Indian J. Fish., 45(5) : 387-397, Oct.-Dec., 1998

Studies on cryogenic preservation of sperm of certain cultivable marine fishes

A. D. DIWAN[1] AND A. NANDAKUMAR

Central Marine Fisheries Research Institute, Cochin - 682 014, India

ABSTRACT

Significant success has been achieved in long-term cryopreservation of sperm at -196°C motility of certain marine fishes like *Liza parsia, Sillago sihama, Mugil cephalus* and *Gerres oyena*. In case of *L. parsia* sperm motility could be preserved successfully for more than 240 days using suitable cryoprotectant. In case of *S. sihama* and *M. cephalus* viable sperm could be cryopreserved for 1 month only, whereas in the case of *G. oyena* viable sperm could be preserved for a period of 3 months. While attempting on short-term preservation of sperm in *L. parsia* at -10°C, viability could be maintained for 24 hrs and beyond this at 48 hrs there was total loss of motility. Analysis of biochemical constituents of seminal plasma in case of *L. parsia* was carried out to know the possible causative factors for the loss of motility of sperm and it was found that the glucose and protein levels decreased significantly in the seminal fluid cryopreserved for 48 hrs whereas lipid content increased. Similarly there was drastic reduction in the levels of Na^+ and K^+ ions in the seminal fluid cryopreserved for 24 hrs.

Introduction

The use of cryopreserved gametes (sperm and eggs) in the research and development programmes of aquaculture was reviewed by Stoss (1983) and Muir and Roberts (1993). The cryopreservation of fish semen has been the subject of many investigations (Scott and Baynes, 1980; Billard, 1988). Successful techniques were devised for short-term and long-term preservation of sperm in a number of teleost fishes particularly in salmonids (Stein and Bayrle, 1985; Legendre and Billard, 1989; Chambeyron and Zohar, 1990). Some attempts have also been made in devising such methods in freshwater carps and mullets (Stein and Bayrle, 1985; Saad *et al.*, 1988). In recent years for enhancing the longevity of viable cryogenic sperm, methods of cryopreservation have been modified by diluting the sperm concentration and increasing the levels of oxygen in preservative media (Erdahl and Graham, 1987; Stoss *et al.*, 1987). Although investigations on structural details and metabolic and ionic changes

Present Address. [1] Central Institute of Fisheries Education, J. P. Road, Versova, Mumbay - 400 061, India.

in frozen spermatozoa could bring further knowledge in progressing the preservation techniques, only limited information is available at present (Billard, 1983; Lahnsteiner *et al.*, 1992).

In the present study cryopreservation methods were developed for preserving the motility of sperms for short-term duration in *Liza parsia* and long-term in *Liza parisa*, *Sillago sihama*, *Mugil cephalus* and *Gerres oyena*. Investigations were carried out on biochemical and ionic concentrations in fresh and frozen seminal fluid of *L. parsia* to assess the effect of freezing on quality of semen.

Materials and methods

Animal collection and maintenance : Mature male fishes of *L. parsia* TL 100-120 mm, *S. sihama* 140-160 mm, *M. cephalus* 400-600 mm and *G. oyena* 60-80 mm were collected from wild with the help of Chinese dip net located at the barmouth of Fort Cochin area. The fishes were transported to the laboratory in big plastic bins and kept in seawater (salinity 30 ‰) in fibre glass tanks (1 tonne capacity) with proper aeration. Except in *L. parsia* the experiment was attempted only to a limited extent in other species due to their high seasonality (breeding season from October to January).

Cryoprotectant and diluents : The cryoprotectants tested were Dimethyl sulfoxide (DMSO, Glaxo India Ltd. 5 and 10 %) and Glycerine (Merck Ltd. 15 %), both prepared in physiological saline (0.9 % NaCl). The different diluents or extenders used were Alsever's solution (a) (Hodgin and Ridgway, 1964), marine teleost ringer and buffer solutions namely b, c, d, e and f (pH 7.5). The details of the ingredients of diluents are given in Table 1.

TABLE 1. *Diluents and cryoprotectants used for cryopreservation of sperms*

Alsever's solution (a)	Sodium citrate ($C_6H_5Na_3O_7$) 0.08 %, dextrose 2.05 % and NaCl 0.4 % (Ratio - STP 1:9, LTP 1:3)
Diluent b	NaCl, 750 mg; $NaHCO_3$, 200 mg; Na_2HPO_4, 53 mg; $MgSO_4.7H_2O$, 23 mg; KCl, 38 mg; $CaCl_2H_2O$, 46 mg; glucose, 100 mg; glycine, 500 mg; H_2O, 100 ml (Ratio - STP 1 : 9, LTP - 1 : 3)
Diluent c	NaCl, 730 mg; $NaHCO_3$, 500 mg; fructose, 500 mg; mannitol, 500 mg; H_2O, 100 ml (Ratio STP - 1:9)
Diluent d	NaCl, 0.6 %; KCl, 0.038 %; $CaCl_2$ $2H_2O$, 0.023 %; $NaHCO_3$, 0.1 %; $Na_2HPO_2H_2O$, 0.041 %; $MgSO_4$ $7H_2O$, 0.023% (Ratio – STP 1:9, LTP1:3)
Diluent e	KCl, 0.75 % Lecithin, 10 % (Ratio – STP 1:9)
Diluent f	NaCl, 800 mg; $NaHCO_3$, 400 mg; Lecithin, 50 mg; H_2O, 100 ml (Ratio – STP 1:9)
Marine teleost ringer	NaCl, 231 mM; KCl, 8 mM; $CaCl_2$, 2.2 mM; $MgCl_2$, 3.7 mM; H_2O, 100 ml (Ratio – LTP 1:1)
Glycerine	15 % prepared in physiological saline (0.9 % NaCl) (Ratio – LTP 1:1)
Dimethyl sulphoxide (DMSO)	10 % prepared in physiological saline (0.9 % NaCl)

(Ratio of mixing of diluent vs DMSO/STP - Short term preservation /LTP-Long term preservation).

Protocol for short-term cryopreservation of sperm (L. parsia) : For storing semen samples, multiple sets of semen chambers (each 5 ml capacity) made of plastic vials were taken in triplicate for each diluent to be tested. The diluents and a cryoprotectant DMSO (5 and 10 %) were prepared as per the formula given in Table 1. All the diluents were prepared in triple distilled water. DMSO and diluents were then mixed in the proportion of 1:9 so as to make the total volume of test solution around 4 ml in each semen chamber. Semen chambers were later kept in a refrigerator for initial cooling (4°C). Once the initial cooling occurred, the semen chambers were taken from refrigerator and fresh semen samples collected by stripping the abdominal portion of fish were transferred to the chambers using a cannula. The semen volume pooled from single individual fish was measured and was found to be in the range of 10 to 20 µl. Semen chambers were then immediately returned to the deep freezers where temperature was maintained at –10°C. Observations on sperm motility were made after 1 and 2 hrs of preservation and subsequently at intervals of 4 hrs until the sperm lost their motility completely by (48 hrs). Prior to observation on sperm motility in the frozen samples, the chambers were thawed at normal room temperature (20°C) for 10 minutes. Motility was determined and expressed by indexed scores, with score 0 representing no motile spermatozoa and scores 1 to 4 representing 0 to 25 %, 25 to 50 %, 50 to 75 % and >75%, respectively. While rating the motility percentage in the present study only active (motile) sperms (50 to 75 %) were taken into account. Fresh semen and the one which is preserved in seawater (G) were treated as control for comparison.

Protocol for long-term cryopreservation of sperm : In order to find a suitable medium to preserve the motility of sperm for long-term duration under cryogenic temperature (-196°C), DMSO (10 %) was mixed with different diluents like teleost marine ringer, (in the ratio 1:1), buffer mixture b, c and Alsever's solution in the ratio 1:3. A mixture containing DMSO (10 %) and glycerine (15 %) in the ratio 1:1 was also prepared. For storing semen samples multiple sets of semen chambers (each 20 ml capacity) taken in triplicate for each diluent to be tested were divided into two groups I and II. Semen chambers of group I contained normal diluents whereas in group II, diluents were oxygenated by releasing oxygen gas for 10-15 minutes through oxygen cylinders. The total volume of diluents and DMSO mixed was around 20 ml in each case. Once the media were ready, antibiotic drug (1 mg, Distreptopenicillin) was added to each semen chamber. The semen chambers were then exposed to the vapours of liquid nitrogen (LN2) for initial cooling (5 minutes). When cooling occurred, the fresh semen obtained by stripping live mature fishes was collected into the cannula and transferred immediately into the semen chambers (10 µl of semen to each semen chamber). Later the semen chambers were further exposed to vapours of LN2 till the media became solid ice.

All the samples were then preserved in LN2 (in cryocan of 20 and 60 l capacity) for long-term preservation. Observations on sperm motility in the case of *L. parsia* were carried out for a total period of 240 days with an interval of 15 and 30 days in the beginning and then after 60, 180 and 240 days. For

S. sihama and *M. cephalus* only two observations were made, first after 15 days and second at 30 days. For *G. oyena* the sperm motility was counted first after 15 days, second and third observations were made after a period of 30 and 90 days, respectively. While rating percentage motility only active (50 to 75 %) sperm were taken into account.

Biochemical and ionic studies : Biochemical profile of the freshly collected and preserved seminal plasma of short duration at −10° C temperature was studied to identify causative factors for the loss of the sperm motility in the fish *L. parsia*. The seminal plasma was obtained by subjecting the samples of semen through micro-centrifuge. In the analysis, glucose content was determined according to the method of Nelson (1994). Protein was estimated by Lowry's method (Lowry *et al.*, 1951) and lipid as per the method of Barnes and Blackstock (1973). Analysis of micro-environment of seminal plasma where certain essential ions like Na+ and K+ were required for motility has also been carried out. Both the ions were estimated by flamephotometric method (APHA, 1975).

Results

Short-term preservation of sperm (-10°C) in **L. parsia**

Observations on sperm motility in 5 % DMSO in combination with different diluents showed that only 5 % of the sperm was active and motile in buffers a, b, c and d at the end of 1hr preservation. All sperms were immotile at the end of 2 hr. In diluents e and f the percentage of motility was around 30-40 % at the end of 1 hr and at the end of 2 hrs it was reduced to 15-20 %. After 3 hrs all the sperms were immotile. As the data obtained was not that significant the same is not presented here. Preservation of sperm motility in 10 % DMSO with different diluents was better than with 5 % DMSO (Fig. 1). In diluents a and b the percentage of motility was 50 and 70 % respectively after 24 hrs of preservation. In diluent b the motility was 50 % until 36 hrs of preservation but reduced later and sperm became inactive within 48 hrs. The percentage motility was around 10 % in diluent d at the end of 30 hrs. In diluents c and f motility upto 50 % could be maintained till the end of 6 hrs preservation and later the sperm became passive at the end of 8 hrs. Diluent e was not a favourable medium as the sperm lost their motility within 4 hrs. Sperm preserved in seawater (S 35 ‰) in combination with 5 and 10 % DMSO lost their motility within 1/2 to 1 hr only. So also the raw sperm kept at −10° showed 60-70 % motility within first 3 hrs of their preservation and later all the sperms were found to be in passive state within 4 to 5 hrs.

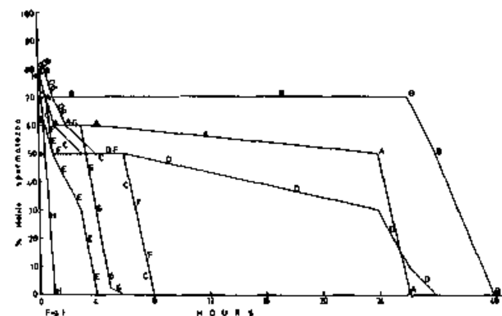

Fig. 1. Percentage of motility of sperm of fish *L. parsia* cryopreserved (−10°C) for different duration of time.

Biochemical and ionic studies of semen plasma of L. parsia

In short-term preservation at –10°C temperature motility of sperm could be maintained hardly upto 48 hrs. For the loss of motility during 48 hrs of preservation time, causative factors related to biochemical and ionic content of seminal plasma were investigated.

Some of the important energy constituents like glucose, protein and lipid were analysed in freshly collected seminal plasma and also in sperm preserved for 6, 24 and 48 hrs at –10°C temperature. Glucose and protein content were reduced drastically in the preserved semen samples of 24 and 48 hrs whereas lipid content increased significantly (Table 2). Similarly in the analysis of

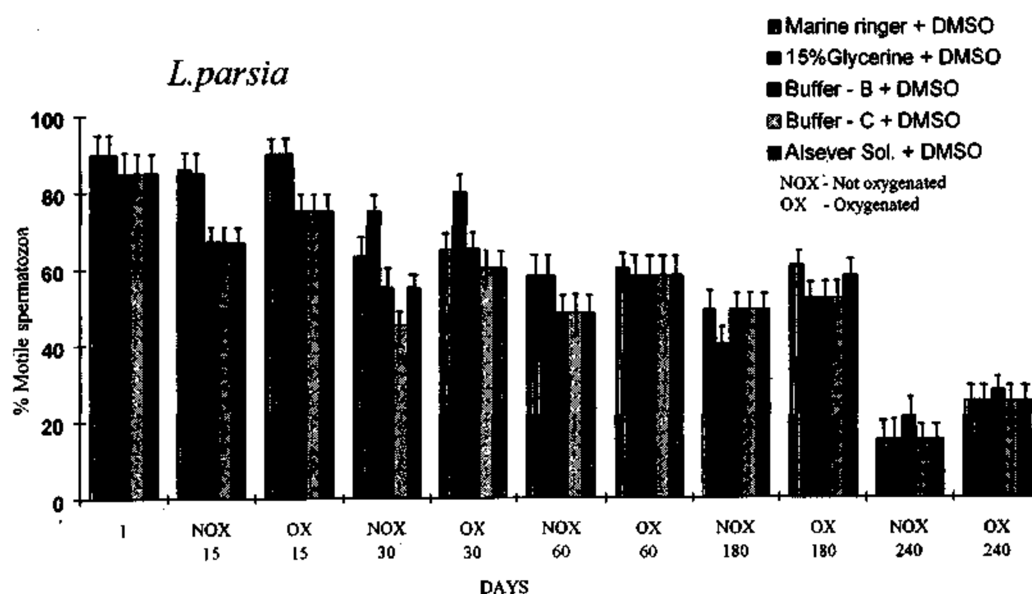

Fig. 2. Percentage of motility of sperm of fish *L. parsia* cryopreserved in different diluents (–190°C) and for different durations.

TABLE 2. *Changes in some biochemical constituents of fresh and preserved semen of* L. parsia

Biochemical constitutents (mg %)	Fresh semen	Preserved semen at –10°C during different time duration		
		6 hrs	24 hrs	48 hrs
a) Glucose	31.66 ± 0.90	29.83 ± 1.70n	23.46 ± 1.25a	18.53 ± 0.77a
b) Protein	83.80 ± 10.13	79.50 ± 7.80n	46.25 ± 7.50a	37.40 ± 2.04a
c) Lipid	38.83 ± 4.79	38.67 ± 9.40n	103.44 ± 5.08a	110.50 ± 3.03a

Each value is the mean of 4 determinations + SD. a = $P < 0.001$. n = not significant compared to values of fresh semen.

ionic contents significant loss was noticed in Na+ and K+ ions in preserved plasma of 24 hrs and beyond (Table 3).

TABLE 3. *Changes in Na^+ and K^+ content in fresh and preserved semen of* L. parsia

	Fresh semen	Preserved semen of 24 hr at −10°C
Na^+(mEq/l)	124.3 + 5.4	54.37 + 4.05a
K^+ (mEq/l)	164.4 + 7.6	41.56 + 3.15a

Each value is the average of 3 determinations + SD. a = P < 0.

Long-term preservation at −196°C temperature

In the long-term preservation, the percentage motility of the sperm of *L. parsia* was tested for a maximum period of 240 days in normal and oxygenated media and the results observed are given in Fig. 2. The percentage motility was reduced as the time of preservation elapsed in all diluents tested. In oxygenated media, there was loss in the motility but percentage loss was comparatively less when compared with the normal media. Among the various diluents tested, marine ringer and 15 % glycerine in combination with 10 % DMSO were better for long-term preservation under cryogenic temperature. Preservative media supplemented with addition of oxygen showed high survival rate of cryopreserved sperm.

In *S. sihama, M. cephalus* and *G. oyena* the motility of cryoperserved sperm was tested for a maximum period of 30 and 90 days, respectively, both in normal and oxygenated media. This test was restricted because of low quantity of semen and high seasonality of brooder males in Cochin backwaters. The results presented in Fig. 3, 4 and 5 showed

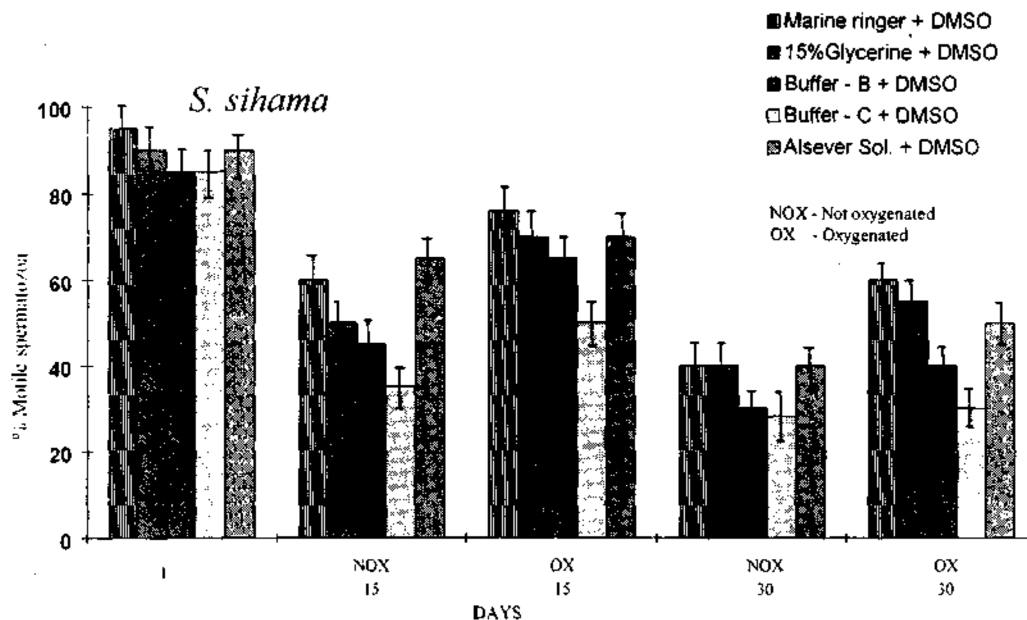

Fig. 3. Percentage of motility of sperm of fish *S. sihama* cryopreserved in different diluents (−196°C) and for different durations.

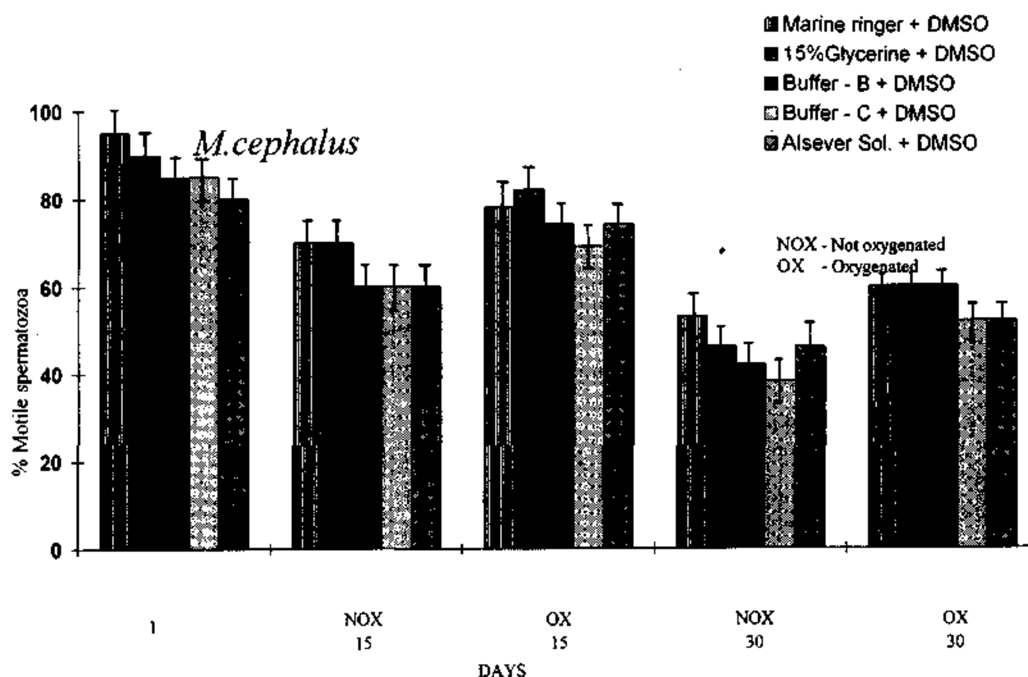

Fig. 4. Percentage of motility of sperm of fish *M. cephalus* cryopreserved in different diluents (–196°C) and for different durations.

that marine ringer and 15 % glycerine are good cryogenic media for long-term preservation. Other media in oxygenated condition are found to be equally good.

Discussion

Sperm motility of *L. parsia* can be retained for a short duration at freezing temperature of –10°C. Use of 10 % DMSO in combination with extender solutions like a and b are found to be good media as the sperm motility could be preserved in these upto 50 % for a period of 36 hrs. The sperms were viable because of their high mobility. Short term preservation methods are very important at places of breeding farms where sperm preservation facility using cryogenic temperature is not available. A number of attempts were made on short-term preservation at freezing temperatures using suitable cryoprotective media in fishes like salmon (Stoss and Refstie, 1983; Wheeler and Thorgaard, 1991), grey mullet *M. cephalus* (Chao *et al.*, 1975), carps *Cyprinus carpio* (Saad *et al.*, 1988), paddlefish (Mims, 1991), rainbow trout (Stoss *et al.*, 1978) and in yellow fin seabream (Gwo, 1994). Hwang *et al.* (1972), could preserve the motility of sperm of *M. cephalus* at –20°C temperature for 1 hr only using suitable cryoprotectant. Chao *et al.* (1975) were able to preserve the sperm of *M. cephalus* for 3-5 days at 5° C by combining 10 % glycerine and 10 % DMSO in different ratios. Saad *et al.* (1988) showed that in *C. carpio* sperm motility could be maintained in liquid state for a short period of 2 days at 4°

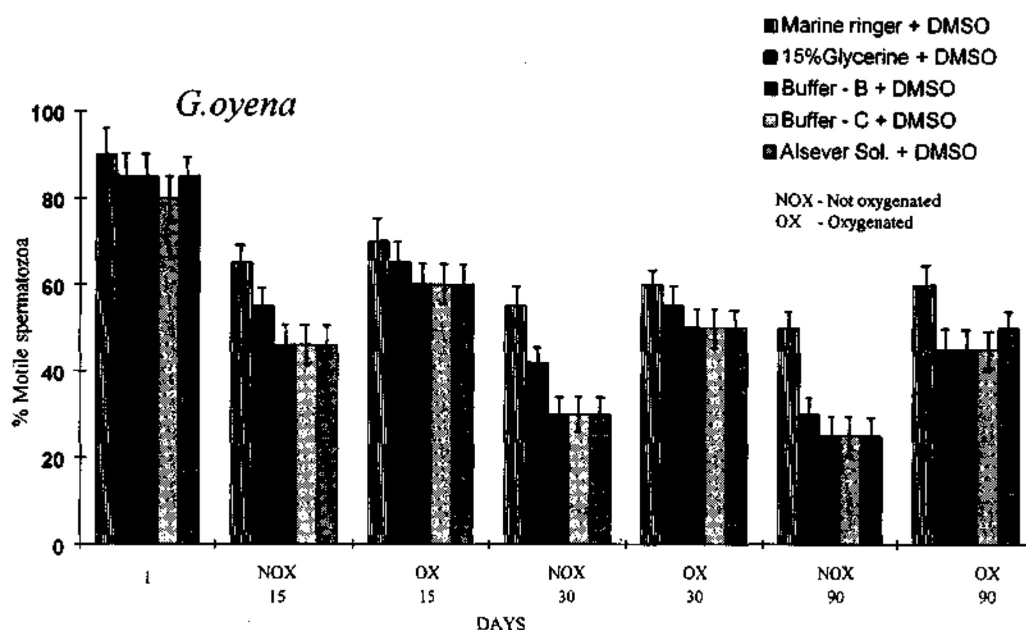

Fig. 5. Percentage of motility of sperm of fish *G. oyena* cryopreserved in different diluents (–196°C) and for different durations.

C but afterwards rapid loss was noticed in the motility. In *Sillago ciliata* the storage of sperm on ice gave greater duration of motility (180 minutes) than kept at room temperature (Goodall *et al.*, 1989). In the present study motility of sperm at normal room temperature (20°C) remained for 5 minutes but it declined continuously with the lapse of time. Even at freezing temperature there was continuous decline in the motility. This decline is presumed to be due to energy loss in the form of organic constituents and disturbance in the ionic composition of seminal fluid. Harvey (1982) described that although fish sperms are not specifically adapted for utilization of exogenous energy sources, they do rely on oxidative metabolism and addition of an energy source that might be expected to improve motility. In the present investigation it was found that glucose and protein contents reduced dramatically in the preserved semen of 24 and 48 hrs whereas, lipid content increased significantly. This indicated that metabolic activity in sperm continued throughout the duration of preservation utilising available energy constitutents. Yoo *et al.* (1987) reported the loss of protein from cryopreserved spermatozoa into outer seminal plasma of salmon due to leakage through cell membrane into the outer medium. Similarly in bovine semen, Pickett and Komarek (1964) also showed leakage of lipid into seminal plasma from cryopreserved spermatozoa. However, in the present study, lipid content was found to be high in frozen

seminal plasma of *L. parsia* after 24 and 48 hrs but glucose and protein content declined.

It has been reported that besides energy constituents, osmolarity of the seminal fluid plays a very significant role in activation of sperm (Goodall *et al.*, 1989). They while working on fish *S. ciliata* found that Na^+ and K^+ ions present in seminal fluid helps in activation of sperm and also enhances the duration of motility. In *L. parsia* significant loss in the levels Na^+ and K^+ ions was recorded in seminal plasma preserved for 24 hrs and beyond. Baynes *et al.* (1981) described inhibitory effect of K^+ ions on initiation of motility in salmonid fish. But Goodall *et al.* (1989) mentioned that K^+ only appears to be inhibitory at extremely high levels. In *S. sihama* activation of sperm by 100 % seawater clearly showed that ionic balance is a very important factor for stimulation of sperm.

The long-term cryogenic preservation of sperm has tremendous potentials in the production of selected strains throughout the year and facilitate genetic selection of beneficial strain for intensive commercial farming, thus creating gene bank. Attempts on such studies are limited particularly in marine fishes. Chao *et al.* (1975) could preserve the sperm of *M. cephalus* for a period of 1 year at cryogenic temperature of –196°C but fertilising capacity of such sperm of was reported to be below 3 %. Pruginin and Cirlin (1976) reported some motility in cryopreserved sperm of *M. cephalus* after a period of 2-4 months. In recent years Rana and McAndrew (1989) described that tilapia spermatozoa protected with 12.5 % methanol in fish ringer and stored in 1.5 ml cryotubes and held in a vapour phase liquid nitrogen refrigerated, remained viable for at least 13 months.

The motility of sperm of *L. parsia*, *S. sihama*, *M. cephalus* and *G. oyena* both in normal and oxygenated media was compared. From the results it was found that oxygenation of diluents helps in enhancing not only the duration of motility but also the survival of sperm at cryogenic temperatures. In oxygen rich atmosphere, the storage time of salmonid semen was found to be prolonged (Billard, 1980) while it was reduced under high levels of CO_2 and nitrogen in the media (Buyukhatipoglu and Holtz, 1978). Many workers have noticed that preservation of semen of Atlantic salmon improved when it was stored in a thin layer with a large volume of air above (Stoss and Holtz, 1983). Saad *et al.* (1988) found that in the case of carp, *C. carpio* incubation of semen under oxygen did not produce better results than storage of the same in open air. Probably open air contributed dissemination of oxygen in a better way.

Recently McNivan *et al.* (1993) while working on rainbow trout semen found that the use of fluorocarbon compounds (solubility of oxygen in this compound is 16 times more than water) for storage of semen showed promise for maintaining sperm viability for several weeks.

Ackowledgment

The authors wish to express their thanks to Dr. P. S. B. R. James, former Director, CMFRI, for providing facilities and encouragements.

References

American Public Health Association 1975. *Standard Methods for the Examination of Water and Wastewater*. 14th edition, Washington, USA.

Barnes, H. and J. Blackstock 1973. Estimation of lipids in marine animals and tissue : Detailed investigation of the sulphophosphovanillin method for total lipids. *Exp. Mar. Biol. Ecol.*, **12** : 103-118.

Baynes, S. M., A. P. Scott and A. P. Dawson 1981. Rainbow trout, *Salmo gairdnerii* Richardson, spermatozoa : effects of cations and pH on motility. *J. Fish. Bio.*, **19** : 259-267.

Billard, R. 1980. Survie des gametes de truite arc-en-ciel apres dilution dans des solutios salines ou du sucrose. *Reprod. Nut. Dev.*, **20** : 1899-1905.

Billard, R. 1983. Ultrastructure of trout spermatozoa : Changes after dilution and deep freezing. *Cell Tissue Res.*, **228** : 205-218.

Billard, R. 1988. Artificial insemination and gamete management in fish. *Mar. Behav. Physiol.*, **14** : 3-21.

Buyukhatipoglu, S. and W. Holtz 1978. Preservation of trout sperm in liquid or frozen state. *Aquaculture*, **14** : 49-56.

Chambeyron, F. and Y. Zohar 1990. A diluent for sperm cryopreservation of gilthead seabream, *Sparus aurata*. *Aquaculture*, **90** : 345-352.

Chao, N. H., H. P. Chen and I. C. Liao 1975. Study on cryopreservation of grey mullet sperm. *Aquaculture*, **5** : 389-406.

Erdahl, A. W. and E. F. Graham 1987. Fertility of teleost semen as affected by dilution and storage in seminal plasma mimicking medium. *Aquaculture*, **60** : 311-321.

Goodall, J. A., A. W. Blackshaw and M. F. Capra 1989. Factors affecting the activation and deviation of motility of the spermatozoa of the summer whiting (*Sillago ciliata*). *Aquaculture*, **77** : 243-250.

Gwo, J. C. 1994. Cryopreservation of yellowfin seabream (*Acanthopagrus latus*) spermatozoa (Teleost, Perciformes, Sparidae). *Theriogenology*, **41** : 989-1004.

Harvey, B. 1982. Crybiology and storage of teleost gametes. *In.* : C. J. J. Richter and H. J. Th. Goos (Compliers). *Proceedings of the International Symposium on Reproductive Physiology of fish*, Wageningen, The Netherlands PUDOC, Wageningen, p. 123-127.

Hodgins, J. O. and G. J. Ridgway 1964. Recovery of viable salmon spermatozoa after fast freezing. *Progressive Fish Culturist*, **26** : 95.

Hwang, S. W., H. P. Chen, D. L. Lee and I. C. Liao 1972. Preliminary results in the cryogenic preservation of grey mullet (*Mugil cephalus*) sperm. *J. Fish. Soc. Taiwan*, **1** : 1-7.

Lahnsteiner, F., T. Weismann and R. A. Patzner 1992. Fine structural changes in spermatozoa of the greyling, *Thymallus thymallus* (Pisces : Teleostei) during routine cryopreservation. *Aquaculture*, **103** : 73-85.

Legendre, M. and R. Billard 1989. Cryopreservation of rainbow trout sperm by deep freezing. *Rep. Nutr. Devp.*, **20** : 1859-1868.

Lowry, D. H., N. J. Rosebrough, A. L. Fair and R. J. Randall 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem.*, **193** : 265-275.

McNivan, M. A., R. K. Gallant and G. F. Richardson 1993. Fresh storage of rainbow trout (*Oncorhynchus mykiss*) semen using a non-aqueous medium. *Aquaculture*, **109** : 71-82.

Mims, S. D. 1991. Evaluation of activator solution, motility duration and short term storage of paddlefish spermatozoa. *J. World Aquacul. Soc.*, **22** : 224-229.

Muir, J. E. and R. J. Roberts 1993. Conservation and preservation of genetic

variation. *In : Recent Advances in Aquaculture,* p. 305-337. IV Blackwell Scientific Publication, London.

Nelson, D. H., 1994. A photometric adaptation of the Somogyi's method for the determination of the glucose. *J. Biol. Chem.,* **153** : 373-380.

Pickett, B. W. and R. J. Komarek 1964. Evidence for loss of lipid from bovine spermatozoa due to freezing. *J. Dairy Sci.,* **47** : 905-908.

Pruginin, Y. and B. Cirlin 1976. Techniques used in controlled breeding and production of larvae and fry in Israel. 1973, 90-100. *EIFAC Workshop on Controlled Reproduction of Cultivated Fish.* Hamburg, Tech paper 25 : 90-100.

Rana, K. J. and B. J. McAndrew 1989. The viability of cryopreserved tilapia spermatozoa. *Aquaculture,* **76** : 335-345.

Saad, A. R. Billard, M. C. Theron and M. G. Hollebecq 1988. Short-term preservation of carp (*Cyprinus carpio*) semen. *Aquaculture,* **71** : 133-150.

Scott, A. P. and S. M. Baynes 1980. A review of the biology, handling and storage of salmonid spermatozoa. *J. Fish. Biol.,* **17** : 707-739.

Stein, H. and H. Bayrle 1985. Gameten and Embryonenkonserverung bei Salmoniden Bayer. *Landwirtsch. Jahrb.,* **2** : 236-244.

Stoss, J. 1983. Fish gamete preservation and spermatozoa physiology. *In: Fish Physiology,* D. J. Hoar, D. J. Randall and E. M. Donaldson (Eds.), **9**B, Academic press, New York, p. 305-350.

Stoss, J., S. Buyukhatipoglu and W. Holtz 1978. Short-term cryopreservation of rainbow trout (*Salmo gairdnerii*) sperm. *Annals of Biology : Anim. Biochem. Biophys.,* **18** : 1077-1082.

Stoss, J., L. Gexies and W. Holtz 1987. The role of sample depth in storing chilled rainbow trout (*Salmo gairdnerii*) semen under oxygen. *Aquaculture,* **61** : 275-297.

Stoss, J. and W. Holtz 1983. Successful storage of chilled rainbow trout (*Salmo gairdnerii*) spermatozoa for upto 34 days. *Aquaculture,* **31** : 269-274.

Stoss, J. and. T. Refstie 1983. Short-term storage and cryopreservation of milt from Atlantic salmon and sea trout. *Aquaculture,* **30** : 229-236.

Wheeler, P.A. and G.H. Thorgaard 1991. Cryopreservation of rainbow trout semen in large strains. *Aquaculture,* **93** : 95-100.

Yoo, B. Y., M. A. Ryan and A. J. Wiggs 1987. Loss of protein from spermatozoa of Atlantic salmon (*Salmo salar* L) because of cryopreservation. *Can. J. Zool.,* **65** : 9-13.