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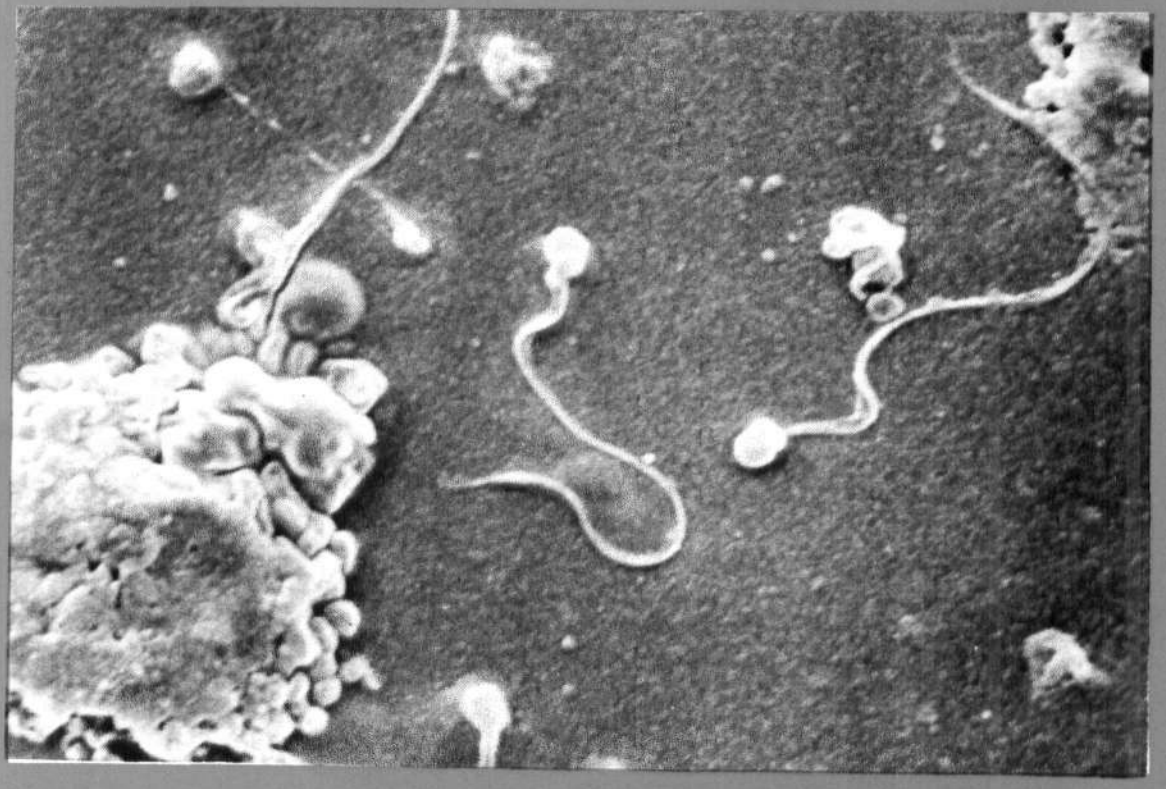

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A STEPPING-STONE IN DEVELOPING MARINE FISH GENE BANK

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The use of cryopreserved sperms in the improvement of R and D programmes of aquaculture/mariculture was emphasized earlier by many workers. Later many attempts have been made on cryopreservation studies in teleost fishes particularly on salmonids and freshwater carps. In India such studies have not gained momentum particularly in fisheries sector. However, cryopreservation techniques if properly planned could be useful in the frontier lines of biotechnology to promote growth and production of animals.

Fig. 1. Scanning electron micrography (SEM) image of the sperms of fish *L. parsia* x 5000.
(Scanning Electron Microscopy work was done at All India Institute of Medical Science New Delhi with help of Prof: G. F. X. DAVID).

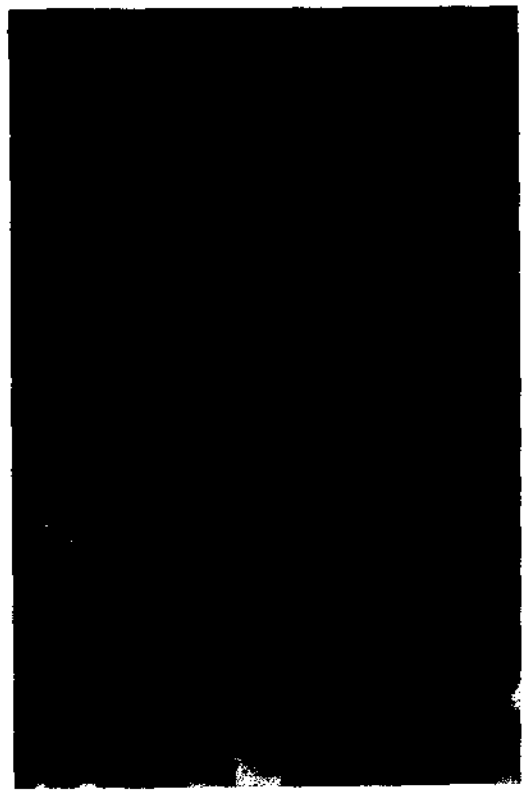

Fig. 2. SEM image of the sperms of fish *M. cephalus* x 2500.
(Scanning Electron Microscopy work was done at All India Institute of Medical Science New Delhi with help of Prof: G. F. X. DAVID).

The history of the science of cryogenic preservation of fertile semen is not only meant for understanding the basic life secret, but also propagating living resources in long run. The developed techniques for the preservation of life and genetic properties at subfreezing temperatures have been successfully attempted in fish spermatozoa. Many remarkable advantages of such applications to animal husbandary, cattle breeding industry and in medical sciences (particularly infertility treatment) have long been demonstrated. It is a fact that the biological response to the freezing process of each indvidual type of sperms varies widely.

In our aquaculture system, for continuous fish seed production one of the major constraints is the non-availability of sufficient spawners at

TABLE 1. *Media used for cryopreservation of sperms*

* Alsever's solution (Hodgins and Ridgeway 1964)		Mixture[B]		Marine Teleost Ringer (Burton 1975)		Mixture[C]	
Sodium citrate	0.08%	NaCl	750 mg	NaCl	231 mM	NaCl	600 mg
($C_6H_5Na_3O7$)		$NaHCO_3$	200 mg	KCl	9 mM	KCl	38 mg
Dextrose	2.05%	Na_2HPO_4	53 mg	CaCl	2.2 mM	$CaCl_2H_2O$	23 mg
NaCl	0.4%	$MgSO_4.7H_2O$	23 mg	$MgCl_2$	3.7 mM	$NaHCO_3$	100 mg
Cryoprotectant	10% DMSO	KCl	38 mg	Cryoprotectants	10% DMSO 15% Glycerine	$NaHPO_4.H_2O$	41 mg
Ratio	1:1	$CaCl_2.2H_2O$	46 mg	Ratio	1:1	$MgSO_4.7H_2O$	23 mg
		Glucose	200 mg			Cryoprotectants	10% 15% Glycerine
		Glycine	500 mg			Ratio	1:1
		H_2O	100 mg				
		Cryoprotectants	10% DMSO				
		Ratio	1:1				

the desired time. In order to solve this problem, efforts are being continuously made world over to evolve suitable methods of obtaining sperms through creation of gamete banks so that through artificial fertilization one will be able to produce the seeds at any time of the year. Success in the development of technology in this area may definitely help in propagation of animals and boosting aquaculture industry in near future.

Preserving viable gametes of cultured fish is desirable as a means of making good any deficiencies of supply, as a means of enabling breeding to occur whether or not maturation of males and females coincides and as a means of establishing a reserve genetic material of known quality for selective breeding programmes. These objectives are in general realized through the storage of sperms and this problem has received most attention in recent past. The storage of ova of cultured species may assume great importance except in instances where gametes of desirable genetic makeup are to be conserved as a means of extending the breeding base. Work in this area is still in the experimental stage only.

For more than a century, scientists have investigated methods to preserve viable gametes of fish. The early studies were directed at prolonging the life of gametes in non-frozen state. Research in recent years has focused on developing cryoprotective techniques for frozen storage of viable spermatozoa. Most of the works in literature relate to fishes of salmonid group or freshwater carps and two or three reports mention about cryopreservation of mullet sperms.

Developments in the cryopreservation of fish sperms depended upon the identification and testing of suitable cryophylactic agents. Reports

TABLE 2. *Changes in some biochemical constituents of fresh and preserved milt of a marine fish* Liza parsia

Bio-chemical constituents		Fresh milt	Preserved milt at —10°C during different time duration		
			6 hrs	24 hrs	48 hrs
*	Glucose (mg %)	31.66 ± 0.904	29.83 ± 1.700	23.46 ± 1.255	18.53 ± 0.776
**	Protein (mg %)	33.80 ±10.136	-	46.25 ± 7.505	37.40± 2.044
***	Lipid (mg %)	38.83 ± 4.790	38.67± 9.404	103.44 ± 5.080	-

* Significant at 1% level between cryo-preserved and fresh.

** Significant at 10% level between 48 hrs cryo-preserved and fresh.

*** Significant at 10% level between 6 hrs cryo-preserved & fresh and 24 hrs cryo-preserved.

(Each value is the average of 4 determinations).

mentioned earlier have stated the use of glycerol as an effective medium in protecting sperm from freeze-thaw damage. Later dimethylsulfoxide (DMSO) has been found to be the most effective protective agent for cryopreserved salmonid sperms. Though extensive studies have been carried out in preserving motility of sperms, the fertilizing ability of the cryopreserved sperms was not tested for many years. The first successful fertilization of freshwater salmonid ova with cryopreserved sperm was reported by Horton and his associates in the year 1967. Later many workers have attempted and succeeded to certain extent in freshwater carps and to a less extent in marine fishes. However, lot need to be explored in this research sector.

The Central Marine Fisheries Research Institute is engaged actively for the past 3 years in cryopreservation studies. Through the extensive research efforts a breakthrough was made in evolving a viable technique for short term and long term preservation of sperm motility of fish *Liza parsia* at —10°C and —196°C temperature respectively. In both the methods, technique involved was the preservation of fresh milt collected from oozing spawner males or by stripping riped males in different diluents alongwith 10% DMSO as a cryoprotectant in different proportions (Table 1). In this methodology while preserving the sperms particularly at — 196°C in liquid nitrogen, standardisation of equilibration time was found to be a very important factor to avoid the effect of temperature shock while effecting sudden cooling on, sperm motility. By using this method, sperm motility could be preserved with greater success and now the motile sperms are available in cryopreserved state in the Institute's laboratory. Among the various diluents tested 15% glycerine alongwith 10% DMSO was found to be the best medium as it could preserve more than 80% motility of the sperms. However, some loss in motility which is likely to occur during equilibration time while preservation could not be prevented.

In short term preservation at —10°C temperature, motility of the sperms could be maintained successfully upto 3 days. To find out the causative factors for some loss of motility of sperms during this 3 days preservation time, investigation was probed to look into some of the biochemical and mineral content of milt. In doing so, some of important energy constitutents like glucose, protein and lipid were analysed in freshly stripped milt and milt preserved for 6, 24 and 48 hrs at —10°C temperature. It was noticed that the glucose and protein content reduced drastically in preserved milt of 24 and 48 hrs whereas lipid content increased significantly (Table 2). Analysis of micro-environment of milt where certain essential ions like Na^+ and K^+ required for motility has been also done. Significant loss in the levels of Na^+ and K^+ ions was noticed in the preserved milt of 24 hrs and beyond (Table 3). The loss of organic constitutents and ions when restored in the preservation media has been found useful in extending the motility of the sperms for longer time at —10°C temperature. The study shows that the motility of the sperms gets affected by some of these factors.

TABLE 3. *Changes in Na^+ and K^+ content in fresh and cryopreserved milt of* Liza parsia

	Fresh milt	Cryopreserved milt of 24 hrs at -10°C
Na^+ (mEq/l)	124.3± 5.4	54.37 ± 4.05
K^+ (mEq/l)	164.4± 7.6	41.56 ± 3.15

Each value is the average of 3 determinations.

The sperms count in *L. parsia* showed an average 9 billion sperms/fish.

Similar studies on preservation of sperms of other marine fishes like *Sillago sihama, Mugil cephalus* and *Gerrus* sp. are being continued. Several samples of fishes were examined to assess the sperm motility. Successful sperm preservation tests were carried out in *Sillago* and *Mugil.*

The sperm morphology of the three different fishes was also investigated through scanning electron microscopy. The studies revealed that morphological features of the sperms of all the three fishes namely *L. parsia,* (Fig. 1) *M. cephalus* (Fig. 2) and *S. sihama* (Front Cover Photo) look similar. The sperms show distinct knob-like head with a long undulated tail. Acrosome formation (cap like structure) is found to be absent here unlike in mammalian sperms. Efforts are also being made to study the viability of the preserved sperms by fertilizing them with the riped ova of spawner female. Further studies are in progress.