CHAPTER II

SPERM CRYOPRESERVATION IN THE EUROPEAN SEA BASS

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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.

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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.