. Aquat Living Resour 16:457–460. https://doi.org/10.1016/S0990-7440(03)00084-6

Horváth Á, Urbányi B (2000) The effect of cryoprotectants on the motility and fertilizing capac- ity of cryopreserved African catfish *Clarias gariepinus* (Burchell 1822) sperm. Aquacult Res 31:317–324. https://doi.org/10.1046/j.1365-2109.2000.00444.x

Horváth, Á and Urbányi, B., 2020. Sperm Cryopreservation of Aquatic Species. M. Yoshida, J. F. Asturiano (eds.), *Reproduction in Aquatic Animals*, https://doi.org/10.1007/978-981-15-2290-1_16

Imsland, A. K., Folkvord, A., Grung, G. L., Stefansson, S. O., & Taranger, G. L. (1997). Sexual dimorphism in growth and maturation of turbot, Scophthalmus maximus (Rafinesque, 1810). *Aquaculture Research*, *28*(2), 101-114.

Jähnichen H, Warnecke D, Trölsch E, Kohlman K, Bergler H, Pluta HJ (1999) Motility and fertil- izing capability of cryopreserved *Acipenser ruthenus* L. sperm. J Appl Ichthyol 15:204–206. https://doi.org/10.1111/j.1439-0426.1999.tb00235.x

Judycka S, Nynca J, Liszewska E, Dobosz S, Grudniewska J, Ciereszko A (2018) Optimal sperm concentration in straws and final glucose concentration in extender are crucial for improving the cryopreservation protocol of salmonid spermatozoa. Aquaculture 486:90–97. https://doi. org/10.1016/j.aquaculture.2017.12.019

Kurokura H, Hirano R, Tomita M, Iwahashi M (1984) Cryopreservation of carp sperm. Aquaculture 37:267–273. https://doi.org/10.1016/0044-8486(84)90159-5

Lahnsteiner, F., Patzner, R. A. & Weismann, T. (1993). Energy resources of spermatozoa of the rainbow trout (*Oncorhynchus mykiss*) (Pisces, Teleostei). *Reproduction, Nutrition, Development* **33**, 349–360

Lahnsteiner F, Berger B, Weismann T, Patzner R (1996) The influence of various cryoprotectants on semen quality of the rainbow trout (*Oncorhynchus mykiss*) before and after cryopreservation. J Appl Ichthyol 12:99–106. https://doi.org/10.1111/j.1439-0426.1996.tb00070.x

Lahnsteiner F. (2000) Semen cryopreservation in the Salmonidae and in the Northern pike. Aquaculture Research 31, 245-258.

Lahnsteiner, F., Berger, B., Weismann, T., & Patzner, R. (1998). Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. *Aquaculture*, **163**(1–2), 163–181. https://doi.org/10.1016/S0044-8486(98)00243-9

Legendre M, Billard R (1980) Cryopreservation of rainbow trout sperm by deep-freezing. Reprod Nutr Dev 20:1859–1868. https://doi.org/10.2331/suisan.46.1493

Liu, Q. H., Ma, D. Y., Xu, S. H., Xiao, Z. Z., Xiao, Y. S., Song, Z. C., & Li, J. (2015). Summer flounder (*Paralichthys dentatus*) sperm cryopreservation and application in interspecific hybridization with olive flounder (*P olivaceus*). *Theriogenology*, *83*(4), 703-710.

Magnotti, C., Cerqueira, V., Lee-Estevez, M., Farias, J.G., Valdebenito, I., Figueroa, E., 2018. Cryopreservation and vitrification of fish semen: a review with special em- phasis on marine species. Rev. Aquac. 10 (1), 15–25.

Martínez-Páramo, S., Horváth, Á., Labbé, C., Zhang, T., Robles, V., Herráez, P., ... & Cabrita, E. (2017). Cryobanking of aquatic species. *Aquaculture*, *472*, 156-177.

Martínez, P., Viñas, A., Sánchez, L., *et al.* (2014). Genetic architecture of sex determination in fish: applications to sex ratio control in aquaculture. *Frontiers in Genetics* **5**, 340.

McNiven MA, Gallant RK, Richardson GF (1993) Dimethyl-acetamide as a cryoprotectant for rainbow trout spermatozoa. Theriogenology 40:943–948

Morisawa M (2008) Adaptation and strategy for fertilization in the sperm of teleost fish. Journal of Applied Ichthyology 24: 362–370.

Morris JP, Berghmans S, Zahrieh D, Neuberg DS, Kanki JP, Look AT (2003) Zebrafish sperm cryopreservation with N,N-dimethylacetamide. Biotechniques 35:956–968

Mounib MS (1978) Cryogenic preservation of fish and mammalian spermatozoa. J Reprod Fertil 53:13–18. https://doi.org/10.1530/jrf.0.0530013

Ottesen OH, Babiak I, Dahle G (2009) Sperm competition and fertilization success of Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture 286: 240–245.

Pan, J. M., Zhu, K. C., Liu, J., Guo, H. Y., Liu, B. S., Zhang, N., ... & Zhang, D. C. (2023). Cryopreservation of black seabream (Acanthopagrus schlegelii) sperm. *Theriogenology*, *210*, 182-191.

Polat, H., Beken, A.T., Aydın, ˙I., Küçük, E., 2014. Spermatological characteristics of hatchery-reared Black Sea turbot (*Psetta maxima*) in spawning

season. In: Aydin, I. (Ed.), FABA 2014: International Symposium on Fisheries and Aquatic Sciences. CFRI press, Trabzon, Turkey, p. 283. https://doi.org/10.13140/RG.2.2.24023.93607, 580 pp.

Polat, H., Kurtoğlu, İ.Z. (2023). Effect of Antioxidants on Cryopreserved Turbot (*Scophthalmus maximus*) Spermatozoa Quality and DNA Damage. *Turkish Journal of Fisheries and Aquatic Sciences*, *23(4)*, *TRJFAS22300.* https://doi.org/10.4194/TRJFAS22300

Pullin R. (1972) The storage of plaice (Pleuronectes platessa) sperm at low temperature. Aquaculture 1, 279-283.

Purchase, C. F., & Earle, P. T. (2012). Modifications to the IMAGEJ computer assisted sperm analysis plugin greatly improve efficiency and fundamentally alter the scope of attainable data. *Journal of Applied Ichthyology*, *28*(6), 1013-1016.

Richardson G.F., Wilson C.E., Crim L.W. & Yao Z. (1999) Cryopreservation of yellowtail flounder (Pleuronectes ferrugineus) semen in large straws. Aquaculture 174, 89-94.

Riesco MF, Raposo C, Engrola S, Martínez-Páramo S, Mira S, Cunha ME, Cabrita E (2017) Improvement of the cryopreservation protocols for the dusky grouper, *Epinephelus margin- atus*. Aquaculture 470:207–213. https://doi.org/10.1016/j.aquaculture.2016.12.027

Robledo, D., Ribas, L., Cal, R., *et al.* (2015). Gene expression analysis at the onset of sex differentiation in turbot (*Scophthalmus maximus*). *BMC Genomics* **16**, 973.

Robles, Vanesa, Elsa Cabrita, and M. Paz Herráez., 2009 "Germplasm cryobanking in zebrafish and other aquarium model species." *Zebrafish* 6, no. 3: 281-293.

Saitoh S. (1996) Cryopreservation of flatfishsh sperm. Science Report Hokkaido Fisheries Experimental Station 48, 9-17.

Sørensen SR, Gallego V, Perez L, Butts IAE, Tomkiewicz J, Asturiano JF (2013) Evaluation of methods to determine sperm density for the European eel, *Anguilla anguilla*. Reproduction in Domestic Animals 48: 936–944

Suquet, M., Chereguini, O., & Fauvel, C. (2008). Cryopreservation of sperm in turbot (*Psetta maxima*). In *Methods in Reproductive Aquaculture* (pp. 485-490). CRC Press.

Suquet, M., Dreanno, C., Petton, B., Normant, Y., Omnes, M. H., & Billard, R. (1998). Long-term effects of the cryopreservation of turbot (Psetta maxima) spermatozoa. *Aquatic Living Resources*, *11*(1), 45-48.

Suquet, M., Omnes, M. H., Normant, Y., & Fauvel, C. (1992). Assessment of sperm concentration and motility in turbot (Scophthalmus maximus). *Aquaculture*, *101*(1-2), 177-185.

Taboada, X., Robledo, D., Bouza, C., Piferrer, F., Viñas, A. M., & Martínez, P. (2018). Reproduction and sex control in turbot. *Sex Control in Aquaculture*, 565-582.

Thilak Pon Jawahar, K., & Betsy, J. (2020). Cryopreservation of fish gametes: An overview. *Cryopreservation of fish gametes*, 151-175.

Viveiros ATM, Nascimento AF, Leal MC, Gonçalves ACS, Orfão LH, Cosson J (2014) Methyl glycol, methanol and DMSO effects on post-thaw motility, velocities, membrane integrity and mitochondrial function of *Brycon orbignyanus* and *Prochilodus lineatus* (Characiformes) sperm. Fish Physiol Biochem 41:193–201. https://doi.org/10.1007/s10695-014-0016-7

Viveiros ATM, Orfão LH, Nascimento AF, Corrêa FM, Caneppele D (2012) Effects of extenders, cryoprotectants and freezing methods on sperm quality of the threatened Brazilian freshwater fish pirapitinga-do-sul *Brycon opalinus* (Characiformes). Theriogenology 78:361–368. https:// doi.org/10.1016/j.theriogenology.2012.02.015

Xin, M., Niksirat, H., Shaliutina-Kolešová, A., Siddique, M. A. M., Sterba, J., Boryshpolets, S., & Linhart, O. (2020). Molecular and subcellular cryoinjury of fish spermatozoa and approaches to improve cryopreservation. *Reviews in Aquaculture*, *12*(2), 909-924.

Yao, Z., Crim, L.W., Richardson, G.F., Emerson, C.J., 2000. Motility, fertility and ultrastructural changes of ocean pout (Macrozoarces americanus L.) sperm after cryopreservation. Aquaculture 181, 361–375.

Yongsheng, T., Jingjing, Z., Zhentong, L., Ziqi, L., & Linna, W. (2020). Cryopreservation of Marine Fish Sperm. *Cryopreservation of Fish Gametes*, 187-210.

Yoshida, M. (2020). Chapter 1 Overview: Reproductive Systems in Aquatic Animals. *Reproduction in aquatic animals: from basic biology to aquaculture technology*, 13-21.

Zidni, I., Lee, Y. H., Park, J. Y., Lee, H. B., Hur, J. W., & Lim, H. K. (2020). Effects of cryoprotective medium composition, dilution ratio, and freezing rates on spotted halibut (*Verasper variegatus*) sperm cryopreservation. *Animals*, *10*(11), 2153.