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Cryopreservation of spermatozoa in the mullet, *Liza parsia*: Effect of cryoprotectants on morphology and motility

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Abstract

A study was conducted to identify the best cryoprotectant for the spermatozoa of the mullet, *Liza parsia* (Hamilton-Buchanan), during cryopreservation, based on changes in ultrastructure and motility. Various cryoprotectants alone and in combinations, at different concentrations have been tried. Morphology of spermatozoa before and after cryopreservation was studied by transmission electron microscopy (TEM). A combination of 5% DMSO and 5% glycerol in V2E extender accorded better protection than 10% DMSO alone.

Introduction

Cryoinjuries to sperm during cryopreservation procedure are bound to occur. Morphological alterations in the spermatozoa of fish following dilution and freezing have been reported (Gwo, 1995; Yao *et al.*, 1995). Different methods are in vogue to assess viability and fitness of spermatozoa following cryopreservation. Sperm motility test is widely used for this purpose. The percentages of motile cells significantly correlate with the fertilization of seabass and turbot spermatozoa (Suquet *et al.*, 2000). Apart from visual motility, the determination of sperm viability can be assessed by computer assisted cell motility analysis (Lahnsteiner *et al.*, 1996) and analysis of flagellar beat frequency (Cosson *et al.*, 1997). Although the ultimate success of cryopreservation depends on the fertilizing ability of cryopreserved sperms, the assessment of efficiency of preservation based on fertilization experiments may be undependable and impracticable. Ultrastructural study of the extent of injuries on milt suspension can be a suitable alternative to evaluate the effectiveness of cryopreservation (Gopalakrishnan *et al.*, 2000)

The extender V2E was found to be the best cryodiluent for *Liza parsia* for storage in liquid nitrogen (Sandhya Sukumaran, 2001). Post-thaw electron microscopic images revealed that about 60% of the sperms had near normal structure and integrity. The choice of cryoprotectant and its concentration is of great importance in minimising injuries. The optimum concentration may, however, vary depending on the type of cryoprotectants, extender and

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equilibration time used. Toxicity of using high concentrations of cryoprotecants can be minimized by using combinations of cryoprotectants. The present work was taken up to compare the effects of different cryoprotectants at different concentrations and combinations in an ideal extender to assess the ability to prevent injuries during preservation and to determine the optimal concentration of a suitable cryoprotectant for the spermatozoa in *L. parsia*.

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Material and methods

The Gold spot mullet, *L. parsia* was the candidate species selected for the cryopreservation studies. The ripe male fishes were stripped manually and the milt obtained was collected in dry and sterile eppendorf tubes and kept over crushed ice. Care was taken to avoid contamination with blood, urine, scales and faeces during stripping. A convenient scale based on type of motility was adopted (Billard, 1984) and motility scores were given from 0-5. The method was standardised by repeating the process with at least four samples. The milt samples, which exhibited rapid progressive movement (score 5) when mixed with seawater were selected for further studies. The maximum duration of motility obtained when induced with seawater was 5 minutes.

Selection of extender and cryoprotectants:

The extender selected for the present study was V2E (Scott and Baynes, 1980). Sandhya Sukumaran (2001) had identified V2E in sea water base as the best diluent for *L. parsia*. The permeating cryoprotectants, dimethyl sulphoxide (DMSO), glycerol, methanol, propylene glycol and its combinations were used in various concentrations. The final concentrations of cryoprotectants used are given in Table 1. The milt was mixed with the cryodiluent (cryoprotectant dissolved in extender) in the ratio 1:3.

Table 1. *Concentrations of cryoprotectants used*

Sl. No.	Cryoprotectants	Concentration (in per cent)
1	DMSO	7.5, 10, 12.5
2	Glycerol	5, 10
3	Methanol	5, 10
4	Propylene glycol	8, 12
	Combinations:	
5	DMSO and glycerol	5+5
6	Methanol and glycerol	5+5
7	DMSO and propylene glycol	5+5
8	Methanol and propylene glycol	5+5

Experimental protocol: One part of milt was mixed with three parts of cryodiluent and all solutions were kept at 20°C; (b) equilibration time of 10 min. over ice was given including time to fill the diluted milt into 0.5 ml French straws and to seal with polyvinyl alcohol powder; (c) exposed to liquid nitrogen vapour (-120°C) for 5 min and (d) plunging the straws into liquid nitrogen after vapour phase.

The duration of preservation was 3 days in liquid nitrogen. After step 1 and 4, samples were fixed for electron microscopic studies using gluteraldehyde as fixative.

Percentage of live spermatozoa: Nigrosine-eosin stain was used to assess the percentage of live and dead spermatozoa in semen sample (Chao *et al.*, 1975). One drop of milt was mixed with two drops of the stain and a thin smear was prepared on clean, grease free glass slides. Two slides from each semen sample were prepared. The slides were air dried and observed under oil immersion(1000X).

The dead sperms took pink stain while the live spermatozoa appeared clear and transparent. The number of live sperms were counted and expressed in relation to total number of sperms. About 3 to 4 fields were counted.

Post-thaw motility assessment: The cryopreserved samples were thawed by rapidly plunging the straws into water bath at 37 °C for 20 sec. Post-thaw motility of the spermatozoa was assessed immediately after thawing.

Transmission Electron Microscopy (TEM): The samples were processed for transmission electron microscopy (TEM). The stained ultra-thin sections were mounted on grids and observed in TEM mode of Hitachi (H 600) electron microscope and recorded normal and abnormal spermatozoa.

Post-thaw intact spermatozoa: In all the experiments, spermatozoa from each sample were studied utilizing TEM images. The ratios of intact and damaged spermatozoa for each treatment were calculated by counting sperms under low magnification (4000X in TEM). Randomly selected portions of at least three grids were taken for each treatment and observed. TEM images of untreated raw milt served as control.

Results

Percentage of live spermatozoa: The percentage of live spermatozoa in raw milt of *L. parsia* was 90%. The live sperms were clear without any stain whereas dead sperms were pink in colour. The abnormalities and immature sperms if any can be made out to some extent. However, the morphological changes were not very clearly visible in nigrosine-eosin stained smears (Plate 1).

Ultrastructural changes due to cryoprotectants

DMSO

The sperms were uniformly shaped with intact plasma membrane when DMSO was used as a cryoprotectant at concentration of 7.5% zero seconds after dilution with motility score of 4. On cryopreservation and subsequent thawing the chromatin showed more aggregation and granulation compared to the raw milt. The nucleus appeared almost intact but there was clear

disintegration of nuclear membrane. The percentage of intact spermatozoa was reduced from 75% to 44% after freezing. In 10% DMSO about 80% of the spermatozoa exhibited near normal structure with motility score of 4. However, slight disorientation of mitochondria and detachment of mid-piece was observed in spermatozoa treated with 10% DMSO on thawing. When the spermatozoa were exposed to 12.5 % DMSO there was a clear indication of rupture of plasma membrane and presence of several vacuoles in the nucleus. About 62% of the spermatozoa were completely deformed. The motility score decreased to 2 (Plate 2).

Glycerol

Maximum changes were observed in plasma membrane when 5% glycerol was used as cryoprotectant. Clear disintegration and rounding up of nuclear material was observed. The flagellum was found intact. Almost 64.8% sperms became abnormal when treated with 5% glycerol and 90% sperms were found deformed when subjected to cryopreservation (Plate 3). On treating with 10% glycerol in zero seconds after dilution the disintegration of chromatin structure was observed in 37.6% of the sperms. The spermatozoa (66.2%) exhibited structural changes and became altogether ruptured. The plasma membrane exhibited clear undulations in frozen thawed spermatozoa.

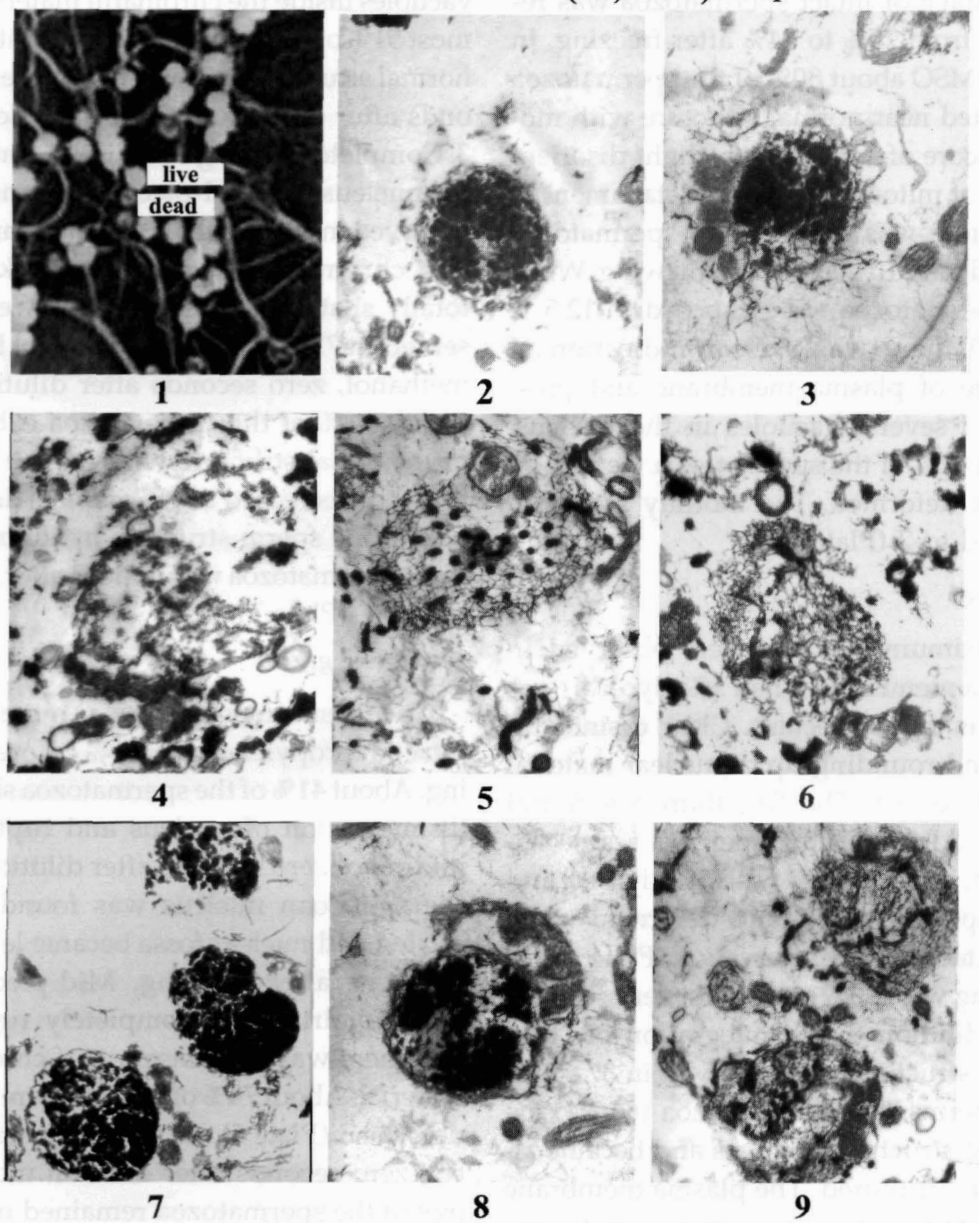
Methanol

In 5% methanol the plasma membrane was ruptured and mid-piece totally damaged even before freezing. The flagella got separated and nucleus exhibited many vacuoles inside the chromatin material. Almost 51% of the spermatozoa exhibited abnormal structure in 5% methanol, zero seconds after dilution with motility score of 3. Complete swelling and disintegration of the nucleus and mid-piece of sperms were observed in 10% methanol treated samples. The chromatin appeared web like and totally shattered. Such changes were observed in 77% of the spermatozoa. In 10% methanol, zero seconds after dilution almost 46.8% of the spermatozoa exhibited near normal structure whereas the motility score decreased to 1. Complete destruction of the sperm structure in almost 82% of the spermatozoa was noticed after freeze-thawing (Plate 4).

Propylene glycol

The plasma membrane was more or less intact in propylene glycol 8% before freezing. About 41% of the spermatozoa showed disintegration of nucleus and rupture of mid-piece zero seconds after dilution. The spermatozoan nucleus was found to be dilated and nuclear fossa became less conspicuous after freezing. Mid-piece and mitochondria were completely ruptured and there was condensation of chromatin material. About 74% of sperms were highly deformed (Plate 5). In 12% propylene glycol, zero seconds after dilution morphology of the spermatozoa remained more or less unchanged. Swelling in the plasma membrane of head, mid-piece and tail region with rupture of mitochondria was observed in 73.7% of spermatozoa after freeze-thawing and motility score of spermatozoa decreased to 2.

TRANSMISSION ELECTRON MICROSCOPIC IMAGES OF SPERMATOZOA (25,000 x) - *L. parsia*



Plates

1. *Dead and live spermatozoa-live ones appear clear and dead darker*
2. *DMSO 12.5% - Rupture of plasma membrane and vacuoles in nucleus*
3. *Gycerol 5% - Disintegration and rounding up of nuclear material*
4. *Methanol 10% - Complete destruction of spermatozoan structure*
5. *Polypropylene glycol 12% - Swelling of plasma membrane with rupture of mitochondria*
6. *Methanol 5% + Propylene glycol 5% - Plasma membrane ruptured with scattered nuclear material*
7. *DMSO 5% + glycerol 5% - Intact plasma membrane and mitochondria without much disintegration*
8. *Glycerol 5% + propylene glycol 5% - Damage and complete destruction*
9. *DMSO 5% + methanol 5% - Chromatin clumped and hypertrophied*

Methanol and propylene glycol

Nucleus was observed to be intact but plasma membrane appeared ruptured immediately after dilution with methanol 5% + propylene glycol 5%. The percentage of damaged spermatozoa was 69.7%. Plasma membrane was totally disrupted with scattering of nuclear material (Plate 6). The percentage of abnormal spermatozoa increased to 92% after freeze thawing.

DMSO and glycerol

Mitochondria and plasma membrane were intact in 86% of the spermatozoa when DMSO 5% and glycerol 5% were used as cryoprotectants. Condensed nucleus with clear nuclear fossa was present. Chromatin was coarsely granular and shape of spermatozoa was uniform in zero seconds after dilution. The motility score of spermatozoa immediately after dilution was 5. The spermatozoa (55.6%) were normal with intact plasma membrane. Mitochondria appeared near normal without much disorientation. Nucleus was dense and bilobed or kidney shaped after freeze thawing (Plate 7).

Glycerol and propylene glycol

Zero seconds after dilution (5% glycerol + 5% propylene glycol), the sperms were found to be damaged. About 86.4% of sperms became damaged and complete destruction was noticed in frozen thawed spermatozoa (Plate 8).

DMSO and methanol

Sperms exhibited near normal structure (30%) with clear nuclear fossa and condensed chromatin material before freezing (DMSO 5% + methanol 5%). After freeze thawing, complete destruction of the bilobed nucleus with highly disrupted plasma membrane was observed. The spermatozoa were almost immotile with a score of 0.5. Chromatin became clumped and hypertrophied. Almost 95% of spermatozoa exhibited abnormal structure (Plate 9).

Discussion

Cryoprotectants have multiple roles. They mainly bind to water molecules and reduce ice crystal formation and also help to stabilise hydrated protein molecules (Jamieson,1991). The ability of cryoprotectants to accord protection from cryoinjuries varies with species, concentration of the protectant extender used, protocol followed and so on. Cryoprotectants are toxic in high concentrations and may cause structural damages resulting in lowered fertility. The ultrastructural observations of spermatozoa are the most reliable method to study the morphological changes. On the basis of the degree of protection accorded from cryoinjuries, a cryoprotectant can be culled or selected for use. A study of changes will give an insight into the acceptability of cryoprotectant and optimal concentration requirement. Structural changes in spermatozoa following dilution and deep-freezing have been reported in several fish species (Billard 1978, 1983; Gwo and Arnold, 1992; Diwan and Nandakumar, 1998; Gopalakrishnan *et al.*, 2000). The sensitiveness of spermatozoa to osmotic pressure may cause morphological alterations including rupture of membrane, swelling and disruption of mid-piece

(Billard, 1983). A good extender-protectant combination (diluent) will keep the structural integrity of spermatozoa at the time of dilution, during the freezing protocol and finally at the thawing process. The percentage of intact spermatozoa (Fig. 1) was highest (86) in DMSO 5%+glycerol 5% followed by DMSO 10% (81) and lowest in DMSO 5%+methanol 5% combination (30). Very fast permeability may be the reason for extensive damages in DMSO-methanol combination while the balanced action DMSO-glycerol combination has provided the best protection. The motility of the spermatozoa also gradually decreased in these treatments (Fig. 2). Fish sperm motility is closely related to the existence of mitochondria in mid-piece (Gwo, 1995). The morphology of spermatozoa did not differ significantly in undiluted milt and sperms diluted with DMSO+glycerol (5+5%). In 10% DMSO+V2E the structural damages were to a lesser extent on dilution.

Maximum cryoinjuries occur during the freezing phase. Exposure to liquid nitrogen vapours and subsequent freezing followed by thawing can cause cold shock to biological systems (Jamieson, 1991). DMSO is rated as a better cryoprotectant for most of the cells due to its fast penetration. On treating the sperms with 7.5% DMSO+V2E and upon freeze thawing the nucleus appeared almost intact but the chromatin showed more granulation compared to the untreated spermatozoa. About 57% of the spermatozoa showed structural changes with several vacuoles in the nucleus and the whole structure exhibited signs of total disintegration in freeze thawed spermatozoa treated with 12.5% DMSO. Maximum structural changes were noticed in DMSO+methanol (5+5%) with V2E. Few spermatozoa also exhibited abnormal structure. Shrinkage of the plasma membrane of the mid-piece was reported for frozen thawed spermatozoa of the Ocean Pout

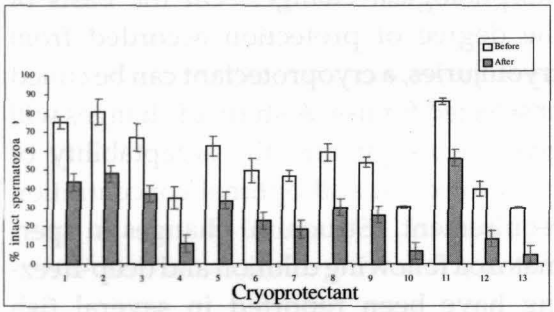


Fig. 1. *Intact spermatozoa of* L. parsia *in different cryoprotectants before and after cryopreservation*

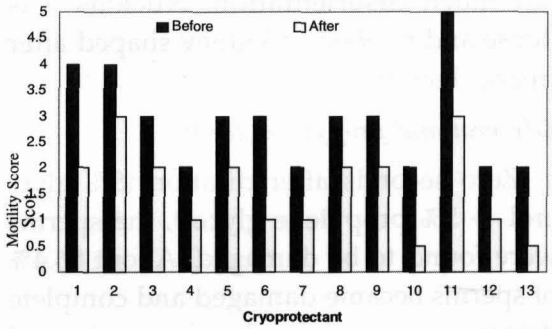


Fig. 2. *Motility score of* L. parsia *spermatozoa in different cryoprotectants before and after cryopreservation*

Cryoprotectants 1: DMSO 7.5%; 2 : DMSO 10%; 3: DMSO 12.5%; 4: Glycerol 5%; 5: Glycerol 10%; 6: Methanol 5%; 7: Methanol 10%; 8:Propylene glycol 8%; 9: Propylene glycol 12%; 10: Methanol + Propylene glycol (5%+5%); 11: DMSO + Glycerol(5%+5%); 12: Glycerol + Propylene glycol (5%+5%); 13: DMSO+ Methanol (5%+ 5%).

(Yao *et al.*, 1995). In frozen thawed Atlantic croaker spermatozoa it was observed that the cristae of mitochondria were disrupted, the plasma membrane swollen and the axoneme coiled (Gwo and Arnold, 1992). Methanol was relatively very poor in according protection both in 5 and 10% concentrations. Frozen thawed spermatozoa treated with 10% methanol with V2E exhibited complete destruction of the spermatozoal structure in almost 82% of the spermatozoa. Even 5% methanol exhibited poor results with 51% abnormal spermatozoa. Within the spermatozoa, probably the plasma membrane is the site most susceptible to damage due to water flux during freezing and thawing (Baynes and Scott, 1987). Almost 66% of the spermatozoa exhibited structural changes and became altogether ruptured. The plasma membrane exhibited clear undulations in frozen thawed spermatozoa treated with 10% glycerol.

In the present investigation, combination of DMSO and glycerol (5+5%) with V2E exhibited fairly good results with almost 55.6% of intact spermatozoa. 10% DMSO in V2E also accorded significant protection with 53% of the spermatozoa exhibiting normal structure after freeze thawing. The study indicated the superiority of combination of dimethyl sulphoxide (DMSO) and glycerol over other cryoprotectants used for *L. parsia* spermatozoa. Though DMSO is rated as a better cryoprotectant owing to its faster penetrability, toxic effects may appear at higher concentrations. Glycerol is least toxic to biological material but also less permeable to the cell membrane and hence takes longer time to equilibrate with glycerol osmolality. Because of the gene activation properties of DMSO (Ashwood-Smith, 1986), possibility of genetic effects in fish cryopreservation cannot be ruled out. Hence any attempt to reduce the concentration of DMSO without sacrificing the viability of spermatozoa will be a desirable one.

The study has succeeded in reducing the concentration of DMSO by combining glycerol with DMSO as cryoprotectant. The concentration of 5% DMSO+5% glycerol maintained the viability of cryopreserved spermatozoa better than 10% DMSO. This not only reduced the possible harmful effects of DMSO but also combined the favourable attributes of both DMSO and glycerol.

References

Ashwood-Smith, M. J. 1986. The cryopreservation of human embryos, *Human Reproduction*, **1**: 319-332.

Baynes, S. M. and A.P. Scott. 1987. Cryopreservation of rainbow trout spermatozoa: The influence of sperm quality, egg quality and extender composition on post thaw fertility. *Aquaculture*, **66**: 53-67.

Billard, R. 1978. Some data on gametes preservation and artificial insemination in teleost fish. *Actes de colloques du* CNEXO., **8**: 59-73.

------, 1983. Ultra structure of trout spermatozoa: changes after dilution and deep freezing. *Cell Tissue Research*, ***228:***205-218.

------, 1984. La conservation des gametes et l' insemination artificielle chez le bar et la daurade, *In*: (Barnabe, G. and Billard, R. (Eds.) L' *Aquaculture du Bar et des sparides*. INRA Publications, Paris: 95-166.

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

Cosson, J., R. Billard, C. Cibert, C. Dreanno, O. Linhart and M. Suquet. 1997. Movements of fish sperm flagella studied by high speed vidieomicroscopy coupled to computer assisted image analysis. *Polskie Archiwum Hydrobiologii*, **44**: 103-113.

Diwan, A. D. and A. Nandakumar. 1998. Studies on cryogenic preservation of sperm of certain cultivable marine fishes. *Indian. J. Fish.*, **45**(5): 387-397.

Gopalakrishnan, A., A. G. Ponniah and Kuldeep, K. Lal. 2000. Fine structural changes of rohu (*Labeo rohita*) sperm after dilution with cryoprotectants, *Ibid*, **47**: 21-27.

Gwo, J. C. 1995. Ultra structural study of osmolality effect on sperm of three marine teleosts. *In:* Goetz, F. W and P. Thomas (Eds.). *Proceedings of the Fifth International Symposium on the Reproductive Physiology of fish.* University of Texas, Austin, pp 119.

------ and C. R.Arnold. 1992. Cryopreservation of Atlantic croaker spermatozoa: evaluation of morphological changes. *J. Exp. Zool.*, **264**: 444-453.

Jamieson, B. G. M. 1991. Fish evolution and systematic evidence from spermatozoa. Cambridge University Press, 167 pp.

Lahnsteiner, F., B. Berger, T. Weismann and R. A. Patzner. 1996. The influence of various cryoprotectants on semen quality of the rainbow trout, (*Onchorynchus mykiss*) before and after cryopreservation. *J. Applied Ichthyology*, **12**: 99-106.

Sandhya Sukumaran. 2001. Cryodiluents and morphological changes in the spermatozoa of fish *Liza parsia* (Hamilton- Buchanan). *M. F. Sc. Dissertation*, CIFE, Mumbai, 50 pp.

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.

Suquet, M., C. Dreanno, C. Fauvel, J. Cosson and R. Billard. 2000. Cryopreservation of sperm in marine fish. *Aquaculture Research*, **31**: 231-243.

Yao, Z., L. W. Crim, G. F.Richardson and C. J Emerson. 1995. Cryopreservation, motility and ultra structure of sperm from the Ocean Pout (*Macrozoarces americanus*. L.) an internally fertilizing marine teleost. *In*: Goetz, F. W and P. Thomas (Eds.). *Proceedings of the Fifth International Symposium Reproductive Physiology of Fish.* University of Texas, Austin, Texas, p. 149.