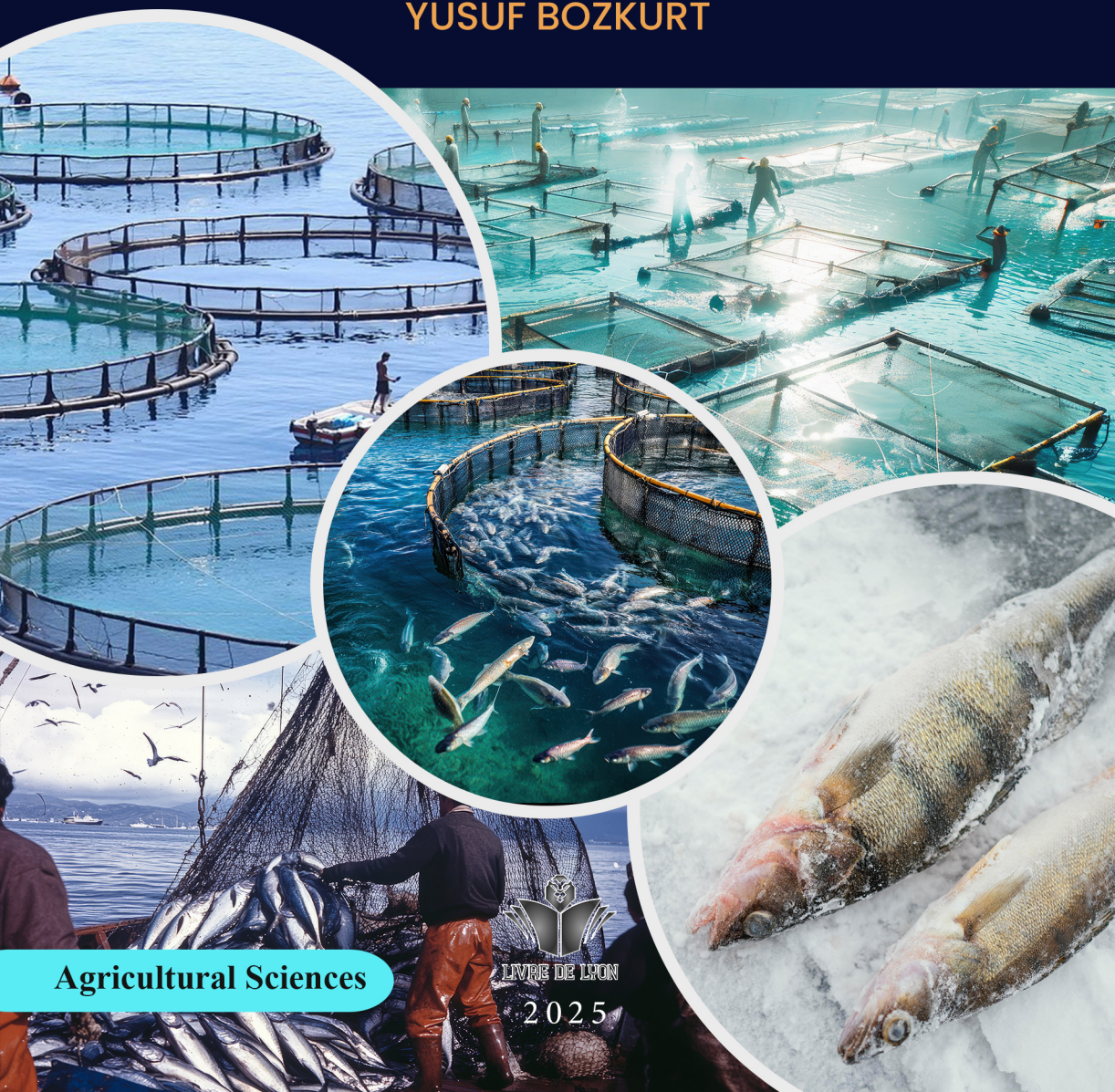
CRYOPRESERVATION IN AQUATIC ANIMALS I

EDITOR
YUSUF BOZKURT



Agricultural Sciences

LIVRE DE LYON
2025

CRYOPRESERVATION IN AQUATIC ANIMALS I

Editor
Yusuf BOZKURT

LIVRE DE LYON
Lyon 2025

CRYOPRESERVATION IN AQUATIC ANIMALS I

Editor
Yusuf BOZKURT

LIVRE DE LYON
Lyon 2025

Cryopreservation in Aquatic Animals I

Editor • Prof. Dr. Yusuf BOZKURT • Orcid: 0000-0002-6162-2466

Cover Design • Motion Graphics
Book Layout • Motion Graphics
First Published • December 2025, Lyon

e-ISBN: 978-2-38236-968-5
DOI: 10.5281/zenodo.18006814

Publisher • Livre de Lyon
Address • 37 rue marietton, 69009, Lyon France
website • http://www.livredelyon.com
e-mail • livredelyon@gmail.com

PREFACE

Advances in reproductive biotechnology have created exceptional opportunities for the conservation, management, and commercial production of aquatic species. Among these technologies, cryopreservation of sperm has emerged as a powerful and versatile tool. It offers long-term genetic preservation, enhanced breeding control, and improved sustainability within aquaculture systems. As global pressures on aquatic biodiversity increase and the demand for reliable aquaculture production rises, the ability to preserve and utilize high-quality genetic material has become more essential than ever.

This book, *Cryopreservation of Sperm in Aquatic Animals*, brings together updated knowledge and practical insights across a wide range of aquatic species and reproductive systems. The review chapters including *Cryopreservation of Sturgeon Sperm: Techniques, Challenges, and Applications in Conservation and Aquaculture*, *Sperm Cryopreservation in the European Sea Bass*, *Cryopreservation of Shabout Barbus grypus* (H, 1843) *Sperm*, *Sperm Cryopreservation in Turbot (Scophthalmus maximus)*, *Cryopreservation of Sperm in Hermaphroditic Fish Species: A Review*, and *Developments on Solutions Used in the Vitrification of Fish Sperm* synthesize current research findings and provide comprehensive evaluations of the methods, challenges, and recent advancements shaping this rapidly evolving field.

I believe that this resource will serve as a valuable guide for academic researchers, technical experts, aquaculture professionals, and conservation scientists working to improve reproductive technologies and conserve the genetic heritage of aquatic organisms. I would like to thank the dedicated researchers who contributed to the preparation of this volume, as well as to the *Livre De Lyon* team for their assistance in publishing of this book.

Yusuf Bozkurt, PhD, Prof. Dr.

CONTENTS

	PREFACE	I
CHAPTER I.	CRYOPRESERVATION OF STURGEON SPERM: TECHNIQUES, CHALLENGES, AND APPLICATIONS IN CONSERVATION AND AQUACULTURE ***Yusuf BOZKURT***	1
CHAPTER II.	SPERM CRYOPRESERVATION IN THE EUROPEAN SEA BASS ***Tülin ARSLAN***	27
CHAPTER III.	CRYOPRESERVATION OF SHABOUT (*BARBUS GRYPUS H*, 1843) SPERM ***Zafer DOĞU & Erdinç ŞAHİNÖZ***	43
CHAPTER IV.	SPERM CRYOPRESERVATON IN TURBOT (*SCOPHTHALMUS MAXIMUS*) ***Atife Tuba BEKEN***	59
CHAPTER V.	CRYOPRESERVATION OF SPERM IN HERMAPHRODITIC FISH SPECIES: A REVIEW ***Filiz KUTLUYER KOCABAŞ & Mehmet KOCABAŞ & Zainal Abidin MUCHLISIN***	79
CHAPTER VI.	DEVELOPMENTS ON SOLUTIONS USED IN THE VITRIFICATION OF FISH SPERM ***Ömer Can ARAL & Faruk ARAL & Zafer DOĞU & Erdinç ŞAHİNÖZ***	95

CHAPTER I

CRYOPRESERVATION OF STURGEON SPERM: TECHNIQUES, CHALLENGES, AND APPLICATIONS IN CONSERVATION AND AQUACULTURE

Yusuf BOZKURT

(Prof. Dr.) Mersin University, Faculty of Fisheries,
Department of Aquaculture, 33160, Mersin, Türkiye
E-mail: yusuf.bozkurt@mersin.edu.tr; yfbozkurt06@gmail.com
ORCID: 0000-0002-6162-2466

1. Introduction

Sturgeons are members of the *Acipenseridae* and *Polyodontidae* families, which are considered crucial freshwater fish in aquaculture. It is believed that they have inhabited the natural aquatic environments of the world's northern hemisphere for 200 million years (Bozkurt and Chebanov, 2024). Among the most valuable fish species worldwide, sturgeons stand out due to their pleasant meat, famous black caviar, and isinglass, which is a kind of gelatin used for various industrial purposes derived from the sturgeon's swim bladder.

Sturgeons represent the most promising temperate freshwater species for aquaculture. Today, sturgeons are considered the ideal candidate species for aquaculture due to their high commercial value (Zhang et al., 2013). However, sturgeon populations and their diversity have suffered declines as a result of environmental pressures like water pollution, dam building, and the development of nearby watersheds for irrigation. Sturgeons, currently known as 'living fossils', are at risk of extinction based on the Red List created by the IUCN (Bozkurt and Chebanov, 2024).

It is apparent that the standard approaches of reducing pollution and conserving habitats have not been effective in preserving and replenishing these populations. Thus, there is an urgent need to develop more stringently measures for the conservation of sturgeon stocks (Pourkazemi, 2006). Considering the

threatened extinction of sturgeon populations, the preservation of their sperm via *ex-situ* methods is critical for their prolonged conservation (Bozkurt and Saber, 2026). This necessitates the rapid integration of biotechnological tools, including cryopreservation, into *ex-situ* conservation programs (Bozkurt, 2023; Bozkurt and Chebanov, 2024). In this context, for critically endangered species like the sturgeon, cryopreservation offers hope for preserving genetic diversity where natural reproduction is severely compromised (Saber and Bozkurt, 2024).

The evolution of sperm management techniques has made cryopreservation an exceptionally valuable instrument within aquaculture. This method, commonly employed in artificial reproduction, provides continuous access to sperm samples, enables the coordination of male and female biological material availability, simplifies the transfer of samples to various research facilities or production locations, and lowers both the necessity and expenses associated with maintaining broodstock in confinement (Martínez- Paramo et al., 2017).

Even though this procedure offers convenience, scientists are persistently seeking innovative approaches to prevent and reverse cellular cryodamage. Such damage is linked to several problems, including membrane defects, DNA breakage, shifts in mitochondrial membrane potential, and a decline in spermatozoa's ability to move and survive, ultimately jeopardizing fertilization rates, future larval survival, and healthy development (Cabrita et al., 2010; Figueroa et al., 2019).

This review summarizes sturgeon sperm cryopreservation, highlighting species-specific protocols, factors affecting post-thaw quality, and applications in aquaculture and conservation, while addressing current challenges and future directions for preserving genetic diversity.

2. Physiological and Biological Features of Sturgeon Sperm

Sturgeon spermatozoa stand out among bony fish because they retain several ancestral traits that teleosts shed during evolution, and comprehending these features appears vital for effective cryopreservation.

The common structure of a spermatozoon includes a lengthy cylindrical head, which is finished with an active acrosome, a concise mid-section containing numerous mitochondria, and a flagellum featuring fin-like projections on both sides (Figure 1). The head's configuration promotes its passage through one of several narrow micropyle canals embedded in the robust, thick egg membrane. The head encompasses the nucleus and an apical acrosome, which is designed like a cap with a rounded summit. The acrosome houses enzymes such as

acrosine and arylsulfatase, which are essential for the acrosome reaction that takes place during fertilization (Mims et al., 2009; Horvath et al., 2006; Linhart and Kudo 1997).

During the acrosome reaction, a fertilization filament forms; this filament is theorized to serve as a signal transducer that transmits information to the egg regarding the arrival of a fertilizing spermatozoon (Alavi et al., 2012). The midpiece includes mitochondria and is attached to a sheath encasing the flagellum's initial section, which possesses opposing lateral fins.

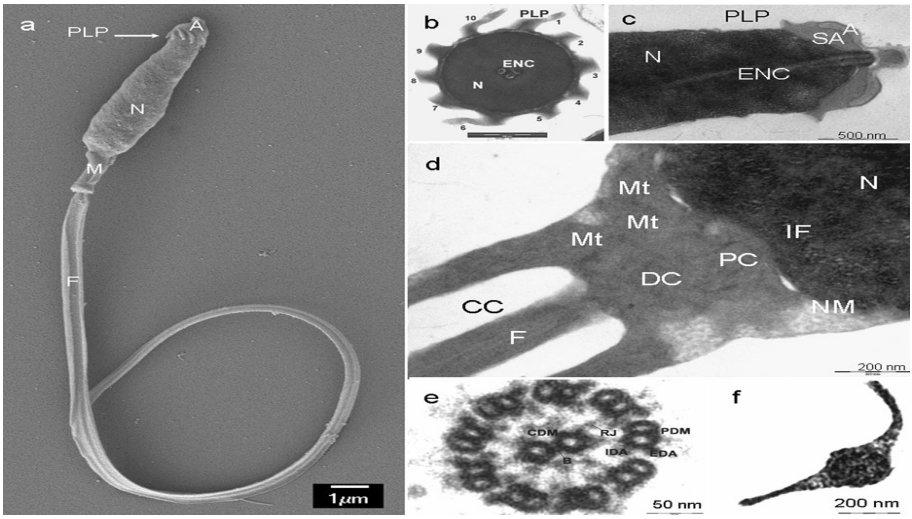


Figure 1. Ultrastructure and morphology of different sturgeon sperm. Microscopic images are selected from research conducted on the Russian sturgeon (a: Hatef et al., 2012), Siberian sturgeon (b, e: Psenicka et al., 2007) and Persian sturgeon (c, d, f: Hatef et al., 2011). A: acrosome, B: bridge, CC: cytoplasmic channel, CDM: central doublets of microtubules, DC: distal centriole, EDA: outer dynein arms, ENC: endonuclear canals, F: Flagellum, IDA: inner dynein arms, IF: implantation fossa, M: midpiece, Mt: mitochondria, N: nucleus, NM: nuclear membrane, PC: proximal centriol, PDM: peripheral doublets of microtubules, PLP: posterolateral projections, RJ: radial joins, SA: Subacrosome (Alavi et al., 2012).

In contrast to teleost fish, whose seminal plasma has an osmolality of approximately 300 mosmol/kg, the seminal plasma of sturgeons exhibits a low osmolality, ranging from 30 to 80 mosmol/kg. This low level persists even though their blood plasma maintains an osmolality near 300 mosmol/kg. Larger male sturgeons are known for generating a considerable quantity of sperm, specifically between 1 and 1.5 liters. However, sperm concentration is recorded at 0.5–2x10^9 spermatozoa/ml, which is a lower level compared to teleosts. The

activated spermatozoa exhibit motility for an extended period of 5–6 minutes, a duration much greater than the 20 seconds to 2 minutes observed in teleosts (Alavi and Cosson, 2006).

Activation of motility depends primarily on osmotic and ionic shifts: for freshwater species, hypoosmotic conditions trigger motility, whereas marine and anadromous species may require specific ionic environments (Alavi and Cosson, 2006). Environmental factors such as temperature, photoperiod, and hormonal state strongly affect semen quality (Dzyuba et al., 2021; Cosson et al., 2008).

3. Main Stages of Cryopreservation Process

3.1. Cryomedium

It is very important to prevent gamete cell's activity in a successful cryopreservation procedure. The cryomedium consists of two main components: an extender and a cryoprotective agent (CPA). The extender is responsible for sustaining the dormant state of gamete cells and supporting their metabolism, whereas CPAs shield cells from harm inflicted by freezing and thawing (Baust et al., 2009).

3.1.1. Extenders

After ensuring gamete quality, the next step in cryopreservation is to dilute the gametes with extenders containing cryoprotectants. This allows the cryoprotectants to penetrate the cells before freezing. For this purpose, there are two main approaches to the development of the extender composition. The first approach is to use complex cryosolution formulations that mimic the composition of fish seminal plasma, while the second approach is to use carbohydrate-based cryosolutions that provide energy to sperm cells. Extenders make it easier to work with gamete cells by increasing the cell volume (Bozkurt, 2020).

An ideal sperm cryopreservation extender should exhibit several key characteristics (Bozkurt, 2017):

-It must preserve the correct electrolytic balance and osmotic pressure within cells to minimize salt-induced harm.

-It should contain metal-complexing agents capable of sequestering heavy metals that are toxic to spermatozoa.

-It needs to be robust, resisting both enzymatic and non-enzymatic degradation.

-The presence of antibiotics is crucial to prevent bacterial contamination of the sample.

-It should also facilitate a substantial increase in sperm volume to optimize fertilization potential.

-It should provide a suitable environment for the normal metabolic activities of the spermatozoa.

Extenders used in sturgeon sperm cryopreservation are designed to maintain osmotic balance, stabilize membranes, and provide a suitable ionic environment before the addition of cryoprotectants. Across Acipenserid species, simple salt- or sugar-based extenders are most common, including variants of glucose, sucrose or lactose solutions, as well as Ringer-type or Tris-buffered media (Judycka et al., 2015; Dadras et al., 2022; Igna et al., 2022).

For instance, in Siberian sturgeon (*Acipenser baerii*) a "glucose–methanol" extender comprising 0.10–0.15 M glucose in combination with 15 % methanol was developed and shown to yield good post-thaw motility and fertilization rates (Judycka et al., 2015).

In another case, for Sterlet (*Acipenser ruthenus*), extenders based on Tris–sucrose–KCl have been widely used, facilitating sperm cryopreservation prior to supplementation of antioxidants (Dadras et al., 2022). In yet another study of the Russian sturgeon (*Acipenser gueldenstaedtii*), two extenders were compared: one containing 50 mM NaCl, 5 mM KCl, 10 mM Tris and 10 % DMSO; the other 30 mM sucrose, 1 mM KCl, 25 mM Tris with 10 % methanol (Igna et al., 2022). In a comprehensive study of Caspian Sea sturgeon species, including beluga sturgeon (*Huso huso*), Persian sturgeon (*Acipenser persicus*), stellate sturgeon (*Acipenser stellatus*), and ship sturgeon (*Acipenser nudiventris*), a standardized protocol employed a Tris buffer (»118 mM Tris, pH 8) with 23.4 mM sucrose as an extender, in combination with 15% DMSO as a cryoprotectant (Saber and Bozkurt, 2024).

These formulations help prevent premature activation of sperm motility while buffering pH and maintaining cell volume during cooling. Many protocols also incorporate cryoprotective supplements, such as egg yolk, bovine serum albumin (BSA), or citrate, which provide additional membrane-stabilising lipoproteins and mitigate cold shock (Rusco et al., 2019; Torres et al., 2022). The "optimal extender" varies between species and even among individual males, but sturgeon research consistently demonstrates that the composition of the extender significantly affects post-thaw motility, membrane integrity, and fertilization success. This underscores the importance of optimizing extenders and tailoring biochemical formulations specifically for each species.

3.1.2. Cryoprotectants

Cryoprotectants are defined as chemicals used to prevent damage in gamete cells during freezing and thawing applications. In general, they show their protective effect by increasing the unfrozen fraction in the medium and reducing the amount of ions. Before the cells are frozen, they are incubated with cryoprotectants and it is ensured that they come to balance in intracellular terms. The most important characteristics of cryoprotectants are that they have low molecular weight (Alcay et al., 2016). On the other hand, most cryoprotectants are toxic to cells depending on their doses. Thus, cryoprotectants should be added slowly to the extenders to minimize this toxic effect. Following this, the extender and cryoprotectant mixture should be stored at a low temperature before being added to the semen (Karimi et al., 2025).

Cryoprotectant substances are classified according to their penetrating properties through the cell membrane such as those that can pass through the cell membrane (permeable-internal) and those that can't pass through the cell membrane (non-permeable-external) (Sieme et al., 2016).

Cryoprotectants that can penetrate into the cell have a low molecular weight and include DMSO, glycerol, ethylene glycol, 1.2 propanediol, 2.3 butanediol, propylene glycol, and some other alcohols. Before freezing occurs, the fluid in the sperm cells is replaced by permeable cryoprotectants due to the osmotic pressure difference. Thus, both the changes in cell volume are reduced and the formation of ice crystals inside sperm cells is minimized. In this way, damage to cells during the freezing process is minimized. Permeable cryoprotectants show their protective effects by binding to H_2O and reducing the toxic effects caused by higher concentrations of other components (Öztürk et al., 2020).

On the other hand, external cryoprotectants that can't penetrate into the cell are divided into two groups. These are low-molecular-weight (glucose, sucrose, trehalose, raffinose, galactose, and some other sugars) and high-molecular-weight (PVA, PVP, and some other polymers) cryoprotectants that can't penetrate into the cell (Öztürk et al., 2020). In this concept, low molecular weight cryoprotectants, which can't penetrate into the cell, show their effects by dehydrating the cells, and reducing the formation of ice crystals formed during the freezing process. On the other hand, high molecular weight cryoprotectants that can't penetrate into the cell show their effects by changing the shapes and sizes of ice crystals formed during the freezing and thawing of cells in such a way that they are harmless (McGann, 1978).

Permeating cryoprotectants (P-CPAs) are essential for conserving sturgeon sperm during freezing, and among these, methanol (MeOH) and dimethyl sulfoxide (DMSO) remain the most widely applied across *Acipenserid* species. For example, in the cold-water species Siberian sturgeon (*Acipenser baerii*), a glucose–methanol extender (0.10–0.15 M glucose + 15% MeOH) produced strong post-thaw motility and fertilization results (Judycka et al., 2015).

In comparative work on the same species, MeOH conserves sperm achieved higher fertilization results than DMSO, despite similar motility (Dietrich et al., 2025). In the Russian sturgeon (*Acipenser gueldenstaedtii*), a study found that MeOH as the cryoprotectant yielded fertilization rates of ~72.7% vs ~37.5% for DMSO in a yolk-free extender (Igna et al., 2022). Conversely, for multiple Caspian Sea species including Beluga sturgeon (*Huso huso*), Persian sturgeon (*Acipenser persicus*) and Stellate sturgeon (*Acipenser stellatus*), 10–15% DMSO paired with sucrose-Tris extenders is commonly reported as a standard approach for cryobanking (Saber and Bozkurt, 2024). Other permeating CPAs (glycerol, ethylene glycol, propylene glycol) have been evaluated in some sturgeons but generally show lower success because of slower permeability or higher cytotoxicity (Kolyada et al., 2023a).

Non-permeating cryoprotectants (NP-CPAs) act extracellularly to control osmotic stress, inhibit ice crystal growth and stabilize membranes. Sugars such as sucrose, trehalose, lactose and maltose are widely used. For instance, disaccharide supplementation (maltose, trehalose, lactose or lactulose) in extenders improved post-thaw motility in both Persian and Beluga sturgeon (Golshahi et al., 2018).

The application of Tris–sucrose extenders (e.g., at 23.4 mM sucrose) in combination with DMSO for Caspian sturgeon species has been documented in sturgeon-related research (Saber and Bozkurt, 2024). The application of glucose (a monosaccharide) within the glucose–methanol extender in Siberian sturgeon provided osmotic and metabolic benefits (Judycka et al., 2015).

Macromolecular NP-CPAs — such as egg yolk components, bovine serum albumin (BSA), citrate — though less frequently detailed in sturgeon literature, are also applied in fish sperm cryopreservation to provide lipoprotein shielding and buffer cold shock (Rusco et al., 2019; Torres et al., 2022). Moreover, recent reviews highlight sugars like trehalose as both NP-CPAs and antioxidant adjuncts in semen cryopreservation because of their ability to reduce reactive-oxygen species (ROS) and stabilize membranes (Jia et al., 2024).

These studies indicate that the selection and concentration of both permeating and non-permeating cryoprotectants vary significantly among sturgeon species. Optimizing the combinations of extenders and cryoprotectants is crucial for maximizing sperm freezability, preserving membrane integrity, recovering motility, and enhancing fertilization capacity.

3.2. Sperm Dilution

Sperm dilution is a critical step in sturgeon sperm cryopreservation because it increases the usable volume for multiple inseminations and regulates sperm exposure to extenders and cryoprotectants. It is imperative to carefully establish the semen-diluent ratio, considering that sperm concentration, osmotic stability, cryoprotectant permeation dynamics, and the inherent buffering capacity of seminal plasma are all critical determinants of subsequent fertilizing potential. Since fertilization requirements vary among *Acipenserid* species, it is crucial to establish an optimal sperm-to-egg ratio before calculating dilution ratios for cryopreservation. It is essential to acknowledge the characteristic reduction in motility and viability that typically occurs after a sample has been thawed. Diluent should be added gradually to avoid osmotic or pH shock, and any extender that induces spontaneous motility should be replaced.

Species-specific studies highlight the importance of optimizing dilution ratios. In Stellate sturgeon (*Acipenser stellatus*), a study comparing dilution ratios of 1:0.5, 1:1, 1:2, and 1:5 found that a 1:1 semen-to-extender ratio produced the best post-thaw motility and motility duration after 30 and 60 days of storage (Sadeghi et al., 2013).

In Persian sturgeon (*Acipenser persicus*), motility duration was greatest at dilution ratios of 1:1 and 1:2, whereas higher dilutions (1:5 or 1:10) significantly reduced sperm survival (Shaluei et al. 2017). Consistent with these findings, the Caspian broodstock programme for Beluga sturgeon (*Huso huso*) and Persian sturgeon (*Acipenser persicus*) routinely employs a 1:1 dilution before cryoprotectant addition and freezing (Saber and Bozkurt, 2024).

Collectively, these data indicate that moderate dilution—typically 1:1 to 1:2—helps maintain protective seminal plasma components while permitting uniform cryoprotectant equilibration, whereas excessive dilution (≥1:5) diminishes membrane protection and increases cryoinjury. Thus, species- and male-specific optimization of dilution ratios remains indispensable for effective sturgeon sperm cryopreservation.

3.3. Equilibration Period

The equilibration period—the interval after cryoprotectant addition and prior to cooling—is critical in sturgeon sperm cryopreservation because it allows permeating cryoprotectants to diffuse into the cells, equilibrate osmotic conditions and stabilize membranes before freezing.

For example, in the cold-water species Siberian sturgeon (*Acipenser baerii*), one study found that a 30-minute equilibration in a glucose-methanol extender produced no significant difference in post-thaw motility or fertilization compared to immediate freezing, suggesting flexibility in handling time in this species (Judycka et al., 2015).

In contrast, in the Persian sturgeon (*Acipenser persicus*), a detailed assessment across equilibration times of 0, 5, 10, 20, 40, and 60 min revealed that motility and fertility outcomes were significantly improved at intermediate times (e.g., 10-20 min) compared with zero or very prolonged equilibration (Shaluei et al., 2017).

Meanwhile, a uniform protocol for Caspian Sea sturgeon species (including Beluga sturgeon (*Huso huso*), Persian sturgeon (*Acipenser persicus*), and Stellate sturgeon (*Acipenser stellatus*) employed an equilibration of one hour (60 min) at 4°C before cryofreezing (Saber and Bozkurt, 2024).

These species-specific differences illustrate that optimum equilibration period depends on factors such as cryoprotectant type and concentration, extender composition, sperm cell membrane permeability and species physiological traits. Too short an equilibration period may lead to inadequate cryoprotectant penetration and increased ice-crystal damage, while excessively long equilibration may increase toxicity or oxidative stress (Rodrigues et al., 2021; Saha et al., 2022). Consequently, achieving species-specific optimisation of the equilibration period is indispensable to maximize post-thaw motility and fertilisation efficacy of cryopreserved sturgeon sperm.

3.4. Packaging Process

Following equilibration, diluted sturgeon semen must be packaged appropriately prior to freezing, and the choice of packaging method can significantly influence post-thaw sperm quality. Three primary approaches are commonly used: a) pellet formation, b) ampoules/vials/visotubes, and c) plastic straws.

a) Packaging in Pellets

In pellet freezing, small droplets of the equilibrated semen–extender mixture are placed into shallow depressions or holes drilled into blocks of solid dry ice (-79°C). Rapid cooling in these depressions forms frozen pellets, which are subsequently transferred into liquid nitrogen (-196°C) for long-term storage. Although this traditional method provides very rapid cooling, it offers limited dose standardization and is used less frequently in modern sturgeon cryopreservation (Billard et al., 2004, Agarwal, 2011).

b) Packaging in Ampoules, Vials, or Visotubes

Glass ampoules, cryovials, or visotubes have historically been used to store diluted milt prior to controlled freezing. This method was widely adopted in early fish cryobiology research (Mounib, 1978), allowing relatively large sample volumes and providing good protection from contamination. However, the slower heat transfer and variable cooling rate within larger containers can reduce post-thaw sperm quality compared with more modern packaging systems (Agarwal, 2011).

c) Packaging in Straws

Straw packaging has become the most widely used method in contemporary fish and sturgeon cryopreservation due to superior thermal properties and improved post-thaw performance. As demonstrated by Erdahl et al. (1984), straws reduce the risk of ice recrystallization during thawing and provide more consistent cooling rates, ultimately enhancing sperm survival. In this system, equilibrated diluted semen is drawn into 0.25–0.5 mL plastic straws, which are then sealed manually—commonly using polyvinyl alcohol (PVA) powder—or with factory-prepared heat seals. Straws are compatible with programmable freezing systems and can be easily labeled, standardized, and stored in liquid nitrogen, making them highly suitable for hatcheries, cryobanks, and long-term conservation programs (Billard et al., 2004, Agarwal, 2011).

3.5. Cooling Process

Cooling—the controlled removal of heat that leads to freezing—is the single most influential physical step in sperm cryopreservation because it determines ice-nucleation dynamics, ice-crystal size, the degree and rate of cellular dehydration, and the magnitude of solute-concentration (solution) effects. Slow cooling allows osmotic equilibration (cell dehydration) and reduces

the risk of lethal intracellular ice, but excessive slowness prolongs exposure of cells to hypertonic residual solution and CPA toxicity; conversely, very rapid cooling can trap water intracellularly and promote intracellular ice formation (Suquet et al., 2000; Thirumala et al., 2005).

Studies emphasize that an intermediate, well-defined cooling profile is required for sturgeon sperm to balance these competing risks (Suquet et al., 2000; Thirumala et al., 2005). Also, literature regarding sturgeon sperm cryopreservation suggests that reproducible cooling rates are best achieved through vapor-phase LN_2 or programmable freezing equipment. Furthermore, it is observed that practical cooling of 0.5-mL straws typically results in effective rates approximated at tens of °C per minute (Billard et al., 2004).

Experimental work in specific sturgeon species confirms that cooling rate markedly affects post-thaw motility, ATP content, oxidative markers and fertilization. For Persian sturgeon (*Acipenser persicus*), systematic testing of freezing rates demonstrated large differences in motility, fertilization and hatching, indicating clear rate-dependence of cryosurvival (Aramli, et al., 2016). In Russian sturgeon (*Acipenser gueldenstaedtii*), studies using simple vapor-phase protocols (straws equilibrated above LN_2) report that controlled distancing/time in the vapor phase prior to plunge into LN_2 gives consistent cooling and good post-thaw function; these practical vapor-phase approaches are widely adopted in hatchery protocols for this species (Igna et al., 2022). Sterlet (*Acipenser ruthenus*) work linking ultrastructural cryoinjury to freezing protocols likewise shows that different cooling profiles produce distinct patterns of membrane, mitochondrial and axonemal damage, supporting species-specific optimisation of cooling curves (Dadras et al., 2022).

In practice, cryobiologists working with sturgeons choose either (a) programmable slow-freezers to apply a precisely controlled multi-step cooling profile, or (b) standardized vapor-phase (straw) methods in which straw size and height above the LN_2 surface are used to reproduce target cooling rates; both approaches have been validated across sturgeon species. Because optimal cooling rates depend on straw/volume, CPA type and species membrane properties, empirical testing (pilot trials comparing post-thaw motility and fertilization under a small set of candidate cooling profiles) remains essential for each species and broodstock population (Horvath et al., 2011).

3.6. Freezing Methods

The freezing stage represents a critical phase in the cryopreservation of sturgeon sperm, as it determines whether cells undergo controlled dehydration

or lethal intracellular ice formation. Sturgeon gametes may be frozen using either slow freezing or vitrification, although slow freezing remains the standard method applied in commercial hatcheries and conservation programs (Cuevas-Uribe et al., 2015). Slow freezing is the most widely used technique because it provides a predictable balance between cooling rate, osmotic responses, and cryoprotectant toxicity, resulting in high post-thaw motility and fertilization rates across numerous sturgeon species (Billard et al., 2004; Aramli et al., 2016).

In slow-freezing protocols, extended semen is first equilibrated—usually at 4°C for 10–15 minutes—before it is packaged into 0.25-mL or 0.5-mL plastic straws, which allow for reproducible cooling conditions. The sealed straws are arranged horizontally on metal or plastic combs and placed approximately 4 cm above the liquid nitrogen (LN_2) surface, exposing them to nitrogen vapor at −110 to −120°C. This vapor-phase exposure typically lasts 10 minutes, during which extracellular ice formation draws water out of the spermatozoa, limiting the risk of intracellular ice crystallization. At the end of this cooling step, straws are plunged directly into LN_2 at −196°C for long-term storage (Dadras et al., 2022). This approach has proven highly effective for Siberian sturgeon (*Acipenser baerii*), where freezing semen in 0.5-mL straws under vapor cooling produced post-thaw motility above 50–70% when used with cryoprotectants such as DMSO or methanol (Tsvetkova et al., 1996). Similarly, Russian sturgeon (*Acipenser gueldenstaedtii*) semen has shown consistently high fertilization rates when frozen in the vapor phase at similar distances, reflecting the species' sensitivity to osmotic stress and the need for a controlled dehydration process (Billard et al., 2004; Alavi et al., 2012).

For Persian sturgeon (*Acipenser persicus*), experimental trials have highlighted that cooling rate is more critical than cryoprotectant concentration in determining post-thaw sperm quality. Studies using 0.25-mL straws demonstrated that moderate vapor-phase cooling produced superior motility and fertilization outcomes compared with faster dry-ice freezing or overly slow programmable cooling steps (Tiersch and Green, 2011; Yang et al., 2010). These findings emphasize the importance of tailoring cooling rates to species-specific differences in membrane composition, osmotic tolerance, and cryoprotectant permeability.

Although pellet freezing on dry ice (−79°C) is one of the earliest methods employed in fish sperm cryopreservation, it is now used far less frequently. In this technique, small depressions are carved into a disk of solid CO_2, and ~0.1 mL drops of equilibrated semen are pipetted directly into these pits, freezing

almost instantly. The hardened pellets are then transferred into LN_2 for storage. This approach has occasionally been applied in small-scale laboratory work with sturgeons such as *Acipenser baerii* or *Acipenser gueldenstaedtii*, but it offers lower control of cooling dynamics, produces more variable post-thaw results, and lacks dose precision compared with straw-based methods. For these reasons, pellet freezing has largely been replaced by standardized vapor-phase straw freezing in both research and hatchery settings (Tsvetkova et al., 1996; Psenicka et al., 2007; Igna et al., 2022).

In contrast to slow freezing, vitrification represents a method of ultra-rapid freezing in which the sample transitions into an amorphous glass-like state without ice crystal formation. While vitrification has attracted interest in fish reproduction, its application in sturgeons has so far been limited predominantly to oocytes, ovarian follicles, and early embryos, rather than sperm. Sturgeon sperm cells have low membrane permeability and are highly sensitive to osmotic shock, making them poor candidates for the extremely high cryoprotectant concentrations (40–60% CPA mixtures) required for vitrification. Attempts to vitrify sturgeon embryos and ovarian tissues have achieved partial structural preservation but very low survival or developmental success post-warming (Cuevas-Uribe et al., 2015).

Overall, slow vapor-phase freezing in sealed plastic straws continues to be the most reliable, practical, and widely adopted method for sturgeon sperm cryopreservation (Tsvetkova et al., 1996). Species-specific differences in membrane physiology and osmoregulatory properties underscore the need for carefully optimized cooling rates for each taxon (Aramli et al., 2016). As conservation efforts expand and hybridization programs grow, developing standardized, species-tailored freezing curves will remain an essential goal in sturgeon reproductive biotechnology (Igna et al., 2022).

3.7. Thawing Process

The thawing process is a critical step in the successful utilization of cryopreserved sturgeon gametes, involving controlled warming of straws or pellets to restore spermatozoa from ultra-low storage temperatures (−196°C) to fertilizing conditions (Tsvetkova et al., 1996). Typically, cryopreserved gametes are immersed in a water bath at temperatures ranging from 20 to 37°C, with immersion times depending on straw size and species (0.25–0.5 mL straws usually require 5–10 seconds). Immediate use of thawed sperm is recommended because a substantial proportion of cells may already have sustained membrane,

mitochondrial, or axonemal damage during freezing, and prolonged exposure in the thawed state can further reduce motility and fertilization success (Tsvetkova et al., 1996; Glodowski et al., 2022).

The effectiveness of thawing is strongly influenced by the cooling history of the sperm. Slowly cooled sperm often experience sufficient dehydration prior to solidification, minimizing intracellular ice formation. In such cases, rapid warming from the frozen state can effectively reduce recrystallization and osmotic stress. For example, Siberian sturgeon (*Acipenser baerii*) semen frozen in 0.5-mL straws and thawed at 35°C for 10 seconds exhibited post-thaw motility of 65–70% and high fertilization rates (>70%) (Tsvetkova et al., 1996). Similarly, Russian sturgeon (*Acipenser gueldenstaedtii*) sperm thawed at 30°C for 8–10 seconds after vapor-phase freezing maintained fertilization rates of 50–80%, highlighting the importance of rapid warming to avoid ice recrystallization (Billard et al., 2004; Alavi et al., 2012).

In Persian sturgeon (*Acipenser persicus*), studies have shown that post-thaw motility and fertilization vary depending on freezing protocol and extender composition; for example, the highest fertilization rate was achieved with a −40°C/min freezing rate (Aramli et al., 2016). Across sturgeon species, the general principle is that thawing should be rapid enough to prevent recrystallization, yet controlled to avoid thermal shock. During thawing, water re-enters the spermatozoa, solute concentrations normalize, and cellular metabolism gradually resumes, allowing the sperm to regain motility and fertilizing capacity (Mazur, 1984; Tsvetkova et al., 1996; Cabrita et al., 2008).

Overall, species-specific optimization of thawing conditions—temperature, time, and straw/pellet size—is essential for achieving high post-thaw sperm quality and fertilization efficiency in sturgeon aquaculture and conservation programs.

3.8. Storage in Liquid Nitrogen

Long-term storage of cryopreserved sturgeon sperm is typically carried out in liquid nitrogen (LN_2) at −196°C, a temperature at which all biochemical and enzymatic processes effectively cease, allowing gametes to remain viable for extended periods. Sperm samples stored in sealed straws or cryotubes can retain motility, membrane integrity, and fertilization potential for decades when proper handling and storage protocols are followed (Tiersch et al., 2007).

Species-specific studies confirm the stability of cryopreserved sturgeon sperm over several years. For example, Siberian sturgeon (*Acipenser baerii*) semen frozen in 0.5-mL straws and stored in LN_2 showed no significant decline

in motility or fertilization capacity even after five years of storage (Tsvetkova et al., 1996). Russian sturgeon (*Acipenser gueldenstaedtii*) and Persian sturgeon (*Acipenser persicus*) have shown substantial post-thaw motility and fertilization potential following cryopreservation and LN_2 storage (Igna et al., 2022; Aramli and Nazari, 2014; Alipour et al., 2009). Long-term success in cryobanking depends on maintaining straw integrity and minimizing temperature fluctuations to avoid ice recrystallization and reduced fertility (Billard et al., 2004; Tiersch et al., 2007).

These findings highlight the effectiveness of LN_2 as a cryogenic medium for both conservation and aquaculture applications, enabling the creation of sperm banks for genetic preservation, selective breeding, and hybridization programs.

4. Challenges in Sturgeon Sperm Cryopreservation

Despite decades of research and significant advances, sturgeon sperm cryopreservation remains technically challenging due to a combination of biological, physiological, and technical factors. The major challenges can be grouped into the following categories:

4.1. Species-Specific Physiological Differences

Sturgeon species exhibit wide variation in sperm size, membrane composition, and osmoregulatory capacity, which affects their sensitivity to freezing and thawing. For example, Siberian sturgeon (*Acipenser baerii*) sperm generally show robust tolerance to standard cryopreservation protocols, whereas Russian sturgeon (*Acipenser gueldenstaedtii*) sperm display greater sensitivity to cryoprotectant composition and handling conditions (Igna et al., 2022; Billard et al., 2004). These species-specific variations complicate the standardization of cryopreservation protocols across diverse taxa.

4.2. Cryodamage

Cryogenic injury can induce a wide range of morphological, physiological, and biochemical alterations in male gametes, which ultimately compromise post-thaw sperm viability and fertilization potential. Major forms of cryodamage include:

-Membrane disruption, leading to loss of selective permeability
-Mitochondrial dysfunction, reducing ATP availability for motility

-DNA fragmentation, compromising genetic integrity

-Oxidative stress, producing reactive oxygen species that damage lipids and proteins (Cabrita et al., 2010; Figueroa et al., 2019). These injuries can lead to reduced motility, viability, fertilization success, and impaired larval development.

4.3. Optimization of Cryoprotectants (CPAs)

The choice and concentration of permeating and non-permeating cryoprotectants critically influence sperm survival. DMSO, methanol, glycerol, and combinations are commonly used, but toxicity varies among species, and suboptimal CPA concentrations can exacerbate cryodamage. Furthermore, equilibration time must be optimized to balance cryoprotection with osmotic stress (Cabrita et al., 2010).

4.4. Cooling and Warming Rate Challenges

The rate of cooling and thawing directly affects sperm survival. Slow cooling can cause solution effects and prolonged exposure to hypertonic environments, while excessively rapid cooling may induce intracellular ice formation. Similarly, thawing must be fast enough to prevent recrystallization but controlled to avoid thermal shock (Tiersch and Green, 2011).

4.5. Dilution and Seminal Plasma Interactions

Semen must be diluted with extenders prior to freezing to allow uniform CPA penetration. However, excessive dilution reduces protective seminal plasma factors, while insufficient dilution can hinder cryoprotectant equilibration. Optimizing sperm-to-extender ratios is often species- and even male-specific (Suquet et al., 2000; Bergeron et al., 2002; Tiersch and Green, 2011).

4.6. Storage and Handling Constraints

While liquid nitrogen storage ensures long-term viability, temperature fluctuations during handling or transfer can compromise sperm quality. Maintaining a consistent −196°C environment across global sperm banks requires careful infrastructure and handling protocols (Tiersch and Green, 2011; Martínez-Páramo et al., 2017).

4.7. Limited Molecular Understanding

Although practical protocols exist, the molecular and biochemical mechanisms governing sperm freezability are not fully understood. Factors

such as membrane lipid composition, antioxidant defenses, and gene expression patterns influencing cryotolerance remain underexplored, limiting the ability to rationally design species-specific protocols (Figueroa et al., 2019).

4.8. Hybridization and Genetic Variability

In hatcheries, the use of cryopreserved sperm for hybrid production (e.g., sterbel hybrids: *Huso huso* × *Acipenser ruthenus*) requires careful genetic management to avoid inbreeding and preserve diversity. Indeed, cryopreserved beluga sperm has been used successfully to produce "sterbel" (*Acipenser ruthenus* × *Huso huso*) hybrids, with molecular verification of hybrid origin and maintained viability (Fopp-Bayat et al., 2023; Fopp-Bayat et al., 2024).

5. Conservation and Aquaculture Applications

Cryopreservation of sturgeon sperm has become a pivotal tool for both conservation biology and aquaculture, enabling long-term preservation of valuable genetic resources while supporting sustainable breeding programs. Sperm-banks have been established for a variety of sturgeon species, including sterlet (*Acipenser ruthenus*), Siberian sturgeon (*Acipenser baerii*), and Beluga sturgeon (*Huso huso*) (Billard et al., 2004; Alipour et al., 2009; Martínez-Páramo et al., 2017). These biobanks not only provide gametes for routine artificial fertilization and hatchery operations, but also facilitate restocking initiatives aimed at recovering endangered wild populations (Comizzoli, 2017).

In aquaculture, cryopreserved sperm has enabled the production of hybrid sturgeon, such as bester hybrids (*Huso huso* × *Acipenser ruthenus*), which combine the rapid growth of Beluga with the hardiness and disease resistance of sterlet. Such hybrids are increasingly used to improve productivity while reducing pressure on wild stocks. Beyond local breeding programs, sperm cryopreservation has fostered international exchange of genetic resources, allowing hatcheries and research institutions worldwide to maintain genetic diversity, conduct selective breeding programs, and mitigate inbreeding risks in cultured and endangered populations (Tiersch and Green, 2011).

Furthermore, cryobanks serve as living genetic repositories, preserving alleles from rare or geographically isolated populations, which can be reintroduced into the gene pool decades later. This capability is especially critical for sturgeon species, many of which face severe declines due to overfishing, habitat loss, and pollution. In this way, cryopreservation integrates conservation and production objectives, bridging aquaculture innovation with species preservation and offering a sustainable strategy to safeguard sturgeon biodiversity globally.

6. Conclusion

Advancements in sperm management techniques have firmly established cryopreservation as an essential tool in both sturgeon aquaculture and conservation programs. This technology enables the creation of genetic repositories, facilitates the combination and preservation of desirable traits, and supports selective breeding programs aimed at developing improved genetic stocks (Tiersch and Green, 2011; Martínez-Páramo et al., 2017). Cryopreservation also ensures the continuous availability of sperm samples, synchronizes the use of male and female gametes, simplifies the transport of genetic material across research or production facilities, and reduces both the costs and logistical challenges associated with maintaining broodstock in captivity (Martínez-Páramo et al., 2017).

Despite these advantages, cryodamage remains a major limitation, affecting plasma membranes, mitochondrial function, and DNA integrity, while often reducing sperm motility and viability. Such damage can compromise fertilization success, larval survival, and normal embryonic development (Cabrita et al., 2010; Figueroa et al., 2019). Sturgeon sperm are particularly sensitive due to their large cell size, low membrane permeability, and species-specific differences in osmotic tolerance, highlighting the need for continued refinement of cryopreservation protocols (Tsvetkova et al., 1996; Glogowski et al., 2002; Cosson, 2019).

Cryoprotectants are used to reduce cryo-injury in sperm, and therefore selecting the most suitable cryoprotectant for a given semen sample is essential. Additionally, various chemicals added to sperm extenders aim to protect the integrity of the sperm plasma membrane. Ongoing research also focuses on preventing sperm DNA damage caused by oxidative stress during the thawing process, as well as minimizing negative effects on sperm kinematics and motility. Similarly, studies continue to optimize sturgeon sperm cryopreservation and to achieve high fertilization rates following thawing and artificial insemination (Tsvetkova et al., 1996; Gil et al., 2017; Cabrita et al., 2010; Badr et al., 2021; Kolyada et al., 2023b; Igna et al., 2022).

Overall, the cryopreservation of sturgeon sperm represents an invaluable tool for both conservation and aquaculture, enabling the preservation of genetic diversity and supporting artificial breeding programs for endangered and commercially important species. Although significant progress has been made in optimizing cryopreservation protocols, our understanding of the molecular and biochemical mechanisms governing sperm freezability remains

incomplete. Future research integrating molecular biology, metabolomics, and bioinformatics holds great promise for enhancing post-thaw sperm quality, improving fertilization outcomes, and safeguarding the genetic heritage of *Acipenseridae* for generations to come.

References

Alavi, S.M.H., Cosson, J. (2006), Sperm motility in fishes. (II) Effects of ions and osmolality: A review. Cell Biology International, 30: 1-14. https://doi.org/10.1016/ j.cellbi.2005.06.004.

Agarwal, N.. (2011). Cryopreservation of Fish Semen. In book: Himalayan Aquatic Biodiversity Conservation & New Tools in Biotechnology (pp.104-127) Edition: First edition, Chapter: 8. Publisher: Transmedia Publication, Srinagar (Garhawal) Uttarakhand, India. Editors: J.P. Bhatt, Madhu Thapliyal, Ashish Thapliyal. https://doi.org/10.13140/ RG.2.1.1215.7209/1.

Alavi, S.M.H., Hatef, A., Pšenička, M., Kaspar, V., Boryshpolets, S., Dzyuba, B., Cosson, J., Bondarenko, V., Rodina, M., Gela, D., Linhart, O. (2012). Sperm biology and control of reproduction in sturgeon: (II) Sperm morphology, acrosome reaction, motility and cryopreservation. Reviews in Fish Biology and Fisheries, 22: 861–886. https://doi.org/10.1007/s11160-012-9270-x.

Alcay, S., Üstüner, B., Nur, Z. (2016). Effects of low molecular weight cryoprotectants on the post-thaw ram sperm quality and fertilizing ability. Small Ruminant Research, 136: 59-64. https://doi.org/10.1016/ j.smallrumres.2016.01.009.

Alipour, A., Baradaran, N.S., Nowruzfashkhami, M.R., Pourkazemi M. (2009). Fertilizing ability of cryopreserved spermatozoa in the Persian sturgeon ((*Acipenser persicus*)) and stellate sturgeon (*Acipenser stellatus*). Iranian Journal of Fisheries Sciences 8(1): 1-12.

Aramli, M.S., Nazari, R.M. (2014). Motility and fertility of cryopreserved semen in Persian sturgeon, *Acipenser persicus*, stored for 30–60min after thawing, Cryobiology, 69 (3): 500-502. https://doi.org/ 10.1016/j.cryobiol.2014.10.006.

Aramli, M.S., Golshahi, K., Nazari, R.M., Sotoudeh, E., Aramli, S., Habibi, E. (2016). Effect of freezing rate for cryopreservation of Persian sturgeon (*Acipenser persicus*) spermatozoa. Theriogenology, 1;85(4):734-739. https://doi.org/10.1016/ j.theriogenology.2015.10.018.

Badr, M.R., Rawash, Z.M., Hassan, H.M., Elaraby, M.E., Amer, M.A., Ahmed, K.M. (2021). Effect of extenders, cryoprotectants, and freezing methods

on sperm quality and fertilisation rate of the European sea bass (*Dicentrarchus labrax*). Egyptian Journal of Aquatic Biology and Fisheries, 25(2): 63-74.

Baust J.G., Gao D., Baust J.M. (2009). Cryopreservation: An Emerging Paradigm Change. Organogenesis, 5: 90–96. https://doi.org/10.4161/org.5.3.10021.

Bergeron, A., Vandenberg, G., Proulx, D., Bailey, J.L. (2002). Comparison of extenders, dilution ratios and theophylline addition on the function of cryopreserved walleye semen. Theriogenology. 57(3):1061-71. https://doi.org/10.1016/s0093-691x (01)00707-5.

Billard, R., Cosson, J., Noveiri, S.B., Pourkazemi, M. (2004). Cryopreservation and short-term storage of sturgeon sperm, a review. Aquaculture, 236 (1-4): 1-9. https://doi.org/10.1016/ j.aquaculture.2003.10.029.

Bozkurt, Y. (2017). The role of cryobiology in conservation of aquatic genetic resources. Lambert Academic Publications, 94p. Saarbrücken, Germany. ISBN: 978-3-330-05346-5.

Bozkurt, Y. (2020). Akuakültürde sperma ve embriyo kriyoprezervasyon biyoteknolojisi. Edited by Aral, F. In: Sperm Cryopreservation in Freshwater Fish. 1st Edition, p. 6-11. Türkiye Klinikleri, Ankara, Türkiye.

Bozkurt Y. (2023). Conservation strategies for the aquatic genetic resources: Opportunities and challenges. Bozkurt, Y. (Ed.). ISBN: 978-625-395-076-7. Modern Reproductive Biotechnologies in the Conservation of Aquatic Genetic Resources. 1st Edition, p. 1-5. Türkiye Klinikleri, Ankara, Türkiye.

Bozkurt, Y., Chebanov, M. (2024). Action before the extinction of endangered sturgeon species: With emphasis on stock enhancement and conservation. In: Bozkurt Y, editor. Aquaculture Industry-Recent Advances and Applications. London, United Kingdom: IntechOpen Publications; 2024. pp. 13–27. https://doi.org/10.5772/intechopen. 113385.

Bozkurt, Y., Saber, M.H. (2026). The crucial role of cryopreservation in *ex-situ* conservation of Caspian Sea sturgeons. IntechOpen. https://doi.org/10.5772/intechopen.1012086.

Cabrita, E., Robles, V., Herraez, P. (Eds.). (2008). Methods in Reproductive Aquaculture: Marine and Freshwater Species (1st ed.). CRC Press. https://doi.org/ 10.1201/9780849380549.

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. J. Appl. Ichthyol., 26: 623-635. https://doi.org/10.1111/j.1439-0426. 2010.01556.x.

Comizzoli, P. (2017). Biobanking and fertility preservation for rare and endangered species. Animal Reproduction, 14 (1): 30-33. https://doi.org/10.21451/1984-3143-AR889.

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: 460-486. https://doi.org/10.1111/j.1439-0426.2008.01151.x.

Cosson, J. (2019). Fish Sperm Physiology: Structure, Factors Regulating Motility, and Motility Evaluation. In: Biological Research in Aquatic Science, Edited by Yusuf Bozkurt. IntechOpen. https://doi.org/10.5772/ intechopen.85139.

Cuevas-Uribe, R., Chesney, E.J., Daly, J., Tiersch, T.R. (2015). Vitrification of sperm from marine fish: effect on motility and membrane integrity. Aquac Res, 46: 1770-1784. https://doi.org/10.1111/are.12337.

Dadras, H., Golpour, A., Rahi, D., Lieskovská, J., Dzyuba, V., Gazo, I., Policar, T. (2022). Cryopreservation of Sterlet, *Acipenser ruthenus* spermatozoa: Evaluation of quality parameters and fine ultrastructure. Front. Mar. Sci. 9: 783278. https://doi.org/10.3389/fmars.2022.783278.

Dietrich, M.A., Kodzik, N., Bakun, M., Szczepkowski, M., Ciereszko, A. (2025). Phosphoproteomic profiling of Siberian sturgeon (*Acipenser baerii*, Brandt, 1869) sperm: insights into cryopreservation-induced changes with DMSO and methanol. Biol Reprod. 113 (4): 820-837. https://doi.org/10.1093/biolre/ioaf128.

Dzyuba, V., Cosson, J., Papadaki, M., Mylonas, C.C., Steinbach, C., Rodina, M., Tučkova, V., Linhart, O., Shelton, W.L., Gela, D., Boryshpolets, S., Dzyuba, B. (2021). Influence of environmental temperature and hormonal stimulation on the ın *vitro* sperm maturation in Sterlet *Acipenser ruthenus* in advance of the spawning season. Animals, 15;11(5): 1417. https://doi.org/10.3390/ani11051417.

Erdahl, A.W., Erdahl, D.A., Graham, E.F. (1984). Some factors affecting the preservation of salmonid spermatozoa. Aquaculture, 43 (1-3): 341-350.

Figueroa, E., Lee-Estevez, M., Valdebenito, I., Watanabe, I., Oliveira, R.P.S., Romero, J., Castillo, R.L., Farías, J.G. (2019). Effects of cryopreservation on mitochondrial function and sperm quality in fish. Aquaculture., 511: 634190. https://doi.org/10.1016/j.aquaculture. 2019.06.004.

Fopp-Bayat, D., Nitkiewicz, A., Sarosiek, B., Kowalski, R.K. (2023). The successful production of "sterbel" hybrids using beluga (*Huso huso*)

cryopreserved sperm. Animal Reproduction Science, 249: 107197. https://doi.org/10.1016/j.anireprosci.2023.107197.

Fopp-Bayat, D., Nitkiewicz, A., Gomułka, P. (2024). The Effect of Cryopreserved Sperm on the Early Development, Survival, and Growth of Intergeneric Sterbel Hybrids (*Acipenser ruthenus* × *Huso huso*). International Journal of Molecular Sciences, 25(11): 5784. https://doi.org/10.3390/ijms25115784v.

Gil, H.W., Lee, T.H., Park, I.S. (2017). Effects of cryoprotectants and diluents on the cryopreservation of spermatozoa from far eastern catfish, *Silurus asotus*. Development and Nutrition, 21(1):71-91. https://doi.org/10.12717/DR.2017.21.1.079.

Glogowski, J., Kolman, R., Mirosław, S., Horváth, A., Urbányi, B., Sieczyński, P., Rzemieniecki, A., Domagała, J., Demianowicz, W., Kowalski, R. (2002). Fertilization rate of Siberian sturgeon (*Acipenser baeri*, Brandt) milt cryopreserved with methanol. Aquaculture. 211. 367-373. 10.1016/S0044-8486(02)00003-0.

Golshahi, K., Aramli, M.S., Nazari, R.M., Habibi, E. (2018). Disaccharide supplementation of extenders is an effective means of improving the cryopreservation of semen in sturgeon. Aquaculture, 486: 261-265. https://doi.org/10.1016/ j.aquaculture.2017.12.045.

Hatef, A., Alavi, S.M.H., Noveiri, S.B., Poorbagher, H., Alipour, A.R., Pourkazemi, M.,Linhart, O. (2011). Morphology and fine structure of *Acipenser persicus* (*Acipenseridae, Chondrostei*) spermatozoon: Inter-species comparison in *Acipenseriformes*. Animal Reproduction Sciences, 123: 81–88. https://doi.org/10.1016/ j.anireprosci.2010.10.013.

Hatef, A., Alavi, S.M.H., Rodina, M., Linhart, O. (2012). Morphology and fine structure of the Russian sturgeon, *Acipenser gueldenstaedtii* (*Acipenseridae*, *Chondrostei*). J. Appl. Ichthyol., 6: 978–983. https://doi.org/10.1111/jai.12056.

Horvath, A., Urbányi, B., Mims, S., Bean, W., Gomelsky, B., Tiersch, T. (2006). Improved cryopreservation of sperm of paddlefish (*Polyodon spathula*). Journal of the World Aquaculture Society, 37 (4), 356-362. https://doi.org/10.1111/j.1749-7345.2006.00048.x.

Horvath, A., Chèvre, P., Urbányi, B. (2011). Sperm cryopreservation in sturgeon with a special focus on *A. sturio*. In: Williot, P., Rochard, E., Desse-Berset, N., Kirschbaum, F., Gessner, J. (eds) Biology and Conservation of the European Sturgeon *Acipenser sturio* L. 1758. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-20611-5_35.

Igna, V., Telea, A., Florea, T., Popp, R., Grozea, A. (2022). Evaluation of Russian sturgeon (*Acipenser gueldenstaedtii*) semen quality and semen cryopreservation. Animals, 12(16): 2153. https://doi.org/10.3390/ ani12162153.

Judycka, S., Szczepkowski, M., Ciereszko, A., Dietrich, G.J. (2015). New extender for cryopreservation of Siberian sturgeon (*Acipenser baerii*) semen. Cryobiology, 70 (2): 184-1899. https://doi.org/10.1016/j.cryobiol. 2015.02.005.

Karimi, I., Mohammad, L.J., Suvitha, A., Haidari, Z., Schiöth, H.B. (2025). Comprehensive overview of the toxicities of small-molecule cryoprotectants for carnivorous spermatozoa: foundation for computational cryobiotechnology. Front. Toxicol. 7:1477822. https://doi.org/10.3389/ftox.2025.1477822.

Kolyada, M.N., Osipova, V.P., Berberova, N.T. (2023a). Use of cryoprotectors and antioxidants in sturgeon semen cryopreservation. Cryobiology, 111: 30-39. https://doi.org/10.1016/j.cryobiol.2023.02.003.

Kolyada, M.N., Osipova, V.P., Pimenov, Y.T. (2023b). Oxidative stress and cryoresistance of sturgeon sperm: A review. Cryobiology. 113:104594. https:// doi.org/10.1016/j.cryobiol.2023.104594.

Linhart, O., Kudo, S. (1997). Surface ultrastructure of paddlefish (*Polyodon spathula*) eggs before and after fertilization. J. Fish Biol., 51(3):573–582. https:// doi.org/10.1111/ j.1095-8649.1997.tb01513.x.

Martínez-Páramo, S., Horvath, A., Labbe, C., Zhang, T., Robles, V., Herraez, P., Suquet, M., Adams, S., Viveiros, A., Tiersch, T.R., Cabrita, E. (2017). Cryobanking of aquatic species. Aquaculture, 472:156-177. https://doi. org/10.1016/j.aquaculture. 2016.05.042.

Mazur, P. (1984). Freezing of living cells: mechanisms and implications. American Journal of Physiology, 247(3Pt1): C125-142. https://doi.org/10.1152/ ajpcell.1984.247.3.C125.

McGann, L. (1978). Differing actions of penetrating and nonpenetrating cryoprotective agents. Cryobiology, 15 (4): 382-390. https://doi. org/10.1016/0011-2240(78)90056-1.

Mims, S.D., Onders, R.J., Shelton, W.L. (2009). Propagation and Culture of Paddlefish. American Fisheries Society Symposium 66:357–383.

Mounib, M. S. (1978). Cryogenic preservation of fish and mammalian spermatozoa. Journal of Reproduction and Fertility, 53(1), 13–18. https://doi. org/ 10.1530/jrf.0.0530013.

Öztürk, A.E., Bucak, M.N., Bodu, M., Başpınar, N., Çelik, İ., Shu, Z., Keskin, N., Gao, D. (2020). Cryobiology and cryopreservation of sperm. In:

Cryopreservation - Current Advances and Evaluations, Edited by Quain, M. IntechOpen. https://doi.org/10.5772/intechopen.89789.

Pourkazemi, M. (2006). Caspian Sea sturgeon conservation and fisheries: Past, present and Future. J. Appl. Ichthyol., 22:12–16. https://doi.org/10.1111/j.1439-0426.2007.00923.x.

Psenicka, M., Alavi, S.M.H., Rodina, M., Gela, D., Nebesarova, J., Linhart, O. (2007). Morphology and ultrastructure of Siberian sturgeon, *Acipenser baerii*, spermatozoa using scanning and transmission electron microscopy. Biology of the Cell. 99: 103–115. https://doi.org/ 10.1042/BC20060060.

Rodrigues, R.B., Uczay, M., Brito, V.B., Godoy, A.C., Moura, D.J., Vogel, C., Vasconcelos, A.C.N., Streit, D.P. Jr. (2021). Oxidative stress and DNA damage of zebrafish sperm at different stages of the cryopreservation process. Zebrafish. 18(2): 97-109. https://doi.org/ 10.1089/zeb.2020.1942.

Rusco, G., Di Iorio, M., Gibertoni, P.P., Esposito, S., Penserini, M., Roncarati, A., Cerolini, S., Iaffaldano N. (2019). Optimization of sperm cryopreservation protocol for Mediterranean brown trout: A comparative study of non-permeating cryoprotectants and thawing rates *in vitro* and *in vivo*. Animals. 31:9(6):304. https://doi.org/10.3390/ani9060304.

Saber, M.H., Bozkurt, Y. (2024). Standardized sperm cryofreezing technique for Caspian Sea sturgeons (*Huso huso, Acipenser persicus, Acipenser stellatus, Acipenser nudiventris*). Genetics of Aquatic Organisms, 8(2):GA782. https://doi.org/ 10.4194/GA782.

Sadeghi, A., Imanpoor, M.R., Shahriari, R., Khalili, M., Abedi, M. (2013). Cryopreservation of stellate (*Acipenser stellatus*) sperm: Effect of different concentrations of DMSO and dilution rates on sperm mobility and motility duration after long-term storage. Global Veterinaria. 10. 26-30. https://doi.org/10.5829/idosi.gv.2013.10.1.7195.

Saha, A., Asaduzzaman, M., Bari, F.Y. (2022). Cryopreservation techniques for ram sperm. Veterinary Medicine International. 30;2022:7378379. https://doi.org/10.1155/ 2022/7378379.

Shaluei, F., Sadeghi, A. and Zadmajid, V. (2017), Cryopreservation of Persian sturgeon (*Acipenser persicus*) sperm: Effects of cryoprotectants, antioxidant, membrane stabilizer, equilibration time and dilution ratio on sperm motility and fertility. Aquaculture Research, 48: 1031-1040. https://doi.org/10.1111/are.12946.

Sieme, H., Oldenhof, H., Wolkers, W.F. (2016). Mode of action of cryoprotectants for sperm preservation. Anim Reproduction Science, 169:2-5. https://doi.org/10.1016/j.anireprosci.2016.02.004.

Suquet, M., Dreanno, C., Fauvel, C., Cosson, J., Billard, R. (2000), Cryopreservation of sperm in marine fish. Aquaculture Research, 31: 231-243. https://doi.org/10.1046/j.1365-2109.2000.00445.x.

Thirumala, S., Huang, C., Dong, Q., Tiersch, T.R., Devireddy, R.V. (2005). A theoretically estimated optimal cooling rate for the cryopreservation of sperm cells from a live-bearing fish, the green swordtail *Xiphophorus helleri*. Theriogenology. 63(9): 2395-2415. https://doi.org/10.1016/j.theriogenology.2004.09.051.

Tiersch, T., Yang, H., Jenkins, J.A., Dong, Q. (2007). Sperm cryopreservation in fish and shellfish. Society of Reproduction and Fertility Supplement, 65: 493-508.

Tiersch, T.R., Green, C.C. (2011). Cryopreservation in Aquatic Species, 2nd Edition. Edited by Tiersch, T. and Green, C.C., World Aquaculture Society. Baton Rouge, Louisiana, U.S.A.

Torres, T.M., de Almeida-Monteiro, P.S., do Nascimento, R.V., Pereira, V.A., Ferreira, Y.M., Lobato, J.S., Pinheiro, R.R.R., Sales, Y.S., Montenegro, A.R., Salmito-Vanderley, C.S.B. (2022). Sperm cryopreservation of *Prochilodus brevis* using different concentrations of non-permeable cryoprotectants. Anim Reproduction, 4:19(1):e20210083. https://doi.org/10.1590/1984-3143-AR2021-0083.

Tsvetkova, L.I., Cosson, J., Linhart, O. and Billard, R. (1996). Motility and fertilizing capacity of fresh and frozen-thawed spermatozoa in sturgeons *Acipenser baeri* and *Acipenser ruthenus*. Journal of Applied Ichthyology, 12: 107-112. https://doi.org/ 10.1111 /j.1439-0426.1996.tb00071.x.

Yang, H., Norris, M., Winn, R., Tiersch, T.R. (2010). Evaluation of cryoprotectant and cooling rate for sperm cryopreservation in the euryhaline fish medaka *Oryzias latipes*. Cryobiology. 61(2): 211-219. https://doi.org/10.1016/j.cryobiol.2010.07.006.

Zhang, X., Wu, W., Li, L., Ma, X., Chen, J. (2013). Genetic variation and relationships of seven sturgeon species and ten interspecific hybrids. Genet. Sel. Evol., 28;45(1):21. https://doi.org/10.1186/1297-9686-45-21.

CHAPTER II

SPERM CRYOPRESERVATION IN THE EUROPEAN SEA BASS

Tülin ARSLAN

(Prof. Dr.), Muğla Sıtkı Koçman Üniversitesi,
E-mail: atulin@mu.edu.tr
ORCID: 0000-0001-9661-045X

1. Introduction

The European sea bass (*Dicentrarchus labrax*) is the first non-salmonid marine fish to be commercially cultivated in Europe (Bagni, 2005). This species is endemic to the coastal waters of the north-eastern Atlantic, the Mediterranean, and the Black Sea. They are typically found at depths shallower than 100 m. They can tolerate wide salinity ranges (0-90 ppt) and temperatures (2-32°C). Sexual maturation occurs earlier in males, usually at two to three years of age, while females mature at three to four years of age (Perez-Ruzafa & Marcos, 2014). The reproductive cycle of the species is seasonal, with the period of spawning occurring between the months of December and March in the Mediterranean region and between March and June in the Atlantic region. Females are highly fecund and release large number of eggs, which are fertilized externally. Juveniles generally occupy estuaries and lagoons from spring to autumn, before migrating with adults to deeper waters in winter, where temperatures remain above 9-10°C (Pickett & Pawson, 1994).

The initial attempts to cultivate sea bass, which entailed the rearing of juveniles from the wild, commenced in the 1970s. In the early 1980s, research focusing on captive breeding and rearing techniques facilitated the development of hatchery methods for juvenile production (Bagni, 2005; Chatain and Chavanne, 2009). This specialized method of fry production, based on the year around voluntary spawning of broodstock in tanks under natural and artificial photoperiod regimes, was adopted in the Mediterranean basin in the 1990s. The accelerated proliferation of this production technique and the consequent escalation in output have precipitated the predominance of sea bass

as the prevailing species within the European mariculture sector. Consequently, since 1992, production of aquaculture has surpassed that of fisheries. In 2023, global European sea bass production reached 292,523.5 tons, with aquaculture accounting for 98.1% of this production (FAO, 2024). The primary contributors to this achievement have been Türkiye (160,802.0 tons), Greece (44,200.8 tons), Egypt (34,077.0 tons), and Spain (24,413.3 tons) (FAO, 2024).

The rapid growth of the European sea bass farming sector, while offering certain benefits, also raises concerns about the sustainability of production. In this context, selective breeding is emerging as a critical management strategy to ensure the sustainability of the sector. Indeed, the first selective breeding programs employing hatchery-raised broodstock were initiated in the 1990s (Chatain and Chavanne, 2009). Subsequent years witnessed the continuation of endeavors aimed at developing lines that exhibited genetically enhanced production performance (Chavanne et al., 2016; Janssen et al., 2017). In recent years, there has been significant progress in the development of tools that can facilitate the implementation of highly effective genome-based selective breeding programs (Vandeputte et al., 2019). However, in contrast to the voluntary spawning technique that is typically utilized in hatcheries, selective breeding programs necessitate the controlled production of specific brooders. This necessity has prompted research initiatives focused on the development of reproductive biotechnologies, encompassing controlled gamete production, insemination, and sperm storage. This chapter provides a comprehensive overview of the current state of research in the field of cryopreservation of sperm in European sea bass. The applications, limitations, and future prospects of this technique as a long-term sperm preservation method are highlighted.

2. Sperm characteristics

Studies on sperm production dynamics of European sea bass have shown that, although the breeding season varies by region from December to June, spawning generally occurs over a two- to three-month period in a particular location (Carrillo et al., 1995). A single 11-ketotestosterone peak initiates and maintains spermatogenesis and spermiogenesis (Prat et al., 1990). Furthermore, gametogenesis begins earlier in males than in females, which ensures availability of sperm before the spawning of females (Prat et al., 1990; Carrillo et al., 1995).

Males produce a highly concentrated milt containing up to 60 x 10^9 spermatozoa/ml, packed in a relatively small amount of seminal plasma that may constitute only 10% of the milt (Billard 1978a; Zohar et al., 1984; Fauvel

et al., 1999). Osmolality and pH of their seminal plasma are 371±9 mOsm/kg and 8.06±0.03, respectively (Fauvel et al., 2012). Furthermore, milt volume and spermatozoa concentration may remain relatively stable throughout the spawning season. Zohar et al. (1984) observed only a slight decline in sperm concentration over a reproductive season lasting more than two months. Sorbera et al. (1996) also reported that weekly stripping of males does not significantly affect sperm production, expressible milt from a particular male is available for 28 days during a spawning period without significant change in spermatozoa density. Additionally, they showed that males treated with GnRHa implants remain fluent for a longer duration of 44 days without variation in sperm concentration. Fauvel et al. (1999) observed only a slight decline in sperm concentration of monthly sampled fish but reported that frequent stripping may shorten the spermiation period and the number of spermiating males declines towards the end of spawning season.

As in most marine teleost, sperm motility in European sea bass is triggered by hyperosmotic shock upon dilution in seawater (Billard, 1984). Differences in motility duration were reported across studies (ranging from 30 s to several min; Billard, 1984; Villani & Catena, 1991; Sorbera et al., 1996) due to methodological variations, including sperm preparation, dilution, and activation procedures. Predilution in a non-activating media and subsequent high-ratio dilution (e.g., 1:1000) have been shown to synchronize activation and provide more accurate motility assessments (Billard & Cosson, 1992). Studies using higher dilution ratios showed that European sea bass sperm exhibits unusually short motility duration compared to other marine fish species, behaving more like freshwater fish sperm in this respect. Motility is typically last around 30-45 s (Dreanno et al., 1999; Fauvel et al., 1999). Dreanno et al. (1999) demonstrated that this brief period of motility is controlled by intracellular energy constraints and ionic regulation. The hyperosmotic shock encountered during activation causes rapid depletion of ATP stores, and rising internal calcium levels disrupt the symmetrical flagellar beating pattern toward the end, ultimately limiting the motility window. Thus, their spermatozoa, which initially swim at high speed and in linear trajectories after activation, gradually lose motility after 45 s (Dreanno et al., 1999). In addition to brief motility duration, progressive declines in sperm motility and viability as the reproductive season advanced was observed by Billard et al. (1977) who evaluated sperm quality at different times during the spawning period, by measuring both motility duration after dilution and survival during cold storage. They demonstrated that sperm diluted

100-fold exhibited decreasing motility duration as the season progressed, from 5 min early in the spawning season to only 30 s at its end. Similarly, sperm survival at 4°C declined from 70 h at the beginning of the season to less than 30 min later in the cycle, suggesting progressive sperm ageing occurs along the reproductive season.

Additionally, it was shown that the salinity or osmolality of the activation solution affects the motility and fertilization capacity of sperm. Physiological saline solution with a pH of 8.5 or seawater with a lowered salinity of 20 ppt and/or a pH of 8-9 increased the percentage of motile sperm, improved the motility duration and fertilization capacity (Billard, 1978a; 1978b; Sansone et al., 2001). However, majority of researchers usually used full strength sea water or artificial seawater in their sperm motility assessments (Table 1).

3. Studies on sperm cryopreservation

Sperm cryopreservation is a crucial biotechnological tool in aquaculture and fisheries research, providing opportunities for selective breeding, genetic resource conservation, and the stabilization of gamete supply in hatcheries. However, limited number of studies were devoted to sperm cryopreservation in marine fish species, including the European sea bass.

The European sea bass milt is thick and highly viscous (Billard 1978a; Zohar et al., 1984; Fauvel et al., 1999). These characteristics make it difficult to work with because the quality of the sperm can deteriorate very quickly, within 30 min to 6 h, after collection due to oxygen depletion (Billard et al., 1977; Sansone et al., 2001; Fauvel et al., 2012). Additionally, the thickness of the milt makes homogeneous mixing with eggs during artificial insemination very difficult (Fauvel et al., 1999). To address these challenges, researchers evaluated various isotonic media for diluting sea bass sperm and/or its chilled liquid storage. In the earliest studies, a physiological saline solution adapted from Mounib (1968) and named B4 was identified as a good candidate for this purpose (Billard 1978b; 1984). In a comprehensive study on the sperm characteristics of captive broodstock, researchers used another isotonic saline solution for diluting sperm before fertilization of eggs stripped from females. They named this solution non-activating medium or as commonly abbreviated NAM (Table 1). Furthermore, they showed that predilution of sperm in NAM up to 128 times does not cause any loss in its initial quality (Fauvel et al., 1999). Taking advantage of the osmolarity and pH sensitivity of sperm, Sansone et al. (2001) used a slightly hypotonic NaCl solution (1%, 297 mOsm/kg), pH

of which was lowered to 5.4 with hydrochloric, citric, or acetic acid. Optimal motility rates were obtained when using the 1% NaCl-citric acid combination and a 1:10 dilution ratio.

The earliest studies on long term sperm preservation in European sea bass revealed that combination of B4 solution adapted from Mounib (1968) in combination with cryoprotectants such as 10% dimethyl sulfoxide (DMSO) or 17% glycerol, and utilizing a relatively slow cooling rate (-10°C/min up to -80°C) during freezing provided satisfactory results (Billard 1978b; 1984). However, an improved diluent (containing a 10 mg/ml bovine serum albumin (BSA)) or a cell culture medium such as Eagle recommended for the preservation of lower quality sperm obtained towards the end of spawning season (Billard 1978a). Later, Fauvel et al. (1998) evaluated a sperm cryopreservation protocol adapted from turbot, using either 0.25 ml straws or 1.5 ml cryovials by utilizing a higher dilution rate and a faster initial cooling rate (Table 2). They also reported highly satisfactory results with post-thaw motilities close to fresh sperm. Sansone et al. (2002) combined 1% NaCl-citric acid solution with 5 distinct cryoprotectants at various concentrations (Table 2). Their basic extender also yielded over 65% post-thaw motility rates when combined with 7% DMSO or 10% ethylene glycol (EG). Combining the egg-yolk-citrate extender at a higher dilution rate of 1: 20 with a 10% DMSO also allowed the high post-thaw motility rates, but unlike previous studies the success of cryopreservation significantly declined when EG, or glycerol was used as cryoprotectant or DMSO was combined with the urea-egg-yolk and 0.9% NaCl solutions (Badr et al., 2021). The egg-yolk-citrate extender combined with 10% methanol (MeOH) also effective and yielded relatively high post-thaw motility rates of 73.33±10.15% (Badr et al., 2021).

Due to observed variability in the results of cryopreservation studies, Peñaranda et al. (2008) evaluated the effects of extenders, previously utilized by other researchers (NAM from Fauvel et al., 1998; 1999 and 1% NaCl-HCl solution from Sansone et al., 2001), on the motility and sperm head morphology of spermatozoa via liquid storage at 4°C. They obtained the best results with the NAM extender or by adding 1 and 2% of BSA to it. The motility of the spermatozoa diluted at 1: 20 ratio and incubated in these extenders was similar to that of fresh sperm until 48 h and was higher than that of undiluted sperm. They also checked the spermatozoa motility and morphology reactions with some of the best performing extenders by combining them with three cryoprotectants at different concentrations (2, 5, 10%). While the combination of extenders with glycerol caused the activation of spermatozoa motility, combining the

2% BSA added NAM with either MeOH or DMSO resulted in a low level of sperm activation and high motility rates 5 min after incubation. Thus, researchers suggested these combinations are good candidates to be used in the cryopreservation of sperm. However, a recent study reported that a 10% DMSO and NAM combination with or without 5% glucose addition yields the highest post-thaw motility kinetics, but MeOH should be avoided as a cryoprotectant (França et al., 2022).

Other researchers evaluated the effects of antioxidant incorporation into cryopreservation media. Cabrita et al. (2011) added 1 mM or 10 mM taurine, hypotaurine, ascorbic acid, 0.1 or 0.5 mM α-tocopherol and 1 ml of a commercial cell antioxidant to a cryomedium consisting of 1%NaCl and 10% DMSO. They observed that these antioxidants neither affect the post-thaw motility kinetics of sperm nor support its viability or provide protection against cryopreservation induced DNA damage. Furthermore, vitamins may even increase the incidence of DNA fragmentation or the commercial cell antioxidant may lower the viability of sperm. In the follow up studies, however, the incorporation of the same vitamins (0.1 mM α-tocopherol or 1 mM ascorbic acid) to NAM and 10% DMSO medium containing 1% BSA improved motility and straight-line velocity of cryopreserved sperm (Martínez-Páramo et al., 2012a). Incorporation of 1 mM hypotaurine and taurine improved the total motility and straight-line velocity of cryopreserved sperm, respectively (Martínez-Páramo et al., 2013). However, taurine and hypotaurine provided only slight protection against DNA damage and none of the antioxidants support the viability or reduce the lipid and protein oxidation levels in cryopreserved sperm (Martínez-Páramo et al., 2012b; 2013).

4. Conclusion and future prospects

The European sea bass exhibits peak sperm concentration and quality at the onset of the spawning season. However, as the season progresses, a marked decline can be observed in motility and fertilization capacity. Despite the potential to sustain sperm production through methods such as regular stripping and hormonal induction, motility rapidly declines after activation and deteriorates further due to aging as the season progresses. These biological constraints present considerable challenges to hatchery operations and selective breeding programs for the species.

The application of cryopreservation technology in hatchery operations and selective breeding programs has the potential to mitigate these limitations. Furthermore, cryopreservation technology provides a pivotal instrument in the

domain of long-term genetic resource banking and conservation. Researchers working on European sea bass sperm cryopreservation have reported significant successes to date. However, as outlined in this chapter, there are some inconsistencies among the studies. Consequently, further research is needed to optimize sperm cryopreservation protocols in this species. In addition to the investigation of effective cryoprotectants and freezing protocols, future studies should include a systematic evaluation of thawed sperm quality. This will facilitate a more comprehensive examination of the effects of the freezing-thawing process and storage in frozen form. Systematic evaluations should include assessments of DNA and membrane integrity, in addition to the degree of fertilization success. The development of effective and robust sperm preservation techniques will ultimately contribute to European sea bass farming, the conservation of biological diversity, and the sustainable use of marine resources.

References

Badr, M., El-Gamal, M., Nasr-Alla, A., & Zaki, M. A. (2021). Effect of extenders, cryoprotectants, and freezing methods on sperm quality and fertilisation rate of the European sea bass (*Dicentrarchus labrax*). *Egyptian Journal of Aquatic Biology and Fisheries, 25*(1), 63–74. http://www.ejabf.journals.ekb.eg

Bagni, M. (2005). Cultured aquatic species information programme: *Dicentrarchus labrax*. FAO Fisheries and Aquaculture Department. http://www.fao.org/fishery/culturedspecies/Dicentrarchus_labrax/en

Billard, R. (1977). Fall in motility and survival time at low temperature of sperm of *Dicentrarchus labrax* L. (Pisces, Teleostei) during the spawning season. *Aquaculture, 11*(4), 363–367.

Billard, R. (1978a). Changes in structure and fertilizing ability of marine and freshwater fish spermatozoa diluted in media of various salinities. *Aquaculture, 14*(3), 187–198.

Billard, R. (1978b). Some data on gametes preservation and artificial insemination in teleost fish. *Actes de Colloques du Centre National de l'Exploitation des Océans (CNEXO), 8*, 59–73.

Billard, R. (1984). La conservation des gamètes et l'insémination artificielle chez le bar et la daurade. In G. Barnabé & R. Billard (Eds.), *L'Aquaculture du Bar et des Sparidés* (pp. 95–116). INRA Publications.

Billard, R., & Cosson, M.-P. (1992). Some problems related to the assessment of sperm motility in freshwater fish. *Journal of Experimental Zoology, 261*(1), 122–131.

Cabrita, E., Robles, V., Rebordinos, L., Sarasquete, C., & Herráez, M. P. (2011). Evaluation of DNA damage in cryopreserved sea bass sperm. *Aquaculture, 311*(1–4), 224–231.

Carrillo, M., Zanuy, S., Prat, F., Cerdá, J., Ramos, J., Mañanós, E., & Bromage, N. (1995). Seabass (*Dicentrarchus labrax*). In N. R. Bromage & R. J. Roberts (Eds.), *Broodstock management and egg and larval quality* (pp. 138–168). Blackwell Science.

Chatain, B., & Chavanne, H. (2009). La génétique du bar (*Dicentrarchus labrax* L.). *Cahiers Agricultures, 18*(2–3), 249–255.

Chavanne, H., Janssen, K., Hofherr, J., Contini, F., Haffray, P., Komen, H., Nielsen, E. E., & Bargelloni, L. (2016). A comprehensive survey on selective breeding programs and seed market in the European aquaculture fish industry. *Aquaculture International, 24*(5), 1287–1307.

Dreanno, C., Cosson, J., Suquet, M., Seguin, F., Dorange, G., & Billard, R. (1999). Effects of osmolality, morphology perturbations and intracellular nucleotide content during the movement of sea bass (*Dicentrarchus labrax*) spermatozoa. *Journal of Reproduction and Fertility, 116*(1), 113–125. https://doi.org/10.1530/jrf.0.1160113

FAO. (2024). *FishStat: Global production by production source, quantity (1950–2023).* Food and Agriculture Organization of the United Nations. https://www.fao.org/fishery/statistics-query/en/global_production/global_production_quantity

Fauvel, C., Dreanno, C., Suquet, M., & Cosson, J. (1998). Cryopreservation of sea bass (*Dicentrarchus labrax*) spermatozoa in experimental and production simulating conditions. *Aquatic Living Resources, 11*(6), 387–394.

Fauvel, C., Savoye, O., Dreanno, C., Cosson, J., & Suquet, M. (1999). Characteristics of sperm of captive seabass in relation to its fertilization potential. *Journal of Fish Biology, 54*(2), 356–369. https://doi.org/10.1111/j.1095-8649.1999.tb00835.x

Fauvel, C., Boryshpolets, S., Cosson, J., Wilson Leedy, J. G., Labbé, C., Haffray, P., & Suquet, M. (2012). Improvement of chilled seabass sperm conservation using a cell culture medium. *Journal of Applied Ichthyology, 28*(6), 961–966. https://doi.org/10.1111/jai.12071

França, T. S., González-López, W. A., Sanchez, M. P., Pérez, L., Mañanós, E. L., Felip, A., Gómez, A., & Asturiano, J. F. (2022). Protocol optimization

for sea bass sperm cryopreservation and assessment of post-thawing dilution to prolong sperm usefulness in aquaculture Mediterranean species. *8th International Workshop on the Biology of Fish Gametes*, Gdańsk, Poland (p. 56).

Janssen, K., Chavanne, H., Berentsen, P., & Komen, H. (2017). Impact of selective breeding on European aquaculture. *Aquaculture, 472*, 8–16.

Martínez-Páramo, S., Diogo, P., Dinis, M. T., Herráez, M. P., Sarasquete, C., & Cabrita, E. (2012a). Incorporation of ascorbic acid and α-tocopherol to the extender media to enhance antioxidant system of cryopreserved sea bass sperm. *Theriogenology, 77*(6), 1129–1136.

Martínez-Páramo, S., Diogo, P., Beirão, J., Dinis, M. T., & Cabrita, E. (2012b). Sea bass sperm freezability is influenced by motility variables and membrane lipid composition but not by membrane integrity and lipid peroxidation. *Animal Reproduction Science, 131*(3–4), 211–218.

Martínez-Páramo, S., Diogo, P., Dinis, M. T., Soares, F., Sarasquete, C., & Cabrita, E. (2013). Effect of two sulfur-containing amino acids, taurine and hypotaurine, in European sea bass (*Dicentrarchus labrax*) sperm cryopreservation. *Cryobiology, 66*(3), 333–338. https://doi.org/10.1016/j.cryobiol.2013.04.001

Mounib, M.S., Hwang, P.C. & Idler, D.R. (1968). Cryogenic preservation of Atlantic cod (*Gadus morhua*) sperm. *Journal of the Fisheries Research Board of Canada*, 25, 2623-2632.

Mounib, M.S. (1978). Cryopreservation of fish and mammalian spermatozoa. *Journal of Reproduction and Fertility*, 53, 13-18.

Peñaranda, D. S., Pérez, L., Fakriadis, G., Mylonas, C. C., & Asturiano, J. F. (2008). Effects of extenders and cryoprotectant combinations on motility and morphometry of sea bass (*Dicentrarchus labrax*) spermatozoa. *Journal of Applied Ichthyology, 24*(4), 450–455.

Pérez-Ruzafa, A., & Marcos, C. (2014). Ecology and distribution of *Dicentrarchus labrax* (Linnaeus 1758). In F. J. Sánchez Vázquez & J. A. Muñoz-Cueto (Eds.), *Biology of European sea bass* (pp. 3–33). CRC Press.

Pickett, G. D., & Pawson, M. G. (1994). *Seabass: Biology, exploitation and conservation*. Chapman & Hall.

Prat, F., Zanuy, S., Carrillo, M., de Mones, A., & Fostier, A. (1990). Seasonal changes in plasma levels of gonadal steroids of sea bass, *Dicentrarchus labrax* L. *General and Comparative Endocrinology, 78*(2), 361–373.

Sansone, G., Fabbrocini, A., Ieropoli, S., Langellotti, A. L., Occidente, M., & Matassino, D. (2002). Effects of extender composition, cooling rate, and freezing on the motility of sea bass (*Dicentrarchus labrax* L.) spermatozoa

after thawing. *Cryobiology, 44*(3), 229–239. https://doi.org/10.1016/S0011-2240(02)00026-3

Sansone, G., Fabbrocini, A., Zupa, A., Lavadera, S. L., Rispoli, S., & Matassino, D. (2001). Inactivator media of sea bass (*Dicentrarchus labrax* L.) spermatozoa motility. *Aquaculture, 202*(3–4), 257–268.

Sorbera, L. A., Mylonas, C. C., Zanuy, S., Carrillo, M., & Zohar, Y. (1996). Sustained administration of GnRHa increases milt volume without altering sperm counts in the sea bass. *Journal of Experimental Zoology, 276*(5), 361–368.

Vandeputte, M., Ganaire, P.-A., & Allal, F. (2019). The European sea bass: A key marine fish model in the wild and in aquaculture. *Animal Genetics, 50*(3), 195–206. https://doi.org/10.1111/age.12779

Villani, P., & Catena, C. (1991). Criopreservazione di gameti maschili di spigola (*Dicentrarchus labrax* L.): Soluzioni e metodologie [Gametes cryopreservation of male sea bass (*D. labrax* L.): Solutions and methodologies]. *Rivista Italiana di Acquacoltura, 6*, 217–222.

Zohar, Y., Billard, R., & Weill, C. (1984). La reproduction de la daurade (*Sparus aurata*) et du bar (*Dicentrarchus labrax* L.): Connaissance du cycle sexuel et contrôle de la gamétogenèse et de la ponte. In G. Barnabé & R. Billard (Eds.), *L'Aquaculture du Bar et des Sparidés* (pp. 3–24). INRA Publications.

Table 1. Activation and sperm extender solutions commonly used for sea bass sperm.

Activation solution	Dilution rate	Extender	Dilution rate	Outcomes	References
20 g/l NaCl, pH=8-9, adjusted with 0.02 M Tris-HCl	1: 100	B4: Modified from Mounib (1968): 19.5 g/l NaCl 6.25 g/l Glycine 0.25 g/l $MgSO_4.7H_2O$ 0.25 $CaCl_2.2H_2O$ Buffered with 0.02 M Tris-HCl to pH=8.5	1: 1	At 20 °C, 2 min motility duration and %78 fertilization success	Billard, 1978a; Billard, 1984
38‰ seawater (1160 mOsm/kg), 10 g/l BSA, pH=8.2	1: 50	NAM: 59.83 mM NaCl 1.47 mM KCl 12.91 mM $MgCl_2$ 3.51 mM $CaCl_2$, 20 mM $NaHCO_3$ 0.44 mM Glucose pH=7.7, 310 mOsm/kg	1: 128	Caused no change in motility parameters	Fauvel et al., 1998; 1999
37‰ seawater (1130 mOsm/kg), pH=7.5 **pH=8.2**	1: 10	1% NaCl-HCl, pH=5.4 **1% NaCl- citric acid, pH=5.4** 1% NaCl- acetic acid, pH=5.4 297±2 mOsm/kg (incubated at 0-2ºC)	1:3, **1: 6**, 1: 10	Help to preserve the motility parameters longer compared to undiluted sperm	Sansone et al., 2001
37‰ seawater (1130 mOsm/kg)	1: 100	1% NaCl (pH=5.4, 297 mOsm/kg)	1: 6	Used for 6 h transfer of samples at 0-2ºC	Sansone et al., 2002
Artificial seawater (2.4 mM $MgCl_2$ 5, 28.2 mM Na_2SO_4, 9.9 mM $CaCl_2$, 9.4 mM KCl, 20 mM $NaHCO_3$, 354.7 mM NaCl) + 0.1% BSA, pH=8.2 adjusted with 0.1 N NaOH	1: 10	**NAM + 1 or 2% BSA** 1% NaCl-HCl + 1 or 2% BSA (incubated at 4°C)	1: 20	Helped to maintain similar motility to fresh sperm up to 48 h	Peñaranda et al., 2008

Table 1 (Continued). Activation and sperm extender solutions commonly used for sea bass sperm.

Activation solution	Dilution rate	Extender	Dilution rate	Outcomes	References
Artificial seawater (513.3 mM NaCl, 10.7 mM KCl, 11.7 mM $CaCl_2$, 54.8 mM $MgSO_4$, 11.6 mM $NaHCO_3$)	1: 40	NAM + 1% BSA, pH 7.7	1: 6	Used for dilution and activation of samples	Martínez-Páramo et al., 2012a; 2012b
Natural (1100 mOsm/kg) or diluted (630 mOsm kg) sea water, 8 g/l BSA, pH 8.2, adjusted with 15 mM Tris-HCl	1: 24	IM: 53 mM NaCl 1.1 mM KC1 4.5 mM $MgCl_2$ 2.0 mM $CaCl_2$ 0.3 mM Glucose 8 g/l BSA, pH 8.2 adjusted with 15 mM Tris-HCl, 150 mOsm/kg	1: 24	Diluted sea water prolonged the motility duration of about 10% of spermatozoa from 45 s to 2 min	Dreanno et al., 1999
-	1: 2000	1. NAM+1 mg/ml gentamycine sulfate, 213 mOsm/kg, pH=7.34 or pH=8.1 2. 63% Leibovitz-15 medium+1 mg/ml gentamycine sulfate, 213 mOsm/kg, pH=7.34 or pH=8.1, adjusted with 1 N NaOH (incubated for 2, 22, 46 h at 4°C)	1: 2, 1: 3, 1: 5	1. Helped to maintain similar motility to fresh sperm up to 24 h 2. Helped to maintain similar motility to fresh sperm up to 48 h	Fauvel et al., 2012

Conditions marked with bold letters yielded the best outcomes presented. B4: Billard, 1984, NAM: Non-activating medium, IM: Immobilizing solution, BSA: Bovine serum albumin.

Table 2. List of studies on the cryopreservation of sea bass sperm.

Cryoprotectant	Extender	Dilution rate	Equilibration time	Freezing protocol	Thawing protocol	Outcomes	References
10% DMSO 17% Glycerol	B4	1: 1	0 min	0.5 ml straws, cooled at a rate of -l0°C/min up to -80°C before plunging into LN	-	-	Billard, 1978b; 1984
10% DMSO	Modified from Mounib (1978): 10.01 g/l $KHCO_3$ 1.99 g/l reduced glutathione 42.78 g sucrose +10 g/l BSA 310 mOsm/kg, pH=7.8	1: 3	0 min	**open end-0.25 ml straws**, kept 6.5 cm above LN for 15 min (64.9°C/min) +1.5 ml vials, kept 6.5 cm above LN for 12 min (64.9°C/min) before plunging into LN	Straws: At 35°C for 5 s Cryovials: At 50°C for 1 min	>80% post-thaw motility and >60% fertilization rates	Fauvel et al., 1998
10% DMSO	63% Leibovitz-15 medium+1 mg/ml gentamycine sulfate, 213 mOsm/kg, pH=7.34 (incubated for 2, 22, 46 h at 4ºC)	1: 3	0 min	open end-0.25 ml straws, kept 6.5 cm above LN for 15 min	At 35°C for 5 s	Showed similar motility to the sperm stored 4ºC	Fauvel et al., 2012
5, 7, 10, 15, 20% **7% DMSO** **10 %EG** PG Glycerol %4, 6, 8, 10 MeOH For 2, 10, 20. 30 min at 25°C with or without 1.25 mM sodium pyruvate	DI2: 1% NaCl- citric acid, pH=5.4 (incubated in for 6 h at 0-2ºC)	1: 6	15 min	0.25 ml straws, cooled at rates of **10**, 12, 15. 24 °C/min up to -150°C before plunging into LN, activated at thawing, 1 and 2 min after thawing	At a rate of 15°C/min up to 15°C	>65% post-thaw motility rates	Sansone et al., 2002

Table 2 (Continued). List of studies on the cryopreservation of sea bass sperm.

Cryoprotectant	Extender	Dilution rate	Equilibration time	Freezing protocol	Thawing protocol	Outcomes	References
10% DMSO	%1 NaCl + 1 mM or 10 mM taurine, hypotaurine, ascorbic acid 0.1 or 0.5 mM α-tocopherol, 1 ml commercial cell antioxidant 320 mOsm/kg, pH=7.4	1: 6		0.5 ml straws, kept 6.5 cm above LN for 15 min before plunging into LN	25°C for 30s	None affected motility parameters, vitamins increased DNA fragmentation, commercial cell antioxidant reduced viability	Cabrita et al., 2011
10% DMSO NAM: 59.83 mM NaCl, 1.47 mM KCL, 12.91 mM MgCl2, 3.51 mM CaCl2, 20 mM NaHCO3, 0.44 mM glucose	NAM+ 1% BSA, 1 mM α-tocopherol or 1 mM ascorbic acid, pH 7.7	1: 6	0	0.5 ml straws, kept 6.5 cm above LN for 15 min before plunging into LN	35°C for 15 s	Antioxidants improved motility and straight-line velocity, none affected viability, lipid or protein oxidation levels	Martínez-Páramo et al., 2012a; 2012b
10% DMSO	NAM+ 1% BSA, 1 ml 1 mM taurine or 1 ml 1 mM hypotaurine pH 7.7	1: 6	0	0.5 ml straws, kept 6.5 cm above LN for 15 min before plunging into LN	35°C for 15 s	Hypotaurine improved total motility, taurine improved straight-line velocity, none affected viability, lipid or protein oxidation levels	Martínez-Páramo et al., 2013
10% DMSO 10% Glycerol 10% EG 10% MeOH	**egg-yolk citrate** urea-egg-yolk 0.9% NaCl	1: 2 1: 4 1: 10 **1: 20**	0 5 **15** 30	PVC sealed-0.5 ml straws	20°C for 1 min	73.33 ± 4.42 post-thaw motility	Badr et al., 2021

Table 2 (Continued). List of studies on the cryopreservation of sea bass sperm.

Cryoprotectant	Extender	Dilution rate	Equilibration time	Freezing protocol	Thawing protocol	Outcomes	References
10% DMSO 10% MeOH	NAM **NAM+** **5% glucose**	-	-	-	-	MeOH showed negative impact on the motility parameters, post-thaw dilution in a BSA added NAM improved the motility parameters and prolong the usability of sperm	França et al., 2022

Conditions marked with bold letters yielded the best outcomes presented. B4: Billard (1984) and NAM: Non-activating medium (see Table 1 for their contents), DMSO: Dimethyl sulfoxide, EG: Ethylene glycol, PG: 1–2 propylene glycol, MeOH: Methanol, BSA: Bovine serum albumin, PVC: Polyvinyl chloride.

CHAPTER III

CRYOPRESERVATION OF SHABOUT (*BARBUS GRYPUS H*, 1843) SPERM

Zafer DOĞU[1] & Erdinç ŞAHİNÖZ[2]

[1](Prof. Dr.) Harran University Birecik Vocational High School
E-mail: zafer_dogu@yahoo.com
ORCID: 0000-0002-5428-8340

[2](Prof. Dr.) Harran University Birecik Vocational High School
E-mail: e_sahinöz@harran.edu.tr
ORCID: 0000-0003-0646-6939

1. Introduction

Freezing preserves genetic material, so promoting biodiversity conservation, population management, selective breeding, and crossbreeding. It facilitates out-of-season breeding by allowing the use of genetic material for breeding purposes. The freezing procedure can be optimized to improve sperm quality, resulting in higher fertilization rates when relied on for artificial insemination. Sperm banking preserves genetic resources from diseases that could wipe out fish populations. It also can help in the adaptation of breeding programs to environmental changes, such as climate change. Cryopreserved sperm is an invaluable resource for researchers studying reproductive biology, genetics, and aquaculture techniques. Furthermore, cryopreservation can reduce costs related to maintaining live broodstock, especially for species that are challenging to culture in captivity. Overall, cryopreservation has various advantages, including higher sperm quality, risk control, and cost-effectiveness in aquaculture.

Endemic fish species play a crucial role in their ecosystems and serve as vital genetic reservoirs. Understanding the various biological aspects of these species, particularly their reproductive traits, is essential for effective management and conservation efforts. In this regard, numerous studies have focused on the biological features and meat quality of the shabut fish, scientifically

known as Barbus grypus. This species is native to the freshwater systems of the Euphrates-Tigris River basin in Türkiye and is also found across Iran, Turkey, Syria, and Iraq (Nikpei, 1996; Abdoli, 2000). *Barbus grypus*, a species found in estuaries, has recently become a candidate species for use in aquaculture. It can attain a maximum length exceeding one meter and can weigh more than 50 kilograms, with spawning generally taking place between May and mid-June (Doğu, 2012). Trying to understand the spermatological characteristics and sperm cryopreservation studies of shabut fish will help their management and protection in nature and may also contribute to their production under controlled conditions. In this chapter, sperm preservation methods, characteristics, some effective factors in freezing, freezing protocol and general methods in frozen sperm thawing protocol were detailed and discussed based on shabut fish sperm.

2. Shabut Sperm Characteristics

Doğu et al (2014) was reported the semen volume of Shabut as 720.71 ±60.88, 829.83 ±44.24, 1013.50 ±93.04 and 1217.33 ±183.23 µL on average in 5, 6, 7 and 8 years old fish, respectively, while the overall average was reported as 886.00 ±78.76 µL. In a study involving 13 parameters, the following ranges were observed: sperm density ranged from 15 to 23 × 10^9; spermatocrit values were between 60% and 90%; the duration of sperm motility varied from 90 to 132 seconds; milt volume was recorded at 2.9 to 4.5 milliliters; seminal plasma pH levels ranged from 7.65 to 8.27; glucose concentrations were between 8.2 and 12.2 mg/dl; cholesterol levels ranged from 21 to 29.3 mg/dl; total protein content varied from 0.97 to 1.9 g/dl; sodium (Na+) levels were between 84.45 and 103 mM/l; potassium (K+) levels ranged from 36.8 to 49.3 mM/l; calcium (Ca++) was measured at 0.86 ± 0.06 mM/l; magnesium (Mg++) levels ranged from 1.2 to 1.55 mM/l; total sperm length was between 3.48 and 4.66 µm; sperm head length varied from 0.88 to 1.33 µm; and the length of the sperm flagellum ranged from 12.6 to 13.3 µm (Khodadadi et al., 2016). The mean fresh sperm concentration and motility duration of shabout were estimated as 19 375 ± 2894 $x10^9$ ml and 1550 ± 413.09 s, respectively (Doğu, 2012).

3. Sperm Preservation Methods

Spallanzani, an Italian priest, first documented in 1776 that exposure to snow and low temperatures affects human sperm, notably impacting sperm motility (Doetsch, 1976). A major breakthrough came with the atificial insemination with

frozen sperm in 1950 (Blaxter, 1953). Today, cryopreservation techniques in fish are mostly applied to sperm. The possibility of cryopreservation of fish sperm has been available for more than 70 years and, due to the large number of fish species, experimental methods are still used in most of them today. Most research efforts have predominantly concentrated on larger aquatic species, including salmon, carp, and catfish. In contrast, there has been limited investigation into aquarium fish, which are typically smaller in size and have sperm volumes restricted to microliter quantities (Tiersch, 2001). Fish sperm storage is typically categorized into two primary approaches: short-term and long-term preservation. Short-term preservation is commonly employed in aquaculture and breeding programs to bridge immediate needs. Long-term preservation methods, by contrast, aim to retain viability over extended periods. Both strategies rely on carefully controlled cooling, extender selection, and, when applicable, cryoprotectants to maintain motility and fertilizing capability. In fact, short-term preservation is also a process used for long-term preservation. Because in long-term preservation, diluents, cryoprotectants, some phospholipids and proteins are added to prevent the sperm from moving before freezing. Typically, storage involves freezing either as dry ice pellets or within straws exposed to liquid nitrogen vapor, as described by Kurokura et al. (1984) and Holtz (1993).

The three studies investigated strategies to optimize cryopreservation of *Barbus grypus* and related species, focusing on cryoprotectant formulations, extenders, and adjuncts.

In the first study, three DMSO concentrations (7%, 10%, 15%) were tested for post-thaw motility and fertility in shabout sperm, with final dilutions incorporating 10% sperm and glucose extender at varying DMSO percentages. Compared to fresh milt, cryopreserved sperm showed reduced motility, and extenders with 7% and 15% DMSO produced lower motile fractions than the 10% DMSO extender, a difference that reached statistical significance. The 10% DMSO extender yielded the best fertility rate among treatments, indicating a mid-range DMSO concentration provides the optimal balance for *Barbus grypus* cryopreservation. Overall, the data underscore the importance of precise cryoprotectant optimization to maximize post-thaw performance and hatchery outcomes (Doğu, 2012).

The second study evaluated long-term cryopreservation feasibility for Shabout's sperm using two extenders and two cryoprotectants, comparing Zhang & Liu versus Hank's HBSS and methanol versus glycerol. Semen collected from mature fish during natural spawning was assessed for prefreezing and

post-thaw motility and survival. Prefreezing motility was higher with Zhang & Liu/methanol than with Hank's/glycerol, but post-thaw motility favored Hank's/glycerol, suggesting Hank's HBSS with glycerol is more advantageous for long-term storage. The results demonstrate that Shabout sperm can be effectively cryopreserved in liquid nitrogen, with the Hank's extender and 10% glycerol being particularly favorable. Short-term preservation may still be possible with Zhang & Liu, but long-term viability favored Hank's HBSS. These findings inform extender and cryoprotectant choices for robust, long-term semen biobanking in this species, with implications for aquaculture and conservation. Additional optimization could further refine processing and dosing to maximize post-thaw viability (Al-Mohammedi et al., 2017).

The third study evaluated oxytetracycline (OTC) supplementation to a glucose-based extender and its impact on oxidative stress markers, antioxidant status, DNA integrity, and apoptosis in Barbus grypus post-thaw semen. OTC at three concentrations (0.78, 1.56, and 3.12 mg) produced a linear enhancement of post-thaw motility and its duration ($P < 0.0001$). Oxidative indicators demonstrated decreases in MDA and total oxidative status, while total antioxidant status (TAS) and GSH rose with OTC, reflecting an improved redox balance ($P < 0.001$). DNA damage measured by 8-OHdG declined with higher OTC levels, and apoptotic sperm decreased linearly to quadratically with OTC ($P < 0.0001$). The results suggest OTC can be a beneficial additive in semen extenders, enhancing freezing-thawing outcomes without compromising overall quality. The findings support incorporating OTC into extenders to improve cryopreservation success, though optimal concentration and potential side effects warrant further study. Future research should assess long-term fertility outcomes and any tissue-level implications of OTC use in aquaculture contexts (Doğu et al., 2022).

Overall, these studies collectively highlight the critical role of extender composition, cryoprotectants, and adjuncts in maximizing sperm viability, motility, and fertility after thaw, with implications for fish conservation and breeding programs.

4. Some effective factors in sperm freezing

In addition, to optimize the cryopreservation of sperm, it is necessary to consider several fundamental parameters, some of which are described below. Antioxidant cryoprotective chemicals such as ascorbic acid, lactic acid, and resveratrol—added to media containing egg yolk and glycerol that protect

sperm cells from osmotic injury—positively influence sperm quality. Below, by reviewing the related studies, the criteria that should be taken into account to achieve optimal outcomes in sperm cryopreservation are elucidated.

4.1 Extenders

For effective cryopreservation, it is crucial to inhibit the activation of spermatozoa during storage. Undiluted sperm is unsuitable for cryogenic preservation; thus, it must be mixed with a suitable medium (Stoss, 1983). Given that the motility of fish spermatozoa typically occurs only once, this medium should not trigger motility and must not compromise the sperm's potential for activation when needed. This type of medium is known as an "Extender" (Routray and Verma, 2011). By diluting the semen, it facilitates the fertilization of a larger number of eggs in a confined space.

An extender is a saline solution designed to sustain cell viability throughout the cryopreservation process. This solution should provide energy sources for the sperm cells, shield them from damage due to temperature fluctuations, and create an optimal environment during the cryopreservation period (Kamaruding et al., 2012). The extender is designed as a buffered physiological saline solution that mirrors the inorganic makeup of seminal plasma (Borchard and Schmidt, 1979), and its exact composition varies across species. Its role is to keep spermatozoa quiescent before dilution by stabilizing their physicochemical properties (Ohta and Izawa, 1996). Typically, sperm are stored in an extender with an osmolality close to isotonic with plasma to prevent premature activation (Bates et al., 1996). Extenders have been developed using saline-based diluents and sugar-based diluents (Urbanyi et al., 1999). Tris-egg yolk has been shown to yield a higher percentage of post-thaw motility (50%) for cryopreserved common carp spermatozoa (Lakra and Krishna, 1997). Sperm diluted with a solution containing 0.3427 g NaCl, 3.4314 g sucrose, 100 ml distilled water, 21 µl NaOH, 0.5 ml antibiotics (penicillin and streptomycin), and DMSO demonstrated optimal post-thaw motility (94.5 ± 3.3%) in *C. carpio* (Irawan et al., 2010). In a study comparing the effectiveness of three extenders—Freshwater Fish Saline, Modified Fish Ringer, and Physiological Saline—for *C. carpio* milt cryopreservation, Modified Fish Ringer achieved the longest motility duration (Betsy and Kumar, 2016).

To mitigate rapid agglutination in fresh Barbus sperm, a glucose-based extender (GBE; comprising 40 mM KCl, 30 mM Tris, and 200 mM glucose) has been utilized, supplemented with 20 µg/mL DNase I (PanReac AppliChem,

Spain, Germany, Italy) (Kojadinović, 2020). The ideal cryoprotectant varies among fish species, and the interactions between the extender and cooling rates are critical (Vuthiphandchai et al., 2014).

In the studies, it has been determined that media formed by mixing DMSO as a cryoprotectant with a glucose-containing diluent best preserve sperm quality at the highest level. When using diluents based on bovine serum albumin (BSA) and fetal bovine serum (FBS), the desired effect was not observed in the cryopreservation of S. balcanicus sperm; however, very favorable results have been reported for sperm cryopreservation in certain freshwater fish species with these media (De Leeuw et al., 1993; Koh et al., 2010; Marco-Jimenez et al., 2006).

4.2. Cryoprotectants

Cryoprotectants employed for fish sperm cryopreservation can be categorized into two primary classes: permeable and non-permeable. This classification is based on the differing characteristics of these substances. Within the permeable category, Dimethyl Sulfoxide (DMSO) is frequently selected by researchers owing to its swift interaction characteristics (Suquet et al., 2000). The Cryoprotectant concentrations used in the cryopreservation medium, which aid in depressing the freezing point of sperm and limiting crystallization, generally span from 5% to 12% (v/v) (Kopeika et al., 2003). Studies have shown that, although this varies by species, both the toxicity of the cryoprotectant and the rate of DNA damage in cells tend to increase with higher dilution and concentration of cryoprotectants in the freezing medium (Stoss, 1983; Said et al., 2010; Boryshpolets et al., 2011; Balamurugan & Munuswamy, 2017).

Determining the optimal mixture of permeating and non-permeating cryoprotectants in the cryopreservation medium is another essential consideration. This combination can help minimize sperm damage caused by ice formation both inside and outside the cells, while also enhancing fertilization rates (Fowler and Toner, 2016).

Recently, there has been an increasing focus on finding alternatives to whole egg yolk in sperm extenders. This shift is primarily due to certain components in egg yolk that can impair sperm respiration and reduce motility. Research has suggested that these components may negatively affect sperm functionality (Kampschmidt et al., 1953; Pace and Graham, 1974), along with the hygienic and practical issues associated with using egg yolk (Pillet et al., 2011). Consequently, low-density lipoproteins (LDLs) extracted from egg yolk and soy lecithin (SL) have been identified as promising substitutes. Soy

lecithin is particularly effective in reducing cryodamage to spermatozoa during cryopreservation by limiting the release of cholesterol and phospholipids, which helps to decrease the formation of intracellular ice crystals. Primarily composed of phosphatidylcholine, soy lecithin is vital for preserving the integrity of the phospholipid membrane throughout the cryopreservation process. Additionally, it functions as an antioxidant, protecting against free radical damage and enhancing sperm viability and motility following thawing (Sun et al., 2021).

Traditionally, egg yolk derived from chicken eggs has served as a cryoprotective additive in semen freezing extenders to mitigate cold-induced stress on sperm, with the protective effects largely ascribed to low-density lipoprotein components. Recent discourse regarding potential cryoprotective antagonists in egg yolk has spurred renewed interest in utilizing exclusively low-density lipoproteins (LDLs) isolated from egg yolk in extender formulations. A range of purified low-density lipoprotein (LDL) concentrations, from 2.5% to 20% (w/v), has been evaluated in bull semen freezing extenders, with outcomes compared against commercial extender formulations. Results indicated that adding LDL to the extender significantly improved sperm motility and other quality parameters compared with commercial extenders containing egg yolk, with an optimal LDL concentration of 8% (Moussa et al., 2002). Substituting egg yolk with LDL (which is derived from egg yolk) or sucrose did not improve the post-thaw quality of Mediterranean trout (Salmo cetti) sperm in vitro, indicating that egg yolk remains the most effective non-permeable cryoprotectant for this species. Notably, increasing LDL concentration from 2.5% to 10% improved cryo-survival rates for Mediterranean trout sperm, while a 15% LDL concentration was associated with decreased post-thaw sperm quality (Rusco et al., 2019). Additionally, Doğu (2012) investigated the motility of thawed shabout spermatozoa after freezing with three different concentrations of DMSO (7%, 10%, and 15%), finding that the highest reduction in motility post-thaw was 41.3 ± 0.8%.

4.3. Equilibration period

The equilibration period is the optimal time for cryoprotectants to penetrate cells while minimizing toxicity, enabling effective protection during freezing. Achieving equilibration during cryopreservation is important to maintain the integrity of sperm membranes (Aral et al., 2009).

When we look at the studies on freezing the sperm of the Shabut, Doğu (2012) conducted a study and summarized the equilibration time as follows. Equilibration serves to harmonize sperm with the chosen diluent-cryoprotectant

system (e.g., 10% methanol or 10% glycerol). This balancing step aims to minimize osmotic and chemical stress prior to freezing. It was conducted for 30 minutes at 4°C, ensuring the sperm attain a stable state with the diluent and cryoprotectants. Proper equilibration is described as critical for optimizing post-thaw viability and motility.

Al-Mohammedi et al. (2017), a 30-minute equilibration at 4°C preceded loading into cryo-straws, with subsequent nitrogen vapor exposure and immersion in liquid nitrogen to mitigate any adverse interactions between semen and diluent that could compromise viability. The equilibration interval is a deliberate 30-minute, 4°C phase intended to align the sperm with the diluent-cryoprotectant matrix, thereby improving outcomes after thawing.

In another study, Doğu et al. (2022) reported that both experimental and control group semen were immediately diluted from each sample at a 1:3 ratio (sperm to diluent) using a Glucose-based extender (0.3 M Glucose) supplemented with 10% dimethyl sulfoxide (DMSO). The prepared semen was loaded into 0.25 ml straws and equilibrated at 4°C for 10 minutes, providing a balancing step before freezing. Subsequently, the straws were frozen by placing them in a styrofoam container 5 cm above the surface of liquid nitrogen for 12 minutes.

4.4 Thawing rate

To improve the cryopreservation protocol, optimizing both thawing rate and thawing temperature are important issues, because the thawing rate is a critical factor for maintaining spermatozoa viability (Moghanloo et al., 2020). This finding comes from Rusco et al. (2019). The researchers reported that the thawing rate significantly influences post-thaw sperm quality and fertilization capacity. Therefore, the thawing rate represents not only a highly sensitive parameter in the cryopreservation of fish sperm but also a further critical determinant influencing sperm preservation outcomes (Wheeler & Thorgard, 1991; Fowler & Toner, 2006).

The motility of sperm is adversely affected following the freezing and thawing process. This impairment is primarily due to the formation of ice crystals both inside and outside the sperm cells, which can cause damage to the organelles and their functions (Woelders et al., 1997; Ba-Awadh et al., 2023). Research conducted by O'Connell et al. (2002) utilizing rhodamine 123 (Rd), a fluorescent marker, demonstrated that mitochondrial function is reduced by nearly 50% post-freezing and thawing. Additionally, oxidative stress during the cryopreservation process plays a significant role in compromising sperm quality

(Amidi et al., 2016; Saleh et al., 2020). The reduction in motility observed after thawing is closely linked to an increase in oxidation-reduction potential, which is associated with damage to the axonal structure and the plasma membrane (Saleh et al., 2020). Moreover, a considerable body of evidence indicates that sperm cryopreservation is associated with increased DNA fragmentation (Thomson et al., 2009). Although sperm DNA fragmentation (SDF) does not markedly impair oocyte fertilization capability, it can significantly influence subsequent embryo development and overall reproductive success. Even when high fertilization rates are achieved, embryo viability tends to be markedly reduced (Gosálvez et al., 2014). Different temperatures and times were applied to thaw sperm. In addition to the differences in fish species, it can be said that sperm size, cell membrane differences, DNA preservation status and their reactions to the freeze-thaw process.

Doğu (2012) thawed frozen Shabut sperm samples in a 40°C water bath for 7 seconds and stated that good results were obtained at the specified temperatures and times. Low thawing rates are considered appropriate for impermeable cryoprotectants. When a low thawing rate (10°C for 30 seconds) was applied after the freeze-thaw process using impermeable cryoprotectants, the quality of the sperm improved after thawing. When low thawing rates (5 °C for 90 s and 10 °C for 30 s) were used, higher fertilization rates were obtained compared to high thawing rates (20 °C for 20 s and 30 °C for 15 s) (Wheeler and Thorgard, 1991). In other studies, when low thawing rates (5 °C for 90 s and 15 °C for 45 s) were used, fertilization rates decreased (Fowler and Toner, 2006; Sarvi et al., 2006). This difference may be due to DNA disorders, ice crystal formation, cell membrane damage, and damage to sperm organelle structure and function.

In a different study, Al-Muohammadi et al. (2017), examined thawing rate effects on Iraqi Shabut sperm preserved with Zhang and Liu's methanol-containing extender and Hank's solution, evaluating post-thaw viability. Sperm were diluted in extenders at controlled ratios, frozen, and then thawed at defined rates to assess motility and survival. Results indicate thawing rate significantly influences post-thaw sperm viability, with methanol-containing extenders supporting higher motility recovery at optimized thaw speeds compared to Hank's. Overall, the thawing rate emerges as a critical parameter guiding extender selection and freezing protocols to maximize post-thaw sperm function.

On the other hand, Doğu et al. (2022) highlighted that thawing rate as a critical factor shaping post-thaw sperm performance when oxytetracycline (OTC) is added to the extender. The data showed that freezing significantly

reduces motility rate and duration, while OTC supplementation mitigates this loss, with effects influenced by the thawing process. Both linear and quadratic trends indicated that higher OTC levels improve motility metrics across thawing scenarios, suggesting an interaction between OTC concentration and thawing rate on post-thaw viability. Overall, optimizing thawing rate in conjunction with OTC supplementation emerges as essential for maximizing post-thaw sperm motility and its persistence.

Interesting results were obtained after the frozen sperm of *B. p. magdalenae* (Pisces, Characiformes) were subjected to a fast thawing rate (60°C for 8 seconds) and a slow thawing rate (30°C for 16 seconds). It was observed that a slow thawing rate led to a reduction in variables such as progressive motility, curvilinear velocity, and straight-line velocity, while when the freezing rate was increased, the decrease in these properties became more pronounced. The independent effects of freezing and thawing rates on motility variables, especially the freezing factor (when slow), were suggested to be positively significant and determinant in the preservation of these properties (Martínez and Carrasco, 2013). Different experimental conditions, including extender composition, freezing rates, and straw volumes, may account for the divergent results observed across studies. It is broadly accepted that the freezing rate should be sufficiently low to promote cellular dehydration, and a moderate to low thawing rate is required to achieve adequate rehydration. Conversely, high freezing rates can induce intracellular ice formation, and a rapid thawing rate is commonly deemed necessary to minimize recrystallization (Rusco et al., 2019).

5. Sperm freezing protocol

The cryopreservation of fish sperm can be achieved through a variety of techniques, including extenders, dilution, cooling, equilibration, freezing and thawing rate. In order to achieve the objective of successful performance of multiple processes in the context of sperm freezing, it is essential to adhere to the requisite protocol.

When reviewing the literature on cryopreservation protocols for Shabut sperm, Doğu (2012) is noted that semen samples were prepared by diluting pooled aliquots at a 1:2 ratio into a glucose-based extender (0.3 M) containing three DMSO concentrations (7%, 10%, and 15% v/v). Each dilution was then loaded into 0.5 ml straws, allocated to goblets on canes, and subjected to a staged freezing process: the straws were first held 1 cm above liquid nitrogen

for 10 minutes before plunge-freezing into liquid nitrogen at -196°C for long-term storage, with three replicates per pool. After two weeks, straws were thawed in a 40°C water bath for 7 seconds, and post-thaw motility was assessed immediately using standard criteria. Activation was performed using a 0.3% NaCl saline solution to evaluate functional viability.

Another study evaluated long-term cryopreservation of Shabout's (*Arabibarbus grypus*) sperm in liquid nitrogen (-196°C) using two extenders and two cryoprotectants to identify the optimal freezing protocol (Al-Mohammedi et al., 2017). Semen was collected manually from mature fish during the 2015 spring spawning season, then divided for testing with two diluent-cryoprotectant combinations: Zhang & Liu with 10% methanol, and Calcium-free Hank's Balanced Salt Solution with 10% glycerol. Each sample was subjected to a controlled freezing process, where extended semen was prepared in the respective diluents, exposed to the cryoprotectants, and then equilibrated as part of balancing before immersion in LN_2, followed by storage at -196°C. Post-thaw motility indicated that Hank's solution with 10% glycerol yielded higher survival and activity than Zhang & Liu with methanol, suggesting this combination as the more effective long-term cryopreservation protocol for Shabout's sperm, though Zhang & Liu may be suitable for short-term preservation.

In a further study, Doğu et al. (2022) outlined a freezing protocol for sperm that includes an experimental design with OTC-supplemented extenders across three dosage groups (0.78, 1.56, and 3.12 mg) and a control/fresh group without OTC. Semen from each sample was diluted at a 1:3 ratio into a Glucose-based extender containing 10% DMSO, then loaded into 0.25 ml straws and equilibrated at 4°C for 10 minutes prior to freezing. Straws were first held 5 cm above liquid nitrogen for 12 minutes in a Styrofoam container and then stored in liquid nitrogen for two weeks before thawing. For post-thaw assessment, straws were rapidly warmed in a 40°C water bath for 8 seconds, following established evaluation procedures.

6. Conclusions

In summary, sperm cryopreservation represents an effective approach for the preservation and storage of the valuable genetic resources of shabut. Recent advancements in this research area have been significant. However, further investigation into extenders and their effectiveness in enhancing preservation is essential. This research should also delve into the mechanisms underlying sperm freezability to optimize the efficiency of shabut sperm cryopreservation.

References

Abdoli, A. (2000). The inland water fishes of Iran. *Iranian Museum of Nature and Wildlife*, Tehran, 88-89.

Al-Mohammedi, K. F., Al-Bayatti, S. A., Lataf, W. Y., Mohammed, O. A., Shaker, A. W., & Mahdi, S. A. (2017). Testing two extenders and cryoprotectants for long-term preservation of shabout fish sperm. *The Iraqi Journal of Agricultural Sciences, 48*(6), 1677–1683.

Amidi, F., Pazhohan, A., Shabani Nashtaei, M., Khodarahmian, M., & Nekoonam, S. (2016). The role of antioxidants in sperm freezing: A review. *Cell and Tissue Bank, 17*, 745–756. https://doi.org/10.1007/s10561-016-9566-5

Aral, F., Şahinöz, E., Doğu, Z., & Demirkol, R. (2009). Effect of equilibration times on sperm cryopreservation of juvenile rainbow trout (*Oncorhynchus mykiss* W., 1792). *International Journal of Natural Engineering Sciences, 3*(1), 9–12.

Ba-Awadh, H. A., Olarinre, I. O., Alowaimer, A. N., Saadeldin, I. M., & Swelum, A. A. (2023). Comparison between rapid and slow cryopreservation protocols for ram semen. *Slovak Veterinary Research, 60*(Suppl 25), 249–257. https://doi.org/10.26873/SVR-1586-2022

Balamurugan, R., & Munuswamy, N. (2017). Cryopreservation of sperm in grey mullet, *Mugil cephalus* (Linnaeus, 1758). *Animal Reproduction Science, 185*, 10.1016/j.anireprosci.2017.08.022

Bates, M. C., Wayman, W. R., & Tiersch, T. R. (1996). Effect of osmotic pressure on the activation and storage of channel catfish sperm. *Transactions of the American Fisheries Society, 125*(5), 798–802.

Betsy, C. J., & Kumar, J. S. (2016). Effect of three different extenders on the spermatological parameters of *Cyprinus carpio* spermatozoa. *Journal of Aquaculture in the Tropics, 31*(1/2), 25.

Blaxter, J. H. S. (1953). Sperm storage and cross-fertilization of spring and autumn spawning herring. *Nature, 172*, 1189–1190.

Borchard, B., & Schmidt, G. W. (1979). Versuche mit Regenbogenforellensperma. IV. Die Tiefkuhlkonservierung. Beobachtungen zum Einsatz bei praktischen und wissenschaftlichen Arbeiten, *Fischwirt, 29*, 49–51.

Boryshpolets, S., Dzyuba, B., Rodina, M., Alavi, S. M. H., Gela, D., Linhart, O. (2011). Cryopreservation of sterlet (*Acipenser ruthenus*) spermatozoa using different cryoprotectants. *Journal of Applied Ichthyology, 27*, 1147–1149.

De Leeuw, F. E., De Leeuw, A. M., Den Daas, J. H. G., Colenbrander, B., & Verkleij, A. J. (1993). Effects of various cryoprotective agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing. Cryobiology, 30, 32–44.

Doetsch, R.N. (1976). Lazzaro Spallanzani's of *Opuscoli* 1776. *Bacteriological Reviews*, 40, 270–275.

Doğu, Z. (2012). Cryopreservation of semen in shabout (*Barbus grypus* Heckel, 1843): sperm motility and fertilization rates. *Journal of Applied Ichthyology*, 28(6), 952-955.

Doğu, Z., Aral, F., & Sahinoz, E. (2014). The determination of some spermatological and hematological parameters of shabbout (*Barbus grypus*) in Atatürk Dam Lake, Şanlıurfa. *Journal of FisheriesSciences.com*, 8(4), 265-277.

Doğu, Z., Şahinöz, E., Aral, F., Koyuncu, I. & Eği, K. (2022). Effects Of Oxytetracycline Supplementation On Cryopreserved Sperm Quality Of Shabout (*Barbus grypus* Heckel 1843): Apoptotic analysis, DNA damage and oxidative stress. *Applied Ecology and Environmental Research,* 20 (3): 2733-2746.

Fowler, A., & Toner, M. (2006). Cryo-injury and biopreservation. *Annals of the New York Academy of Sciences*, 1066, 119–135. https://doi.org/10.1196/annals.1363.010

Gosálvez, J., López-Fernández, C., Hermoso, A., Fernández, J.L., & Kjelland, M.E. (2014). Sperm DNA fragmentation in zebrafish (*Danio rerio*) and its impact on fertility and embryo viability — Implications for fisheries and aquaculture. *Aquaculture*, 433, 173-182. https://doi.org/10.1016/j.aquaculture.2014.05.036

Holtz, W. (1993). Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) sperm: practical recommendations. *Aquaculture*, 110(1), 97-100.

Irawan, H., Vuthiphandchai, V., & Nimrat, S. (2010). The effect of extenders, cryoprotectants, and cryopreservation methods on common carp (*Cyprinus carpio*) sperm. *Animal Reproduction Science*, 122(3-4), 236-243.

Kamaruding, N.A., Embong, W.K., & Abdullan, R.B. (2012). Frozen-thawed sperm motility characteristics of African catfish (*Clarias gariepinus*) by using glycerol or DMSO based extender. *International Journal of Environmental Science and Development*, 3(1), 49.

Kampschmidt, R.F., Mayer, D.T., & Herman, H.A. (1953). Lipid and lipoprotein constituents of egg yolk in the resistance and storage of bull spermatozoa. *Journal of Dairy Science*, 36, 733–742. https://doi.org/10.3168/jds.S0022-0302(53)91553-7

Khodadadi, M., Arab, A., & Jaferian, A. (2016). Turkish Journal of Fisheries and Aquatic Sciences, 16, 947-951.

Kojadinović, R., Zoran, N.M., Tijana, V., Aleksandra, Ö., Marija, J., Ákos, H., & Vladica, S. (2020). Cryopreservation of Danube barbel *Barbus balcanicus* sperm and its effects on sperm subpopulation structure. *Archives of Biological Sciences*, 72, 46-46. https://doi.org/10.2298/ABS200831046K

Kopeika, J., Kopeika, E., Zhang, T., & Rawson, D.M. (2003). Studies on the toxicity of dimethyl sulfoxide, ethylene glycol, methanol, and glycerol to loach (*Misgurnus fossilis*) sperm and the effect on subsequent embryo development. *CryoLetters*, 24, 365–374.

Kurokura, H., Hirano, R., Tomita, M., & Iwahashi, M. (1984). Cryopreservation of carp sperm. *Aquaculture*, 37(3), 267-273.

Lakra, W.S., & Krishna, G. (1997). Preliminary trials for cryopreservation of spermatozoa of selected carps and catfishes. *Indian Journal of Animal Sciences*, 67(1), 90-92.

Martínez, J., & Carrasco, S.C.P. (2013). Effect of freezing and thawing rates on sperm motility in Bocachico *Prochilodus magdalenae* (Pisces, Characiformes). *Revista MVZ Córdoba*, 18(1), 3295-3303.

Moghanloo, M., Vaghefi, M., & Ghodsian, M. (2020). Experimental investigation on the effect of increasing the collar thickness on the flow pattern around the oblong pier in 180˚ sharp bend with balanced bed. *Journal of Applied Fluid Mechanics*, 13, 245-260. https://doi.org/10.29252/jafm.13.01.30164

Moussa, M., Martinet, V., Trimeche, A., Tainturier, D., & Anton, M. (2002). Low density lipoproteins extracted from hen egg yolk by an easy method: Cryoprotective effect on frozen-thawed bull semen. *Theriogenology*, 57(6), 1695-1706. https://doi.org/10.1016/S0093-691X(02)00682-9

Nikpei, M. (1996). Research project report: Biological study of *Barbus grypus* and *Barbus sharpie*. *Iranian Fisheries Research Institute*, 1, 52-64.

Ohta, H., & Izawa, T. (1996). Diluent for cool storage of the Japanese eel (*Anguilla japonica*) spermatozoa. *Aquaculture*, 142(1-2), 107-118. https://doi.org/10.1016/0044-8486(96)01358-0

Pace, M. M., & Graham, E. F. (1974). Components in egg yolk which protect bovine spermatozoa during freezing. *Journal of Animal Science*, 39, 1144–1149. https://doi.org/10.2527/jas1974.3961144x

Pillet, E., Duchamp, G., Batellier, F., Beaumal, V., Anton, M., Desherces, S., Magistrini, M. (2011). Egg yolk plasma can replace egg yolk in stallion

freezing extenders. *Theriogenology*, 75, 105-114. https://doi.org/10.1016/j.theriogenology.2010.07.015

Routray, P., & Verma, D. K. (2011). Utilization of milt cryopreservation technology in aquaculture: A strategy. In *National Workshop On "Portable FRP Carp Hatchery Technology"* (pp. 28).

Rusco, G., Di Iorio, M., Gibertoni, P. P., Esposito, S., Penserini, M., Roncarati, A., Cerolini, S., & Iaffaldano, N. (2019). Optimization of sperm cryopreservation protocol for Mediterranean brown trout: A comparative study of non-permeating cryoprotectants and thawing rates in vitro and in vivo. *Animals (Basel)*, 9(6), 304. https://doi.org/10.3390/ani9060304.

Said, T.M., Gaglani, A. & Agarwal, A. , 2010. Implication of apoptosis in sperm cryo -injury. Reproduction Biomedical Online, 21, 456 -462.

Saleh, R., Elsuity, M., Henkel, R., & Agarwal, A. (2020). High levels of oxidation-reduction potential in frozen-thawed human semen are significantly correlated with poor post-thaw sperm quality. *Andrologia*, 52, e13608. https://doi.org/10.1111/and.13608

Stoss, J. (1983). Fish gamete preservation and spermatozoan physiology, In *Fish Physiology,* 9, 305-350.

Sun, L., He, M., Wu, C., Zhang, S., Dai, J., & Zhang, D. (2021). Beneficial influence of soybean lecithin nanoparticles on rooster frozen-thawed semen quality and fertility. *Animals (Basel)*, 11(6), 1769. https://doi.org/10.3390/ani11061769

Suquet, M., Dreanno, C., Fauvel, C., Cosson, J., & Billard, R. (2000). Cryopreservation of sperm in marine fish. *Aquaculture Research*, 31(3), 231-243. https://doi.org/10.1046/j.1365-2109.2000.00485.x

Thomson, L. K., Fleming, S. D., Aitken, R. J., De Iuliis, G. N., Zieschang, J. A., & Clark, A. M. (2009). Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Human Reproduction*, 24, 2061–2070. https://doi.org/10.1093/humrep/dep214

Tiersch, T. R. (2001). Cryopreservation in aquarium fishes. *Marine Biotechnology*, 3, S212-S223. https://doi.org/10.1007/s10126001-0044-z

Urbányi, B., Horváth, Á., Varga, Z., Horváth, L., Magyary, I., & Radics, F. (1999). Effect of extenders on sperm cryopreservation of African catfish, *Clarias gariepinus* (Burchell). *Aquaculture Research*, 30(2), 145-151. https://doi.org/10.1046/j.1365-2109.1999.00334.x

Vuthiphandchai, V., Wilairattanadilok, K., Chomphuthawach, S., Sooksawat, T., & Nimrat, S. (2014). Sperm cryopreservation of silver barb

(*Barbodes gonionotus*): Cryoprotectants, cooling rate, and storage time on sperm quality. *Aquaculture Research*, 46, 2443-2451. https://doi.org/10.1111/are.12214

Wheeler, P. A., & Thorgard, G. H. (1991). Cryopreservation of rainbow trout semen in large straws. *Aquaculture*, 93, 95-100. https://doi.org/10.1016/0044-8486(91)90208-O

Woelders, H., Matthijs, A., & Engel, B. (1997). Effects of trehalose and sucrose, osmolality of the freezing medium, and cooling rate on viability and intactness of bull sperm after freezing and thawing. *Cryobiology*, 35(2), 93-105. https://doi.org/10.1006/cryo.1997.2028

CHAPTER IV

SPERM CRYOPRESERVATON IN TURBOT (*SCOPHTHALMUS MAXIMUS*)

Atife Tuba BEKEN

(Dr.), Su Ürünleri Merkez Araştırma Enstitüsü Müdürlüğü,
E-mail: atifetuba.beken@tarimorman.gov.tr
ORCID: 0000-0002-4859-3568

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).

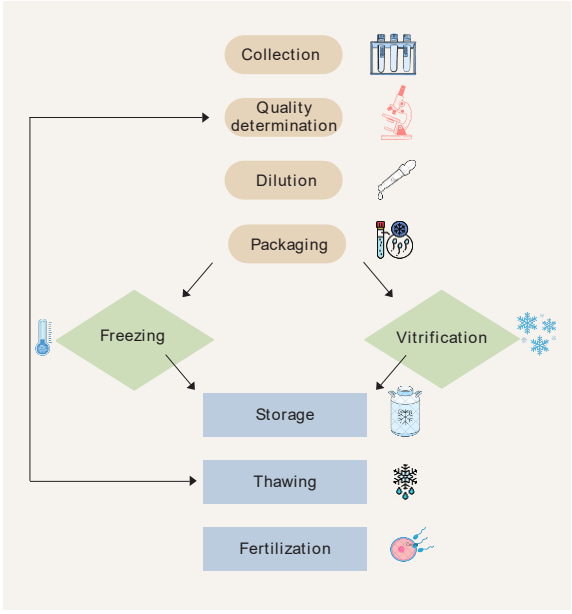


Figure 1. Procedures for the cryopreservation of fish sperm (modified from Horváth and Urbányi 2020)

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.

CHAPTER V

CRYOPRESERVATION OF SPERM IN HERMAPHRODITIC FISH SPECIES: A REVIEW

Filiz KUTLUYER KOCABAŞ[1*] & Mehmet KOCABAŞ[2] & Zainal Abidin MUCHLISIN[3]

[1]Prof. Dr. Munzur University, Fisheries Faculty, Tunceli, Türkiye.
e-mail: filizkutluyer@hotmail.com
ORCID: 0000-0001-8334-5802

[2]Karadeniz Technical University Faculty of Forestry, Department of Wildlife Ecology and Management, Trabzon, Türkiye.
e-mail: mkocabas@hotmail.com.
ORCID: 0000-0002-7934-6500.

[3]Department of Aquaculture, Faculty of Marine and Fisheries, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia.
e-mail: muchlisinza@unsyiah.ac.id.
ORCID: 0000-0003-4650-7483.

[*]Corresponding author

1. Introduction

Artificial reproduction in fish aquaculture encompasses a range of methods designed to generate high-quality offspring for future cultivation or restocking efforts (Zhu et al., 2023). In aquaculture, the later reproductive phases are chiefly guided by human control, where efforts are placed on prompting oocytes to reach full maturity, ensuring that ovulation occurs properly, and managing the release of milt during spermiation. It also involves the handling of gametes and other procedures prior to hatching. A key challenge in artificial reproduction is the mismatch in timing between estrus in

females and the hormonal response in both male and female broodstocks (Billard, 1999; Alavi et al., 2012; Zhu et al., 2023). This challenge can be successfully mitigated through the use of sperm cryopreservation. In addition, by maintaining stored sperm, hatcheries gain a dependable source of male gametes, genetic material can be exchanged more easily between different facilities, overall genetic variation within populations can be strengthened, and the likelihood of inbreeding in vulnerable fish species can be reduced (Lahnsteiner et al., 2002; Gao et al., 2019).

In hermaphroditic species, a single organism fulfills both reproductive functions, generating eggs and sperm either at the same time or at different stages of its life cycle. This trait is observed across major plant classifications and is common among numerous Metazoan species (Lewis 1942; Leonard 2013; Pla et al., 2021). Simultaneous hermaphroditism is particularly prevalent among invertebrates, with an estimated 65,000 hermaphroditic species, accounting for roughly 5–6% of all animal species. However, if insects are excluded, this proportion increases to approximately 30% (Jarne and Auld, 2006; Pla et al., 2021).

Among vertebrates, the occurrence of hermaphroditism is limited to fish, where it is expressed in two fundamental ways. One of these is sequential hermaphroditism, a strategy in which an organism alters its sex over the course of its life. This group consists of: a) species that begin their reproductive phase as females and later adopt the male function permanently (protogynous), b) species that first operate as males and then shift to the female role for the remainder of their lifespan (protandrous), and c) species that are able to switch between male and female roles in both directions, particularly when population numbers decline (bi-directional sequential hermaphroditism) (Pla et al., 2021). In the case of simultaneous hermaphroditism, both types of gametes—sperm and eggs—can be produced by the same organism, whether this occurs within the same moment or over a very brief span of time (Pla et al., 2021). An extensive research literature has emerged evaluating the effectiveness of cryogenic sperm preservation protocols in fish species exhibiting hermaphroditic reproductive strategies. This review highlights studies on sperm cryopreservation in hermaphroditic fish species, offering a unique perspective for future research.

2. Sperm Quality

Ensuring high-quality sperm remains a critical issue within aquaculture, as it underpins the reliable production of existing cultured species and supports

the successful domestication and commercialization of emerging species with significant market value. The establishment of robust and reproducible biomarkers of sperm quality would substantially advance multiple research domains and simultaneously strengthen the technological development capacity of biotechnology-oriented industries. Estimating sperm quality accurately has been a major challenge and linking these quality indicators to the sperm's ability not only to reach and fertilize the egg but also to support successful early embryonic development, as will be shown, is still difficult. To better understand how sperm is affected and to manage factors that influence overall gamete quality, evaluating sperm quality is essential. These determinants can be influenced by the biological and behavioral background of male spawners, as well as by the conditions under which they are maintained. Factors such as broodstock dietary regimes, alterations to the rearing environment, approaches used to stimulate spawning, and the specific methods applied during sperm collection and manipulation all play important roles (Cabrita et al., 2014).

Evaluating sperm quality commonly entails determining the sperm's capacity to initiate successful egg activation and its ability to contribute to the production of healthy, well-developing larvae (Bobe and Labb´e, 2010). Motility and velocity are the primary biomarkers used to assess sperm quality, but these kinematic factors only evaluate one specific trait of the sperm – its capacity to travel toward the egg (Gallego and Asturiano, 2019). Therefore, metrics derived solely from sperm movement do not reveal deeper molecular impairments—including alterations in DNA integrity or heightened oxidative stress—that can compromise overall sperm functionality (Cabrita et al., 2014). New analytical strategies for determining sperm quality now surpass the limits of standard microscopy, placing greater attention on molecular indicators—ranging from cellular stress signatures and antioxidant defenses to DNA integrity, lipid oxidation processes, mitochondrial health, and broader genomic characteristics (Cabrita et al., 2014; Hess et al., 2024).

3. Sperm Cryopreservation

By enabling sperm cells to be stored at extremely low temperatures, cryopreservation provides a means to mitigate several reproductive constraints, including the limited presence of suitable males, fluctuations in when and how individuals reach reproductive maturity, and skewed male–female proportions in cultured populations (Oh et al., 2013). Sperm cryopreservation provides the advantage of selecting sperm from disease-free fish through reducing the risk

of contamination (Lubzens et al., 1997; Oh et al., 2013). Optimizing various essential factors, including extender composition, cryoprotectants, packaging methods, dilution rates, equilibration duration, and cooling rates, is crucial for establishing an effective sperm cryopreservation protocol (Tiersch et al. 2007; Yang et al., 2020; Yang et al., 2022). The composition of the extender is considered a critical determinant among the factors influencing the success of cryopreservation. To prevent activation before freezing, an ideal extender should effectively preserve sperm viability while offering a strong buffering capacity (Yang et al., 2022).

In fish, sperm cells are typically preserved using liquid nitrogen, which supports both regulated and non-regulated freezing procedures during the cryopreservation process. Under certain conditions, a steady supply of liquid nitrogen (LN) may not be guaranteed. Furthermore, improper handling of liquid nitrogen (LN) can pose risks to operators. An LN-free freezing method provides a compelling alternative for practical use (Yang et al., 2021).

In the context of aquatic organisms, cryogenic storage of sperm ensures that male gametes remain continuously accessible for reproductive interventions, thereby offering substantial support to commercial aquaculture, safeguarding endangered species, and advancing various biomedical studies (Martínez-Páramo et al., 2017). Although cryopreservation provides clear advantages for fish reproduction, the freeze–thaw cycle can adversely affect sperm performance. This process may compromise structural integrity and cellular function, resulting in membrane disruption, oxidative degradation of lipids, breaks in DNA, impaired mitochondrial activity, and ultimately a decline in the sperm's capacity to fertilize eggs (Sandoval-Vargas et al., 2021). One of the key factors believed to contribute to sperm cryodamage during cryopreservation is the overproduction of reactive oxygen species (ROS) (Alevra et al., 2022). The combination of a PUFA-rich membrane composition and a limited amount of cytoplasm renders fish spermatozoa exceptionally sensitive to oxidative stress, especially under conditions that elevate reactive oxygen species (ROS) (Cabrita et al., 2014). The protection against ROS is insufficient because of the low antioxidant content in seminal plasma after semen dilution, despite the inherent antioxidant defense system in fish semen that helps neutralize free radicals (Lahnsteiner and Mansour, 2010). In spermatozoa, cryopreservation-induced perturbations to cellular homeostasis can shift the redox balance, allowing reactive oxygen species to accumulate beyond the neutralizing capacity of endogenous antioxidant systems, thereby promoting oxidative damage.

For successful sperm cryopreservation and induced spawning, it is vital to assess quality using standardized methods (Torres et al., 2016; Liu et al., 2018). In studies designed to refine cryopreservation protocols for aquatic species, sperm quality serves as a central criterion for assessing how various procedures influence critical stages of the process. These stages include the selection of extenders, the mechanisms used to trigger motility, the potential cytotoxicity of cryoprotectants, the specific cooling and thawing regimes applied, and the outcomes of fertilization trials (Tiersch, 2011). Because motility percentage can be measured quickly—without waiting for fertilization or embryo development—it is frequently used as a primary indicator of sperm quality in aquatic species and is considered a strong predictor of fertilization success (Cosson et al., 2008). The proportion and persistence of sperm motility can be quantified either through direct microscopic observation or by employing computer-assisted sperm analysis (CASA) systems. If assessment techniques are applied inconsistently, both the reliability of experimental outcomes and the stability of production performance can be compromised (Torres et al., 2018). The diverse methods of motility assessment from different sources hinder direct comparison of research outcomes, while inadequate checkpoints during large-scale cryopreservation processes result in unpredictability regarding the quality of the final products. Evaluating sperm quality also requires considering sperm concentration when samples are collected. While sperm concentration has a major impact on motility, the level of clumping in thawed sperm, and fertilization outcomes, many studies on aquatic species' sperm cryopreservation fail to standardize or report this parameter (Bart and Dunham, 1996; Dong et al., 2007; Nynca et al., 2017).

Thanks to commercial cryopreservation techniques—which help ensure sperm is available precisely when needed—frozen sperm can be used once females enter their optimal spawning period, improving the chances of successful fertilization (Hu et al., 2011, 2014). The benefits of cryopreservation include establishing cryobanks for species preservation or genetic improvement, enabling genetic resource transport, simplifying breeding management, maximizing sperm efficiency through optimized insemination doses, and decreasing gonadal asynchrony (Cabrita et al., 2010; Motta et al., 2022).

4. Hermaphroditism in Fish

Fish represent over half of the world's extant vertebrate species, comprising more than 32,000 out of a total of 60,000 species (Nelson et al.,

2016; Kuwamura et al., 2020). With nearly 99% of vertebrate species having individuals that are strictly male or female, gonochorism clearly demonstrates how separate sexes dominate vertebrate evolution. In contrast, only 1% of vertebrate species exhibit hermaphroditism with most fish (Avise 2011; Ashman et al. 2014; Kuwamura et al., 2020). In hermaphroditic fish, four distinct sexual strategies are observed: protandry, bidirectional sex change—also known as reversed sex change in protogynous species—simultaneous (or synchronous) hermaphroditism, and protogyny, reflecting the diverse ways these fishes can alter or express their sex (Sadovy de Mitcheson and Liu 2008; Munday et al. 2010; Kuwamura et al. 2014; Kuwamura et al., 2020). Though uncommon among fish, the ability of simultaneous hermaphrodites to form both eggs and sperm within the same gonads illustrates a reproductive adaptation that can be advantageous when potential mates are limited (Avise 2011; Kuwamura et al., 2020). Since the 1990s, there has been an increase in reports of bidirectional sex change occurring within certain species. Throughout their lives, sequential hermaphrodites—predominantly protogynous species—may undergo sex transitions, with protogyny (PG) allowing females to become males and protandry (PA) enabling males to turn into females, providing flexibility in reproductive roles (Kuwamura and Nakashima 1998; Munday et al. 2010; Kuwamura et al. 2014, 2016; Kuwamura et al., 2020).

Across 156 genera, 41 families, and 17 teleost orders, more than 450 species have been shown to exhibit functional hermaphroditism—including simultaneous (SH), protandry (PA), protogyny (PG), and bidirectional sex change (BS)—according to Nelson et al. (2016). Remarkable variation exists in the prevalence of hermaphroditic species: Cichlidae show only 0.1% (2 of 1,762 species), whereas Giganturidae and Eleginopsidae consist entirely of hermaphroditic species. In families with over 30 species, Sparidae stands out with 84%, followed by Scaridae (35%), Ipnopidae (28%), Lethrinidae (26%), Pomacanthidae (26%), Labridae (19%), Cirrhitidae (18%), and Serranidae (17%), emphasizing how hermaphroditism is unevenly distributed across teleost lineages (Kuwamura et al., 2020).

4.1. Sperm Cryopreservation in Hermaphroditic Fish

Hermaphroditic fish sperm cryopreservation has become important in fry production through artificial breeding. Hermaphroditic fish may face challenges with natural mating due to the inability to coordinate male and female spawning, which stems from reproductive strategy factors (He et al., 2011; Che-Zulkifli et

al., 2020). In captive fish, natural spawning is often not possible due to fact that the maturation of male and female gonads occurs at different times. By using cryopreserved sperm, it is possible to achieve successful reproduction of various hermaphroditic fish species year-round (Che-Zulkifli et al., 2020).

To date, numerous researchers have conducted cryopreservation of sperm from hermaphroditic fish with variable results. Different cryoprotectants and extenders have been applied in the cryopreservation techniques for hermaphroditic fish. The protocol and success rates of sperm cryopreservation of hermaphroditic fish species studied by researches are presented Table 1.

5. Conclusions

Sperm cryopreservation is a powerful tool for managing and conserving hermaphroditic fish species. While challenges remain, advances in cryopreservation techniques and species-specific protocols are paving the way for its broader application in aquaculture and conservation. In the farming industry, artificial insemination has proven to be highly effective. Hatchery managers control breeding schedules during artificial insemination by stimulating the maturation of female broodstock with reproductive hormones. In the aquaculture sector, some hermaphroditic fish species are economically important, and sperm cryopreservation is crucial for these species for the success of production. By reducing the financial burden associated with maintaining male broodstock, breeders can prioritize the maintenance of female broodstock through the commercial application of cryopreservation techniques in hatcheries. A small number of male broodstock is sufficient for collecting milt for cryopreservation, which reduces maintenance needs and eliminates the need for synchronization with female broodstock. The development of new cryopreservation methods for hermaphroditic fish species will support artificial breeding, improve fertilization outcomes, and increase aquaculture production. By preserving genetic diversity and enabling flexible breeding strategies, cryopreservation holds great promise for the future of hermaphroditic fish species.

References

Alavi, S. M. H., Hatef, A., Mylonas, C. C., Gela, D., Papadaki, M., Rodina, M., and Linhart, O. (2012). Sperm characteristics and androgens in *Acipenser ruthenus* after induction of spermiation by carp pituitary extract or GnRHa implants. *Fish Physiology and Biochemistry*, 38, 1655-1666.

Alevra, A. I., Exadactylos, A., Mente, E., and Papadopoulos, S. (2022). The protective role of melatonin in sperm cryopreservation of farm animals and human: lessons for male fish cryopreservation. *Animals*, 12(6), 791.

Ashman, T. L., Bachtrog, D., Blackmon, H., Goldberg, E. E., Hahn, M. W., Kirkpatrick, M., Kitano, J., Mank, J. E., Mayrose, I., Ming, R., and Otto, S. P. (2014). Tree of sex: A database of sexual systems. *Scientific Data,* 1, 140015.

Avise, J. C. (2011). *Hermaphroditism: A primer on the biology, ecology, and evolution of dual sexuality*. Columbia University Press.

Bart, A., and Dunham, R. (1996). Effects of sperm concentration and egg number on fertilization efficiency with channel catfish (*Ictalurus punctatus*) eggs and blue catfish (*I. furcatus*) spermatozoa. *Theriogenology,* 45, 673-682.

Billard, R. (1999). *The carp: Biology and culture*. Springer Science & Business Media.

Bobe, J., and Labbé, C. (2010). Egg and sperm quality in fish. *General and Comparative Endocrinology, 165*, 535–548. https://doi.org/10.1016/j.ygcen.2009.02.011

Cabrita, E., Robles, V., Herráez, P. (2009). -Sperm quality assessment. In Methods in Reproductive Aquaculture (pp. 115-170). CRC press.

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Perez-Cerezales, S., and Herraez, M. P. (2010). Cryopreservation of fish sperm: Applications and perspectives. *Journal of Applied Ichthyology, 26*, 623-635. https://doi.org/10.1111/j.1439-0426.2010.01556.x

Cabrita, E., Martínez-Páramo, S., Gavaia, P. J., Riesco, M. F., Valcarce, D. G., Sarasquete, C., Herráez, M. P., and Robles, V. (2014). Factors enhancing fish sperm quality and emerging tools for sperm analysis. *Aquaculture, 432*, 389–401. https://doi.org/10.1016/j.aquaculture.2014.04.034

Casaccia, E. N. E. A. (1998). Preliminary experiences for cryopreservation of *Sparus aurata* and *Diplodus puntazzo* semen. *Cahiers Options Méditerranéennes*, 34, 281-287.

Che-Zulkifli, C. I., Koh, I. C. C., Shahreza, M. S., and Ikhwanuddin, M. (2020). Cryopreservation of spermatozoa on grouper species: A review. *Reviews in Aquaculture*, 12(1), 26-32.

Chen, Y. K., Liu, Q. H., Li, J., Xiao, Z. Z., Xu, S. H., Shi, X. H., and Ma, D. Y. (2010). Effect of long-term cryopreservation on physiological characteristics, antioxidant activities and lipid peroxidation of red seabream (*Pagrus major*) sperm. *Cryobiology*, 61(2), 189-193.

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

DeGraaf, J. D., King, W., Benton, C., and Berlinsky, D. L. (2004). Production and storage of sperm from the black sea bass *Centropristis striata* L. *Aquaculture Research*, 35(15), 1457-1465.

Dong, Q., Huang, C., and Tiersch, T. R. (2007). Control of sperm concentration is necessary for standardization of sperm cryopreservation in aquatic species: Evidence from sperm agglutination in oysters. *Cryobiology*, 54, 87-98.

Fabbrocini, A., Lavadera, S. L., Rispoli, S., and Sansone, G. (2000). Cryopreservation of seabream (*Sparus aurata*) spermatozoa. *Cryobiology*, 40(1), 46-53.

Fan, B., Liu, X. C., Meng, Z. N., Tan, B. H., Wang, L., Zhang, H. F., and Lin, H. R. (2014). Cryopreservation of giant grouper *E pinephelus* lanceolatus (B loch, 1790) sperm. *Journal of Applied Ichthyology*, *30*(2), 334-339.

Frankel, T. E., Theisen, D. D., Guthrie, H. D., Welch, G. R., and Woods III, L. C. (2013). The effect of freezing rate on the quality of striped bass sperm. *Theriogenology*, 79(6), 940-945.

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

Gao, Y., Yang, C., Huang, C., Zhang, G., Ji, H., and Dong, W. (2019). The effects of melatonin supplement on paddlefish (*Polyodon spathula*) sperm quality and ATP content in sperm during in vitro storage. *Aquaculture*, 503, 475-482. https://doi.org/10.1016/j.aquaculture.2019.01.010.

Gwo, J. C. (1993). Cryopreservation of black grouper (*Epinephelus malabaricus)* spermatozoa. *Theriogenology*, *39*(6), 1331-1342.

He, Q., Lu, G., Che, K., Zhao, E., Fang, Q., Wang, H., et al. (2011). Sperm cryopreservation of the endangered red spotted grouper, *Epinephelus akaara*, with a special emphasis on membrane lipids. *Aquaculture*, 318(1–2), 185–190.

Hess, H. N., Strader, M. E., Montague, H. R., Bruce, T. J., Aguilar, G. L., Sørensen, S. R., ... and Butts, I. A. E. (2024). Molecular biomarkers for sperm quality in an economically important fish: Blue catfish, *Ictalurus furcatus*. *Aquaculture*, 579, 740220.

Hu, E., Yang, H., and Tiersch, T. R. (2011). High-throughput cryopreservation of spermatozoa of blue catfish (*Ictalurus furcatus*): Establishment of an

approach for commercial-scale processing. *Cryobiology*, 62, 74–82. https://doi.org/10.1016/j.cryobiol.2010.12.006

Hu, E., Bosworth, B., Baxter, J., and Tiersch, T. R. (2014). On-site evaluation of commercial-scale hybrid catfish production using cryopreserved blue catfish sperm. *Aquaculture*, 426–427, 88–95. https://doi.org/10.1016/j.aquaculture.2014.01.024

Jarne, P., and Auld, J. R. (2006). Animals mix it up too: The distribution of self-fertilization among hermaphroditic animals. *Evolution*, 60(9), 1816-1824. https://doi.org/10.1111/j.0014-3820.2006.tb00525.x

Jenkins-Keeran, K., and Woods III, L. C. (2002). The cryopreservation of striped bass *Morone saxatilis* semen. *Journal of the World Aquaculture Society*, 33(1), 70-77.

Jia, P. Y., Guo, H. Y., Liu, B. S., Guo, L., Zhang, N., Zhu, K. C., ... and Zhang, D. C. (2022). Effects of cryopreservation on the physiological characteristics and enzyme activities of yellowfin seabream sperm, *Acanthopagrus latus* (Houttuyn 1782). *Aquaculture*, 549, 737739.

Kerby, J. H. (1983). Cryogenic preservation of sperm from striped bass. *Transactions of the American Fisheries Society*, 112(1), 86-94.

Klaiwattana, P., Srisook, K., Srisook, E., Vuthiphandchai, V., and Neumvonk, J. (2016). Effect of cryopreservation on lipid composition and antioxidant enzyme activity of seabass (*Lates calcarifer*) sperm. *Iranian Journal of Fisheries Sciences*, 15(1), 157-169.

Koh, I. C. C., Yokoi, K. I., Tsuji, M., Tsuchihashi, Y., and Ohta, H. (2010). Cryopreservation of sperm from seven-band grouper, Epinephelus septemfasciatus. *Cryobiology*, *61*(3), 263-267.

Kuwamura, T., Kadota, T., and Suzuki, S. (2014). Testing the low-density hypothesis for reversed sex change in polygynous fish: Experiments in *Labroides dimidiatus*. *Scientific Reports*, 4, 4369.

Kuwamura, T., and Nakashima, Y. (1998). New aspects of sex change among reef fishes: Recent studies in Japan. *Environmental Biology of Fishes*, 52, 125-135.

Kuwamura, T., Sunobe, T., Sakai, Y., et al. (2020). Hermaphroditism in fishes: an annotated list of species, phylogeny, and mating system. *Ichthyological Research*, 67, 341-360. https://doi.org/10.1007/s10228-020-00754-6

Lahnsteiner, F., Mansour, N., and Weismann, T. (2002). The cryopreservation of spermatozoa of the burbot, *Lota lota* (Gadidae, Teleostei). *Cryobiology*, 45, 195-203. https://doi.org/10.1016/S0011-2240(02)00140-2.

Lahnsteiner, F., & Mansour, N. (2010). A comparative study on antioxidant systems in semen of species of the Percidae, Salmonidae, Cyprinidae, and Lotidae for improving semen storage techniques. *Aquaculture*, 307(1-2), 130-140.

Leonard, J. L. (2013). Williams' paradox and the role of phenotypic plasticity in sexual systems. *Integrative and Comparative Biology*, 53(4), 671-688. https://doi.org/10.1093/icb/ict088

Lewis, D. (1942). The evolution of sex in flowering plants. *Biological Reviews*, 17(1), 46-67. https://doi.org/10.1111/j.1469-185X.1942.tb00431.x.

Lim, H. K., and Le, M. H. (2013). Evaluation of extenders and cryoprotectants on motility and morphology of longtooth grouper (*Epinephelus bruneus*) sperm. *Theriogenology*, 79(5), 867-871.

Liu, Q., Li, J., Zhang, S., Ding, F., Xu, X., Xiao, Z., and Xu, S. (2006). An efficient methodology for cryopreservation of spermatozoa of red seabream, *Pagrus major*, with 2-mL cryovials. *Journal of the World Aquaculture Society*, 37(3), 289-297.

Liu, Q., Wang, X., Wang, W., Zhang, X., Xu, S., Ma, D., and Li, J. (2015). Effect of the addition of six antioxidants on sperm motility, membrane integrity and mitochondrial function in red seabream (*Pagrus major*) sperm cryopreservation. *Fish Physiology and Biochemistry*, 41, 413-422.

Liu, Y., Torres, L., and Tiersch, T. R. (2018). Quality evaluation of sperm from livebearing fishes: Standardized assessment of sperm bundles (spermatozeugmata) from *Xenotoca eiseni* (Goodeidae). *Theriogenology*, 107, 50-56.

Lubzens, E., Daube, N., Pekarsky, I., Magnus, Y., Cohen, A., Yusefovich, F., & Feigin, P. (1997). Carp (*Cyprinus carpio* L.) spermatozoa cryobanks—strategies in research and application. *Aquaculture*, 155(1-4), 13-30.

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

Motta, N. C., Egger, R. C., Monteiro, K. S., de Oliveira, A. V., and Murgas, L. D. S. (2022). Effects of melatonin supplementation on the quality of cryopreserved sperm in the neotropical fish *Prochilodus lineatus*. *Theriogenology*, 179, 14-21.

Nelson, J. S., Grande, T. C., and Wilson, M. V. H. (2016). *Fishes of the world* (5th ed.). John Wiley & Sons.

Nynca, J., Judycka, S., Liszewska, E., Dobosz, S., and Ciereszko, A. (2017). Standardization of spermatozoa concentration for cryopreservation of rainbow trout semen using a glucose-methanol extender. Aquaculture, 477, 23-27.

Oh, S. R., Lee, C. H., Kang, H. C., Song, Y. B., Kim, H. B., and Lee, Y. D. (2013). Evaluation of fertilizing ability using frozen thawed sperm in the longtooth grouper, *Epinephelus bruneus*. Development & reproduction, 17(4), 345.

Palmer, P. J., Blackshaw, A. W., and Garrett, R. N. (1993). Successful fertility experiments with cryopreserved spermatozoa of barramundi, *Lates calcarifer* (Bloch), using dimethylsulfoxide and glycerol as cryoprotectants. Reproduction, Fertility and Development, 5(3), 285-293. https://doi.org/10.1071/rd9930285

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

Pan, J. M., Zhu, K. C., Liu, J., Guo, H. Y., Liu, B. S., Zhang, N., and Zhang, D. C. (2024). Cryopreservation of Goldlined seabream *Rhabdosargus sarba* (Forsskål, 1775) sperm: CASA observation and enzyme activity evaluation. Aquaculture, 582, 740494.

Pla, S., Maynou, F., and Piferrer, F. (2021). Hermaphroditism in fish: incidence, distribution and associations with abiotic environmental factors. Reviews in Fish Biology and Fisheries, 31, 935–955. https://doi.org/10.1007/s11160-021-09681-9.

Sadovy de Mitcheson, Y., and Liu, M. (2008). Functional hermaphroditism in teleosts. Fish and Fisheries, 9, 1–43.

Sandoval-Vargas, L., Dumorne, K., Contreras, P., Farias, J. G., Figueroa, E., Risopatron, J., and Valdebenito, I. (2021). Cryopreservation of coho salmon sperm (*Oncorhynchus kisutch*): Effect on sperm function, oxidative stress and fertilizing capacity. Aquaculture, 533, 736151.

Sean, N., Yahsiro, R., Tunkijjanukij, S., & Ponchunchoovong, S. (2009). Efficient cryopreservation of humpback grouper, Cromileptes altivelis (Valenciennes, 1828) spermatozoa. *Journal of Fisheries and Environment*, *33*(2), 12-23.

Thirumala, S., Campbell, W. T., Vicknair, M. R., Tiersch, T. R., and Devireddy, R. V. (2006). Freezing response and optimal cooling rates for cryopreserving sperm cells of striped bass, *Morone saxatilis*. Theriogenology, 66(4), 964-973.

Tian, Y., Qi, W., Jiang, J., Wang, N., Wang, D., Zhai, J., and Chen, S. (2013). Sperm cryopreservation of sex-reversed seven-band grouper, *Epinephelus septemfasciatus*. *Animal reproduction science*, *137*(3-4), 230-236.

Tiersch, T. R. (2011). Process pathways for cryopreservation research, application and commercialization. Cryopreservation in aquatic species, 2, 646-671.

Tiersch, T. R., Yang, H., Jenkins, J. A., & Dong, Q. (2007). Sperm cryopreservation in fish and shellfish. Society of Reproduction and *Fertility supplement*, 65, 493.

Tirpan, M. B., Özgöray, E. D., Alemdar, H., and Akçay, E. (2016). Cryopreservation of seabream (*Sparus aurata*) semen and fertility. *Turkish Journal of Veterinary & Animal Sciences*, 40(2), 200-206.

Torres, L., Hu, E., and Tiersch, T. R. (2016). Cryopreservation in fish: current status and pathways to quality assurance and quality control in repository development. *Reproduction, Fertility and Development, 28*, 1105-1115.

Torres, A., Fernández, N., Zu Ermgassen, S., Helmer, W., Revilla, E., Saavedra, D., and Pereira, H. M. (2018). Measuring rewilding progress. Philosophical Transactions of the Royal Society B: *Biological Sciences*, 373(1761), 20170433.

Widyaningsih, W., and Susandi, D. (2021, April). Fertilization rate of giant grouper (*Epinephelus lanceolatus*, Bloch 1790) sperm post-cryopreservation and application on hybridization in different grouper species. In *IOP Conference Series: Earth and Environmental Science* (Vol. 748, No. 1, p. 012001). IOP Publishing.

Yang, S., Fan, B., Chen, X., and Meng, Z. (2020). Cryopreservation of sperm in brown-marbled grouper (*Epinephelus fuscoguttatus*). *Aquaculture International, 28*, 1501-1516.

Yang, T., Deng, L., Sun, B., Zhang, S., Xian, Y., Xiao, X., and Qiu, Y. (2021). Semen quality and windows of susceptibility: A case study during COVID-19 outbreak in China. Environmental Research, 197, 111085.

Yang, S., Chen, X., Fan, B., Hua, Y., and Meng, Z. (2022). Successful short-term sperm cryopreservation in brown-marbled grouper (*Epinephelus fuscoguttatus*) with the utility of ultra-freezer (−80°C). *Reproduction in Domestic Animals, 57*(4), 444-449.

Yusoff, M., Hassan, B. N., Ikhwanuddin, M., Sheriff, S. M., Hashim, F., Mustafa, S., and Koh, I. C. C. (2018). Successful sperm cryopreservation of the

brown-marbled grouper, *Epinephelus fuscoguttatus* using propylene glycol as cryoprotectant. *Cryobiology, 81*, 168-173.

Zhu, C., Liu, Q., Deng, Y., Zheng, L., Wang, Y., Zhang, L., and Dong, W. (2023). Selenium nanoparticles improve fish sperm quality by enhancing glucose uptake capacity via AMPK activation. *Theriogenology, 208*, 88-101.

Zilli, L., Bianchi, A., Sabbagh, M., Pecoraro, L., Schiavone, R., and Vilella, S. (2018). Development of sea bream (*Sparus aurata*) semen vitrification protocols. *Theriogenology, 110*, 103-109.

Table 1. The Protocol and Motility Rate of Sperm Cryopreservation of Hermaphroditic Fish Species Studied by Researches.

Ordo	Family	Sexual pattern	Species	Cryoprotectant	Sperm to extender ratio	Thawing method	Motility rate	References
Moroni-formes	Moronidae	PA	*Morone saxatilis*	Gly (10.0 23.8%), EG (10.0%), PG (10.0%), and DMSO (5.0-25.0%).	1:1, 1:3, 1:9	50-60 C	13.7%	Kerby (1983)
			Morone saxatilis	DMSO (5-7.5%)	2:1	25C and 12 s	26.1%	Jenkins-Keeran and Woods (2002)
			Morone saxatilis	DMSO (5%)	1:4	40 C and 10 s.	7-20%	Thirumala et al. (2006)
			Morone saxatilis	DMSO (7.5%)	1:3	40 C and 6 s	7.14-10.06%	Frankel et al. (2013)
Perciformes	Latidae	PA	*Lates calcarifer*	DMSO (5%), Gly (10%)	1:4	25°C and 2-3 min		Palmer et al. (1993)
			Lates calcarifer	DMSO (20%)	1:1	70°C and 30 s	55%	Klaiwattana et al. (2016)
	Serranidae	PA	*Epinephelus malabaricus*	DMSO (20%)		20 C		Gwo (1993)
			Epinephelus marginatus	DMSO (20%)+ BSA (10 mg/ml)	1:9	25°C and 30 s		Cabrita et al. (2009)
			Cromileptes altivelis		1:1, 1:3, 1:4			Sean et al. (2009)
			Epinephelus septemfasciatus		1:49	25°C and 30 s		Koh et al. (2010)
			Epinephelus akaara	DMSO (10, 20%), BSA (10 mg/ml)	1:9	25°C/30 s, 40°C/7 s and 20°C/30 s, 40°C/7s		He et al. (2011)
			Epinephelus septemfasciatus	DMSO (10%), PG (10%)	1:1	38°C 70–80 s		Tian et al. (2013)
			Epinephelus lanceolatus	DMSO (12%)	1:1 and 1:2	37°C in 1.5 min		Fan et al. (2014)

			Epinephelus bruneus	Gly (10%)				Lim & Le (2013)
			Epinephelus bruneus	DMSO (5, 7.5, 10%)	1:49			Oh et al. (2013)
			Epinephelus fuscoguttatus	DMSO, DMA, EG, PG (5, 10, 15, 20%)	1:49	27°C and 15 s	90%	Yusoff et al. (2018)
			Epinephelus fuscoguttatus	DMSO, DMF, GLY, MeOH, PG (10%)	1:3	38°C 20–30 s	94%	Yang et al. (2020)
			Epinephelus lanceolatus	Gly (6%)			66%	Widyaningsih et al. (2021)
			Epinephelus fuscoguttatus	DMSO (10%)	1:3	40°C and 7 s	10-48%	Yang et al. (2022)
	Serranidae	PG	*Centropristis striata*	DMSO (10%)	1:1, 1:3	20°C and 30 s	39-73%	DeGraaf et al. (2004)
	Sparidae	PA	*Acanthopagrus latus*	EG (5%)	2:1	37°C	80%	Jia et al. (2022)
			Acanthopagrus schlegelii	EG (5%)	1:1, 1:2, 1:3, 1:4, 1:5, 1:7, 1:9	37°C	85%	Pan et al. (2023)
		PG, G	*Pagrus major*	DMSO, Gly, DMA, EG, PG (6, 9, 12, 15%)	1:3	90-110 s	36-84%	Liu et al. (2006)
			Pagrus major	DMSO (15%)	1:3	40°C	75-87%	Chen et al. (2010)
			Pagrus major	DMSO (15%)	1:3	37°C and 90-110 s	3-79%	Liu et al. (2015)
			Rhabdosargus sarba	DMSO, EG, MeOH, (5, 10, 15, 20%)	1:1	37°C	13-84:%	Pan et al. (2024)
		PA, G	*Sparus aurata*	DMSO (15%)	1:1.5	26°C and 2 min	22-27½	Casaccia (1998)
			Sparus aurata	DMSO, EG, PG, Gly, MeOH (5-15%)	1:6	30°C	10%	Fabbrocini et al. (2000)
			Sparus aurata	DMSO (10%)	1:2	26°C and 20 s	12-54%	Tirpan et al. (2016)
			Sparus aurata	DMSO (10%)+Gly (10%)	1:3	37°C and 60 s	5-40%	Zilli et al. (2018)

*PA: protandry, PG: protogyny, G: gonochorism, DMSO: Dimethyl sulfoxide, EG: ethylene glycol, PG: propylene glycol, Gly: Glycerol, MeOH: Methanol, DMA: Dimethylacetamide, DMF: Dimetil formamid, BSA: Bovine serum albumin

CHAPTER VI

DEVELOPMENTS ON SOLUTIONS USED IN THE VITRIFICATION OF FISH SPERM

Ömer Can ARAL[1] & Faruk ARAL[2] &
Zafer DOĞU[3] & Erdinç ŞAHİNÖZ[4]

[1](VM.) Aral Veteriner Kliniği Çivril/Denizli
E-mail:omercanaral@hotmail.com
ORCID: 0000-0002-1461-8798

[2](Prof. Dr.) Retired Faculty Member
E-mail: faral6@hotmail.com
ORCID: 0000-0003-2297-6509

[3](Prof. Dr.) Harran University Birecik Vocational High School
E-mail: zafer_dogu@yahoo.com
ORCID: 0000-0002-5428-8340

[4](Prof. Dr.) Harran University Birecik Vocational High School
E-mail: e_sahinöz@harran.edu.tr
ORCID: 0000-0003-0646-6939

1. Introduction

Vitrification typically requires relatively high cooling rates, resulting in non-uniform temperature distributions throughout the samples. Cryopreservation by vitrification is achieved by perfusion, diffusion or a combination of these after the biological solutions have been replaced by cryoprotectant solutions (CPA).

Because vitrification relies on ultra-rapid cooling rather than specialized instrumentation, it can be applied with ease both in hatchery-scale operations and in remote field settings; in this method, the abrupt exposure of the sperm suspension to liquid nitrogen causes a marked rise in medium viscosity, preventing water within the cells and extender from arranging into ice crystals

and instead driving the formation of a glass-like amorphous solid phase (Cuevas-Uribe et al., 2011a, 2011b). Preventing ice crystal formation is only one of the requirements for successful cryopreservation by vitrification; other important factors affecting successful sample recovery from cryopreservation are CPA toxicity, thermo-mechanical stresses caused by sample preparation and cryopreservation protocols, and natural degradation. A concise description of the physicochemical mechanisms underlying vitrification is presented, with the aim of clarifying how these processes operate during the cryopreservation of fish spermatozoa.

This chapter has been prepared to give the properties of the various solutions and their constituents used in fish sperm vitrification, and to show the concentrations and combinations of substances used.

2. Ultra-rapid cooling or vitrification

In vitrification-based cryopreservation of fish sperm, minute aliquots of semen are rapidly plunged into liquid nitrogen, enabling ultra-fast cooling rates required for glass-state formation. This technique has been employed in the study of spermatozoa from various fish species, including rainbow trout, swordtail, and Atlantic salmon (Merino et al., 2011, 2012; Cuevas-Uribe et al., 2011a, 2011b, 2015; Figueroa et al., 2013, 2015).

This technique is distinguished by its simplicity and expeditiousness when compared with conventional cryopreservation methods. The efficacy of vitrification is contingent on numerous factors, including the precise steps involved in the addition and removal of cryoprotectants, the method employed for vitrification, and the viscosity and proportions of the solutions (Yavin & Arav, 2007; Rabin & Steif, 2006). The primary discrepancy between vitrification and slow cryopreservation can be attributed to the distinct mechanisms involved in each process. During direct immersion in liquid nitrogen, an increase in medium viscosity hinders the organisation of water molecules within the sample, thereby preventing the formation of a crystalline structure. Instead, the molecules adopt an amorphous form of solid, resulting in the characteristic vitrified water behaviour. The objective is to facilitate the glass transition by accelerating it through rapid cooling or by increasing the concentration of cryoprotectants (Cuevas-Uribe et al., 2011a, 2011b). evertheless, elevating cryoprotectant levels during the cryopreservation of fish spermatozoa may impose substantial cellular stress, manifested as osmotic imbalance, disruptions in membrane transport properties, and an increased incidence of DNA damage.

It is widely recognized that using a combination of cryoprotective agents with complementary physicochemical characteristics provides a more effective approach for the cryopreservation of fish spermatozoa. The utilisation of cryoprotectants in low concentrations has been demonstrated to facilitate vitreous formation with minimal toxicity, as evidenced by Saragusty and Arav's (2011) research. For instance, human serum albumin in association with sucrose has been successfully used as an impermeable cryoprotectant for human sperm vitrification by means of ultrafreezing (Isachenko et al., 2008). Cuevas-Uribe et al. (2015) conducted a series of tests on different concentrations of cryoprotectants, finding that the combination of permeable and impermeable substances yielded superior results when compared with the utilisation of a single cryoprotectant at a high concentration. In the course of these experiments, it was established that a combination of 15% DMSO + 15% G + 10% Gli + 1% X-1000™(X) + 1% Z-1000™(Z) yielded the optimal levels of vitrification rate, motility, and membrane integrity in semen samples from three species of fish: *Lutjanus campechanus*, *Cynoscion nebulosus*, and *Sciaenops ocellatus*. However, favourable outcomes were observed for motility and membrane integrity when other combinations of permeable and impermeable compounds were employed (for example, 10% DMSO + 30% EG + 0.25 M trehalose).

Seminal plasma has been shown to be a successful cryoprotectant in the vitrification of salmon semen. Researchers such as Merino et al. (2011, 2012), Figueroa et al. (2013, 2015) have utilised a solution composed of 10% DMSO, 1-2% BSA, 0.13 M sucrose, and 30%-50% seminal plasma from rainbow trout and Atlantic salmon, to vitrify spermatozoa. When the Cortland solution was used in a combination with BSA, and its permeable cryoprotectant was replaced with 40% seminal plasma, enhanced outcomes were observed in terms of >80% motility, >82% plasma membrane integrity, and 55% mitochondrial membrane potential and fertility.

It is evident that the majority of studies address exclusively technical issues, encompassing the replacement of diluents and cryoprotectants, and the presentation of outcomes pertaining to sperm motility and fertility. The utilisation of cellular and molecular techniques is advocated for the characterisation of cryodamage. As posited by Figueroa et al. (2013), a range of techniques is recommended for the purpose of verifying plasma membrane integrity. These include flow cytometry (utilising SYBR-14/PI) and optical microscopy (eosin), in addition to techniques for testing DNA fragmentation (e.g. TUNEL) and

mitochondrial integrity (e.g. epifluorescence microscopy and flow cytometry (Rhodamine and JC-1).

3. Vitrification solutions

Although a range of vitrification media with diverse chemical profiles has been formulated for the cryopreservation of fish spermatozoa, most approaches continue to employ only a few traditional base solutions (Massaldi, 1984; Merino et al., 2011, 2012; Figueroa et al., 2013, 2015). Nevertheless, the performance of these formulations shows pronounced interspecific variability, limiting their universal applicability.

Vitrification techniques typically employ ultra-rapid cooling/warming rates, minimal sample volumes, specialized carriers, and high concentrations of permeable cryoprotective agents (CPAs). However, successful vitrification demands precise optimization, requiring careful balancing of CPA mixtures, carrier systems, and thermal protocols. One of the major obstacles in vitrification-based protocols for fish sperm is the cellular toxicity induced by high levels of cryoprotective agents, which has driven researchers to investigate alternative methodological approaches that reduce CPA burden while maintaining post-thaw functionality.

Recent advances have introduced cryoprotectant-free vitrification methods as a promising approach to mitigate toxicity while maintaining post-thaw sperm viability in aquatic species.

3.1 Cryoprotectants (CPAs)

Cryoprotectants (CPAs) are essential additives in sperm cryopreservation, mitigating freezing-induced cellular damage by inhibiting ice crystal formation. Their protective mechanism relies on increasing the overall solute concentration in the medium, thereby depressing the freezing point of water and minimizing ice nucleation at subzero temperatures. CPAs must exhibit sufficient membrane permeability while maintaining low cytotoxicity to ensure cell viability during deep cryogenic storage and post-thaw recovery.

Common permeating CPAs, such as ethylene glycol (EG), dimethyl sulfoxide (DMSO), and glycerol, function by permeating cells and disrupting hydrogen bonding in water, thereby suppressing crystallization. These compounds, often termed “antifreeze” agents in broader applications, are typically employed at concentrations of 5–15% for effective cellular preservation at liquid nitrogen

temperatures (Pegg, 2007). Despite the emergence of alternative CPAs, glycerol and DMSO remain the most widely utilized due to their proven efficacy.

However, cryopreservation-induced alterations in membrane carbohydrate composition, ion channel distribution, and protein localization can compromise spermatozoal integrity, impairing fertilization capacity (Di Santo et al., 2012; Parks & Graham, 1992). CPAs counteract these effects through multiple mechanisms: (1) depressing the freezing point of intra- and extracellular water (Royere et al., 1996), (2) interacting with cytoplasmic macromolecules, and (3) stabilizing the sperm plasma membrane via surface shielding.

Based on their cellular permeability, CPAs are broadly categorized into two classes:

1. Permeating CPAs (e.g., DMSO, glycerol, EG): Small, neutral molecules that traverse the plasma membrane, protecting both intracellular and extracellular compartments.
2. Non-permeating CPAs (e.g., sucrose, trehalose): Large or charged molecules that exert osmotic effects extracellularly, dehydrating cells to reduce intracellular ice formation.

Cryoprotective agents (CPAs) are categorized based on their membrane permeability. Permeable cryoprotectants, such as glycerol (GLY), dimethyl sulfoxide (DMSO), ethylene glycol (EG), dimethylacetaldehyde (DMA), and propylene glycol (PROH), traverse the plasma membrane, displacing intracellular water. However, these compounds exhibit cytotoxicity at elevated concentrations, with studies demonstrating a detrimental impact on sperm fertility when used in high concentrations (Gilmore et al., 1997). Permeating cryoprotective agents used in the cryopreservation of fish spermatozoa can be categorized according to the speed at which they traverse the plasma membrane. Compounds such as methanol, ethanol, ethylene glycol, propylene glycol, dimethylformamide, methylacetamide and DMSO exhibit rapid membrane permeation, whereas glycerol is generally regarded as a slow-penetrating CPA. Additionally, some CPAs permeate only the cell wall (CW) (e.g., mono- and disaccharides, amino acids), while others penetrate both the CW and cytoplasmic membrane (CM) (e.g., DMSO, glycerol). High-molecular-weight polymers (e.g., proteins, polysaccharides, dextran) are impermeable to the CW.

Non-permeable cryoprotectants, including raffinose (RF), sucrose (SUC), trehalose (TRE), egg yolk, albumin, polyethylene glycol (PG),

polyvinylpyrrolidone (PVP), and hydroxyethyl starch (HES), do not cross the plasma membrane but still confer cryoprotection (Di Santo et al., 2012).

An alternative classification divides CPAs into osmotic and non-osmotic types. Osmotic cryoprotectants (e.g., glycerol, DMSO, EG, acetamide, PROH) depress the freezing point of aqueous solutions, equilibrate osmotic pressure across cell membranes, and minimize cellular damage from shrinkage or swelling during thermal transitions. In contrast, non-osmotic cryoprotectants (e.g., PVP, sucrose, glucan, albumin, PEG) remain extracellular, reducing solute concentration and maintaining a supercooled state. Sucrose, for instance, has been effectively employed in the vitrification of amphibian sperm (Luyet & Hodapp, 1938).

3.1.1 Sulphoxides

Sulphoxides are oxidised thioethers containing one oxygen atom per molecule) and are water soluble (McGann & Walterson, 1987). Dimethylsulfoxide (DMSO, Me_2SO) has been found to be a very effective and universal CPA that is rapidly gaining acceptance in cryobiology. It was first used for cryoprotection and freezing of spermatozoa (Lovelock & Bishop, 1959).

The optimal concentration of Me_2SO was used for vitrification of spermatozoa from sex-reversed rainbow trout (Figueroa et al., 2013). In the study, the vitrification solution consisted of a standard buffer for fish spermatozoa (Cortland® medium) + 10% DMSO + 2% BSA + 0.13 M sucrose + SP (seminal plasma) at concentrations of 30-50%. Fertilisation rates were higher for spermatozoa vitrified in the medium with the highest percentage of seminal plasma (40%).

For vitrification of *A. ruthenus* and *A. gueldenstaedtii* ovarian tissue, 1.5 M Me_2SO was used (Lujić et al., 2023). For sperm vitrification of *L. campechanus*, *S. ocellatus* and *C. nebulosus*, 15% DMSO was found to be suitable (Cuevas-Uribe et al., 2015). For sperm vitrification of *S. salar*, 10% DMSO was found to be sufficient (Figueroa et al., 2015). However, only for sperm vitrification of *I. punctatus* was more than 20% dimethylsulfoxide found to be toxic (Cuevas-Uribe et al., 2011b).

3.1.2 Alcohol and its derivatives

Alcohols and derivatives include 1) Monohydric alcohols and their derivatives: Methanol, ethanol and polyvinyl alcohol, 2) Diols and derivatives: Ethylene glycol, propylene glycol, polyethylene glycol, 1,2-propanediol, methyl

glycol, 3) Triols: Glycerol 4) Polyalcohols: Mannitol and sorbitol have been used for freezing and vitrification of sperm from fish and many other species.

Polyhydric alcohols, especially glycerol, as well as glycols and sugar alcohols, are widely used as CPAs, while monovalent alcohols are rarely used due to their toxicity to many biological systems. However, methanol has been used for the vitrification of many fish sperm. For vitrification of *A. ruthenus* and *A. gueldenstaedtii* ovarian tissue, 1.5 M methanol (MeOH) and propylene glycol (PG) are as effective as 1.5 M Me_2SO (Lujić et al., 2023). For *I. punctatus* sperm vitrification, methyl glycol and 1,2-propanediol were used at 10% in the cryoprotectant mixture. For *A. anguilla* sperm vitrification, a 40% cryoprotectant mixture (20% MeOH and 20% PG) was preferred (Kása et al., 2017).

Glycerol(Gly), propane-1,2,3-triol, is a symmetrical polyol containing three hydroxy (OH) groups: two identical primary OH and one secondary OH with a similar pKa. Glycerol ($C_3H_8O_3$), systematically termed propane-1,2,3-triol, is a trihydric alcohol featuring two primary and one secondary hydroxyl groups. This small polyol occurs ubiquitously in biological systems, primarily esterified in triglycerides (storage lipids) and as a structural backbone in phospholipid bilayers (Plasseraud, 2024). Its physical characteristics include high viscosity, optical transparency, neutral olfactory properties, a saccharine flavor profile, and extreme hygroscopicity. Demonstrating low acute toxicity, it exhibits complete miscibility with hydrophilic solvents (e.g., water, ethanol) but negligible solubility in nonpolar media such as benzene or chloroform.

The molecule's polar nature facilitates extensive hydrogen bonding with aqueous solutions and short-chain alcohols (Sillanpää & Ncibi, 2017). While its limited membrane permeability reduces cellular toxicity, hypertonic glycerol solutions may induce deleterious osmotic imbalances (Warner et al., 2021). As a colligative cryoprotectant, it depresses the freezing point of aqueous systems, with binary glycerol-water mixtures achieving maximal freezing point depression at approximately -46°C.

Synergistic cryoprotection is achieved when Ethylene glycol ($C_2H_6O_2$) (EG) is formulated with glycerol and dimethyl sulfoxide (DMSO), as these compounds exhibit complementary interactions with cellular water and macromolecular structures (Cuevas-Uribe et al., 2011a,b; 2015). This ternary combination enhances vitrification efficiency while mitigating solute toxicity.

Propylene glycol (PG) is another liquid commonly used in the cryopreservation process. PG is metabolised to oxalic acid and behaves like EG. PG usually shows toxicity when used as a CPA. Studies have demonstrated

that propylene glycol concentrations exceeding 2.5 M can compromise embryo developmental capacity, largely due to a reduction in intracellular pH (Damien et al., 1990). Although this evidence originates from mammalian systems, it underscores the potential for high PG levels to induce comparable intracellular acidification risks in fish spermatozoa during cryopreservation. Propylene glycol 15% in combination with 15% methanol has been used as a cryoprotectant in the vitrification of fish sperm (Kása et al., 2017).

3.1.3 Amides, N-Alkylamides, Imides

N,N-dimethylacetamide, dimethylacetamide (DMA) is a synthetic organic compound. For sperm vitrification in teleost species, dimethylacetamide (DMA) levels in the range of 10–20% have been identified as effective, whereas exceeding this threshold has been associated with cytotoxic effects in Ictalurus punctatus spermatozoa. This cryoprotectant was found to alter mitochondrial and microtubule organisation in rat spermatozoa, leading to loss of motility. Such exposure adversely affects sperm quality performance, leading to declines in both overall motility and forward-directed movement. Consequently, the use of DMA resulted in a marked impairment of fertilization capacity (Khera et al., 2020).

Following its designation in 2011 as a substance of very high concern in the EU due to documented reproductive toxicity, dimethylacetamide was subsequently subjected to a regulatory reassessment under REACH, leading to the European Commission's 2014 action to restrict its use (Commission Regulation (EU) No 895/2014; Member State Committee Agreement, 2011). DMA exposure induces marked disruptions in mitochondrial architecture and axonemal microtubule organisation within spermatozoa, leading to impaired motility patterns characterized by reduced total and progressive movement, ultimately culminating in diminished fertilisation capacity (Khera et al., 2020).

3.1.4 Other substances used as cryoprotectants

Monosaccharides: Glucose; Disaccharides: Sucrose, Lactose, Maltose, Trehalose; Polysaccharides: Dextran, Manan; Heterocyclic compounds: Polyvinylpyrrolidone; Amino acids and carboxylic acids: Proline, Glycine; Proteins, peptides, polypeptides and glycoproteins: Blood serum, albumins; Complex substrates: Skimmed milk

In fish sperm cryopreservation, cellular injury is mitigated through the incorporation of appropriate cryoprotective agents; however, their intrinsic

cytotoxicity necessitates rapid elimination during thawing, a requirement that considerably increases both the technical complexity and the operational cost of the procedure.

Spermatozoal uptake of cryoprotective agents exhibits species- and compound-specific variability, with membrane permeability differing according to the particular cryoprotectant applied. Although the permeability of cryoprotectants in fish sperm is within the expected range for most sperm (~10 -4 cm/min) and dimethyl sulfoxide penetrates sperm within a few minutes. However, some cryoprotectants (10% methanol and 10% N,N-dimethylformamide) cause no change in cell volume when entering and leaving the cell. The absence of detectable volumetric shifts in the presence of formamide or methanol is likely attributable either to the rapid efflux of these solutes matching the osmotic influx of water or to inherently low membrane permeability of spermatozoa to these compounds (Raju et al., 2021). In the presence of an impermeable solute, the volume of the cells remains constant rather than shrinking. The demonstrated success of cryoprotectants in fish sperm cryopreservation confirms their intracellular activity, even though certain agents within this group are categorized as high-molecular-weight, membrane-impermeable CPAs that do not traverse the plasma membrane (Raju et al., 2021). The most commonly used CPAs are: trehalose (TRE), sucrose (SUC), hydroxyethyl starch and polyvinyl pyrrolidone (PVP) (Zamecnik et al., 2021).

The precise molecular pathways underlying cryoprotection remain incompletely characterized. Contemporary theoretical frameworks indicate that cryoadditives—whether capable of traversing the plasma membrane or restricted to the extracellular milieu—primarily exert their protective action by reducing the availability of free water, thereby lowering the likelihood of intracellular ice formation during the cryopreservation of fish spermatozoa (Mazur, 1984). Permeable compounds exhibit additional protective effects by:

1. Acting as cosolvents that mitigate solution effects during freeze-induced dehydration
2. Maintaining macromolecular hydration shells through direct hydrogen bonding

Notably, biophysical studies indicate certain cryoprotectants may stabilize membrane phospholipid arrays and protein tertiary structures through specific molecular interactions (Anchordoguy et al., 1987). However, this stabilization

exhibits concentration-dependent duality – while effective at optimal levels, prolonged exposure or excessive concentrations can induce cytotoxic effects through membrane fluidity disruption and protein denaturation (Fahy, 1986).

3.1.4.1 Non-Penetrating Cryoprotective Agents

High-molecular-weight additives including:

- Oligosaccharides (raffinose, sucrose)
- Protein complexes (egg yolk lipoproteins, albumin)
- Synthetic polymers (PEG, PVP)

demonstrate cryoprotective efficacy despite lacking membrane permeability (Di Santo et al., 2012; Zamecnik et al., 2021). These agents function through:

1. Extracellular supercooled state
2. Colligative depression of ice crystal growth kinetics
3. Macromolecular crowding effects that stabilize membrane interfaces

A review of vitrification practices indicates that sucrose represents the earliest non-penetrating cryoprotectant successfully employed for the vitrification of amphibian gametes. (Luyet & Hodapp, 1938). Modern cryobiology recognizes a spectrum of membrane interaction potentials, with certain sugars exhibiting intermediate permeability characteristics. Recent advances continue to expand the repertoire of identified cryoprotective molecules, including novel biomimetic compounds (Zamecnik et al., 2021).

Carbohydrate-based additives facilitate osmotic dehydration during vitrification and are incorporated into CPA formulations to support the stabilization of sperm plasma membranes under near-anhydrous conditions (Acker & McGann, 2003).

Glucose penetrates the cell membrane more rapidly than sucrose due to its molecular size. In fish sperm freezing, 5.85% glucose and 6.55% glucose, 0.13 M sucrose and 0.25 M trehalose are the most commonly used sugars (Isachenko et al. 2008; Viveiros et al. 2012; Figueroa et al., 2013, 2015). In addition to these agents, non-permeating sugars such as raffinose and sucrose are frequently incorporated into vitrification media due to their extracellular protective functions (Di Santo et al., 2012).

The vitrification of different mammalian spermatozoa after vitrification/thawing with different ice-blocking media (X-1000™ and Z-1000™) was

investigated. Because highly concentrated vitrification media depress the homogeneous nucleation point to temperatures lower than the glass transition, the principal barrier to achieving a fully vitreous state is the activity of heterogeneous nucleators, a challenge mitigated through the incorporation of ice-blocking polymers such as Super Cool X-1000—a polyvinyl-alcohol/vinyl-acetate copolymer—and Super Cool Z-1000, as reported by Ting et al. (2012). In contrast to penetrating CPAs, which interfere with cryopreservation by interacting with water, X-1000 in particular acts at the nucleation point of the growing ice, preventing other water molecules from becoming blinded on the flat surface and subsequently preventing the formation of spicules in the ice crystals, which could cause major cell damage during the process. In this respect, studies have shown that the polymer (Badrzadeh et al., 2010).

These ice blocks were evaluated for vitrification of sperm from different fish species. Sperm in 1% X-1000™ + 1% Z-1000™ had an average post-thaw motility of 58% and membrane integrity of 19% for spotted seatrout, 38% and 9% for *L. Campechanus,* 30% and 19% for *S. ocellatus* and *C. nebulosus* (Cuevas-Uribe et al., 2015).

3.1.5. Seminal plasma

Previous research group has been working on the application of human sperm vitrification without seminal plasma, and the first successful results in the absence of cryoprotectant were obtained in the early 2000s (Isachenko et al., 2003).

Supplementation of the vitrification medium with butylhydroxytoluene (BHT), a synthetic analogue of vitamin E, was associated with a reduction in ROS production. It significantly preserved DNA integrity. BHT concentrations of 0.5 to 1.0 mM appear to be optimal for cryopreservation of human semen by vitrification (Aitken & Clarkson, 1989). The proposed protective mechanism of BHT is its incorporation into the sperm membrane and increasing the fluidity of the membrane (Uribe et al., 2017).

Antioxidant components in the seminal plasma, including both enzymatic and non-enzymatic factors, contribute to safeguarding fish spermatozoa against oxidative stress during cryopreservation (Cosson, 2007, Ciereszko, 2008). Proteins present in the seminal plasma may contribute to mitigating freezing-related injuries by interacting with the selectively permeable plasma membrane of fish spermatozoa. (Barrios et al., 2000). Seminal plasma, functioning as an endogenous antioxidant, has been shown to markedly mitigate cryo-

induced damage in fish spermatozoa, with a 40% inclusion level providing notable protection against DNA fragmentation and loss of membrane integrity (Lahnsteiner, 2007; Figueroa et al., 2013). During cryopreservation procedures such as freezing and vitrification, excessive dilution of fish spermatozoa can reduce the protective influence of seminal plasma proteins associated with the plasma membrane, which in turn may adversely affect key performance traits including motility, viability, fertilization capacity, and resistance to cryogenic stress. (Lahnsteiner, 2007). Results indicate that spermatozoa can be effectively preserved by direct plunging into liquid nitrogen, achieving significant post-warming motility and cytoplasmic membrane integrity, with optimal results seen using 1% BSA combined with 40% seminal plasma(Figueroa et al., 2013).

According to Rall (1987), incorporating BSA into vitrification media can contribute to enhancing the stability of the amorphous phase during the cryopreservation of fish spermatozoa.

Protocols involving direct ultra-rapid cooling of fish spermatozoa in liquid nitrogen have incorporated 1–2% bovine serum albumin (BSA) together with 30–50% seminal plasma, both serving as extracellular cryoprotective additives in vitrification procedures (Merino et al., 2011, 2012; Isachenko, 2013; Figueroa et al., 2013, 2015).

4.Cryoprotectant mixtures in fish sperm vitrification

Cryoprotectant mixtures have been preferred for sperm vitrification in some fish species. To limit toxicity effects, it is reasonable to use the least toxic CPA at the lowest possible concentration that will prevent vitrification. The simultaneous use of different cryoprotectants may also result in different thermal expansion properties of the different components of the CPA agents during cryopreservation, which may further increase thermo-mechanical stresses.

A combination of 40% glycerol and 20% glycerol + 20% ethylene glycol has been tried for the vitrification of *X. hellerii* sperm (Cuevas-Uribe et al., 2011a). The combination of 15% DMSO + 15% ethylene glycol + 10% glycerol was used for *L. campechanus, S. ocella*tus and *C. nebulosus* (Cuevas-Uribe et al., 2015). The vitrification method for *P. fluviatilis* sperm was 30% cryoprotectant consisting of 15% methanol + 15% propylene glycol (Kása et al., 2017).

5. Media used for sperm vitrification

The vitrification medium contained a standard buffer for sex-reversed rainbow trout spermatozoa, a standard culture medium, Cortland® medium,

was used. The composition of the Cortland® medium per litre is: 1.88 g NaCl, 0.23 g CaCl 2, 7.2 g KCl, 0.41 g NaH 2 PO 4, 1 g NaHCO3, 0.23 g MgSO 4 ×7H 2 O, 1.0 g glucose, 10% (0.02 M) glycol and 10% Tris (0.02 M), pH 9.0; 261 mOsm. (Figueroa et al., 2013). For *S. aurata* semen vitrification protocols, the best vitrification medium includes Mounib buffer containing 10% Me2SO and 10% glycerol (Zilli et al., 2018).

6. Conclusion

Vitrification allows long-term storage of sperm in a glassy state at ultra-low temperatures in liquid nitrogen. This suppresses the sperm's biochemical processes. The aim of this method is to vitrify sperm and prevent ice crystal formation during cooling and heating. Although cryoprotectants and mixtures are useful substances in sperm vitrification, the physico-chemical process and cryoprotectant toxicity of sperm, vitrification solutions should be taken into account. Many new cryoprotectants are entering this field. Vitrification has advantages and disadvantages. Although the disadvantages cannot be reduced to zero, the difference between the advantages and disadvantages can be increased in the favour of the advantages.

References

Acker, J.P. & McGann, L.E. (2003) Protective effect of intracellular ice during freezing? *Cryobiology*, 46:197–202, doi: 10.1016/S0011-2240(03)00025-7.

Aitken, J.& Clarkson, S. (1989). Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *Journal of Andrology,* 9, 367-376. doi: 10.1002/j.1939-4640.1988.tb01067.x

Anchordoguy, T.J., Rudolph, A.S., Carpenter, J.F., & Crowe, J.H., (1987) Modes of interaction of cryoprotectants with membrane phospholipids during freezing, *Cryobiology,* 24, 4, 324-331, https://doi.org/10.1016/0011-2240(87)90036-8.

Badrzadeh, H., Najmabadi, S., Paymani, R., Macaso, T., Azadbadi, Z., & Ahmady, A. (2010). Super cool X-1000 and Super cool Z-1000, two ice blockers, and their effect on vitrification/warming of mouse embryos, *European Journal of Obstetrics & Gynecology and Reproductive Biology*, 151, 1, 2010, 70-71, https://doi.org/10.1016/j.ejogrb.2010.03.028.

Barrios, B., Pérez-Pé, R., Gallego, M., Tato, A., Osada, J., Muiño-Blanco, T., & Cebrián-Pérez, J.A. (2000). Seminal plasma proteins revert the cold-shock

damage on ram sperm membrane. *Biol Reprod*. 63(5), 1531-1537. doi: 10.1095/biolreprod63.5.1531. PMID: 11058562.

Ciereszko, A. (2008). Chemical composition of seminal plasma and its physiological relationship with sperm motility, fertilising capacity and cryopreservation success in fish. In: S.M.H. ALAVI, J. COSSON, K. COWARD and G. RAFIEE eds. *Fish spermatology* Oxford, UK: Alpha Science International Ltd., pp. 215-240.

Commission Regulation (EU) No 895/2014. (2014). *Official Journal of the European Union*. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX%3A32014R0895

Cosson, J.J. (2007). The motility apparatus of fish spermatozoa. In: Fish spermatology. *Alpha Science*, Oxford, pp 281–316.

Cuevas-Uribe, R., Chesney, E.J., Daly, J., & Tiersch, T.R. (2015). Vitrification of Sperm from Marine Fishes: Effect on Motility and Membrane Integrity. *Aquaculture Research,* 1;46(7):1770-1784. doi: 10.1111/are.12337.

Cuevas-Uribe, R., Leibo, S.P., Daly, J., & Tiersch, T.R. (2011b). Production of channel catfish with sperm cryopreserved by rapid non-equilibrium cooling. *Cryobiology,* 63, 3, 186-197, https://doi.org/10.1016/j.cryobiol.2011.06.004.

Cuevas-Uribe, R., Yang, H., Daly, J., Savage, M.G., Walter, R.B., & Tiersch, T.R. (2011a). Production of F1 offspring with vitrified sperm from a live-bearing fish, the green swordtail *Xiphophorus hellerii*. *Zebrafish.* 8(4):167-79. doi: 10.1089/zeb.2011.0704. PMID: 21883000; PMCID: PMC3241736.

Damien, M., Luciano, A. A., & Peluso, J.J. (1990). Propanediol alters intracellular pH and developmental potential of mouse zygotes independently of volume change. *Human reproduction,* 5, 212–216.

Di Santo, M., Tarozzi, N., Nadalini, M., & Borini, A. (2012). Human Sperm Cryopreservation: Update on Techniques, Effect on DNA Integrity, and Implications for ART. *Advances in Urology,* 2012:854837. doi: 10.1155/2012/854837.

Figueroa, E., Merino, O., Risopatrón, J., Isachenko, V., Sánchez, R., Effer, B., Isachenko, E, & Farias, J.G. (2015). Valdebenito I, Effect of seminal plasma on Atlantic salmon (*Salmo salar*) sperm vitrification, Theriogenology, 83, 2, 238-245. https://doi.org/10.1016/j.theriogenology.2014.09.015.

Figueroa, E., Risopatron, J., Sanchez, R., Isachenko, E., Merino, O., Isachenko, Farias, J.G., & Valdebenito, I. (2013). Spermatozoa vitrification of sex-reversed rainbow trout (*Onchorhyncus mykiss*): effect of seminal plasma on physiological parameters. *Aquaculture.* 372 (5):119–126. https://doi.org/10.1016/j.aquaculture.2012.10.019.

Gilmore, J.A., Liu, J., Gao, D.Y. & Critser, J.K. (1997). Determination of optimal cryoprotectants and procedures for their addition and removal from human spermatozoa, *Human Reproduction,* 12, 112–118.

Isachenko, E., Isachenko, V., Weiss, J.M., Kreienberg, R., Katkov, I.I., & Schulz, M. et al. (2008) Acrosomal status and mitochondrialactivity of human spermatozoa vitrified with sucrose. *Reproduction,* 136, 167–173. DOI: 10.1530/REP-07-0463

Isachenko, E., Isachenko, V., Katkov, I., Dessole, S., & Nawroth, F.(2003). Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. *Reprod BioMed Online*, 2003; 6: 191-200. DOI: 10.1016/s1472-6483(10)61710-5

Isachenko, E. (2013). Spermatozoa vitrification of sex-reversed rainbow trout (*Oncorhynchus mykiss*): Effect of seminal plasma on physiological parameters. *Aquaculture.* 6, 2, 191-200, https://doi.org/10.1016/j.aquaculture.2012.10.019

Kása, E., Bernáth, G., Kollár, T., Żarski, D., Lujić, J., Marinović, Z., Bokor, Z., Hegyi, Á., Urbányi, B., Vílchez, M.C., Morini, M., Peñaranda, D.S., Pérez, L., Asturiano, J.F. & Horváth, A., (2017) Development of sperm vitrification protocols for freshwater fish (Eurasian perch, *Perca fluviatilis*) and marine fish (European eel, *Anguilla anguilla*, *General and Comparative Endocrinology,* 245, 102-107, https://doi.org/10.1016/j.ygcen.2016.05.010.

Khera, N., Ghayor, C., Lindholm, A., Pavlova, E., Atanassova, N., & Weber, F.E. (2020) N, N-Dimethylacetamide, an FDA approved excipient, acts post-meiotically to impair spermatogenesis and cause infertility in rats. *Chemosphere,* 256(2),127001, DOI:10.1016/j.chemosphere.2020.127001

Lahnsteiner, F. (2007).Characterization of seminal plasma proteins stabilizing the sperm viability in rainbow trout (*Oncorhynchus mykiss*). *Animal Reproduction Science,* 97, 151–164. DOI: 10.1016/j.anireprosci.2006.01.003

Lovelock, J.E., & Bishop, M.W.H. (1959). Prevention of freezing damage to living cells by dimethylsulphoxide, *Nature,* 183, 1394–1395, doi: 10.1038/1831394a0.

Lujić, J., Franěk, R., Marinović, Z., Kašpar, V., Xie, X., Horváth, Á., Pšenička, M., & Urbányi, B. (2023). Vitrification of the ovarian tissue in sturgeons, *Theriogenology*, 196, 18-24, https://doi.org/10.1016/j.theriogenology.2022.11.009.

Luyet, B.J. & Hodapp, E.L. (1938). Revival of Frog's Spermatozoa Vitrified in Liquid Air. *Proceedings of the Society for Experimental Biology and Medicine*, 39, 433-434. https://doi.org/10.3181/00379727-39-10229P.

Massaldi, H.A. (1984). The physico-chemical mechanism of mediated transport: II. Osmotic and isosmotic volume flow, *Journal of Theoretical Biology,* 110 (1), 35-57, https://doi.org/10.1016/S0022-5193(84)80014-4.

Mazur, P. (1984). Freezing of living cells: mechanisms and implications. *American Physiological Society,* 247(3), 125-42. doi: 10.1152/ajpcell.1984.247.3.C125.

McGann, L.E. & Walterson, M.L. (1987). Cryoprotection by dimethylsulfoxide and dimethylsulfone, *Cryobiology,* 24, 11–16. https://doi.org/10.1016/0011-2240(87)90003-4

Merino, O., Risopatrón, J., Sánchez, R., Isachenko, E., Figueroa, E., Valdebenito, I., & Isachenko, V. (2011). Fish (*Oncorhynchus mykiss*) spermatozoa cryoprotectant-free vitrification: stability of mitochondrion as criterion of effectiveness. *Animal Reproduction Science,* 124(1-2), 125-131. doi: 10.1016/j.anireprosci.2011.02.023.

Merino, O., Sanchez, R., Risopatron, J., Isachenko, E., Katkov, I., & Figueroa, E. et al. (2012). Cryoprotectant-free vitrification of fish(*Oncorhynchus mykiss*) spermatozoa: first report. *Andrologia,*44,390–395. doi: 10.1016/j.anireprosci.2011.02.023.

Parks, J.E. & Graham, J.K. (1992). Effects of cryopreservation procedures on sperm membranes, *Theriogenology*, 38, (2), 209-222, https://doi.org/10.1016/0093-691X(92)90231-F.

Raju, R., Bryant, S.J., Wilkinson, B.L., & Bryant, G. (2021). The need for novel cryoprotectants and cryopreservation protocols: Insights into the importance of biophysical investigation and cell permeability, *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1865, 1, 129749, https://doi.org/10.1016/j.bbagen.2020.129749.

Rabin, Y., & Steif, P.S. (2006): Solid mechanics aspect of cryobiology, *In: Advances in Biopreservation* (Baust, J.G., and Baust J.M., Eds, Chap). 13, 359-382.

Rall, W. (1987). Factors affecting the survival of mouse embryos cryopreserved by vitrification, *Cryobiology,* 24, 387–402. doi: 10.1016/0011-2240(87)90042-3.

Royere, D., Barthelemy, C., Hamamah, S., & Lansac J. (1996). Cryopreservation of spermatozoa: a 1996 review. *Hum Reprod Update.* 2(6), 553-9. doi: 10.1093/humupd/2.6.553. PMID: 9111188.

Saragusty, J. & Arav, A. (2011). Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction*, 141(1), 1-19. doi: 10.1530/REP-10-0236. Epub 2010 Oct 25. PMID: 20974741.

Sillanpää, M., & Ncibi, C. A. (2017). Sustainable Bioeconomy. Springer; Berlin/Heidelberg, Germany: *Biochemicals,* 141–183.

Ting, A.Y., Yeoman, R.R., Lawson, M.S., & Zelinski, M.B. (2012). Synthetic polymers improve vitrification outcomes of macaque ovarian tissue as assessed by histological integrity and the in vitro development of secondary follicles, *Cryobiology,* 65 (1), 1-11, https://doi.org/10.1016/j.cryobiol.2012.04.005.

Uribe, P., Rojas, C., Meriño, J., Zambrano, F., Villegas, J.V., Treulen, F., Boguen, R., Isachenko, V., Isachenko, E. & Sánchez, R. (2017). Effect of incubation temperature after devitrification on quality parameters in human sperm cells. *Cryobiology*, 79, 78-81, doi: 10.1016/j.cryobiol.2017.10.005.

Viveiros, A.T.M., Orfão, L.H., Nascimento, A.F., Corrêa, F.M., & Caneppele, D. (2012). Effects of extenders, cryoprotectants and freezing methods on sperm quality of the threatened Brazilian freshwater fish pirapitinga-do-sul *Brycon opalinus* (Charachiformes),*Theriogenology*,78 (2), 361-368, https://doi.org/10.1016/j.theriogenology.2012.02.015.

Warner, R.M., Ampo, E., Nelson, D., Benson, J.D., Eroglu, A. & Higgins, A.Z.(2021). Rapid quantification of multi-cryoprotectant toxicity using an automated liquid handling method. *Cryobiology,* 98, 219–232. doi: 10.1016/j.cryobiol.2020.10.017.

Yavin, S. & Arav, A. (2007). Measurement of essential physical properties of vitrification solutions. *Theriogenology,* 67, 81–89.

Zamecnik, J., Faltus, M., & Bilavcik, A. (2021). Vitrification Solutions for Plant Cryopreservation: Modification and Properties. *Plants (Basel)*,10(12):2623. doi: 10.3390/plants10122623.

Zilli, L., Bianchi, A., Sabbagh, M., Pecoraro, L., Schiavone, R., & Vilella, S. (2018) Development of sea bream (*Sparus aurata*) semen vitrification protocols. Theriogenology, 110, 103-109, https://doi.org/10.1016/j.theriogenology.2017.12.039.

ISBN 978-2-38236-968-5

livredelyon.com
livredelyon
livredelyon
livredelyon