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Cryopreservation of sperm in marine fish

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Abstract: Since the first work of Blaxter in 1953, fish sperm cryopreservation has been attempted on about 30 marine species. The present paper reviews the techniques used and the results published in these species. Particular attention is paid to the handling procedure of sperm before freezing, the problems of semen ageing and semen contamination with urine. The quality of frozen-thawed semen was evaluated using previously standardized biotests, such as a two-step motility activation technique adapted for the different species and fertilization assays using a discriminating insemination technique. Most extenders used in marine fish are saline or sugar solutions. From the investigated cryoprotectants, dimethyl sulphoxide (DMSO) generally leads to the best results. Cooling rates range from 8 °C to 99 °C min-1; the thawing rate is generally high. Compared with freshwater species, a high percentage of spermatozoa survives cryopreservation. Therefore, and because of the simplicity of the techniques, the cryopreservation of marine fish sperm is suited for application in aquaculture.

Keywords: Fish sperm, cryopreservation, fertilization, insemination, DMSO

Introduction

Techniques of sperm management have been established in some freshwater fish species such as cyprinids (Billard, Cosson, Crim & Suquet 1995) or siluroids (Legendre, Linhart & Billard 1996) and in salmonids (Scott & Baynes 1980; Billard 1992). Among these techniques, sperm storage and cryopreservation are of special interest. At 0°C conditions, spermatozoa can be stored for a few hours up to several days, depending on the species while cryopreserved gametes can be theoretically stored between 200 and 32 000 years without deleterious effect (Ashwood-Smith 1980).

The use of cryopreserved spermatozoa can be delayed from the date of collection and adjusted to the moment of ova processing. The benefits of this technique include :

-Synchronization of gamete availability of both sexes : ovulations are only noticed when sperm production declines in cross fertilization of different strains like spring and autumn spawning herring (*Clupea harengus* L. : Blaxter 1953).

-Use of the total volume of available semen: this is useful for sperm economy in species where semen is difficult to obtain (Japanese eel, *Anguilla japonica* Temminck & Schlegel) (Ohta & Izawa 1996), but also in species where only low volume of semen can be stripped in captivity (yellowtail flounder, *Pleuronectes ferrugineus* L. : Clearwater & Crim 1995 or turbot, *Psetta maxima* L. : Suquet, Billard, Cosson, Dorange, Chauvaud, Mugnier & Fauvel 1994).

-Simplifying broodstock maintenance: off season spawning can be induced in most cultured fish species, by the manipulation of photoperiod and temperature cycles (Bromage 1995). However, the technique is cost intensive. When cryopreserved sperm is available all year round, the manipulation of the spawning season could be restricted to females.

-Transport of gametes: useful when male and female gametes are collected in different locations. This enables also the introduction of genes from the wild into hatchery stocks.

-Avoiding aging of sperm: the senescence of sperm during the course of the spawning season has been reported for many fish species and results in a decrease of milt quality (Rana 1995a). Cryopreservation allows the collection of sperm when it has the highest quality.

-Experimental programs: for genetic studies, in comparing the breeding performances of successive generations in the same experiment and for experiments where the use of identical sperm samples is necessary over an extended period *e.g* study of short term storage of ova.

-Conserving genetic variability in domesticated populations: the use of a limited number of breeders leads to a reduction of heterozygosity. The cryopreserved semen of selected strains or genetically improved populations can be introduced in domesticated stocks *e.g.* the sperm of sex reversed gynogenetic hirame (*Paralichthys olivaceus* Temminck & Schlegel) female (Tabata & Mizuta 1997). Gene banks of cryopreserved semen can also be used to maintain genetic diversity of fish populations that are endangered and protect against inbreeding. In protogynous hermaphrodite species such as Black grouper (*Epinephelus malabaricus* Bloch & Schneider), sperm can only be collected in 5 to 10 years old animals (Gwo 1993). As a consequence, success in breeding is greatly enhanced by the use of frozen sperm.

Under consideration of the many benefits of this technique, the sperm of over 200 fish species has been cryopreserved (for review see Billard *et al*. 1995). These techniques have been reviewed in detail by Stein & Bayrle (1978), Scott & Baynes (1980), Stoss (1983), Chao (1991), Jamieson (1991), Tiersch (1995), Rana (1995a & b), Maisse (1996) and Maisse, Labbé, Ogier de Baulny, Leveroni & Haffray (1998). Most publications are devoted to salmonids, tilapias and carps. In marine fish, the first report was by Blaxter (1953) in herring. As marine fish farming expands, there is an increasing need to apply sperm cryopreservation techniques. Due to the increasing knowledge in sperm biology also spermatozoa of other marine fish have been recently cryopreserved successfully. This paper reviews the techniques and results obtained for cryopreservation of marine fish species. It emphasizes the high survival and fertilization

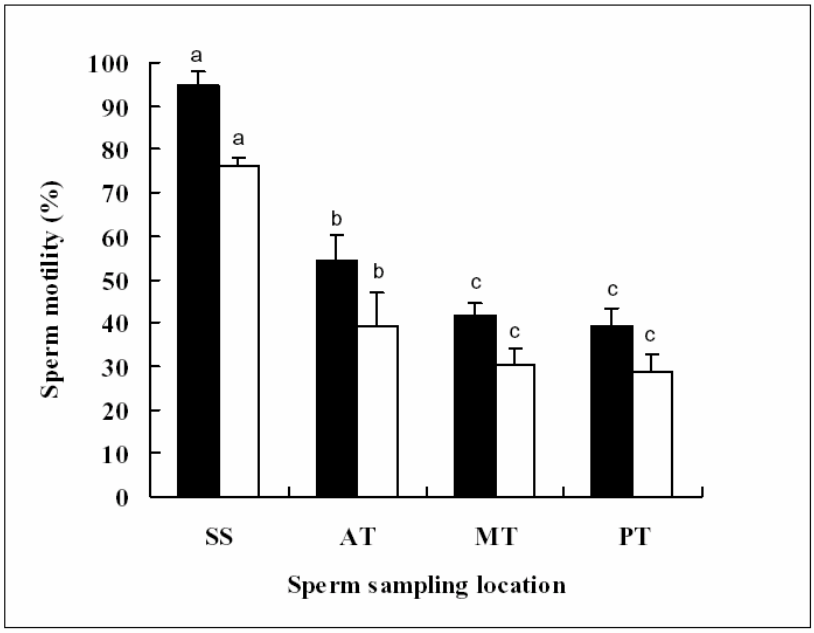
capacity generally obtained in frozen-thawed spermatozoa of marine fish in comparison to freshwater species. Special attention was paid on individual variations of sperm quality and on biotests useful to determine the quality of thawed cells.

Individual variations of sperm quality

High individual variations of milt quality are frequently reported (Rana 1995a). This may be due to genetic variability but also to sampling location (from testes to the genital pore), contamination of sperm samples by urine and aging of spermatozoa during the spawning season.

Similar to the Salmonidae (Morisawa & Morisawa 1988), a maturation process of spermatozoa has been reported for several marine fish species. Compared to samples collected by stripping, the percentage of motile spermatozoa of turbot decreases when intratesticular milt is collected (Figure 1). In Japanese eel, testicular spermatozoa exhibited only a very slight motility after activation, while spermatozoa of stripped sperm could be successfully activated (Ohta, Ikeda & Izawa 1997a). Furthermore, testicular spermatozoa of this species, incubated in saline solutions with high concentrations of K^+ and $HCO3^-$, artificially acquire their motility potential and show a similar fertilization capacity as stripped milt (Ohta, Tanaka, Kagawa, Okuzawa & Iinuma 1997b).

Figure 1. Differences in sperm motility rates of turbot collected by stripping (SS), from the anterior testicule (AT), the median testicule (MT) and the posterior testicule (PT) (black bars : 10 s post-activation, white bars : 60 s post-activation). For each time post-activation, values followed by different letters are significantly different ($P<0.05$).

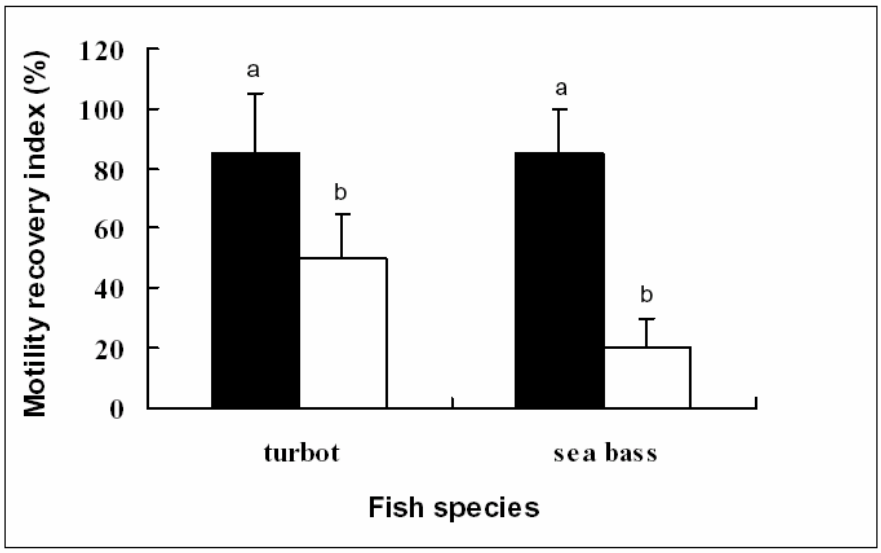


Because of the close vicinity of sperm ducts and urinary ducts, sperm samples are frequently contaminated with urine. This phenomenon is described in freshwater fish species (Rana 1995a), but more scarcely in marine species. In turbot, a mean contamination rate of 15.3% (urine volume : sperm volume) was reported, basing on the determination of urea concentration (Dreanno, Suquet, Desbruyères, Cosson, Le Delliou & Billard 1998). Artificial urine contamination of milt decreased the percentage of motile spermatozoa, sperm velocity, the fertilization capacity and the storage ability. These effects increased with urine concentration and incubation time. Catherization of the ureter prior to sperm collection significantly decreased the urine contamination to 9.3%. In species with high sperm densities such as sea bass (*Dicentrarchus labrax* L.), urine contamination is more easily detected because of the lower viscosity and colour changes of portions of sperm samples containing urine (Fauvel, Savoye, Dreanno, Cosson & Suquet 1999).

Intratesticular aging of sperm has been reported for many fish species and it affected sperm quality at the end of the milting period (Rana 1995a). In marine fish, in the sea bass the concentration of spermatozoa decreased as the spawning season progressed (Fauvel *et al*. 1999). Spermatozoa of this species maintained their swimming duration for a longer period at the beginning of the milting season than at the end (Billard, Dupont & Barnabé 1977; Sorbera, Mylonas, Zanuy, Carillo & Zohar 1996). Furthermore, lower motility rates, fertilization rates and reduced short-term storage capacity were recorded at the end of the reproduction period. As revealed by electron microscopy, also the structure was changed in aged sea bass sperm (Dreanno, Suquet, Fauvel, Le Coz, Dorange, Quemener & Billard in press). In the Atlantic halibut (*Hippoglossus hippoglossus* L.), sperm motility was reduced at the end of the reproductive period (Methven & Crim 1991; Shangguan 1998). The highest motility rates of winter flounder (*Pleuronectes americanus* Walbaum) sperm were found at the beginning of the milting period (Shangguan & Crim, 1995). A decline of motility parameters, storage and fertilization capacities as well as alterations of the plasma membrane were reported in turbot as the milting season progressed (Suquet, Dreanno, Dorange, Normant, Quemener, Gaignon & Billard 1998a). As recorded in sea bass and turbot, senescence of spermatozoa can result in a decrease of freezing ability (Figure 2).

Subsequently, when sampling sperm for cryopreservation, one must take care of the three described factors (intra testicular maturation, contamination by urine and aging of sperm) as they can decrease the initial quality of spermatozoa.

Figure 2. Changes in motility recovery index (defined as in table 6) of frozen-thawed spermatozoa of turbot (assessed at 60 s *post-activation*) and sea bass (assessed at 30 s *post-activation*) as a function of the sampling date (black bars : beginning of the spawning period of the females, white bars : end of the spawning period of the females). For each species, values followed by different letters are significantly different ($P<0.05$).



Biotests

Several biological tests are used to improve the cryopreservation techniques and to assess the sperm viability of frozen-thawed spermatozoa. An efficient quality test for frozen-thawed spermatozoa must be correlated with the fertilization capacity. The test must allow objective and sensitive measurements of a large number of cells and give a rapid answer because of the fast deterioration of ova quality immediately after collection. The presently applied tests describe physiological parameters (percentage of motile spermatozoa and fertilization capacity). Complementary biochemical tests (ATP, O_2 consumption) or morphological ones (electron microscopy) precisely localize damages due to the cryopreservation process.

-Motility assessment: Billard & Cosson (1992) reviewed and improved early techniques used to describe fish sperm motility: because of a high sperm concentration in milt, the assessment of sperm motility requires a high dilution rate. A two-step procedure is necessary to initiate simultaneous motility of the maximum number of spermatozoa : sperm is first diluted in a

medium that does not initiate motility. Then, sperm movement is activated, by mixing spermatozoa directly under the microscope with an activating solution.

Cosson, Billard, Cibert, Dreanno, Linhart & Suquet (1997) developped the techniques allowing a fine description of motility. This observation is greatly enhanced using dark field optics microscopy attached to a camcorder. Spermatozoa can be visualised on a video monitor. Using stroboscopic illumination, the flagellar beat frequency can be measured by reference to the calibrated frequency of the flash illuminator. The percentage of motile cells is estimated using an arbitrary scale, each step representing a wide range of spermatozoa showing progressive movement. CASA (computer-assisted sperm analysis) system allows an analysis of videotapes using software which set up parameters are adjusted for the studied species sperm characteristics. Straight or curvilinear velocities are rarely assessed using CASA system (turbot, Dreanno, Suquet, Quemener, Cosson, Fierville, Normant & Billard 1997; sea bass, Fauvel, Suquet, Dreanno, Zonno & Menu 1998a). Among the criteria used to estimate sperm motility, the percentage of motile cells is significantly correlated with the fertilization capacity of sea bass and turbot spermatozoa. Because no significant correlation was reported between fertilization rate and the velocity of spermatozoa in these species, the success of fertilization was assumed to depend on the number of motile cells rather than on the quality of sperm movement (Dreanno 1998). However in cod (*Gadus morhua* L.), the proportion of spermatozoa exhibiting a progressive movement accounted for only a small percentage of the fertilization success (Trippel & Neilson 1992), and spermatozoa showing non progressive vibrating motion could fertilize ova of this species. As a consequence, complementary tests must be used to assess the quality of frozen-thawed spermatozoa.

-Assessment of energetic status: the determination of intracellular ATP concentration has proven to be informative in freshwater fish species, because a drastic decrease of nucleotide content has been reported in frozen-thawed spermatozoa compared to fresh sperm (Ogier de

Baulny, Le Vern, Kerboeuf, Heydorff & Maisse 1996). Furthermore, a significant positive correlation was reported between the ATP content of turbot spermatzoa and the percentage of motile cells. In sea bass, Adenylate Energy Charge (AEC) is correlated with the percentage of motile cells and the fertilization capacity (Dreanno 1998). The measurement of intracellular ATP content at various times *post-activation* and the assessment of oxygen consumption allow to assess the degree of cell integrity before and after the cryopreservation (Dreanno *et al*. 1997).

-Seminal fluid composition: in rainbow trout (*Oncorhynchus mykiss*) seminal plasma pH and osmolality have been selected to determine the semen suitability for cryopreservation (Malejac, Loir & Maisse 1990; Lahnsteiner, Berger, Weismann & Patzner 1996a). A high content of 42kd protein in the seminal fluid was also correlated with a low fertilization capacity of frozen-thawed rainbow trout spermatozoa (Maisse, Pinson & Loir 1988). The oxaloacetic transaminase activity of the seminal fluid of thawed rainbow trout spermatozoa was also considered as a quality test (Malejac *et al*. 1990). The leakage of these different components into the seminal fluid reflects the alteration of the sperm membrane during the cryopreservation.

-Swelling tests: This technique derives from rainbow trout. Sperm samples were incubated in hypo-osmotic solutions and their resistance to the osmotic shock correlated with the cryopreservation ability (Malejac *et al*. 1990). However, this test is difficult to use in turbot, because no lysis of spermatozoa was observed after dilution in freshwater (Dreanno 1998).

-Fertilization capacity: because of the difficulty to collect good quality ova, the fertilization capacity of frozen-thawed spermatozoa has only been tested in a few studies. However, it represents a major step forward in the management of gametes. A standardized experimental insemination procedure is necessary for reproducable evaluation of the fertilization capacity (Suquet, Gaignon, Quemener & Normant 1995a). The problem of replicate variation is illustrated by the sampling technique of ova. When a standard pipette was used, more ovarian fluid than ova was sucked in, resulting in a large methodological error (coefficient of variation :

66.5% in nine successive 800µl samples) in the number of sampled ova. This variation was largely reduced using a positive displacement pipette (5.1%). Weighing ova samples may also be useful. However, correlations between the number of ova and their weight must be calculated for each species and variations could also be due to individual fish conditions or seasonal changes.

The fertilization capacity of frozen-thawed spermatozoa has to be tested using discriminating conditions. In rainbow trout, a significant correlation was reported between fertilization rate and motility estimates using a low number of spermatozoa per ovum (<200,000), whereas a supraoptimal ratio did not allow to detect this correlation (Moccia & Munkittrick 1987). Therefore, knowledge of the optimal sperm to ovum ratio is necessary to improve the cryopreservation technique. Optimal sperm to ovum ratios are only reported for few marine fish species in the literature (Table 1).

Table 1. Optimal sperm to ovum ratio in marine fish species.

Species	Optimal sperm : ovum ratio	number of ova /ml diluent	Reference
Pacific herring	24 : 1	unknown	Hourston &Rosenthal 1976
Atlantic croaker	1,000 : 1	5,000	Gwo, Strawn, Longnecker & Arnold 1991
turbot	6,000 : 1	2,000	Suquet, Billard, Cosson, Normant & Fauvel 1995b
Atlantic halibut	10,000 : 1	unknown	Rana unpublished result
sea bass	66,000 : 1	2,000	Fauvel *et al.* 1999
wolffish	200,000 : 1	unknown	Mokness & Pavlov 1996

Development of the cryopreservation protocol

To establish a cryopreservation protocol, the extender, the cryoprotectant as well as the cooling and thawing conditions must be investigated. This is difficult as all parameters may interact with each other.

Extender

Pure semen is usually not suitable for freezing. The composition of the most successfull extenders used in marine fish species is reported in Table 2. Except for the ocean pout (*Macrozoarces americanus* L.), which is an internally fertilizing marine teleost and for which a diluent mimmicking seminal plasma is used (Yao, Crim, Richardson & Emerson 1995), most diluents used in marine fish are saline (concentration 1-10%) or sugar (5-10%) solutions. As motility depends on internally stored ATP which can be resynthesized only at very low rates, the extender must inhibit sperm motility before freezing. However in turbot, addition of cryoprotectants such as DMSO increased osmolality up to 1100 mOsmol/kg and induced the movement of spermatozoa for a period of less than 1 min. This activation before freezing did

not affect sperm motion capacity, probably because turbot spermatozoa are able to resynthetise energy during the period of sperm motion (Dreanno *et al*. 1997). The Mounib extender (Mounib 1978) is suitable for the cod, sea bass and turbot spermatozoa. The presence of reduced gluthatione prevents deleterious effects of free radicals (Maracine & Segner 1998) and avoids peroxidation of lipids, protecting the sperm membranes (Ogier de Baulny 1997). The Mounib diluent was also successfully used for spermatozoa of freshwater fish (Mounib, 1978; Legendre & Billard 1980). But, Mounib's medium was not appropriate for freezing Atlantic croaker (*Micropogonias undulatus* L.) spermatozoa (Gwo, Strawn, Longnecker & Arnold 1991).

Dilution ratios of sperm in extender range from 1 : 1 to 1 : 20 (volume of semen : volume of diluent). Lower survival of frozen-thawed spermatozoa was recorded for dilution ratios larger than 1 : 20 in Atlantic croaker (Gwo *et al*. 1991) and larger than 1 : 50 in seabream (Chambeyron & Zohar 1990). The motility duration of black grouper spermatozoa decreased from 40 to 2 minutes when increasing the semen dilution ratio from 1 : 10 to 1 : 100 (Gwo 1993). Increasing the dilution rate from 1 : 1 up to 1 : 9 did not modify the percentage of motile frozen-thawed turbot spermatozoa (Dreanno *et al*. 1997). It is suggested that seminal plasma proteins protect sperm viability and higher dilution ratios than 1 : 10 may reduce this effect. This was observed in freshwater fish species (Billard 1983) and in turbot (Chauvaud, Cosson, Suquet & Billard 1995).

Table 2. Composition of the extenders successfully used for freezing of spermatozoa of marine fish species.

Species	Extender composition	Reference
Atlantic croaker	NaCl, Glucose or sucrose	Gwo *et al*. 1991
Atlantic halibut	NaCl-Glycine-$NaHCO_3$	Bolla, Holmefjord & Refstie 1987
barramundi	Ringer solution for freshwater fish	Leung 1987
black grouper	NaCl	Gwo 1993
cod	sucrose, reduced glutathione, $KHCO_3$	Mounib 1978
grey mullet	Ringer solution for marine fish	Chao, Chen & Liao 1975
grouper	NaCl, $NaHCO_3$, fructose, lecithin, mannitol	Withler & Lim 1982
hirame	Ringer for freshwater fish	Tabata & Mizuta 1997
mullet	Ringer for marine fish	Joseph & Rao 1993
ocean pout	Medium mimmicking seminal fluid	Yao, Crim, Richardson & Emerson 1995
Pacific herring	Ringer for marine fish	Pillai, Yanagimachi & Cherr 1994
plaice	NaCl	Pullin 1972
puffer	Glucose	Gwo, Kurokura & Hirano 1993
sea bass	sucrose, reduced glutathione, $KHCO_3$	Fauvel *et al.* 1998a
sea bream	NaCl	Barbato, Canese, Moretti & Misiti 1996
turbot	sucrose, reduced glutathione, $KHCO_3$	Dreanno *et al.* 1997

Cryoprotectant:

The multiple roles of cryoprotectants during the cooling process were reviewed by Jamieson (1991). Cryoprotectants are classified in permeating and non-permeating, according to their ability to pass through the cell membrane. Permeating cryoprotectants such as ethylene,

propylene glycol, glycerol, DMSO and methanol were tested for cryopreservation of spermatozoa of marine fish. DMSO generally gave the best results (Table 3) and its success can be explained by the fast penetration into spermatozoa and by its interaction with the phospholipids of the sperm membrane (Ogier de Baulny *et al*. 1996). Flow cytometric analysis revealed a high percentage of turbot spermatozoa presenting no cryo-injuries of the plasma membrane and mitochondria in the presence of DMSO (Ogier de Baulny 1997). However, DMSO is toxic at high concentrations: the motility duration of frozen-thawed barramundi (*Lates calcarifer*, Bloch) spermatozoa was reduced when the DMSO concentration was higher than 5% (Leung 1987) and also in the black grouper, sperm motility was decreased at a concentration of 30% (Gwo 1993).

Table 3. Optimal DMSO concentrations for cryopreservation of sperm of marine fish species.

Species	Concentration (%)	Reference
Atlantic croaker	15	Gwo *et al.* 1991
barramundi	5	Leung 1987
black grouper	20	Gwo 1993
grouper	10	Withler & Lim 1982
ocean pout	20	Yao *et al.* 1995
Pacific herring	15	Pillai *et al*. 1994
sea bream	10	Maisse *et al*. 1998
turbot	10	Dreanno *et al.* 1997
yellowtail flounder	10	Richardson, Crim, Yao & Short 1995

Methanol has low (barramundi : Leung 1987; turbot : Dreanno *et al.* 1997) or no (Atlantic croaker : Gwo *et al*. 1991; black grouper : Gwo 1993) cryoprotective efficiency in frozen spermatozoa of marine fish. Ethylene glycerol and propylene glycerol gave intermediate results

in Atlantic croaker (Gwo *et al.* 1991) and yellowfin seabream (Gwo 1994). In striped trumpeter (*Latris lineata* Schneider) and in yellowfin bream (*Acanthopagrus australis* Guenther), the *post-thaw* motility of spermatozoa was higher with glycerol than with DMSO (Thorogood & Blackshaw 1992; Ritar & Campet 1995). Glycerol gave good protection in yellowfin seabream (Gwo 1994) but it provided no protection in black grouper (Gwo 1993) and low protection in the turbot (Dreanno *et al*. 1997). Therefore and because of the species specific cryopreservation requirements, the suitability of each potential cryoprotectant must be evaluated when developping a method for new fish species.

In freshwater fish, non-permeating cryoprotectants such as proteins (BSA) or lipoprotein (egg yolk) have been commonly used to prevent damages to the plasma membrane (Scott & Baynes, 1980). Cabrita, Alvarez, Anel, Rana & Herraez (1998) suggested that they increase the membrane resistance to osmotic stress and the motility rate of frozen-thawed rainbow trout spermatozoa. In marine fish, in the Pacific herring, addition of 10% hen's egg yolk did not improve the survival of frozen-thawed spermatozoa (Pillai, Yanagimachi & Cherr 1994). No significant difference was observed in the motility of frozen-thawed turbot spermatozoa when egg yolk (10%), BSA (10%) or a combination of both (5% + 5%) was used (Dreanno *et al*. 1997). Furthermore, increasing the egg yolk concentration from 10 to 20% or substituting egg yolk with milk, did not change the survival of frozen-thawed spermatozoa in this species (Chereguini, Cal, Dreanno, Ogier de Baulny, Suquet & Maisse 1997). Permeating and non-permeating cryoprotectants can interact : in barramundi sperm, the protective ability of skim milk gave better cryoprotection than egg yolk when glycerol or methanol was used instead of DMSO (Leung 1987).

Because spermatozoa are small, the penetration of cryoprotectants is rapid (Jamieson 1991), and no equilibration period is required. Therefore the toxic effect of DMSO can be minimized. Increasing the equilibration period from 5 to 60 minutes and the DMSO concentration from 10

to 30% lowered the *post-thaw* motility of yellowfin seabream spermatozoa (Gwo 1994). In seabream, the fertilizing ability of frozen-thawed sperm decreased in DMSO extender when the equilibration period exceeded two minutes (Billard 1978). A similar effect was observed after one hour in grey mullet (Chao, Chen & Liao 1975). An equilibration time of 10 to 60 minutes had no effect on the fertility of frozen-thawed spermatozoa of hirame (Tabata & Mizuta 1997). In rainbow trout, Ogier de Baulny (1997) observed that DMSO needed 10 minutes to penetrate into spermatozoa althought the fertilization capacity of frozen-thawed spermatozoa was the same with or without equilibration period. This observation suggests that the protective role of DMSO does not depend on its penetration into spermatozoa. With glycerol as cryoprotectant, the motility rate of frozen-thawed spermatozoa of bluefin tuna (*Thunnus thynnus* L.) was increased at long equilibration periodes (30 minutes) compared to short ones (10 minutes), but the opposite was recorded for DMSO (Doi, Hoshino, Taki & Ogasawara 1982). Since penetration of glycerol is slow, an equilibration time may be necessary.

Cooling rate

For freezing of fish semen, generally a two step procedure is applied : milt is cooled in nitrogen vapour on a floating tray or in the neck of a container and then the straws are plunged into liquid nitrogen. The cooling rate is determined by the height of the tray or the depth at which canisters are placed. The cooling rates most frequently used in marine fish are shown in Table 4. In Atlantic halibut (Bolla, Holmefjord & Refstie 1987), freezing has also been carried out by pelleting diluted semen onto dry ice. This approximately corresponds to a cooling rate of 35°C.min^{-1} (Stoss & Donaldson 1982).

Motility of thawed barramundi spermatozoa was not affected by a freezing rate between 1 and 30°C.minute^{-1} (Lo unpublished result), suggesting high freezing tolerance. On the other hand, a lower freezing tolerance was recorded in seabream as it was optimal only at 10

°C.minute^{-1} compared to 1, 5, 20, 50 and 100 °C.minute^{-1} (Billard 1978). In Atlantic cod, a freezing rate of 5 °C minute^{-1} was successfully applied, whereas 1 °C.minute^{-1} resulted in low *post-thaw* motility (Mounib, Hwang & Idler 1968). Freezing conditions for turbot were optimal at a cooling rate of 99 °C.minute^{-1}, but at 46 °C.minute^{-1} and 148 °C.minute^{-1} the *post-thaw* motility rate assessed at 10 seconds *post-activation* decreased for 8 and 30 % respectively (Dreanno *et al*. 1997). As a consequence, the optimal cooling rate is highly dependent on the fish species.

Table 4. Freezing rates used for cryopreservation of spermatozoa of marine fish species.

Species	Freezing rate (°C.min^{-1})	Reference
barramundi	31	Leung 1987
cod	5	Mounib, Hwang & Idler 1968
hirame	8	Tabata & Mizuta 1997
sea bass	10	Villani & Catena 1991
sea bass	65	Fauvel *et al*. 1998a
sea bream	10	Barbato *et al*. 1996
turbot	99	Dreanno *et al*. 1997

Thawing rate

Rapid thawing is necessary to avoid recrystallisation. Thawing rates used in marine fish are shown in Table 5 and are lower than those reported for freshwater fish (30-80°C : Rana 1995a). In striped trumpeter, there was no difference in the percentage of motile frozen-thawed spermatozoa using thawing temperatures between 10 and 30°C (Ritar & Campet 1995). In turbot thawing temperature between 20 and 40°C did not affect *post-thaw* sperm motility (Dreanno *et al*. 1997). But, an increase in the thawing temperature from 1 to 30°C reduced the *post-thaw* motility of ocean pout spermatozoa (Yao *et al*. 1995). When Atlantic croaker

spermatozoa were thawed at 0°C, the fertilization rate was significantly lower than at 25 and 50°C (Gwo *et al*. 1991). In the Atlantic halibut, the optimal thawing temperature was 10°C for straws and 40°C for pellets (Bolla *et al*. 1987).

Thawed spermatozoa must be rapidly used: 60 minutes after thawing, the percentage of motile turbot spermatozoa stored on crushed ice decreased for 35% (Dreanno *et al*. 1997). This indicates that cryopreservation induced damages in spermatozoa. Diluting frozen-thawed spermatozoa of this species in a medium mimicking the seminal fluid improved their short term storage capacity. Also, in halibut, short term storage ability of thawed sperm was lower than for fresh sperm (Billard, Cosson & Crim 1993).

Table 5. Thawing rates used in marine fish species

Species	Thawing rate (°C.min^{-1})	Reference
Atlantic halibut	10-40	Bolla *et al*. 1987
barramundi	30	Leung 1987
bluefin tuna	40	Doi *et al*. 1982
cod	38	Mounib 1978
grouper	25	Withler & Lim 1982
hirame	20	Tabata & Mizuta 1997
ocean pout	1	Yao *et al*. 1995
puffer	25	Gwo *et al*. 1993
sea bass	35	Fauvel *et al*. 1998a
sea bream	26	Barbato *et al*. 1996
turbot	30	Dreanno *et al*. 1997
yellowfin bream	20	Thorogood & Blackshaw 1992
yellowtail flounder	30	Richardson *et al*. 1995

The quality of frozen-thawed spermatozoa.

Generally, the motility rate of frozen-thawed spermatozoa is very high in marine fish species (Table 6) when compared to freshwater species: 21% in rainbow trout (Lahnsteiner, Berger, Weismann & Patzner 1996b), 25% in carp (Babiak, Glogowsky, Brzuska, Szumiec & Adamek 1995), and from 40 to 85% in tilapias (Chao, Chao, Liu & Liao 1987). In marine species, the high motility rates of frozen-thawed sperm result also in high fertilization rates as recorded in sea bass (fertilization expressed as a percentage of fresh semen control : 65%; Fauvel *et al*. 1998a) and turbot (83%; Dreanno *et al*. 1997), using limiting quantities of sperm. The decrease in the fertilization capacity of frozen-thawed semen probably reflects the changes in motility rates observed after freezing and thawing. Other parameters describing the movement of thawed

spermatozoa are rarely assessed. In sea bass, the flagellar beat frequency of swimming spermatozoa was not affected by the cryopreservation process, but straight line and curvilinear swimming velocities were significantly decreased (Fauvel *et al*. 1998a). The straight line velocity of frozen-thawed turbot spermatozoa did not significantly differ from that of fresh sperm (Dreanno *et al*. 1997).

Table 6. Mean motility recovery of frozen-thawed spermatozoa of marine fish species.

Species	Motility recovery (%)[1]	Reference
barramundi	100	Leung 1987
black grouper	100	Gwo 1993
bluefin tuna	100	Doi *et al*. 1982
cod	39	Mounib 1978
halibut	65	Billard *et al*. 1993
ocean pout	50	Yao *et al*. 1995
sea bream	85	Maisse *et al*. 1998
striped trumpeter	49	Ritar & Campet 1995
turbot	70	Dreanno *et al*. 1997

[1] Motility recovery : motility rate of frozen-thawed semen expressed as percentage of fresh semen motility rate.

Cryo-injuries have been reported for thawed spermatozoa of many freshwater fish species (Rana 1995a). In marine fish, the fine structure of the head region of 90% of the thawed black grouper spermatozoa was similar to untreated ones (Gwo 1993). Also in the puffer, 80% of the frozen-thawed spermatozoa had similar ultrastructure as untreated ones (Gwo, Kurokura & Hirano 1993). Shrinkage of the plasma membrane of the midpiece was reported for frozen-thawed spermatozoa of the ocean pout (Yao *et al*. 1995). In frozen-thawed Atlantic croaker spermatozoa, the cristae of mitochondria were disrupted, plasma membrane was swollen or disrupted and the axoneme coiled (Gwo & Arnold 1992). Flow cytometric analysis of frozen-

thawed turbot spermatozoa revealed a high percentage (up to 93%) of intact plasma membranes and mitochondria (close to 80%; Ogier de Baulny 1997) and the mitochondrial respiratory activity was not altered (Dreanno *et al*. 1997).

The development of fish produced with frozen-thawed sperm was not frequently investigated. Fertilizing ova with cryopreserved sperm did not affect the hatching percentages in cod. Furthermore, the morphology of larvae produced with frozen-thawed spermatozoa was similar to those produced with fresh sperm (Mounib 1978). Also, the rate of malformed hirame larvae was not significantly different when using fresh or frozen-thawed spermatozoa (Tabata & Mizuta 1997). In the yellowtail flounder, the hatching rate and the percentage of malformed larvae were not affected by the cryopreservation process (Richardson, Crim, Yao & Short 1995) and also 29 days old barramundi larvae, produced with frozen-thawed spermatozoa showed no malformalities (Palmer, Blackshaw & Garrett 1993). The hatching rate, the larval survival rate and the larval weight of 10 days old turbot were not significantly different when ova were inseminated with fresh compared to frozen-thawed sperm (Suquet, Dreanno, Petton, Normant, Omnes & Billard 1998b). But, the hatching rates of eggs inseminated with frozen-thawed sea bass sperm (69%) were significantly lower than those obtained with fresh sperm (81%) (Fauvel, Zonno, Suquet, Storelli & Dreanno 1998b).

Compared to fresh water fish, the high quality of cryopreserved spermatozoa of marine fish species has already been emphasized by Scott & Baynes (1980). Ogier de Baulny (1997) found a significant correlation between the percentage of motile frozen-thawed turbot spermatozoa and the membrane integrity as determined by flow cytometric analysis. Furthermore, this author recorded that up to 93% of frozen-thawed spermatozoa had intact plasma membranes in the turbot, 45% in the trout, 80% in tilapia and 90% in catfish. Drokin (1993) proposed that the cryoresistance of marine fish spermatozoa could be due to the lipid composition of sperm membranes, mainly to the molar ratio of cholesterol to phospholipids which is 2-3 times higher

than in freshwater fish. Contrary in rainbow trout spermatozoa, a lower cholesterol content of the plasma membrane was correlated with a higher fertilizing capacity of frozen-thawed sperm (Labbé & Maisse 1996), and the ratio of cholesterol to phospholipids was not higher in turbot than in trout (Ogier de Baulny *et al*. 1996). According to Drokin (1993), phosphatidylcholine may increase the cryoresistance of the sperm membrane and in sperm of marine fish this component has higher levels than in freshwater fish. Its protective role against osmotic and cold stress has been also reported by Simpson, Swan & White (1986).

The process of cryopreservation reduces the decrease of intracellular ATP content of turbot spermatozoa for 20-40% (Ogier de Baulny 1997; Dreanno *et al*. 1997). This decline is lower than in trout (50-90%) and similar to catfish (25%; Ogier de Baulny 1997). This low rate of ATP consumption could explain the higher motility percentages observed in frozen-thawed spermatozoa of turbot (70%) and catfish (60%) compared to trout (21%). Furthermore, the significant decrease of cryoresistance of sea bass spermatozoa recorded at the end of the milting season could be explained by the decrease in endogeneous ATP (Dreanno *et al*. in press). Contrary, ATP content decreased only slightly in tilapia, *Oreochromis niloticus* (L.) spermatozoa during cryopreservation (0-25%). However, only 22% of the frozen-thawed spermatozoa of this species could be activated (Ogier de Baulny 1997). Before movement, ATP content of frozen-thawed trout spermatozoa is about 1.85×10^{-2} mmole/10^9 spermatozoa. When calculating the internal cell volume as 0.16 µl/10^7 spermatozoa (Christen, Gatti & Billard 1987), this leads to a 1.2 mmole/l concentration. According to Saudrais, Fierville, Cibert, Loir, Le Rumeur & Cosson (1998), the demembranated trout spermatozoa move also at much lower concentration of ATP (0.2 mmole/l). Therefore, the lower losses of intracellular ATP levels can not explain the higher survival of frozen-thawed turbot spermatozoa compared to trout spermatozoa. Complementary studies on the cryoprotectant toxicity and on thermal and osmotic

stress should be carried out in marine fish species to understand their high capacity to tolerate the cryopreservation.

Conclusions

Simple freezing protocols are available for the cryopreservation of marine fish semen. The composition of the extender is generally less complex than the seminal fluid and consists of DMSO containing saline or sugar solutions. Simple methodology as straws on a tray in the vapour of liquid nitrogen and thawing in a bath are used for freezing and thawing.

To date, the semen of about 30 different marine fish species has been cryopreserved and compared to freshwater species, a high survival of frozen-thawed spermatozoa is often recorded. In the literature, the species specific differences in cryoresistance are related to species specific differences in ATP consumption and to various grades of damages of frozen-thawed spermatozoa especially of their mitochondria and plasma membranes. However, studies on the cryopreservation of spermatozoa of marine fish species were mostly concentrated on the improvement of freezing technique protocols, but lacked a complete description of morphological and metabolic changes. Also, the quality of sperm sample before freezing should be investigated in more details as the problems of semen aging and of urine contamination can alter the biological features of spermatozoa and their suitability for freezing. The possible improvement of sperm fitness for cryopreservation by modifying rearing parameters during spermatogenesis (e.g. water temperature and food composition) has not yet been studied in marine fish species. The effect of the process of domestication on the quality of sperm and its ability to be frozen has also not been described.

Therefore, the cryopreservation techniques for semen of marine fish are applicable for production purposes in aquaculture and for establishement of sperm banks. Coupled with

insemination and short term storage techniques, cryopreservation will lead to an improvement of gamete management in marine fish species.

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