

Note

Short-term storage and cryopreservation of turbot (*Scophthalmus maximus*) sperm

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Abstract

Short-term storage over several days as well as cryopreservation of turbot (*Scophthalmus maximus*) sperm were studied. Two extenders, Ringer 200 and artificial seminal liquid (ASL2), are suggested for semen collection in order to avoid the deleterious effect of urine contamination, and for the purpose of short-term storage between 0 and 15°C. Oxygen atmosphere is not suitable for turbot sperm storage. Turbot spermatozoa undergo cryopreservation with a high rate of success especially in a sucrose solution with 10% dimethyl sulfoxide (DMSO) and 10% egg yolk.

Keywords: Turbot, sperm, storage, cryopreservation.

INTRODUCTION

Development of fish farming increases the need for selective breeding. In this framework, control of artificial reproduction in fish requires gametes of high quality and often their storage for several hours, days or years; moreover, cryopreservation of semen is the chief tool required for genebanking and many authors agree that the marine fish semen is more resistant to freezing-and-thawing than that of freshwater fish. This assertion

is subject to restrictions, given that few experiments have previously been carried out on marine species (Maisse, 1996). There have been few studies on turbot (*Scophthalmus maximus*) sperm; nevertheless, its general characteristics have been described in previous works. Sperm concentration varies between 2.00×10^9 and 5.46×10^9 spermatozoa.ml⁻¹. (Suquet *et al.*, 1992). The composition of the seminal fluid was analysed by Suquet *et al.* (1993); mean values of osmolality, pH and total protein content were found to be respectively

306 mOsm.l⁻¹, 7.31 and 8.8 mg. ml⁻¹. Concentration of adenosine-triphosphate (ATP) in turbot sperm was measured by Geffen and Frayer (1993), mean value was 9.2 nmol ATP/10⁸ spermatozoa. The percentage of motile spermatozoa is a function of the osmotic pressure of the activating medium, the maximum being observed in a range of 300-1 100 mOsm.l⁻¹ (Chauvaud *et al.*, 1995). Compared to most freshwater species, the duration of turbot spermatozoa motility is long (1-17 min) (Suquet *et al.*, 1994).

In the present work we have compared the effect of different diluents on turbot spermatozoa survival after short-term storage (several days) under different conditions and after freezing. The optimal short-term storage and freezing conditions have been validated by artificial fertilization studies.

MATERIAL AND METHODS

Collection of milt

Two-year-old turbot males bred at the experimental fish farm of the Spanish Institute of Oceanography in Vigo (Spain) were maintained in natural sea water in large tanks and subjected to a natural photoperiod. The urinary bladder was cleared out by gentle abdominal pressure and the genital papilla was dried with absorbant paper, then the milt of each individual male ($n = 6$ for each experiment) was carefully collected into a 1 ml syringe.

Short-term storage

In the first experiment, the survival rate of turbot spermatozoa after storage under O₂, N₂ or air atmosphere were compared. The experiment was carried out with 6 individual sperms (500 µl) kept horizontally in plastic tubes at 6°C up to 28 hours.

The goal of the second experiment was to test the survival of turbot spermatozoa at 6°C after dilution in different media, whose compositions are given in Table 1. After our data concerning seminal fluid pH, which was 7.9 versus 7.31 by Suquet *et al.* (1993), the pH of the different media was stabilized near 8. In this experiment, the samples of each individual sperm were mixed with each medium (1 vol. sperm/9 vol. medium), just after collection and stored in an air atmosphere.

In the third experiment, the influence of the storage temperature was studied at 0, 6, 15 and 20°C with the optimal extenders selected from the second experiment.

Cryopreservation

Two different media were used to freeze sperm: the Mounib (1978) medium, designed especially for atlantic salmon (*Salmo salar*) sperm and successfully tested

with cod (*Gadus morhua*) sperm, using dimethyl sulfoxide (DMSO) as a cryoprotectant, and the Ringer medium modified by Rana and McAndrew (1989) for tilapia (*Oreochromis sp.*) sperm, and 2/3 diluted in distilled water (Ringer 200). In a preliminary study on turbot (*Scophthalmus maximus*) sperm, Ogier de Baulny *et al.* (1996) have tested different cryoprotectants with the Mounib (1978) extender. They showed by flow cytometry that the optimal cryoprotection of plasma membrane was obtained with DMSO, dimethylacetamide or glycerol at a concentration of 10%. Finally, after a number of preliminary trials, DMSO (10% of final volume) was chosen as a cryoprotectant and egg yolk or skimmed milk were compared (10% and 20% of final volume) as a membrane stabilizer.

Each sperm (1 volume) was mixed with each freezing extender (3 volumes) and a micropipette was used to fill 500 µl French straws (IMV, L'Aigle, France). Straws were kept in the vapour phase of a liquid nitrogen bath - just 3 cm above the liquid for 20 min - and then plunged directly in liquid nitrogen where they were stored for a few hours. The straws were thawed by immersion in a 37°C water bath for 5 seconds.

Checking of spermatozoa motility

Spermatozoa motility in sea water containing 5 mg.ml⁻¹ bovine serum albumin (Chauvaud *et al.*, 1995) was observed by means of a video camera and scored independently by 5 persons on a scale from 0 to 5, 0 being « no activation » and 5 being « total activation ». The motility score for each sample was the mean of these 5 scores.

Validation

These trials were conducted with turbot broodstock from the IFREMER experimental fish farm in Brest (France). The artificial insemination conditions were described by Suquet *et al.* (1995) and remained the same for fresh, stored or frozen sperm. Each sperm fertilization test was carried out in triplicate using 6 000 spermatozoa per egg. In the framework of a short-term storage, fertilization trials were performed the day of collection and after 3 to 6 days of storage.

Analysis of data

The data are expressed as mean egg quality among females, the fertilization rates of eggs were expressed as a percentage of the control sample (fertilization rate of the same batch of eggs with a pool of 3 fresh sperms the motility of which had been previously checked). In all experiments, the data have been systematically analysed by paired sample Wilcoxon T-test ($p = 0.05$) (Schwartz, 1969).

RESULTS AND DISCUSSION

Short-term storage

Figure 1 shows a rapid decrease in the motility score of turbot sperm whatever the gas used. After 20 hours, the survival rate was significantly higher with air than with oxygen or nitrogen. Contrary to rainbow trout (*Oncorhynchus mykiss*) sperm (Billard, 1981), the spermatozoa of turbot cannot be stored under an oxygen atmosphere. In a review on fish gamete preservation, Stoss (1983) reported the case of the sperm of several species in which anaerobic storage conditions appear favourable and concludes that a large liquid-to-gas interface may not be necessary for all species'sperm. Based on our observations turbot sperm cannot be stored under anaerobic conditions and, consequently, the influence of O₂ concentration in storage gas (21% in air) could be high.

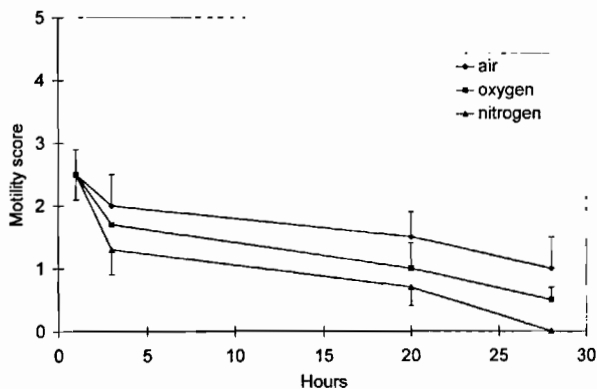


Figure 1. – Mean motility score of 6 individual turbot sperm stored at 6°C in air, oxygen or nitrogen atmosphere in relation with time after collection.

In Figure 2, control data confirm the rapid decrease of motility score, after a few hours of storage, already observed in Figure 1; Ringer 200 and ASL2 extenders significantly improved the sperm motility score after 4 hours of storage. As under our experimental conditions turbot semen is usually collected by manual stripping and syringe aspiration (Chauvaud *et al.*, 1995), but urine contamination is not completely suppressed. In the case of most species, the collection of semen void of urine is very difficult when stripping is carried out and this contamination often causes sperm quality and storage duration to decrease. Rana (1995) suggested the collection of salmon (*Salmo salar*) milt by catheterizing the gonad and Linhart *et al.* (1987) ameliorated fertilization rate in european catfish (*Silurus glanis*) by collecting the sperm in an immobilizing solution. On the basis of our results, Ringer 200 and ASL2 can be suggested as solutions for turbot sperm

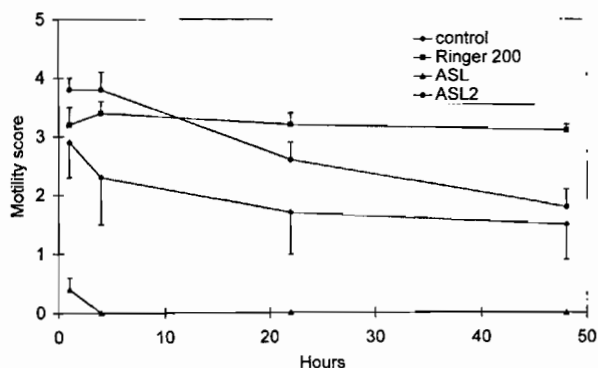


Figure 2. – Mean motility score of 6 individual turbot sperm diluted in different media and stored at 6°C until 48 hours after collection. Control is undiluted sperm.

collection. Moreover, the survival of spermatozoa was maintained in Ringer 200 for at least 45 hours. The toxic effect of ASL was noted after as little as 1 hour of storage. The comparison between ASL and ASL2 compositions (Table 1) tends to suggest the deleterious effect of Tris-buffer.

Table 1. – Composition of the different media used for short-term preservation trials; Ringer 200 is modified fish Ringer's solution, Artificial seminal liquid (ASL) is from Dreanno and Billard (unpublished data) and ASL2 is modified ASL.

Tris: Tris(hydroxymethyl)aminomethane; BSA: bovine serum albumin.

	Ringer 200	ASL	ASL2
NaCl (mM)	74.0	85.0	70.0
KCl (mM)	27.0	1.5	1.5
NaHCO ₃ (mM)	1.6		25.0
CaCl ₂ (mM)	1.8	2.7	2.7
MgCl ₂ (mM)		6.1	6.1
Glucose (mM)		0.4	0.4
Tris (mM)		20	
BSA (mg.ml ⁻¹)		10.0	10.0
pH	8.1	8.2	7.9
Osmolality (mOsm.l ⁻¹)	204	200	205

The influence of storage temperature is shown in Figure 3. The initial motility score of undiluted sperm is significantly improved after 6 hours of storage in Ringer 200 or in ASL2, whatever the temperature. After 18 hours of storage, the survival rate is not significantly different at 0, 6 and 15°C. At 20°C the motility score was significantly higher when sperm was stored in Ringer 200 than in the case of ASL2, but for other temperatures there is no significant difference between the two media.

The results of fertilization trials with short-term stored sperm are given in Figure 4. They confirm that it is possible to store turbot sperm in Ringer 200 or ASL2 extenders at 6°C while maintaining a good fer-

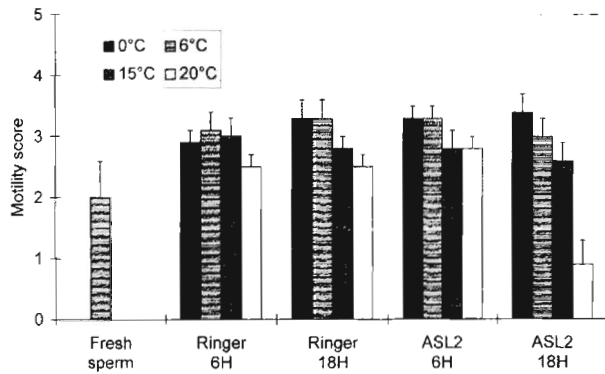


Figure 3. – Mean motility score of 6 individual turbot sperm stored in Ringer 200 or ASL2 (artificial seminal liquid) at different temperatures until 18 hours after collection. Initial motility score is checked just after collection before addition of storage medium.

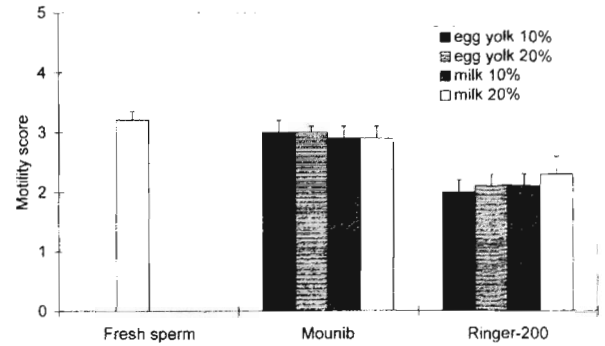


Figure 5. – Mean motility score of 6 individual turbot sperm frozen in Mounib medium or Ringer 200 with 10% DMSO and 10% or 20% of egg yolk or skimmed milk.

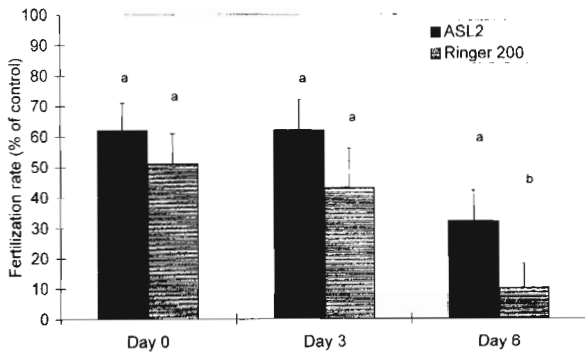


Figure 4. – Mean fertilization rate of 6 individual turbot sperm stored at 6°C in Ringer 200 or ASL2 (artificial seminal liquid) until 6 days after collection (bars not marked by the same letter are significantly different, $p < 0.05$).

tilizing ability after 3 days storage. Data show a decline in sperm quality after 6 days. ASL2 is significantly better than Ringer 200 after 6 days' storage.

Cryopreservation

After thawing, the motility score of frozen turbot sperm was generally good and significantly better with the Mounib medium than with Ringer (Fig. 5). The present results show that Mounib (1978) extender modified by Legendre and Billard (1980) for trout sperm (the extender includes 10% egg yolk) is suitable for turbot sperm. The increase of egg yolk concentration or the substitution of egg yolk by milk has no effect. The significant superiority of Mounib medium, by comparison with Ringer 200, could be explained by the protective effect of sucrose (Crowe *et al.*, 1988).

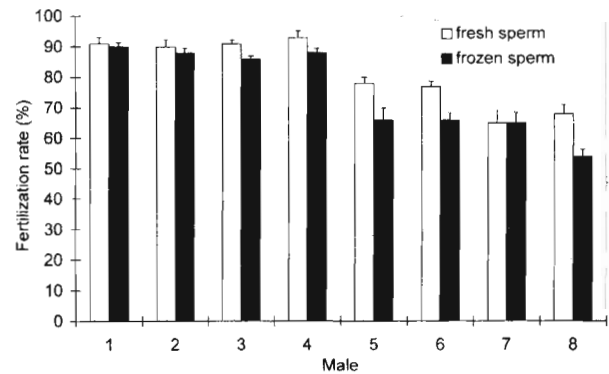


Figure 6. – Fertilization rate of fresh or frozen turbot sperm for 8 males

After freezing in the Mounib extender with 10% DMSO and 10% egg yolk, the fertilizing potential of frozen sperm was slightly, but significantly, below that of fresh sperm (Fig. 6).

CONCLUSION

The present results show that Ringer 200 and ASL2 are suitable extenders for collection and short-term storage of turbot sperm. For a few hours' storage, the temperature (between 0°C and 20°C) has no influence, but for a longer time it is better to store extended semen in a fridge, under air atmosphere. The improvement of this method for several days' storage has to take prevention of bacterial growth into account, by means of the addition of antibiotics to medium (Stoss et Holtz, 1983).

The good aptitude for cryopreservation of turbot sperm is demonstrated and a freezing extender based on Mounib medium, 10% DMSO and 10% egg yolk

can already be considered as providing satisfactory results both in terms of motility and fertilizing ability after thawing. However, the standardization of the

method, from the collection of milt to fertilization with thawed sperm will be necessary before suggesting this tool for aquaculture.

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REFERENCES

- Billard R. 1981. Short-term preservation of sperm under oxygen atmosphere in rainbow trout (*Salmo gairdneri*). *Aquaculture* **23**, 287-293.
- Chauvaud L., J. Cosson, M. Suquet, R. Billard 1995. Sperm motility in turbot, *Scophthalmus maximus*: initiation of movement and changes with time of swimming characteristics. *Environ. Biol. Fish* **43**, 341-349
- Crowe J. H., L. M. Crowe, J. F. Carpenter, A. S. Rudolph, C. Aurel Wistrom, B. J. Spargo, T. J. Anchordoguy 1988. Interaction of sugars with membranes. *Biochim. Biophys. Acta* **947**, 367-384.
- Geffen A. J., O. Frayer 1993. Retention of sperm motility in turbot, *Scophthalmus maximus* L.: the effects of time from activation, thermal shock and adenosine triphosphate levels. *Aquac. Fish. Manag.* **24**, 203-209.
- Legendre M., R. Billard 1980. Cryopreservation of rainbow trout sperm by deep-freezing. *Reprod. Nutr. Dévelop.* **20**, 1859-1868.
- Linhart O., J. Kouril, J. Hamackova 1987. Increased rate of egg fertilization in artificial propagation of sheatfish (*Silurus glanis* L.) by means of suppressing the movements of spermatozoa with immobilization solution. *Aquaculture* **65**, 353-358.
- Maisse G. 1996. Cryopreservation of fish semen; a review. *In: Proc. Refrigeration and Aquaculture, Bordeaux, France, 20-22 March 1996*, 443-466.
- Mounib M. S. 1978. Cryogenic preservation of fish and mammalian spermatozoa. *J. Reprod. Fert.* **53**, 13-18.
- Ogier de Baulny B., Y. Le Vern, D. Kerboeuf, M. Heydorff, G. Maisse 1996. Flow cytometric analysis of plasma membrane damages of rainbow trout and turbot frozen spermatozoa. *In: Proc. Refrigeration and Aquaculture, Bordeaux, France, 20-22 March 1996*, 65-72.
- Rana K. J. 1995. Cryopreservation of aquatic gametes and embryos: recent advances and applications. *In: Proc. 5th Int. Symp. Reproductive Physiology of Fish.* F.W. Goetz, P. Thomas eds. Austin, Texas, U.S.A., 2-8 July 1995, 85-89.
- Rana K. J., B. J. McAndrew 1989. The viability of cryopreserved tilapia spermatozoa. *Aquaculture* **76**, 335-345.
- Stoss J. 1983. Fish gamete preservation and spermatozoan physiology. *In: Fish Physiology*, 9b. W.S. Hoar, D. J. Randall, E. M. Doalson eds. Academic Press, London, 305-350.
- Stoss J., W. Holtz 1983. Successful storage of chilled rainbow trout (*Salmo gairdneri*) spermatozoa for up to 34 days. *Aquaculture* **31**, 269-274.
- Suquet M., M. H. Omnes, Y. Normant, C. Fauvel 1992. Assessment of sperm concentration and motility in turbot (*Scophthalmus maximus*). *Aquaculture* **101**, 177-185.
- Suquet M., G. Dorange, M. H. Omnes, Y. Normant, A. Le Roux, C. Fauvel 1993. Composition of the seminal fluid and ultrastructure of the spermatozoon of turbot (*Scophthalmus maximus*). *J. Fish Biol.* **42**, 509-516.
- Suquet M., R. Billard, J. Cosson, G. Dorange, L. Chauvaud, C. Mugnier, C. Fauvel 1994. Sperm features in turbot (*Scophthalmus maximus*): a comparison with other freshwater and marine fish species. *Aquat. Living Resour.* **7**, 283-294.
- Suquet M., R. Billard, J. Cosson, Y. Normant, C. Fauvel 1995. Artificial insemination in turbot (*Scophthalmus maximus*): determination of the optimal sperm to egg ratio and time of gamete contact. *Aquaculture* **133**, 83-90.
- Schwartz D. 1969. Méthodes statistiques à l'usage des médecins et des biologistes. Flammarion ed., 318 p.