Motility of fresh and aged halibut sperm

Roland Billard [1], Jacky Cosson [2] and Laurence W. Crim [3]

[1] *Muséum National d'Histoire Naturelle, Laboratoire d'Ichtyologie Générale et Appliquée, Unité de Recherches marines, 43, rue Cuvier, 75231 Paris Cedex 05, France.*
[2] *CNRS, Station Zoologique UA 671, 06230 Villefranche-sur-Mer, France.*
[3] *Ocean Sciences Centre, Memorial University of Newfoundland, St. Johns, Newfoundland A1C 5S7, Canada.*

Abstract

Preliminary studies of freshly collected halibut (*Hippoglossus hippoglossus*) sperm by light microscopy indicated that sperm motility, *i.e.* the percentage of motile sperm and the duration of forward progressive movement, was optimized under conditions of osmotic pressure ranging between 400 and 1 100 mOsmol/kg and pH for 6.5-8.5. The quality of sperm, stored *in vitro* on ice, deteriorated rapidly within a few hours, a characteristic particularly evident in sperm after freezing and thawing tests. Studies of several cryoprotectants indicated that halibut sperm could be successfully preserved frozen when diluted with 3 parts extender (sucrose: 150 mM, $CaCl_2$: 1.7 mM, $MgSO_4$: 7 mM, glycine: 86 mM and Tris: 30 mM at pH: 8), 10% of 1-2 propanediol and one part sperm (3:1). Additional studies of the motility of seawater-activated halibut sperm were conducted by dark field microscopy under stroboscopic illumination. These observations indicated that fast forward-moving sperm motility, which lasts 60-70 s, is correlated with propagating waves on the full length of the sperm flagellum with an initial beat frequency of 45-50 Hz which abruptly drops to about 10 Hz after 60-80 s. The beat frequency is blocked by KCN (5 mM) and NaN_3 (1.2 mM), on intact sperm. The flagellum beat frequency of demembranated/reactivated sperm is dependent on the ATP concentration in the reactivation solution.

Keywords: *Hippoglossus*, halibut, sperm, motility, cryopreservation.

Motilité du spermatozoïde frais et âgé de flétan.

Résumé

Des études préliminaires en microscopie optique de spermatozoïdes de flétan (*Hippoglossus hippoglossus*) ont montré que la motilité (le pourcentage de cellules mobiles et la durée de mouvement propulsif) était optimale dans une gamme de pression osmotique allant de 400 à 1 100 mOsmol/kg et de pH de 6,5 à 8,5. Une détérioration rapide de la qualité du sperme apparaît au cours de sa conservation *in vitro* sur glace et plus particulièrement après tests de congélation/décongélation. Les essais portant sur plusieurs cryoprotecteurs ont montré que le sperme de flétan pouvait être congelé en présence de 1-2 propanediol ajouté à 10 % d'un dilueur constitué de glucose: 150 mM, $CaCl_2$: 1,7 mM, $MgSO_4$: 7 mM, Glycine: 86 mM, Tris 30 mM, pH: 8) dans la proportion de 3 volumes pour un volume de sperme. Des études en microscopie à fond noir avec éclairage stroboscopique ont indiqué que la motilité progressive durait environ 60-70 s avec des ondulations affectant toute la longueur du flagelle dont la fréquence initiale de 45-50 Hz chutait brutalement à 10 Hz après 60-80 s. Les battements

(¹) Correspondence should be addressed to Dr. R. Billard.

flagellaires de spermatozoïdes intacts sont bloqués par KCN (5 mM) et NaN$_3$ (1,2 mM) ajoutés dans la solution d'activation. La fréquence de battement flagellaire de spermatozoïdes démembranés-réactivés est dépendante de la concentration en ATP dans le milieu de réactivation.

Mots-clés : *Hippoglossus*, flétan, motilité, sperme, cryoconservation.

INTRODUCTION

In most fish species which exhibit external fertilization, the spermatozoa are immotile in the testes and the genital tract and are activated only after release into the external medium for a short period of forward motility (from 30 s to several minutes) (Scott and Baynes, 1980). The mechanisms involved in the immobilization in the testes or the sperm duct and in the initiation of motility are not well understood. Most of the currently available information was obtained from the sperm of a few freshwater fish species. In trout the inhibition of motility is due to low calcium concentration in late spermatids and early spermatozoa in the testes (Billard and Cosson, 1992) and to high concentration of K$^+$ in the seminal fluid for mature spermatozoa in the testes and in the sperm duct (Schlenk and Kahmann, 1938; Morisawa and Suzuki, 1980; Baynes *et al.*, 1981). Spermatozoa are activated after dilution in freshwater or saline solutions of osmotic pressure of up to 300 mOsmol/kg. Initiation of motility is due to an entrance of Ca^{++} into the cell through Ca^{++} channel which raises internal calcium concentration (Tanimoto and Morizawa, 1988; Cosson *et al.*, 1989, Okuno 1991, Boitano and Omoto, 1992). The period of progressive motility is short, 20-25 s at 20°C and the flagellar beat frequency declines from 60 to 20 Hz during this period (Cosson *et al.*, 1985). The same pattern is observed at a lower temperature with a lower beat frequency and a longer duration (Billard and Cosson, 1986). The short duration of motility and the decline of the flagellum beat frequency are due to a parallel exhaustion of the endogenous stores of ATP (Christen *et al.*, 1987; Robitaille *et al.*, 1987). In carp the blockage of sperm motility in the testes and in the seminal fluid is due to the high osmotic pressure (300 mOsmol/kg). Motility is initiated after dilution in freshwater or saline solution of osmotic pressure lower than 200 mOsmol/kg and lasts for 45 s (Redondo-Muller *et al.*, 1991) with, as in trout, a regular decline of the flagellar beat frequency (Billard and Cosson, 1992) and the intracellular stores of ATP (Perchec, 1992). Although less information is available in marine fish species, total motility durations of 3 to 26 min have been reported in the sea bass *Dicentrarchus labrax* (Billard *et al.*, 1977) and turbot *Scophthalmus maximus* (Suquet *et al.*, 1992).

The present work was undertaken to obtain some basic information on sperm motility in halibut and to characterize sperm quality with respect to several parameters such as *in vitro* survival of spermatozoa stored straight or in diluted semen and the capacity of the spermatozoa to withstand the freezing and thawing procedures.

MATERIALS AND METHODS

Halibut broodstock and sperm sampling procedures

Atlantic halibut were collected throughout the year by commercial vessels using long lines and by bottom trawls on Department of Fisheries and Oceans research vessels. After collection of the halibut along the south and west coast of Newfoundland or from the Scottian Shelf, the fish were transported to the Marine Laboratory and maintained in 5 m diameter tanks under controlled water temperatures in the range 4-12°C. Throughout most of the year except the spawning season, from January to May, the fish were offered a diet composed of vitamin-supplemented chopped herring three times per week. In males, maturity was investigated by abdominal compression and spermiation was detected in some males from January to May. Although sperm production continues for several months in individual male halibut, sperm quality (motility) undergoes a seasonal decline particularly towards the end of the spermiation period when spermatocrit values approach 100% (Methven and Crim, 1991). Milt was stripped and stored on ice until motility analysis or freezing.

Evaluation of motility

The halibut sperm motility was measured according to several methods: for evaluation of the percentage of motile spermatozoa and duration of motility, 1 µl of semen was diluted in 100 µl immobilizing solution (full seawater [FSW] diluted 1/4) buffered at pH 8.0 with 30 mM Tris-HCl. 1 µl of this mixture was diluted in a 20 µl drop of activating solution which consists of FSW buffered at pH 8.0 with 30 mM Tris-HCl previously placed on a glass slide on the microscope stage. The first examination of this sperm preparation is possible within a few seconds after the second semen dilution. For routine tests, a direct dilution

technique may be used and consisted of mixing directly a microdrop of semen taken on the extremity of a fine wire or a micropipette tip with 20 µl the activating solution previously placed on the slide. This high dilution allows individual motile and non-motile sperm heads to be clearly seen and recorded yielding a good estimate of the percentage of actively swimming sperm. For semen diluted for cryopreservation or exposure to various solutions, the direct dilution was used and the amount of semen was adjusted according to the initial dilution rate. With time and continuous observations, the change in the percentage of motile spermatozoa may be estimated. The duration of motility was measured at the same time; usually the duration of the more progressive movement was taken into account, *i.e.* from the time at which the majority of spermatozoa stopped showing forward motion. In some cases, the total duration of motility including the sperm activity without forward displacement (quivering in place) was also measured. Observations were made at room temperature (18-20°C).

Measurement of beat frequency and swimming speed

Sperm was diluted as above and observed in a hanging drop by dark field microscopy using an inverted microscope (Zeiss IM 35) under stroboscopic illumination (Strobex Chadwick Helmut model 8440-3). A dark field condenser was combined with 20 x or 40 x non-immersion lenses for quick routine observations. A 100 x oil immersion lens was combined with an oil immersion condenser for detailed records of preparations in between the glass slide and a coverslip. Records of sperm swimming were obtained either through a video camera or through photography using Kodak Tmax film exposed for 1 s and processed in Tmax developer to 1 600 ASA sensitivity.

Effects of pH, osmotic pressure and respiration inhibitors on sperm motility

For testing the effect of osmotic pressure, sperm samples were diluted in activating solutions ranging from 90-750 mM NaCl, sucrose or choline chloride with the addition of 30 mM Tris-HCl, at pH 8.0 (final osmotic pressure ranging from 200 to 1 525 mOsmols).

The effect of pH on motility was tested in solutions of NaCl-sucrose solutions containing 1.7 mM $CaCl_2$, 7 mM $MgSO_4$ and 30 mM Tris-HCl ranging in pH from 6.5-10.0. The final osmotic pressure of the solution was about 800 mOsmol/kg. In one experiment the effect of varying pH between 6.5 and 9.5 was tested on sperm survival after dilution during a period of 4 h in a 300 mM sucrose solution containing 1.7 mM $CaCl_2$, 7 mM $MgSO_4$ and 30 mM Tris-HCl (final osmotic pressure 385 mOsmol/kg). The dilution rate of semen to extender was 1:3 v/v. In all cases motility was evaluated by the % motile spermatozoa

and the duration of forward movement both measured at room temperature.

Two respiration inhibitors were mixed in FSW buffered at pH 8 with Tris, 30 mM; KCN: 0 to 5 mM, NaN_3 0 to 1.2 mM. Sperm was diluted as described above in these solutions and the flagellar beat frequency was measured during the first minute following the activation.

Short-term storage of sperm and its capacity to survive freezing

The change in the ability of the halibut spermatozoa to be frozen and thawed was followed on semen collected from 2 males sampled in the morning. The semen was processed immediately or was stored in 3 ml portions in 20 ml vials on ice for various lengths of time up to 7 h before dilution and freezing. Freezing of sperm into pellets was accomplished by combining semen with extender (1:3 v/v) containing 10% cryoprotectant and placing 50 µl drops directly onto depressions made in dry ice. After 3-5 min for freezing on dry ice, pellets were placed into 0.5 ml precooled plastic vials and transferred directly to liquid nitrogen for storage. After several trials the following extender was found to produce good results: 150 mM sucrose, 1.7 mM $CaCl_2$, 7 mM $MgSO_4$, 86 mM glycine and 30 mM Tris-HCl buffered at pH 8.0 (final osmotic pressure 250 mOsmol/kg). The concentration of ions and Tris-glycine was similar to the DCSB4 extender used for dilution and freezing of sea bass and sea bream sperm (Billard, 1984; Chambeyron and Zohar, 1990) and also mimicked the composition of the halibut seminal plasma (L. W. Crim, unpubl.). 1,2 propanediol (10%) was used as the cryoprotectant and found more suitable than DMSO and glycerol. The final osmotic pressure of extender combined with propanediol was 1 780 mOsmol/kg; after adding the semen (3:1), the osmotic pressure dropped to 1 375 mOsmol/kg.

No equilibration time was allowed. Indeed, long exposure of the semen to the extender and propanediol was found deleterious for the spermatozoa and the time of mixing semen with extender was less than 1 min. Initially, frozen pellets were warmed for 10-12 s in plastic vials in a 40°C water bath before transferring the pellet to the glass slide under the microscope for final thawing. A microdrop of thawed material was diluted in buffered FSW to estimate the % motile sperm and the duration of the progressive movement. In order to check the integrity of axoneme of the exhausted sperm following activation, sperm were demembranated and reactivated according to the technique of Gibbons and Gibbons (1972), adapted for fish sperm by Cosson and Gagnon (1988). Sperm membrane was first permeabilized by exposure to a demembranation (extraction) solution: 150 mM K^+ acetate, 20 mM Tris-HCl, pH 8.0, 1 mM EGTA, 1 mM DTT, 1 mM Mg-ATP, 0.5 mM $CaCl_2$ and

0.04% Triton X100. For this procedure, 2 µl of sperm were diluted into 200 µl FSW and 20-40 µl of this mixture was placed for 30 s in 160-180 µl extraction solution. 10 µl of the mixture was transferred to 150 µl of the reactivating solution: 150 mM K^+ acetate, 20 mM Tris-Cl pH 8.0, 1 mM EGTA, 1 mM DTT, and 1 mM Mg-ATP (ATP was omitted in the control).

RESULTS

Analysis of motility of halibut sperm

Direct observation of semen by dark field microscopy shows that spermatozoa in the seminal plasma are immotile. They become activated after dilution in seawater with the flagellum showing large undulating waves when the beat frequency was 40-60 Hz. Most of the males samples provided 90 to 100% motile sperm immediately after sperm dilution. The percentage of motile sperm declined slowly in the first min
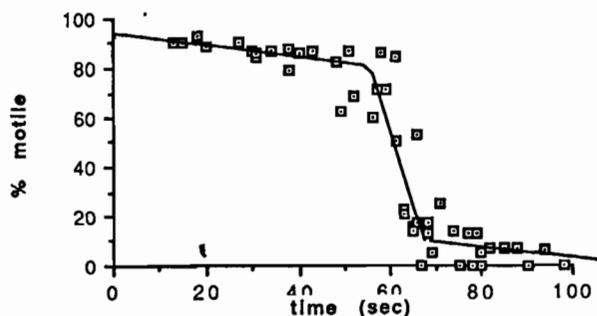
Figure 1. — Changes in the percentage of motile halibut sperm with time after dilution with full seawater. Motility is defined as sperm with speed exceeding 40 µm/s. Data combined from 3 independent motility sequences. Data were collected from video sequences analysed on successive still frames.
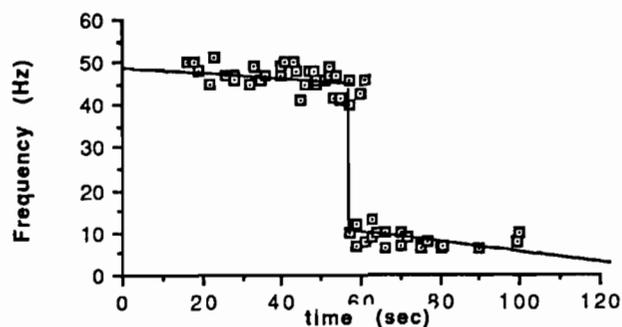

Figure 2. — Beat frequency of sperm flagellum with time after dilution with full seawater. Data were obtained from visual observations of free swimming sperm and adjustment of the strobe frequency in register with the flagellar beating. Data refer to the major population of cells observed and it is noteworthy that a few % of the cells keep a frequency higher than 40 Hz at time periods up to 3 or 4 min.
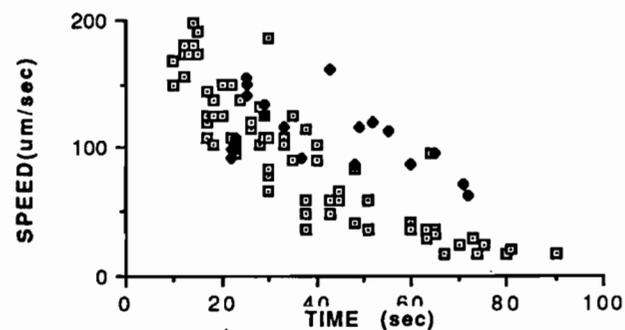

Figure 3. — A comparison of control and frozen/thawed halibut sperm swimming speed with time after dilution with FSW. Open squares refer to control untreated sperm while diamonds refer to frozen/thawed spermatozoa.

of motion and abruptly thereafter (*fig.* 1). Direct observations at high magnification at the end of the motility phase show drastic distortions of flagella which could indicate serious damage to membranes. The duration of mass forward progressive movement was 60-70 s and 1 to 5% of spermatozoa remain active after 90 s. The beat frequency was in the range of 50 Hz and homogeneous for all spermatozoa immediately after dilution and most of them kept a frequency of 45-50 Hz for a period of up to 60 s (*fig.* 2). After 60-80 s the beat frequency dropped suddenly to 10 Hz. However, a few spermatozoa exhibit a high beat frequency for a long time, up to 8 min. Soon after activation (about 10 s), the swimming speed of sperm was approximately 200 µm/s and it declined steadily over the interval of 90 s to about 10 µm/s (*fig.* 3). After demembranation and reactivation in

solution saturated with ATP-Mg (1 mM), the maximum beat frequency did not exceed 50 Hz; in decreasing ATP-Mg concentration the beat frequency decreased proportionally (*fig.* 4).

Effect of the osmotic pressure and pH on motility

The spermatozoa were motile in a range of osmotic pressure from 380 to 1 150 mOsmol/kg (*fig.* 5). The duration of motility and the percentage of motile cells were lower at the extreme range of osmotic pressure. There was a tendency of an increase of the percentage of motile sperm (60 to 90%) and duration of motility (50 to 70 s) with the rise of osmotic pressure from 400 to 1 100 mOsmol/kg in the case of NaCl and choline.
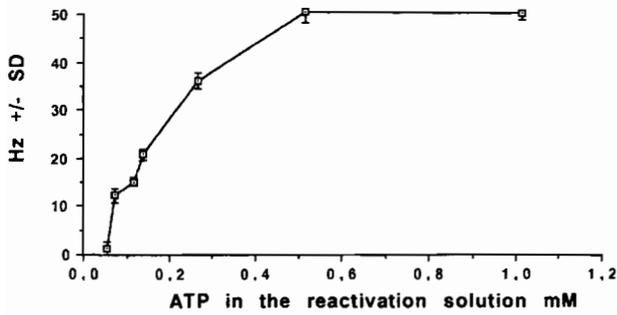
Figure 4. — Changes in the beat frequency (Hz) of freshly collected demembranated (permeabilized) sperm with increasing amount of ATP in the reactivation medium; mean values ± SD, $n = 10$.

The spermatozoa were active in the range of pH tested and no total inhibition was observed. With sperm freshly collected the percentage of motile cells progressively increased from pH 6.5 to 8.5 and declined above pH 8.5 as shown for two different males (*fig.* 6). The duration of forward movement increases also from pH 6.5 to 7.5 and reaches a maximum of 60 s at pH 7.5-8.0. It declines progressively to 45 s at pH 10.0. In aged semen stored on ice during 24 h the percentage of motile spermatozoa was lower, never exceeding 40% at pH 7.0 to 8.0 and was 30% at pH 8.5 to 9.5. The duration of progressive motility was maximum (60 s) at pH 8.0 to 9.0 (*fig.* 7). The experiment on semen stored in the sucrose solution buffered at different pHs (*see* materials and methods) showed that the optimum pH for sperm survival *in vitro* was in the range of 7.5 to 8.5 both measured by the percentage of motile cells (*fig.* 8) and by the duration of motility (*fig.* 9).

Effect of respiration inhibitors on motility

Increasing amount of NaN_3 and KCN in the activating solution resulted in a regular decline of the flagellum beat frequency; motility is entirely blocked at concentration of 1 mM NaN_3 and 5 mM KCN (*fig.* 10).

Short term changes in the ability of the sperm to withstand freezing

All semen studied showed a good motility after sampling with 80-90% of motile cells and a duration of forward motility of 60-80 s. After dilution in the extender including 10% propanediol, the motility parameter was not affected. After freezing and thawing, the motility performances were slightly reduced, with the percentage of motile spermatozoa dropping to 50-60%, and the swimming speed remained unaffected (*fig.* 3). The freezing trials carried out on semen ageing on ice during a 7 h period showed a constant and regular decline of motility performances

Effects of the osmotic pressure of the diluting medium on halibut sperm preservation
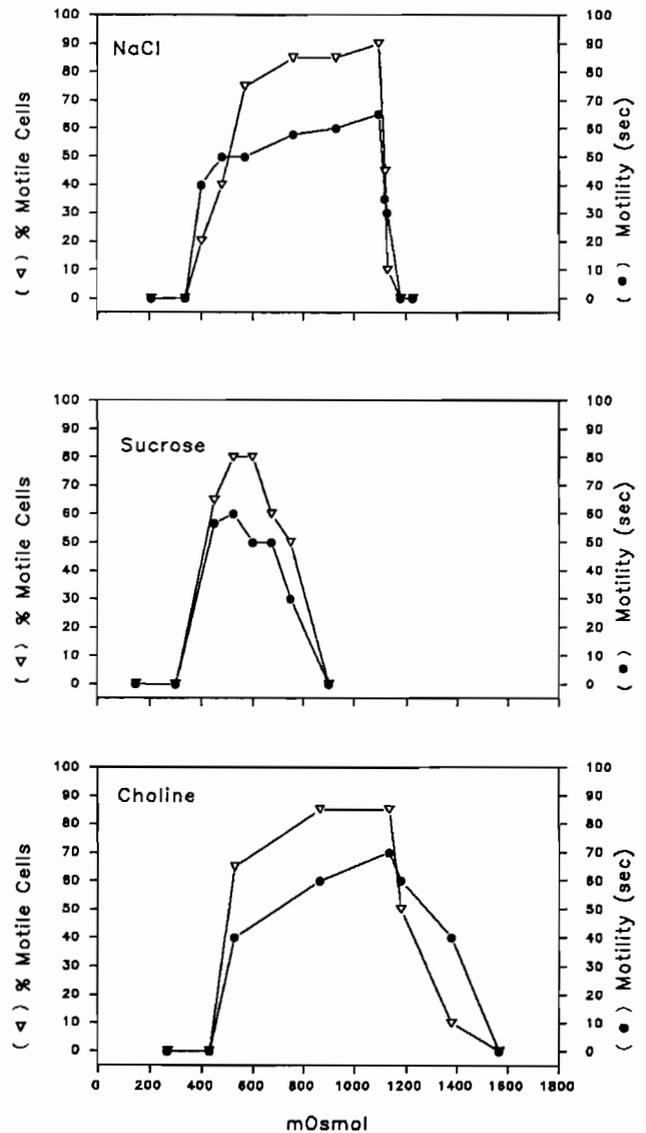
Figure 5. — Representative changes in motile sperm % and duration of sperm motility (sec) with changes in osmotic pressure of activating medium. Tests conducted with freshly collected semen from the same male.

after freezing and thawing (*fig.* 11). In male 3 there was no recovery of motility. On the contrary, semen left intact on ice preserved in most cases their initial motility performances. Sperm diluted in the extender including the cryoprotectant but not frozen and thawed showed a good motility and was not much affected by the process of ageing. Demembranated sperm which were immotile after freezing and thawing were reactivated following exposure to reactivation solution containing 1 mM ATP-Mg with a beat frequency of 50 Hz which is comparable to reactivation of freshly collected sperm (*fig.* 4).

Effects of the pH of the Diluting Medium
on Halibut Spermatozoa Motility

Short term preservation of halibut sperm diluted
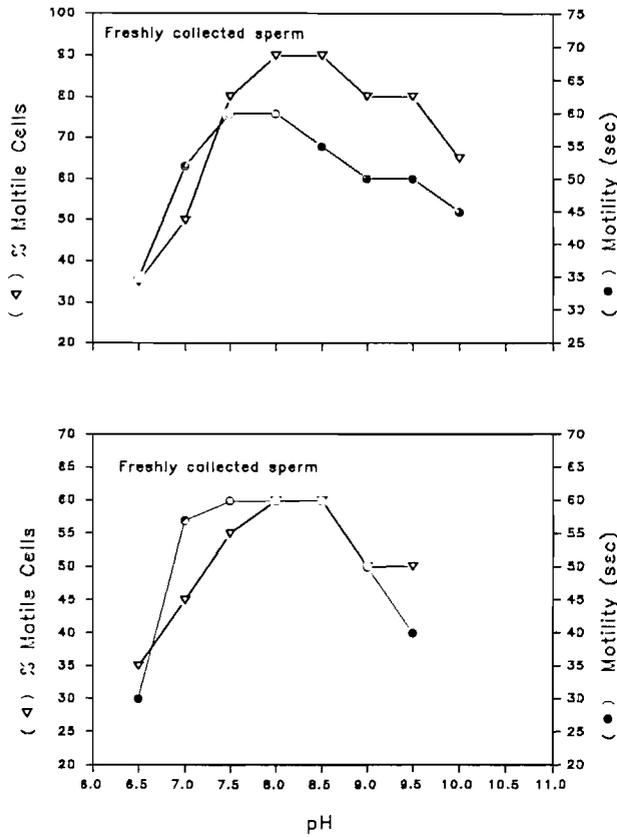in sucrose solution buffered at different pH

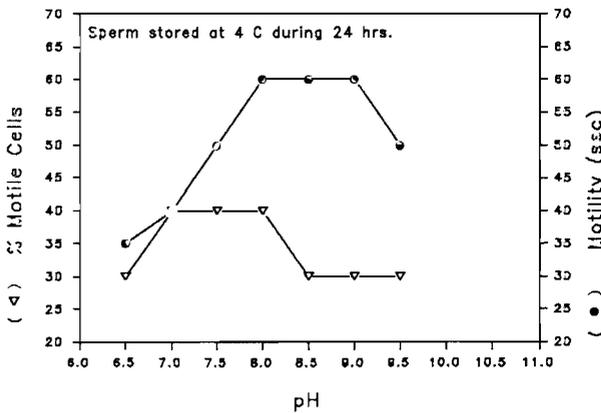**Figure 6.** — Representative changes in % motile sperm and duration of sperm motility (s) with variation in pH of the activating medium. Tests conducted on freshly collected semen. top: male 1, bottom: male 2.
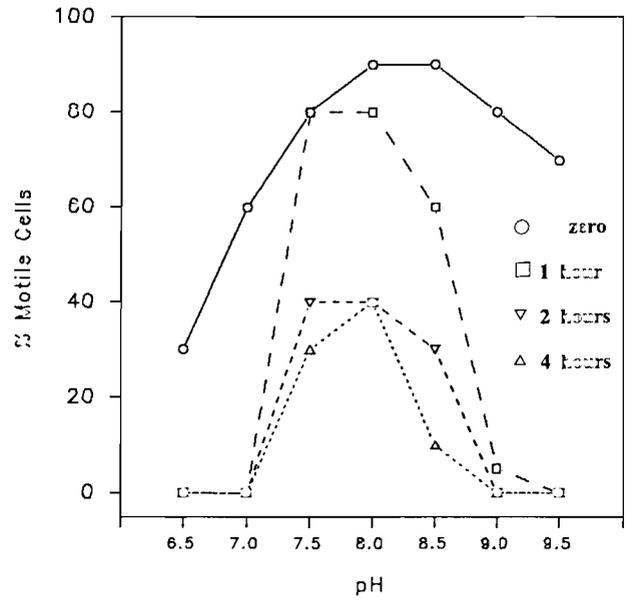
Figure 8. — Representative changes in sperm survival (% motile sperm after sea water activation) from individual male with time of storage on ice with variations in pH of the diluting medium.

Effects of the pH of the Diluting Medium
on Halibut Spermatozoa Motility

Short term preservation of halibut sperm dilute
in sucrose solution buffered at different pH

Figure 7. — Representative changes in motile sperm % and duration of sperm motility with (s) from one male with variation in pH of the activating medium. Tests conducted on semen stored on ice for 24 h.
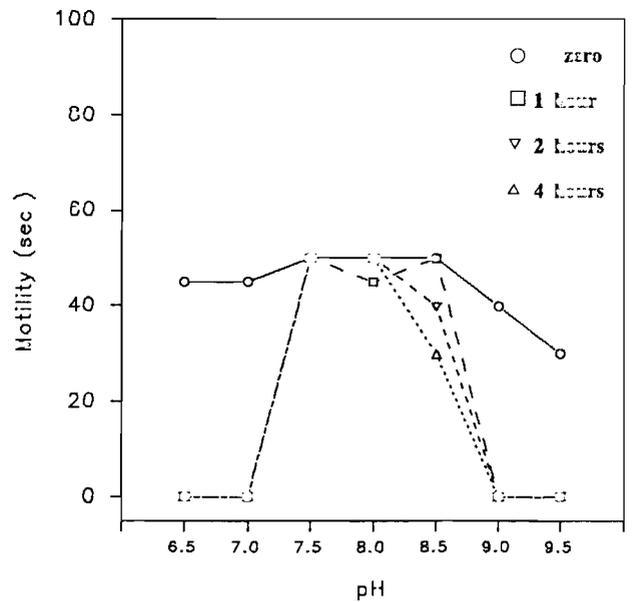
Figure 9. — Sperm survival (motility in s) from one male after FSW activation, with time of storage on ice with variations in pH of the diluting storage medium.
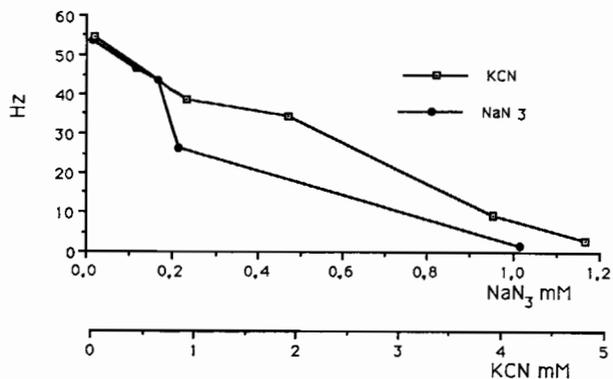
Figure 10. – Changes in flagellum beat frequency of halibut sperm diluted in an activating solution (FSW buffered at pH 8) containing increasing concentration of respiration blockers (NaN_3 and KCN).

DISCUSSION

The motility of halibut sperm is initiated by a rise in osmotic pressure as in some other marine fish species (Morisawa and Suzuki, 1980). The precise mechanisms which trigger the initiation of sperm motility are not yet fully understood. Preliminary evidence from work on halibut sperm shows that sucrose and choline solutions initiate motility in the same range of osmotic pressure as 500 mM NaCl. Likewise, Lee *et al.*, (1992) reported that glucose and sucrose solutions with osmolarity >400 activate the sperm of striped mullet. These results suggest that external Na^+ is not involved in the blockage of motility.

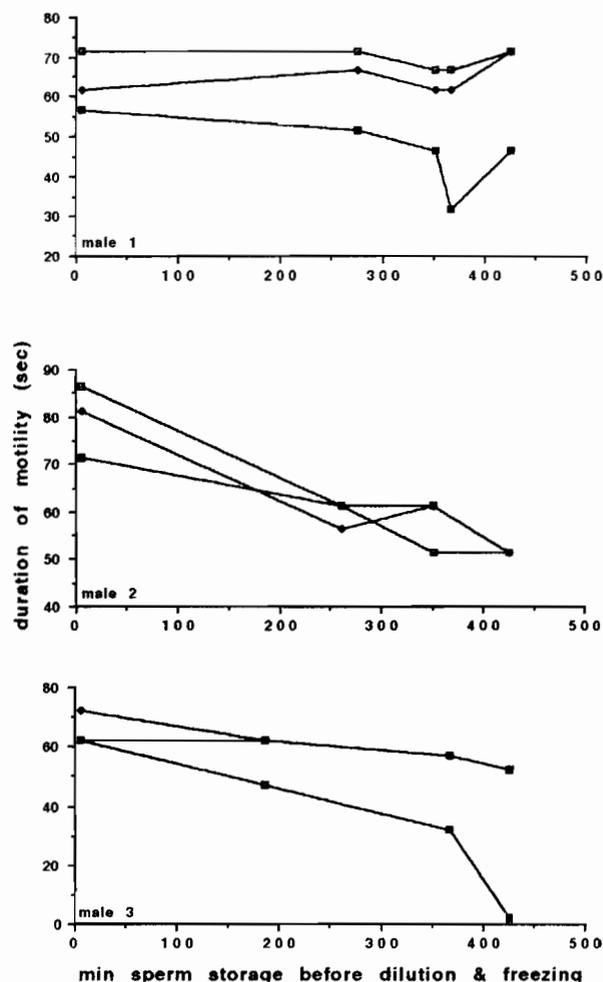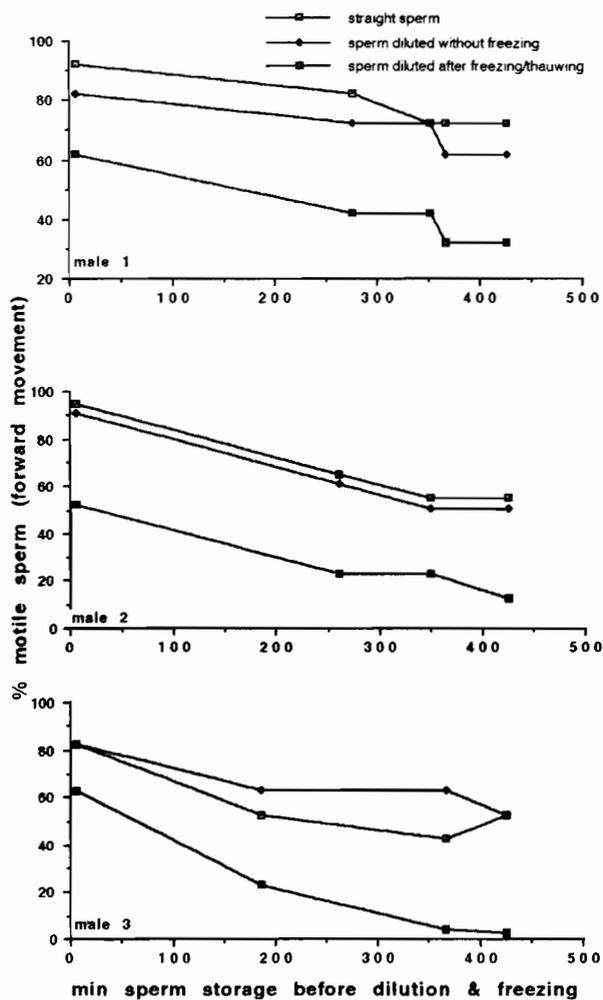The duration of halibut sperm motility is short similar to other fish species such as carp but the

Figure 11. – The influence of time of storage on ice on changes in the motility of halibut sperm collected from 3 males following freezing/thawing. Motility was assessed by motile sperm % and duration of motility (min). Control sperm included non-diluted (straight sperm) and diluted sperm without freezing.

pattern of motility is entirely different with an abrupt change in flagellar beat frequency for the majority of sperm 60-80 s after dilution and activation. The mechanism of such a phenomenon is not yet known. It seems different than in carp or trout where the endogenous store of ATP is progressively consumed during motility with a progressive decrease of the flagellar beat frequency (*see* introduction). The stability in the frequency may result from higher amounts of accumulated ATP providing energy for a full efficiency of function of the flagellum. After demembranation and reactivation by 1 mM concentration of ATP the maximum frequency did not exceed 50 Hz and a decreasing ATP-Mg concentration does decrease frequency proportionally. In contrast, it was also shown that inhibitors of respiration could block motility immediately after addition suggesting that there is not such a store and that the ATP is provided entirely by an endogenous production by the mitochondria during the motility period. Then the initiation of motility would include a concomitant stimulation of the process of respiration and its cessation would be due to an abrupt stop of mitochondrial production of ATP. The reason as to why its production of ATP suddenly stops is not known but is currently under study to elucidate this point.

The problems of ageing and change in quality of fish sperm has often been discussed (Stoss and Holtz, 1981; 1983; Billard, 1992). It is clear that the "quality" of sperm declines during the reproductive season in several species. This includes morphological changes with deterioration of the structure of the spermatozoa for example in the sea bass (Billard, 1984) and in the halibut (Emerson and Crim, unpubl.), a decline in the duration of sperm motility as in sea bass (Billard *et al.*, 1977) and halibut (Methven and Crim 1991), a reduction of the fertilizing capacity in trout (Yazbek-Chemayel, 1975) and the capacity to be cryopreserved (Stoss, 1983). The present data also shows that the capacity of the halibut sperm to be frozen declines

rapidly after collection and storage *in vitro* during a period of 7 h. At the same time sperm left *in vivo* in the genital tract or in the testes were successfully frozen immediately after sampling. So the ageing was much more rapid *in vitro* than *in vivo*, *i.e.*, the sperm deteriorate rapidly as soon as they are extracted from the fish. It should be pointed out that this deterioration of sperm "quality" was undetectable except for being clearly demonstrated by the freezing test.

The decline of sperm quality during *in vitro* storage in the cold is not clearly understood; several factors are probably involved. The deteriorating sperm structures are not precisely identified but it was shown from demembranation and reactivation experiments with ATP that the axoneme remained functional in, at least, 20% of the spermatozoa after freezing. Since added ATP reactivated these spermatozoa, it indicates that the endogenous amounts of ATP were insufficient and that sperm membranes were badly damaged. The damage to sperm was due to the process of freezing and thawing since the control, undiluted sperm and sperm diluted in the extender with cryoprotectant did not show any reactivation after adding ATP. In this respect propanediol exhibits lower toxicity than DMSO which was shown to induce spontaneous motility in rainbow trout sperm probably by opening some Ca^{++} channel favouring Ca^{++} entry into the cell and the initiation of some motility (Billard unpubl.).

As often reported in the literature (Stoss, 1983; Stoss and Holtz, 1983) a large variability of sperm quality was observed here for sperm samples taken from individual males.

The present work on freezing and thawing of diluted sperm in the presence of 10% propanediol shows that there are good prospects for improving the technology of cryopreservation of halibut sperm indicated already by the work of Bola *et al.* (1987) and Rana *et al.* (1992).

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