CRYODILUENTS AND MORPHOLOGICAL CHANGES IN THE SPERMATOZOA OF FISH *LIZA PARSIA* (HAMILTON-BUCHANAN)

DISSERTATION SUBMITTED
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF FISHERIES SCIENCE
(MARICULTURE)

OF THE
CENTRAL INSTITUTE OF FISHERIES EDUCATION
(DEEMED UNIVERSITY)

MUMBAI-400 061

by

Sandhya Sukumaran
(Mari. 51)

CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
INDIAN COUNCIL OF AGRICULTURAL RESEARCH
COCHIN - 682 014
INDIA.

JUNE 2001

DEDICATED TO
MY PARENTS

भा कृ अनु प
ICAR

केंद्रीय समुद्री मात्स्यिकी अनुसंधान संस्थान
पोस्ट बॉक्स सं 1603, एरणाकुलम, कोचीन-682 014

CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
POST BOX No. 1603, ERNAKULAM, COCHIN-682 014
(भारतीय कृषि अनुसंधान परिषद)
(Indian Council of Agricultural Research)

Phone (Off) : 394867/....Ext. 391407
Telegram : CADALMIN EKM
Telex : 0885-6435 MFRI IN
Fax : 0484-394909
E-mail : mdcmfri@md2.vsnl.net.in

Dated: 30 June 2001

CERTIFICATE

Certified that the thesis entitled **"CRYODILUENTS AND MORPHOLOGICAL CHANGES IN THE SPERMATOZOA OF FISH *LIZA PARSIA* (HAMILTON-BUCHANAN)"** is a record of independent bonafide research work carried out by **Ms. Sandhya Sukumaran** during the period of study from September 1999 to August 2001 under our supervision and guidance for the degree of **Master of Fisheries Science (Mariculture)** and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

Major Advisor/Chairman

(D. Noble)
Senior Scientist
PNP Division
CMFRI

Advisory committee

(A. Gopalakrishnan)
Scientist (Senior Scale)
NBFGR, Cochin unit

(N. K. Sanil)
Scientist
PNP Division
CMFRI

DECLARATION

I hereby declare that the thesis entitled **"CRYODILUENTS AND MORPHOLOGICAL CHANGES IN THE SPERMATOZOA OF FISH *LIZA PARSIA* (HAMILTON-BUCHANAN)"** is an authentic record of the work done by me and no part thereof has been presented for the award of any degree, diploma, associateship, fellowship, or any other similar title.

Sandhya.S.

30 June 2001
Cochin

(Sandhya Sukumaran)
M. F. Sc. Student
Central Marine Fisheries
Research Institute

ACKNOWLEDGEMENTS

I sincerely acknowledge my extreme sense of gratitude to my guide **Dr. D. Noble**, Senior Scientist for his inspiring guidance, timely help and advise, to successfully carry out this work. My sincere thanks go to my co-guide **Dr. A. Gopalakrishnan**, Scientist (Senior Scale) for the ideas, generous suggestions and helps given by him which were valuable for the successful completion of this work. I am extremely indebted to **Shri. N. K. Sanil**, Scientist, who is also my co-guide for all his wholehearted efforts for getting the electron micrographs needed for this thesis, at the right time.

I am also grateful to **Dr. Mohan Joseph Modayil**, Director, CMFRI for providing me with the facilities to carry out the work in the institute. I express my sincere thanks to **Dr. R. Paul Raj**, O.I.C, Post graduate Programme in Mariculture for his helps and encouragements throughout the M. F. Sc. course.

My deep-felt gratitude goes to **Mr. M. Ayyappan Pillai**, Technical Officer, CMFRI, who was instrumental in doing the electron mocroscopic works which were of immense help to me when I was really running short of time.

The timely supply of Liquid Nitrogen by **Kerala Livestock Development Board Ltd.** is also duly acknowledged.

I am also indebted to **Dr. Reeta Jayasankar** and **Dr. L. Krishnan** for all the hatchery facilities made available during the course of this work. Words cannot express my deep-felt gratitude to **Dr. K. S. Sobhana** who was a constant source of encouragement and help to me.

My sincere thanks go to **Mr.Prashanth, Mr. P.M. Muneer, S.R.F** and **Mr. K. Musammilu, S. R. F** for all the helps extended by them.

I am also indebted to **Ms.Nisha, S. R. F., Mr. Unnikrishnan, S.R.F., and Mr. Anikkuttan (Ph.D , Scholar), Mr. Ramalinga (Ph.D Scholar)** ,and **Ms. Juliet** for all the helps.

My deep felt gratitude also goes to **Mr. Rajanna (M.F.Sc.Second semester)** who readily helped me whenever I needed.

I express my thanks to all my **classmates,**especially **Kiran, Subodh, Chandrasekhara Rao, Laxmikant and Liya** for all the helps and moral support to carry out this work. I also express my thanks to my room-mate, **Mini Thomas** who encouraged me a lot.

I acknowledge my sincere thanks to **I.C.A.R.**, New Delhi, for awarding me with the Junior Research Fellowship throughout the tenure of my M. F. Sc. course.

Last but not the least I express my extreme gratitude to my **parents** to whom this work is dedicated.

सारांश

अत्यधिक पालन शक्यता वाली पृथुलवणी मछली लिज़ा पारसिआ के बिजाणुओं का निम्नतापपरिरक्षण (क्रयोप्रिसरवेशन) करने पर होने वाली चोट पर जानकारी प्राप्त होने तथा सबसे अच्छा निम्नतापतनुकारी यानी क्रयोडायलुएन्ट पहचानने का प्रयास इस अध्ययन में किया गया है.

ट्रान्समिशन इलक्ट्रोन माइक्रोस्कोपी (टी ई एम) द्वारा द्रव नाइट्रजन में -196°c तापमान में विभिन्न स्तरों में बीजाणु का शीतीकरण करने पर निम्नतापतनुकारी द्वारा बीजाणु के आकृतिक परिवर्तन तथा हानियों पर परासंरचनात्मक अध्ययन किया गया.

इस तरह अध्ययन करने पर वी 2 ई विस्तारक (एक्स्टेन्डर) शुक्राणु को अधिक रक्षण प्रदान करनेवाला, कम हानिकारक और मानक ताप में अधिकतम पश्च हिमद्रवण गतिशीलता वाला डायलुएन्ट देखा गया. अत: वी 2 ई विस्तारक (एक्स्टेन्डर) लिज़ा पारसिआ के शुक्राणु के लिए उत्कृष्ट निम्नतापतनुकारी माना जाता है. इस परिणाम की पुष्टि के लिए और भी उर्वरता परीक्षण आवश्यक हैं.

ABSTRACT

An attempt has been made to get an insight into cryoinjuries that occur during the process of cryopreservation and to identify the best cryodiluent for the milt of *Liza parsia*, one of the euryhaline fishes of immense culture potential. The morphological changes or damages caused by the cryodiluent at various stages of sperm preservation in liquid nitrogen at -196^{0}C were studied at ultrastructural level by Transmission Electron Microscopy (TEM). V2E+DMSO diluent accorded maximum protection to the spermatozoa and inflicted least damages, retaining maximum post-thaw motility. Hence, V2E+DMSO appear to be the best cryodiluent for the milt of *Liza parsia*. The present findings need further confirmation with actual fertility trials.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

INTRODUCTION

1. INTRODUCTION

For more than a century, scientists have investigated methods to preserve gametes of fish. The early studies were directed at prolonging the life of gametes in non-frozen state. With the advances in the field of Cryobiology, the focus has shifted to develop cryoprotective techniques for preservation of viable gametes in frozen condition at $-196^{0}C$ in Liquid Nitrogen. Most of the work in literature relate to fishes of Salmonid group or freshwater species. Not much work has been reported on marine cultivable fishes of commercial importance like seabass, mullets, seabreams etc.

Developments in the cryopreservation of fish sperms depend upon the identification and testing of suitable cryodiluents. Undiluted sperms cannot withstand the rigours of freezing and thawing. They must be diluted with a suitable extender which is a solution of balanced salts and sometimes organic compounds. Although there have been many extender media reported in literature, it has been claimed that there is little difference in post-thaw fertility between them, while some workers claim superiority of certain extenders.

When biological cells are cooled in an aqueous solution, both cells and solution super cool to some extent. Then heterogeneous nucleation takes place usually in the extracellular solution. As water is frozen out the extracellular solution becomes progressively more concentrated. If cooling rate is low there is sufficient time for the cells to lose enough water to remain in osmotic equilibrium with the concentrating extracellular solution. This causes dehydration. If cooling rate is high, there is insufficient time for water to diffuse out of the cells to the ice crystal. The cells will equilibrate by intracellular freezing initiated either by homogenous or heterogeneous nucleation. Intracellular freezing is also fatal.

A balanced situation may exist which allows survival when the cooling rate is high enough to minimise the time of exposure to concentrated

solutions and yet is low enough to minimise the intracellular ice below a damaging level. Certain chemicals can increase the dimensions of this balance between the effects of intracellular ice and concentrated solution thus improving survival. These chemicals are termed cryoprotectants. There are permeating and non-permeating cryoprotectants. Dimethyl sulphoxide(DMSO) is a permeating cryoprotectant while glycerol is a non permeating one. Cryoprotectants suppress most of the cryoinjuries and the effectiveness of cryoprotectants in suppressing cryoinjuries varies at different concentrations and along with different extenders. The prevention of gamete activation is most critical in both short and long-term storage as fertility declines soon after sperm activation. Reduction of fertility because of loss of motility by exhaustion has been postulated. DMSO at concentrations of 5-20% has been used successfully for cryopreservation of fish spermatozoa and appears to be better cryoprotectant than glycerol or methanol. Ten percent DMSO appears to be the optimal level.

The ultimate success of cryopreservation depends on the fertilising ability of cryopreserved sperms. Apart from the extender and cryoprotectant, standardisation of equilibration time is also found to be an important factor to avoid the effect of temperature shock on sperm viability. Although cooling rate is the most critical factor in cryopreservation, it is the least standardised variable in fish sperm cryopreservation studies and no result yet on the theoretical optimum-freezing rate. Hence, sperm injuries are bound to occur.

Despite advances to date, at best, only 30-50% of the spermatozoa retain their post-thaw motility and have little insight into when the damage occurs. It is imperative to study the structural changes of the spermatozoa in order to arrive at the right extender, cryoprotectant, equilibration time and freezing rate to retain post-thaw sperm fitness and fertility. Hence the present work is taken up to study the morphological changes in the spermatozoa of the brackishwater fish *Liza parsia* (Hamilton-Buchanan, 1822) using Transmission Electron Microscopy (TEM) in different diluents with 10% DMSO as a cryoprotectant during various stages of cryopreservation protocol.

Liza parsia is a smaller variety of mullet with restricted distribution commonly found in south west coast of Indian peninsula south of Bombay. In Kerala they are found mainly in Vembanad and Kayamkulam lakes. The maximum size attained by the species is 330 mm. The size at first maturity is found to be 120 mm in males and 129 mm in females. Females are the dominant sex.

Liza parsia is having immense culture potential and a preferred table fish commanding premium price in the market. Identification of a cryodiluent that accord maximum protection to the spermatozoa of *Liza parsia* will greatly enhance the farming potential of this species by ensuring constant supply of 'seed' for culture. Hence the present work has been taken up.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The history of cryogenic preservation of gametes is a long one. The application of the same for fish sperms is of recent origin. Studies on the cryopreservation of milt of marine and brackish water fishes are limited (Chao *et al.*, 1975). Since the first work of Blaxter in 1953, fish sperm cryopreservation has been attempted on about 30 marine species (Jamieson, 1991). Compared with freshwater species, a high percentage of spermatozoa survives *cryopreservation in marine fishes* (Suquet *et al.*, 2000).

Dilution of milt with cryodiluents, freezing and subsequent thawing result in structural changes such as deformed nucleus and acrosome and rupture of membranes in the spermatozoa of various teleosts including rainbowtrout, Atlantic croaker and grayling (Billard, 1983). Although cellular damage during cryopreservation of freshwater fish spermatozoa has been reported in several studies, there is a lack of correlation between this damage and the fertility rates of eggs with post thawed milt (Cabrita *et al.*, 1998).

The ultra-structural images of fish sperm are useful in (1) preliminary screening and selection of suitable cryodiluents for freezing. (2) determining the quality and freezing ability of milt (3) predicting post – thaw sperm fitness and fertilizing ability (Gopalakrishnan *et al.*, 2000).

2.1 SPERM STRUCTURE AND MILT COMPOSITION

Mullet spermatozoan is composed of a head part measuring 2.3micron X1.4 micron and a tail part four to five times as long as head. There were about 5.3^{10} sperms in each ml of semen and the pH value of mullet semen is 7.4 on the average (Chao *et al.*,1975) . The biochemical constituents of mullet

semen are glucose 31.66 %, protein 33.8%, lipid 38.83%, sodium 124.3 +/- 5.4 and potassium 164 .4+/- 7.6 m Eq / l in fresh milt (Diwan and Nandakumar, 1992).

The sperm of mugiloids (Liza and Galeoids) studied are similar. (Fig.1), described by Jamieson (1991).

2.1.1. Nucleus: The nucleus, bilobed or kidney shaped in longitudinal section is tilted relative to the axoneme. The chromatin is very coarsely granular. In *Liza dumerilii* a dense body has been observed lying in contact with the nucleus in some sperm; the nuclear and overlying plasma membrane covering the ventral and dorsal tip of the nucleus forms bulbous evaginations containing chromatin.

2.1.2. Mitochondria: Four sub spherical mitochondria are arranged in a ring but eccentrically around the base of the axoneme from which they are seperated by a long cytoplasmic canal.

2.1.3. Centrioles: Two fully developed centrioles are present. Despite the eccentric emergence of the flagellum, the proximal centriole and in one plane the distal centriole lies in the nuclear fossa.

2.1.4. Axoneme: Two classes of flagella were observed. Eighty percent varied between 30 and 43 micrometers. in length, while the remainder, considered to indicate abnormal sperm, were much shorter and varied in length (21-30 micrometers).

2.2 CRYOPRESERVATION OF SPERMATOZOA

Sperm motility was preserved for up to 23 days in the case of raw milt at 5^{0}C (Chao *et al.*, 1975). Undiluted milt with or without a cryoprotectant added directly to it is not suitable for

freezing. Milt must be diluted with a suitable extender (Scott & Baynes, 1980; Gopalakrishnan *et al.*, 1999). Motility was used to evaluate the quality of raw milt and to verify the effect of *extender – cryoprotectant combination on pre-freezing and frozen* thawed samples (Chao *et al.*, 1986) .The intensity of motility decreased when the osmotic pressure of diluent was reduced, and was zero or significantly inhibited when osmotic pressure of diluent was close to that of fish seminal plasma (Chamberyon & Zohar,1990). A diluent in which the sperm remains immotile would always be preferable for cryopreservation (Jamieson, 1991; Gopalakrishnan *et al.*, 2000).

Ability of the diluent used for cryopreservation to maintain the spermatozoa in quiscent state is a critical requirement as activation prior to cryofreezing can result in loss of capacity to fertilize (Jameson, 1991). Sperm cell density, motility and viability are the potential predictors related to fertility of sperm after cryopreservation (Paul Wheeler and Thorgaard, 1991). Milt of European catfish stripped into an immobilizing solution was successfully cryopreserved after a stepwise freezing and fast thawing procedure (Linhart *et al.*, 1993). Sperm motility is a routine indicator of the fertilization ability of fresh semen (Babiak *et al.*, 1994). The motility of frozen thawed semen is a reliable parameter for quality determination since there is a statistically greater correlation with post- thaw fertilization rate (Lahnsteiner *et al.*, 1995). Sperm suspended in extender solution retained motility significantly longer than did sperm in intact testis (Michael christensen & Terrence Tiersch, 1996). In case of *Liza parsia* sperm motility could be preserved successfully for more than 240 days using a suitable cryoprotectant (Diwan & Nandakumar, 1998). An increase in viable motile spermatozoa to egg would result in better fertilization and hatching of paddle fish (George Brown

and Steven Mims, 1999). The extenders used for cryopreservation of spermatozoa of various cyprinid and salmonid fishes were found to be unsuitable for *Tenualosa ilisha* as they activated the sperm on dilution (Kuldeep Lal *et al.*, 1999). Osmolality of seminal plasma and concentrations of sodium, potassium and magnesium ions had low variability, which suggests that they are important for creating a stable environment for sperm storage in sperm duct (Glogowski *et al.*, 1999).

Cryoprotective agents are needed at the ultra low temperature of preservation in liquid nitrogen (Chao *et al.*, 1975). DMSO repeatedly provides the best protection of salmonid spermatozoa from damage by freezing (Horton and Ott, 1976; Gopalakrishnan *et al.*, 1999). Glycerol and DMSO bind electrolytes thereby preventing these substances from concentrating in the residual unfrozen solution in and around the cell during freezing (Scott and Baynes, 1980). No fertilization was obtained when methanol was the cryoprotectant (Gwo *et al.*, 1991). The mechanism of cryoprotection of fish sperm by DMSO lies in its fast penetration into the sperm and increase of osmotic pressure of the sperm in Atlantic croaker, *Micropogonias undulatus*(Zhang *et al.*, 1994). Methanol and DMSO have proven most satisfactory as cryoprotective agents, often yielding post - thaw motility value of greater than 40% in many species (Terrence Tiersch, 1995). Studies in turbot spermatozoa showed that a high rate of success obtained in a sucrose solution with 10% DMSO and 10% egg yolk (Olvidio Chereguini *et al.*, 1997). In tilapia spermatozoa the optimum concentrations of cryoprotectant glycerol was found to be between 10 to 20 %, while maximum post-thaw motility of spermatozoa was observed in 20% concentration (Rexaline Sumathi and Sukumaran, 1998). Spermatozoa frozen with a combination of an intracellular cryoprotectant (methanol) and an extracellular

cryoprotectant (skim milk) produced no fertilization in blue catfish (Bart *et al.*, 1998). Fertilization and larval hatch rates were higher for milt frozen with a diluent containing DMSO than containing glycerol in striped trumpeter, *Latris lineata*(Ritar *et al.*, 1999). Forty percent post- thaw motility was achieved using a cryodiluent of 5% glucose as an extender and 5% glycerol as a cryoprotectant in African catfish sperm cryopreservation (Urbanyi *et al.*,1999). Presence of DMSO in milt extender was essential for protecting the sperm from dying during freeze and thaw and 20% of DMSO yielded the highest post thawed sperm motility of 20-25 % of the total cells (Yao *et al.*, 2000).

Most workers use a dilution ratio of one part semen to 3-4 parts extender (Scott & Baynes, 1980). The post-thaw fertilisation rates decrease at dilution ratios of less than 3 fold in *Onchorhynchus mykiss* and of less than five to seven fold in *Salmo trutta fasciatre* and *Salmo trutta fario* which demonstrates that the optimal dilution rate must not exceed a critical value estimated to 2.1 to 2.5x10^8 spermatozoa/ml. dilutes. Higher cell concentrations in the extender significantly decrease the post-thaw fertility of milt. lower sperm concentrations do not affect its post-thaw fertility (Weismann *et al.*, 1995).

Equilibration time should be kept to a minimum (minutes to less than one hour) to avoid exhaustion (Billard, 1978; Chao *et al.*, 1975). The rate of cooling is a critical variable during cryopreservation (Scott & Baynes, 1980). Optimal cooling rate is 30^{0}C per minute (Harvey, 1982). French straw method was designed to speed up the collection, freezing and distribution of sperm (Scott and Baynes, 1980). Fresh semen diluted with cryoprotective agents was dispensed into 0.5 ml straws which were then sealed. These pre-treatments prior to cryopreservation had to be done within the correct equilibration time of the

hours or less. The optimum effect of cryoprotective agents was found with 6-10 % glycerine /DMSO at 1:1, 1:5, 1:10 dilution. In this solution both good motility and fertility before freezing and cryoprotection were obtained (Chao et al., 1985).

Fertilizing ability of previously frozen and thawed rainbow trout sperm was high when pH was 7-8 prior to addition of DMSO and semen. Fertilization rates of eggs from individual females with frozen thawed sperm were found to be significantly different (Snow and Holtz ,1981).

2.3 MORPHOLOGICAL CHANGES OF FISH SPERM

Cryoinjuries occure to biological material during cryopreservation. In trout sperm morphological changes occur when they are diluted in fresh water. Within seconds the plasma membrane around the head becomes swollen and blebbs form at the insertion of flagellum and midpiece. The flagellum and subsequently becomes coiled up within its membrane. These changes are due to rapid uptake of water, as a consequence of the osmotic imbalance of the sperm and the medium. This is evidenced by the fact that physiological salines result in less pronounced changes (Billard, 1978).

In rainbow trout and brown trout in thawed spermatozoa after cryopreservation considerable ultra-structural alterations was observed in chromatin structure (Billard, 1983).

Within the spermatozoan, plasma membrane is probably the site most susceptible to damage due to water flux during freezing and thawing (Baynes and Scott ,1987).

In rainbow trout Oncorhynchus mykiss, spermatozoa went through morphological changes during storage, like winding of

flagella, detachment of nuclear envelope and plasma membrane from the nucleus of the sperm head. There were 1% abnormal spermatozoa in fresh sperm and about 15% during storage (Park and Yoon, 1992). Electron microscopy indicated obvious structural damage, especially in the trout spermatozoa (Gwo *et al., 1993*).

In ocean pout, cryopreservation caused sperm ultra structural changes including the shrinkage of cell membranes leading to the exposure of mitochondria and death of the sperm (Yao *et al., 1995*).

Osmolality is an external trigger for the initiation of sperm motility in marine teleosts. Following the activation of the 3 marine fish sperm in artificial seawater, the mitochondria of the sperm were shrunken and completely disappeared at the end of motility duration, with no obvious change in other sperm ultrastructures. On exposure to a hypotonic solution (distilled water) severe morphological distortions including swelling of the nucleus, mitochondria completely disappeared and bursting of plasma membranes occurred. After 1-minute activation in artificial seawater, the intra cristal spaces were grossly dilated, the matrix space reduced to a minimum, and bud like evaginations of cristae membranes grew into intra cristae spaces. Marine fish sperm motility is closely related to existence of mitochondria in the mid-piece (Gwo, 1995).

In rainbow trout (*Oncorhyncus mykiss*) semen, after cryopreservation, about 10-20% of the spermatozoa had an unchanged structure that was similar to untreated spermatozoa; about 20-40% showed intensive signs of swelling of the head and midpiece regions and of the mitochondria. The remaining spermatozoa were damaged in some way. Creatine phosphate

and adenosine tri phosphate levels were significantly lower in frozen thawed semen than in untreated semen (Lahnsteiner *et al.*, 1996).

In rainbow trout non-damaged cryopreserved spermatozoa presented the same characteristics of fresh spermatozoa, except for chromatin, which was extensively clumped. Spermatozoa that were damaged by cryopreservation exhibited a swelled and non-continuous membrane with a clumpered chromatin, showing high electron density zones. The flagella of fresh spermatozoa showed a continuous and well organized membrane which surrounds the typical 9+2 structure of the axoneme, while the same flagella structure was observed in non-damaged cryopreserved spermatozoa. In spermatozoa damaged by cryopreservation, a break and a displacement of axoneme, along with changes in flagella membrane were observed. The shape of mitochondria was not altered by cryopreservation (Conget *et al.*, 1996).

A low membrane cholesterol or phospholipid ratio was strongly correlated with better freezing resistance of the corresponding sperm (Labbe *et al.*, 1996). Most of the damage occurred within 10 minutes of adding DMSO to the sperm suspension in carps (Lubzens *et al.*, 1997).

In freeze-fracture electron microscopy studies of rainbow trout (*Oncorhynchus mykiss*), it was revealed that the changes were induced in the organization of the plasma membranes of spermatozoa when they were cryopreserved. Electron micrographic images of spermatozoa that had not been exposed to cryopreservation showed a surface with particles homogenously distributed. The concentration of particles was low in the median portion of the tail. The authors obtained longitudinal strips, consisting of particles along the bands. In the neck of

the spermatozoon the particles were aggregates in chaotic manner. Electron micrographs of trout spermatozoa that had been cryopreserved showed particles grouped in rounded clusters on the protoplasmic surface of both head and tail. In some spermatozoa, folding of protoplasmic membrane with the particle free sites were found (Drokin *et al.*, 1995).

Studies in European catfish sperm, the best protection was given by dimethylacetamide (10and15 %) in sucrose solution. Under these conditions, the percentage of cells with intact membrane was high (80%) and the protection of the activity of the mitochondria was complete (97%). It was shown that addition of dimethylacetamide increased the ATP content of spermatozoa (Ogier-de-baulney *et al.*, 1999).

In Rohu spermatozoa damages were observed in head midpiece, and tail of about 51% of spermatozoa immediately after dilution in one of the cryodiluents. After 10 minutes of equilibration period, the frequency of damaged sperm increased to 81% (Gopalakrishnan *et al.*, 2000).

2.4 POST-THAW MOTILITY AND FERTILITY

In a series of fertility tests in carps, the chemical composition of the medium in which fertilization was attempted influenced the fertility of cryopreserved sperm (Kurokura *et al.*, 1984). Inclusion of egg yolk in the extender improved the fertilization rates of thawed spermatozoa (1983). Cryopreserved sperm showed motility comparable between 5% and 30% (Chao *et al.*, 1990). A strong positive correlation was found between post-thaw motility and fertility, indicating high fertilizing ability of cryopreserved sperm (Chao *et al.*, 1990). Experiments on cryopreservation of spermatozoa, motility and stabilization of spermatozoa motility

after thawing of frozen spermatozoa with Na super (+) and K super (+) ions in solutions were initiated with common carp, Cyprinus *carpio* for the purpose of long-term preservation of spermatozoa. The maintenance of capacity for motility of post-thawed spermatozoa was tested with KCl and NaCl solutions 200 –400 mM with 30mM Tris –HCl buffer, pH 7. The 275 mM NaCl solution appeared to be the best one with 80% pos -thaw spermatozoa motility against 23% spermatozoa motility of the control. (Linhart and Cosson, 1997). In *Liza macrolepis* spermatozoa, which displayed a post- thaw motility of 30-40%, were selected for inseminating the ova (Sultana *et al., 1998*).

In studies in Muskellunge spermatozoa, although spermatozoa cryopreserved with extender containing 10% methanol had only traces of motility or no motility at all after thawing, fertilization trials indicated that their fertilizing ability was preserved (Cierezko *et al.*, 1999).

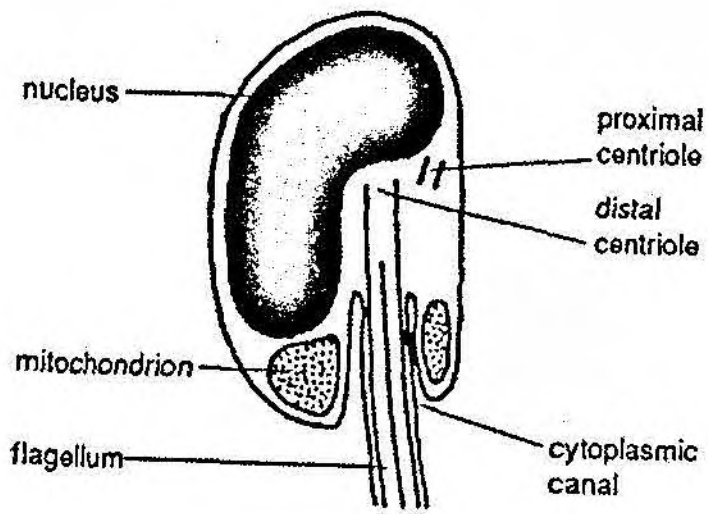


Fig 1. *Diagrammatic representation of Spermatozoa of* ***Liza*** *species* (Jamieson, 1991)

MATERIALS AND METHODS

3.MATERIALS AND METHODS

The candidate species for this cryopreservation experiments was the gold spot mullet, *Liza parsia*. Ripe males with milt oozing on slight pressure of the abdomen were collected from Chinese dipnets operated in Vypeen region. The collected fishes were then transported in small bins with aeration and stocked in fibre tanks. They were maintained at a salinity of 20 ‰.

3.1 COLLECTION OF MILT

The stocked *Liza parsia* males were given an intramuscular injection of Ovaprim (Syndel laboratories, Canada, marketed in India by Agrivet Farm Care Division of Glaxo) at the rate of 0.3 ml per kg body weight. The fishes after injection were given a brief disinfection treatment in povidone iodine solution. After 24 hours they were dry stripped manually and milt collected in vials, without contamination of blood, urine and faeces.

3.2 PRELIMINARY MOTILITY STUDIES

One drop of the milt collected was placed on a glass slide and its motility checked in fresh water and seawater. For this a compound microscope was used and motility observed in 10x10x magnification. The sperms were immotile in fresh water. The sperms after treatment in freshwater were collected and fixed in glutaraldehyde for studying the effect of osmotic shock on the morphology of sperms electron microscopically.

Sperms were actively motile in seawater of salinity 35‰. Motility in seawater lasted for about 6 minutes .The milt

samples for further treatments were tested for their quality taking motility as the criteria.

A drop of milt taken from a live ripe specimen of *Liza parsia* was placed on a clean dry glass slide with a drop of seawater. A cover slip was carefully placed over it and the material was immediately observed under a compound microscope at a magnification of 10x10x. Three main types of movements could be observed.

- Rapid progressive or shooting movement.
- Sluggish or lethargic movement.
- Vibration in loco.

A quick eye estimation of the approximate percentage of spermatozoa belonging to each of the above categories was done. On the basis of this a motility score was given to each sample as per the table below. The method was standardised by repeating the process with a number of samples.

CRITERIA	MOTILITYSCORE
• 90% or above of the sperms exhibiting rapid progressive or shooting movement.	5
• 75% or more exhibiting rapid progressive movement, 10% sluggish and the rest immotile.	4
• 50% exhibiting rapid progressive movement, 25% sluggish and 10% vibrating in loco and the rest immotile.	3
• 25% exhibiting shooting movement, 50% moving sluggishly, 10% vibrating in loco and the rest immotile.	2
• Occasional sperm shooting, 10% Showing sluggish movement, 50% Vibrating in loco and the rest immotile.	1
• No shooting movement, occasionally sperm Moving sluggishly. About 10% vibrating in Loco. Majority immotile.	0.5
• *Completely immotile.*	0

Only samples with a motility score of 4 and above were chosen for treatments.

3.3 PREPARATION OF EXTENDERS

Extenders are simple salt solutions which keep the sperms in viable but in inactive condition. The chemical composition of different extenders is presented in Table 1.

Table 1. Chemical composition of different extenders

Extender / Chemical composition (mg)	CC1 (Kuroku - ra et al, 1984)	Rana and Mc Andrew (1989)	Chao (1975)	Marine teleost ringer solution	Mixture B (Eliza- beth, 1987)	Mixture C (Eliza- beth, 1987)	V2E (Scott & Baynes, 1980)
NaCl	750	650	1350	1350	600	600	750
KCl	20	300	60	60	38	38	38
$CaCl_2$	20	30	-	-	-	23	-
$NaHCO_3$	20	20	20	20	200	100	200
$NaHPO_4.H_2O$	-	-	-	-	-	41	-
$MgSO_4.7H_2O$	-	-	35	35	23	23	-
$MgCl_2$	-	-	-	-	-	-	-
Na_2HPO_4	-	-	-	-	53	-	-
Glucose	-	-	5000	-	-	-	100
Egg yolk	-	-	-	-	-	-	20
Distilled water/seawater	100	100	100	100	100	100	100
pH	7.3	7.3	7.2	6.8	7.0	7.3	7.0

As the sperms were motile in seawater only, seawater was identified as the spawning medium and all these extenders (except Chao's extender and 0.9% NaCl) were also prepared with seawater as base solution.

Motility of sperms in these extender solutions was observed using a compound microscope, and extenders were selected based on their capacity not to activate spermatozoa after mixing. The sperms were actively motile in Marine Fish Ringer solution (pH 6.8), Chao's Extender (pH 7.2), CC 1 Extender (pH 7.3), CC1 Extender with seawater as base solution (pH 7.3), Rana and Mc Andrew Extender (pH 7.2), Mixture B (pH 7), Mixture B with seawater as base solution (pH 7.2), Mixture C (pH 7.3) and V2E Extender (pH 7).

The sperms were feebly active in (motility score 1 and 0.5) V2E with seawater as base solution, Mixture C with seawater as base solution and Rana and Mc Andrew extender with seawater as base solution. These extenders were selected for the present experiments.

Chao's extender was also selected even though sperms were motile when diluted, as it is one of the commonly used extenders for cryopreserving sperms of mullets. Dimethyl sulphoxide (DMSO) at a final concentration of 10% was used as the cryoprotectant .

3.4 PROCEDURE

The ratio of milt: cryodiluent (Extender + DMSO) for this experiment was fixed as 1:3, with a final concentration of DMSO, 10%. So for every 1ml of milt, 2.6ml of extender and 0.4 ml of DMSO was taken.

3.5 TREATMENTS

The spermatozoa were treated with cryodiluents as follows:

1) Zero seconds after mixing with cryodiluents.
2) After an equilibration time of 10 minutes with cryodiluents including the time taken for filling diluted milt into 0.5ml French straws.
3) Exposing to liquid nitrogen vapours (-100^{0}C) after step 2.
4) After vapour phase, plunging straws into liquid nitrogen and keeping overnight.

After each treatment the diluted milt was thawed for 20 seconds in a water bath maintained at 37^{0}C and processed for

Transmission electron microscopy studies following the method of Elizabeth (1987), Diwan & Nandakumar (1998) and Gopalakrishnan *et al.*, (2000).

3.6 PROCESSING OF SPERMS FOR ELECTRON MICROSCOPY STUDIES

The samples after all the treatments were primarily fixed in 2% buffered glutaraldehyde for 2 hours at 4^0C following immersion fixation . Then the supernatant was decanted and the pellets were washed thrice for 15 minutes each using sodium cacodylate buffer. Then the samples were centrifuged for 10 minutes at 5,000 rpm. The pellets were post – fixed in 1% osmium tetroxide for 1 hour at 4^0C and centrifuged at 5000 rpm for 10 minutes . After the fixations the pellets were transferred to 2% agar. Then the agar blocks were trimmed to $1mm^3$ size. The agar embedded samples were transferred stepwise through a concentration series of acetone (30%, 50% , 70% , 90% and 100 % v/v) following standard time schedule . Following the dehydration steps, infiltration is carried out in spurr medium with acetone in 3 steps. First in Spurr / acetone ratio 1:3 for 1 hour, second step in 1:1 and finally in 3:1 for I hour each at 4^0C. Embedding was carried in Spurr's medium in plastic mould and kept in incubator for 12 hours at 70^0C , for polymerisation.

The polymerised blocks were cut into ultra thin sections in the LKB ultra tome Nova .The thin sections were double stained in uranyl acetate and lead citrate for 10 minutes. The stained thin sections were mounted on the grid and observed in the STEM model Hitachi (H 600) electron microscope and recorded the image.

3.7 COUNTING OF POST-THAW INTACT SPERMATOZOA

The ratio of intact and damaged spermatozoa for each treatment was calculated by counting sperms under low magnification (4000 x) in TEM. In this process, randomly selected portions of grids (at least 3grids for each treatment) were brought under magnification and all together 150 spermatozoa were screened for each treatment. TEM images of untreated raw milt served as control.

RESULTS

4. RESULTS

4.1 ULTRASTRUCTURE OF UNTREATED SPERMATOZOA

The sperm of *Liza parsia* is a typical anacrosomal aquasperm. The nucleus is bilobed or kidney shaped in longitudinal section (Plate 1). It is also tilted relative to the axoneme. Chromatin is very coarsely granular and not condensed. Matrix spaces are clearly visible.

A cytoplasmic collar, which extends around the base of the flagellum, is present and it is separated from the flagellum by a periaxonemal space, the cytoplasmic canal (Plate 1). Small cristate mitochondria are present and they may be situated in the cytoplasmic collar. The flagellum is parallel to the base of the nucleus and a depression is present at this point, the nuclear fossa (Plate 1). A plasma membrane surrounding the whole structure is also present (Plate 2).

4.2 THE ULTRASTRUCTURAL CHANGES OF *Liza parsia* SPERM AFTER OSMOTIC SHOCK. (ZERO SECONDS AFTER DILUTION WITH FRESH WATER)

The most conspicuous change after the osmotic shock is bursting of plasma membrane and it's subsequent loss (Plates 3 & 4). The nucleus becomes rounded in 80% of the sperms and the nuclear fossa becomes less conspicuous (Plates 3 & 4). Flagellum gets separated from the nucleus and gets coiled up within its membrane (Plates 3 & 4).

4.3 THE ULTRASTRUCTURAL CHANGES AFTER TREATMENTS

4.3.1 CHAO'S EXTENDER AND DMSO

***4.3.1.1* Zero seconds after dilution (Plate 5):** Mitochondria and plasma membrane is intact in 80% of sperms. A condensed nucleus with a clear nuclear fossa is present.

***4.3.1.2* 10 minutes after dilution- after an equilibration time (plate6)** Nucleus is dilated and nuclear fossa became less visible. Plasma membrane is present. The chromatin material has become more condensed. About 33% of sperms exhibited such abnormalities.

***4.3.1.3* After exposure to Liquid Nitrogen vapours (Plate7):** The gross morphology of the spermatozoa remained more or less similar as compared to the second step, but the percentage of damaged spermatozoa increased to 42%.

***4.3.1.4* After plunging into Liquid Nitrogen (Plate8):** The same intact nucleus is present as in step 3.But the plasma membrane appeared to be ruptured. The percentage of damaged sperms increased to 52%.

4.3.2 MIXTURE C AND DMSO

4.3.2.1 Zero seconds after dilution (Plate 9): The spermatozoa appeared similar to the raw milt in 70% of sperms. Rest 30% of sperms exhibited slight disruption of mid-piece.

4.3.2.2 10 minutes after dilution- after an equilibration time (plate10): After an equilibration time mitochondria are less conspicuous. The plasma

membrane exhibited corrugated appearance. The chromatin got more condensed. These damages occurred in almost 60% of sperms.

4.3.2.3 After *exposure to Liquid Nitrogen vapours* (Plate11): *Plasma* membrane is totally disrupted but the shape of the nucleus remained more or less same as in the above case. The mid-piece and mitochondria became altogether *ruptured.*

4.3.2.4 After plunging into Liquid Nitrogen (Plate12): Almost 75% of spermatozoa became abnormal and exhibited structural changes as in step 3. In addition the nucleus exhibited vacuoles inside chromatin material.

4.3.3 RANA AND MC ANDREW EXTENDER AND DMSO

4.3.3.1 Zero seconds after dilution (Plate 13): Almost 70% of spermatozoa became abnormal and exhibited following structural alterations. Nucleus remained intact but the plasma membrane exhibited undulations and the chromatin material got condensed and vacuoles were present in it. The mid-piece and mitochondria were deformed.

4.3.3.2 10 minutes after dilution- after an equilibration time (plate14): The structure is almost similar to the above treatment (1), but the rate damaged sperms increased to 85%.

4.3.3.3 After exposure to Liquid Nitrogen vapours(Plate15): Mitochondria in the midpiece are totally damaged and the plasma *membrane showed severe undulations.*

4.3.3.4 After plunging into Liquid Nitrogen (Plate16): almost 95% of *sperms exhibited complete damages in head and mid-piece* region as in previous treatment (3).

4.3.4 V2E EXTENDER AND DMSO

4.3.4.1 Zero seconds after dilution (Plate17): Almost all the spermatozoa appeared similar to sperms in raw milt without much damages. Appearance of flagellum, mitochondria, nucleus, nuclear fossa etc. are more or less similar to the raw milt in 90% of the sperms.

4.3.4.2 10 minutes after dilution- after an equilibration time (plate18): All the structures remained more or less similar to the above step in 76% sperms. Rest of the sperms showed disintegration of plasma membrane and rupture mid-piece.

4.3.4.3 After exposure to liquid nitrogen vapours (Plate19): All most 60% sperms near normal structure. The plasma membrane is more or less intact and did not exhibit much evaginations. Nucleus is intact. Mitochondria also appeared near normal.

4.3.4.4 After plunging into liquid nitrogen (Plate 20): Structural feature of spermatozoa did not differ further after freezing and thawing protocol. The plasma membrane appeared to be more disrupted compared to the previous stage and the mitochondria detached. The percentage of damaged sperms increased to 46%.

Table 2. Percentage of intact spermatozoa of *liza parsia* and their motility score after each step in various extenders+DMSO (10%)

Extender / Treatments	CHAO'S EXTENDER +DMSO	MIXTURE C +DMSO	RANA & MC ANDREW +DMSO	V2 E +DMSO
Step-1 (0 seconds after dilution)	80.67±5.33 (5)	70.34±5.86 (4)	29.98±5.11 (3)	89.23±6.68 (5)
Step-2 (10 min after exposure)	66.38±5.61 (4)	40.04±3.34 (3)	15.56±1.67 (2)	76.04±2.98 (5)
Step-3 (After vapour phase)	58.10±3.89 (4)	30.66±6.94 (3)	10.89±5.32 (1)	59.87±4.91 (4)
Step-4 (After Liquid N_2 exposure)	47.83±0.64 (3)	25.06±9.91 (2)	5.01±0.2 (5)	54.0±1.76 (4)

Figures in parenthesis indicate motility score

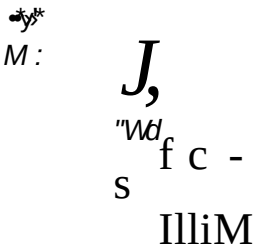
nucleus
nuclear fossa
cytoplasmic *a*
flagellum

Untreated spermatozoa of *Liza parsia*
(20000 x)

PLATE 2

plasma
Membrane

Untreated spermatozoa of *Liza parsia*
(40000 x)



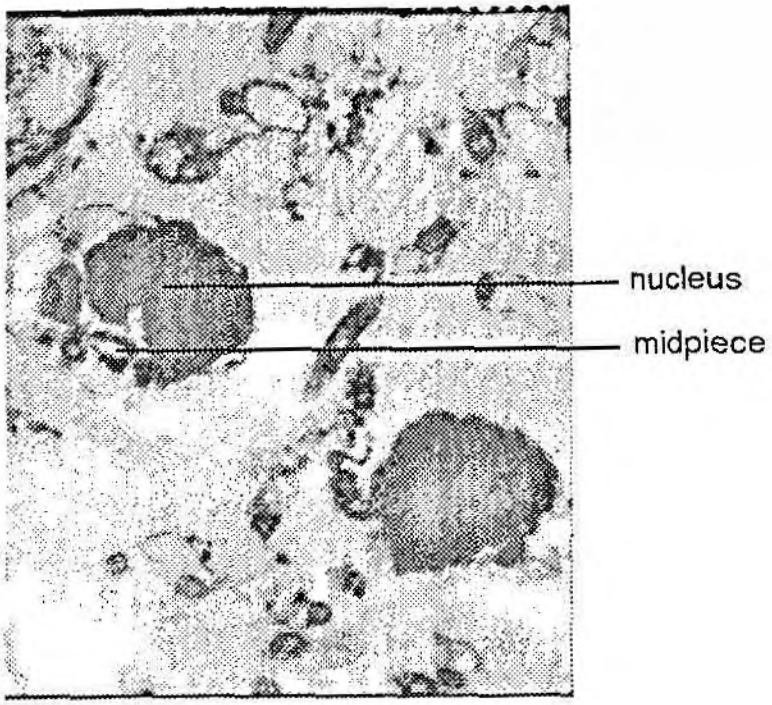
&

Spermatozoa of *Liza parsia*
zero seconds after Osmotic Shock
(12000X)

PLATE 4

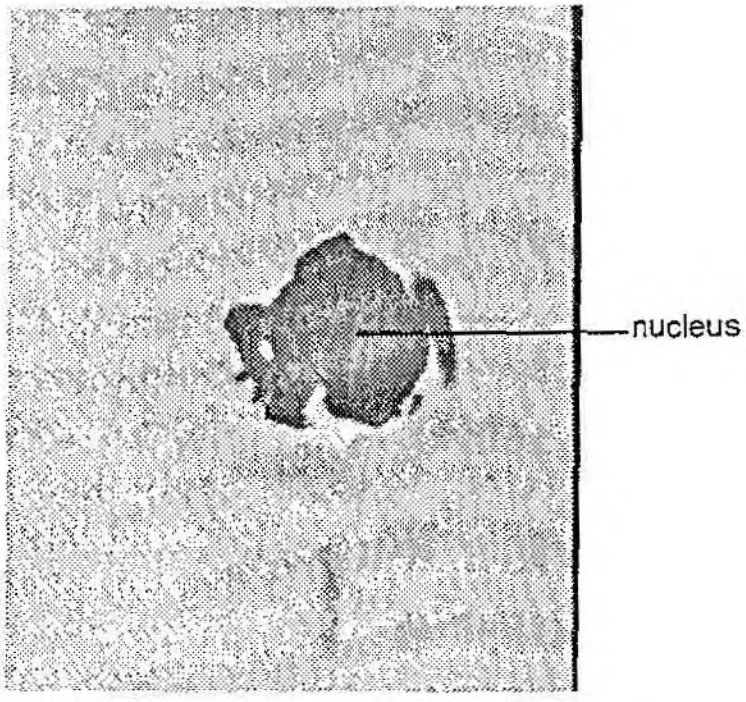
Spermatozoa of *Liza parsia*
1 Minute after Osmotic Shock
(12000X)

PLATE 5



Spermatozoa treated with Chao's extender + DMSO
(zero seconds after dilution)
(20000 x)

PLATE 6



Spermatozoa of *Liza parsia*
(after 10 minutes equilibration time)
(20000 x)

Head

Mid piece

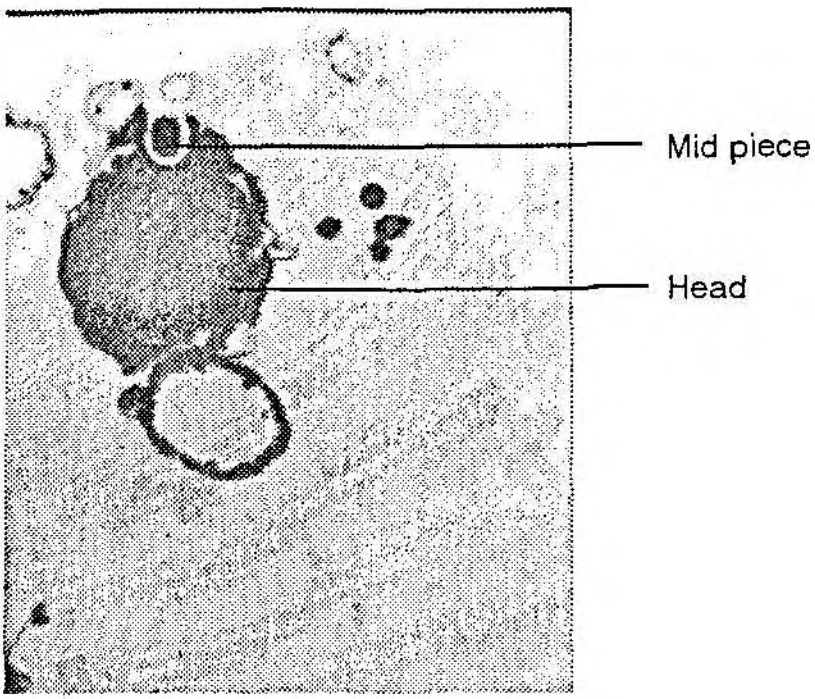
Tail

Spermatozoa treated with Mixture C + DMSO
(Zero seconds after dilution)
(20000X)

PLATE 10

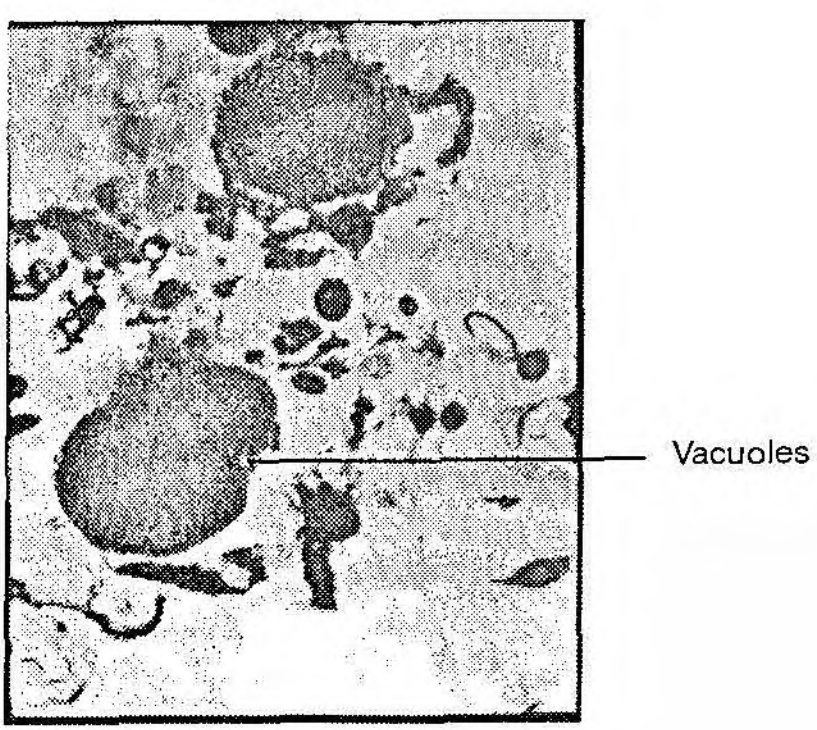
Head

PLATE 11



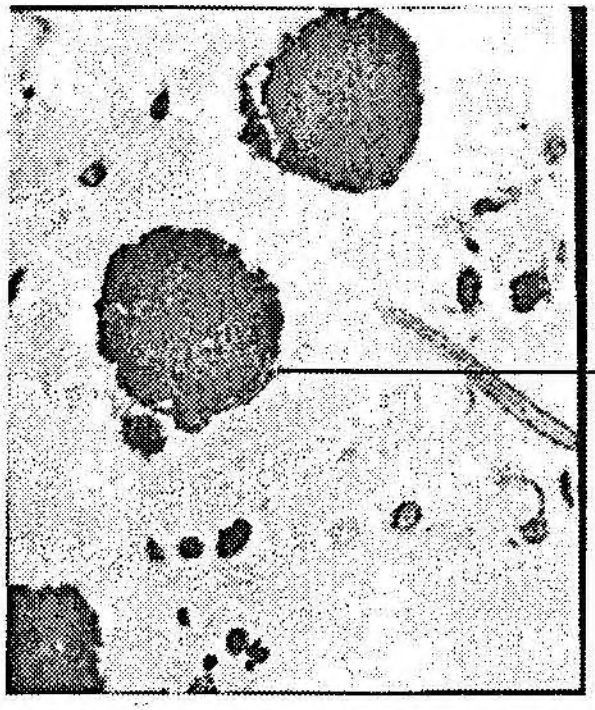
Spermatozoa treated with Mixture C + DMSO
(After exposure to Liquid Nitrogen Vapours)
(20000 x)

PLATE 12



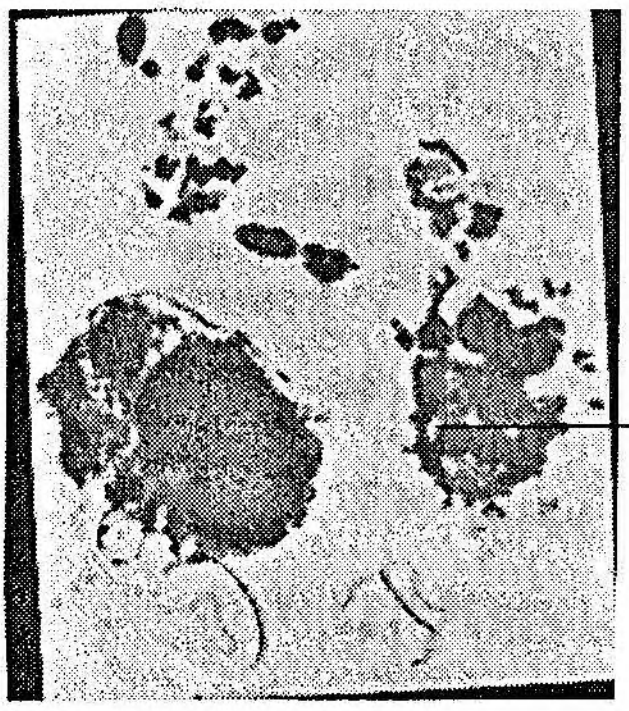
Spermatozoa treated with Mixture C + DMSO
(After plunging into Liquid Nitrogen)
(20000 x)

PLATE 13



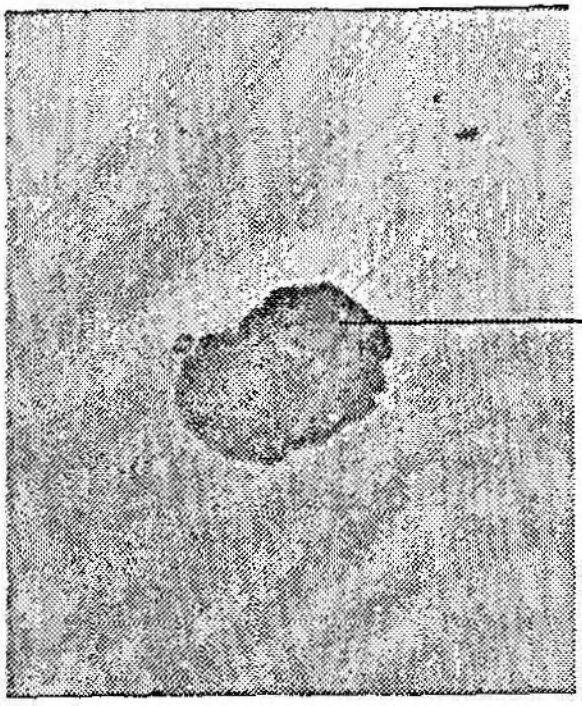
Spermatozoa treated with Rana and Mc Andrew extender + DMSO
(Zero seconds after dilution)
(20000 x)

PLATE 14



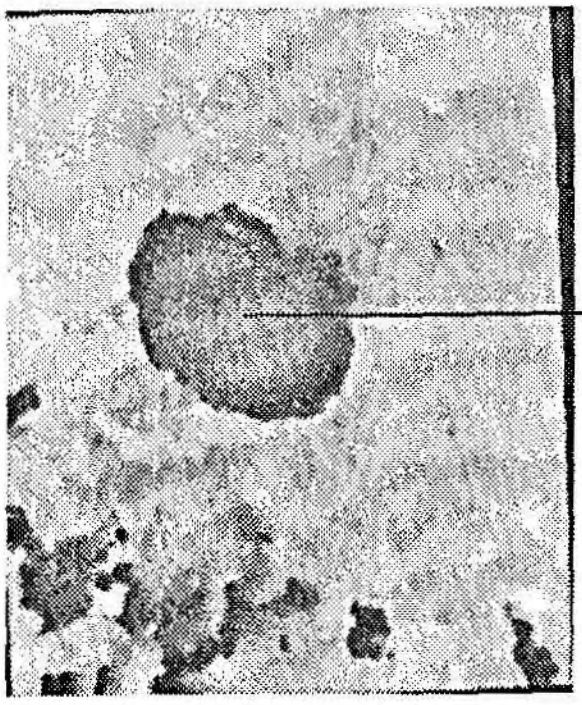
Spermatozoa treated with Rana and Mc Andrew extender + DMSO
(After 10 minutes equilibration time)
(20000 x)

PLATE 15



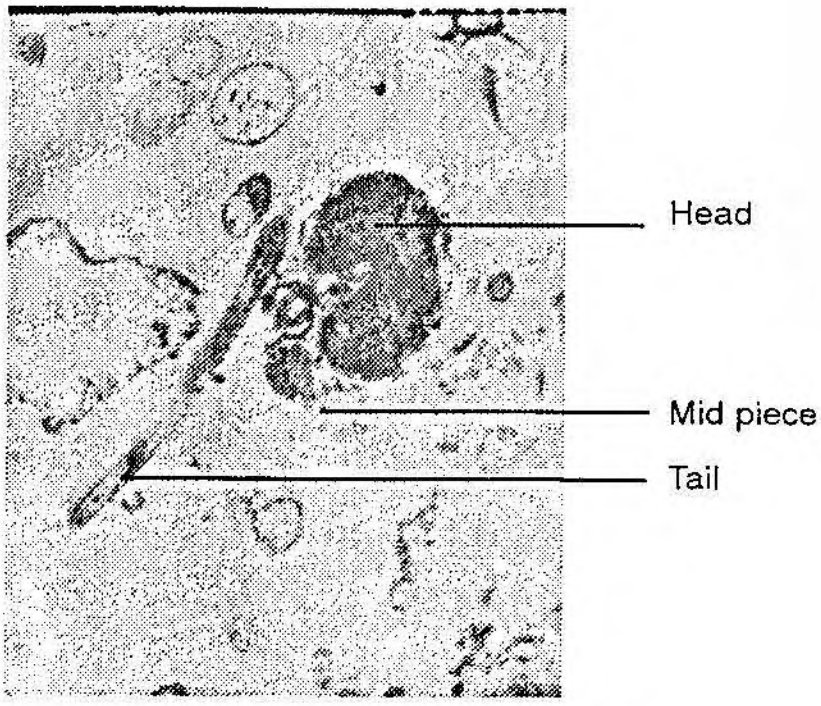
Spermatozoa treated with Rana and Mc Andrew extender + DMSO
(After exposure to Liquid Nitrogen Vapours)
(20000 x)

PLATE 16



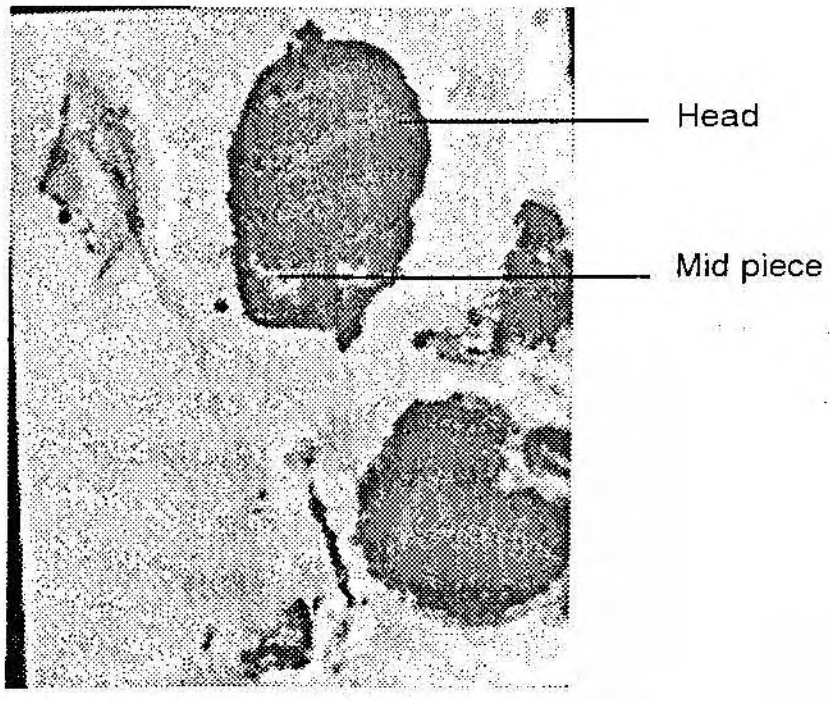
Spermatozoa treated with Rana and Mc Andrew extender + DMSO
(After plunging into Liquid Nitrogen)
(20000 x)

PLATE 17



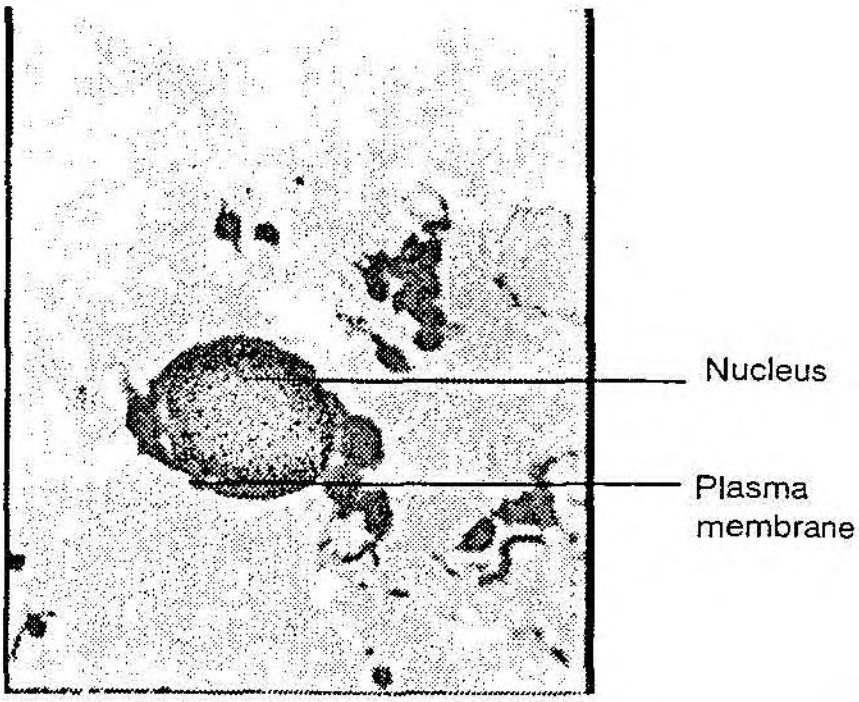
Spermatozoa treated with V2E extender + DMSO
(Zero seconds after dilution)
(20000 x)

PLATE 18



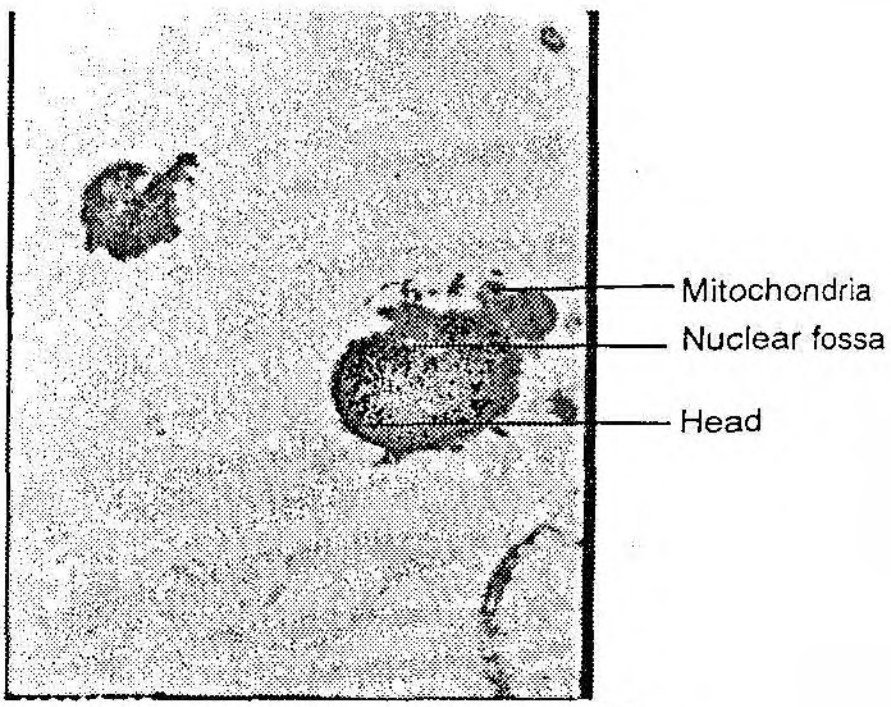
Spermatozoa treated with V2E extender + DMSO
(After 10 minutes equilibration time)
(20000 x)

PLATE 19



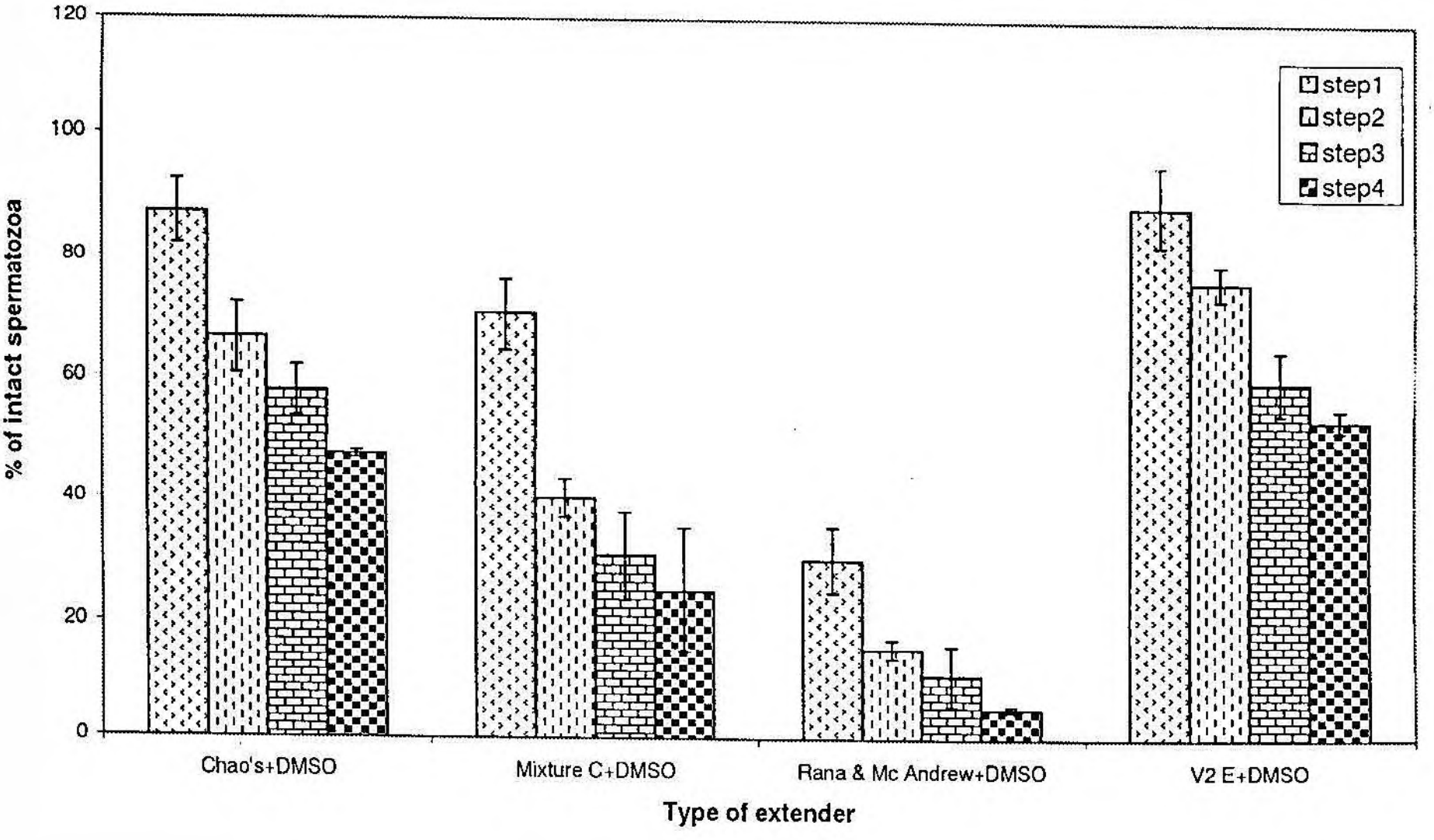
Spermatozoa treated with V2E extender + DMSO
(After exposure to Liquid Nitrogen Vapours)
(20000 x)

PLATE 20



Spermatozoa treated with V2E extender + DMSO
(After plunging into Liquid Nitrogen)
(20000 x)

Fig. 2 Percentage of intact spermatozoa of *Liza parsia* after each step in various externders+DMSO (10%)



DISCUSSION

5. DISCUSSION

The success of cryopreservation mainly depends on the prevention of cryoinjury during the freezing phase. The injuries generally discernible in 1) zero seconds after dilution 2) 10 minutes after the equilibration time 3) after exposure to the liquid nitrogen vapour and 4) after plunging into liquid nitrogen stages. A comparison of the extent of damages at the above milestones in relation to various diluents will give an in depth knowledge into the relative merits of the cryoextenders and the stages in which maximum damages occur.

5.1 Zero seconds after dilution:

Maximum damages occurred to spermatozoa in the milt diluted in Rana and Mc Andrew extender+DMSO. Almost 70% spermatozoa became abnormal in this stage. The plasma membrane exhibited undulations and the chromatin material got condensed and vacuoles were present in it. The mid piece and mitochondria were deformed but nucleus remained intact (Plate 13). In Mixture C + DMSO, 30% sperms exhibit slight disruption of mid piece (Plate 9). In Chao's extender + DMSO, a condensed nucleus with clear nuclear fossa is present, mitochondria and plasma membrane is intact in 80% of sperms (Plate 5). In V2E extender + DMSO, almost all the spermatozoa appeared similar to sperms in raw milt without much damages. Appearance of flagellum, mitochondria, nuclear fossa, nucleus etc. are more or less similar to the raw milt in 90% of sperms (Plate 17). So V2E Extender is found to be the best cryodiluent among 4 studied. Chao's Extender is the second best cryodiluent. In Rana and Mc Andrew most damages occurred among 4 cryodiluents studied.

5.2 10 minutes after dilution:

More damages occurred in Rana and Mc Andrew Extender +DMSO, the extend of damaged sperm is observed to be 85%. The plasma membrane exhibited undulations and the chromatin material got condensed and vacuoles were present in it. The mid piece and mitochondria were deformed (Plate 14). In Mixture C + DMSO the damages occurred in almost 60% sperms. The mitochondria are less conspicuous. The plasma membrane exhibited corrugated appearance. The chromotin got condensed more (Plate 10). In Chao's extender and DMSO, about 33% of sperms exhibited abnormality like nucleus dilated and nuclear fossa became less visible (Plate 6). In V 2 E extender and DMSO, most spermatozoa (76%) appeared similar to sperms in raw milt retaining flagellum, mitochondria, nucleus, nuclear fossa etc. Rest (24%) sperms showed disintegration of plasma membrane and rupture of mid piece (Plate 18). So V 2 E extender is found to be the best diluent at 10 minutes after dilution among 4 diluents studied. More damages are observed in Rana and Mc Andrew in this step.

5.3 After exposure to Liquid Nitrogen vapour:

In Rana and Mc Andrew extender and DMSO, mitochondria in the mid piece are totally damaged and the plasma membrane showed severe undulations (Plate 15). In Mixture C and DMSO, plasma membrane is totally disrupted, the mid piece and mitochondria became altogether ruptured (Plate 11). In Chao's extender and DMSO the percentage of damaged spermatozoa is 42%, the gross morphology remained more or less similar as compared to second step. Plasma membrane is present, nucleus is dilated and nuclear fossa became less visible. Chromatin material became condensed (Plate 6). In V 2 E extender and DMSO, almost 60% of sperms exhibited near normal structure. Plasma membrane is more or less intact and did not exhibit much invagination. Nucleus is intact, mitochondria also appeared near normal (Plate 19). So V 2 E is found to be the best cryodiluent after exposure to Liquid Nitrogen vapour. Severe damages were observed in Rana and Mc Andrew extender.

5.4 After plunging into Liquid Nitrogen:

Very severe damages were observed in Rana and Mc Andrew extender and DMSO, where in almost 95% of sperms exhibited complete damages in head and mitochondria in the mid piece are totally damaged (Plate 16). In mixture C and DMSO, almost 75% of spermatozoa became abnormal, plasma membrane is totally disrupted, the nucleus exhibited vacuoles inside chromatin material (Plate 12). In Chao's extender and DMSO, the percentage of damaged sperms is 52. Plasma membrane appeared to be ruptured, the same intact nucleus is present (Plate 8). In V 2 E extender and DMSO the percentage of damaged sperms is 46.Sturctural features of spermatozoa did not differ further after freezing and thawing protocol. The plasma membrane appeared to be more disrupted compared to the previous step and the mitochondria detached (Plate 20). V 2 E extender is the best cryodiluent among 4 studied after plunging into liquid nitrogen.

Structural changes in spermatozoa following dilution and deep freezing have been reported in several fish species (Billard, 1978, 1983; Gwo& Arnold, 1992; Lahnsteiner et al., 1992; Diwan & Nandakumar, 1998; Gopalakrishnan et al., 2000). The ultrastructural changes in *Liza parsia* following dilution in different extenders after 10 minutes equilibration time and fresh water are similar to those described in other species like rohu, salmonids and guppy (Lahnsteiner et al., 1992; Gopalakrishnan et al., 2000). The spermatozoan is very sensitive to changes in osmotic pressure due to dilution (Billard, 1983). This would cause morphological alterations including rupture of membrane, swelling and disruption of mid-piece (Billard, 1983). The abnormalities in *Liza parsia* sperm were more pronounced when diluted with fresh water, Rana & Mc Andrew Extender+DMSO and mixture C+DMSO. The rate of abnormal damaged sperms increased progressively in the extenders mentioned above in different treatments followed. The motility score also gradually decreased in these extenders as the exposure time advanced(Table 2).

Considerable alterations particularly those visible in the nucleus (condensation of chromatin) and mid piece deserve attention especially in connection with selection of a suitable cryodiluent for mullet sperms. Fish sperm motility is closely related to the existence of mitochondria in mid piece (Gwo, 1995). Disruption of mitochondria and the probable exhaustion of energy supply, condensation of chromatin, broken plasma membrane in almost 60 & 85% spermatozoa (step 2) after dilution in extenders C and Rana & Mc Andrew respectively prior to freezing – all these can be factors in addition to cryoinjuries, responsible for poor motility performance of frozen thawed *L. parsia* milt in these extenders. The morphology of spermatozoa did not differ significantly in-undiluted milt and sperms diluted with V2 E and to a lesser extent in Chao's extender. This may be due to exposure of milt to a suitable acceptable extender having proper concentration of salts and DMSO and the ideal dilution ratio. The motility studies carried out supported the above observation indicating the suitability of V2 E and 10% DMSO as an ideal cryodiluent for mullet milt.

Exposure to liquid nitrogen vapours and subsequent freezing followed by thawing can cause cold shock to biological systems (Jamieson, 1991). Cold shock is mainly caused by the change of membrane lipids from the liquid to the solid phase. Ice crystal formation during freezing will effectively dehydrate and create phase transition of the lipids in biological membranes. This would increase the membrane tension leading to its rupture. In addition the likelihood of formation of intra cellular ice increases with cooling rate and the degree of injury is proportional to the size of ice crystals. Recrystalisation of small ice crystals during thawing inside and outside the cell also is detrimental. All these reactions will end up with mechanical destruction of membrane structures and cell death. As water is frozen out during freezing, both extra and intracellular solutions become progressively more concentrated leading to osmotic shock (Jamieson, 1991). This would denature the membrane lipoproteins and cause shrinkage of cells. The addition of cryoprotectant DMSO is reported to minimize cell damages associated with ice formation and accumulation of salts. DMSO is

rated as a better cryoprotectant for most of the cells than glycerol probably because its rate of penetration to cells is much faster than glycerol (Jamieson, 1991). DMSO at concentrations 5-20% (v/v) has been successfully used for cryopreservation of sperms of a variety of species (Scott & Baynes, 1980). In the present study, a final volume of 10% DMSO was selected as this was found to be an optimum concentration for many teleost species (Gopalakrishnan et al., 2000). Relatively high percentage of intact sperms in 10% DMSO –V2 E combination. The present investigation supports the view that concentration of DMSO is optimum for this species. Yao et al., (1995) in ocean pout reported that the degree of ultra structural integrity of spermatozoa can be a indicator of semen quality and fertilization ability. They compared the ultra structural images of unfrozen raw milt and frozen- thawed diluted milt in various extenders and found a positive correlation between fertility percentage and percentages of normal mitochondria, nucleus, mid piece and perforatoria. In the present study, similar results are obtained with raw milt and frozen-thawed milt in V2 E (10%) DMSO combination. DMSO diluted in V2 E has provided a better protection to spermatozoa as evidenced by less damages in TEM images, comparatively higher rate of intact spermatozoa (54.0±1.76) after thawing and better motility score (4). The Chao's extender-10% DMSO combination offered cryoprotection to a lesser extent compared to V2 E indicating that property of a cryoprotectant can vary with different extenders. Similar results are reported in rohu (Gopalakrishnan et al., 2000) and graylings (Drokin et al., 1998).

The study also indicated the necessity to give proper emphasis on developing suitable extenders in cryopreservation experiments. The basic criteria used in selection of an extender are its ability to retain the sperms alive but in inactive condition. All the extenders (except Chao's extender) in the present study retained spermatozoa of *L. parsia* in inactive condition, but failed to produce comparable results after freeze-thaw protocol. This indicated that extenders inhibiting the sperm motility alone need not always be an ideal one for species. To assess the efficacy of cyroprotectants and

extenders, fertilization experiments may become undependable (due to varying egg quality) expensive (brood-fish maintenance) and impractical (annual breeders with short breeding season). Trypan blue exclusion tests and application of supra vital fluorochromes to discriminate live and dead sperms (in both diluted and undiluted milt suspensions) have been developed for "acrosomal" sperms of higher vertebrates. Lack of acrosome and the small size make teleosts sperms unsuitable for above test (Billard, 1983). Even though not as fast as vital staining method, ultra-structural observation of milt suspensions can be a suitable and reliable alternative not only to cull extenders but also to understand the freeze-thaw effects.

From the present investigations, V2 E with seawater as base solution and 10% DMSO is selected as the best cryodiluent for *Liza parsia* sperms, as in this cryodiluent the spermatozoa exhibited structures similar to raw milt at each step of cryopreservation protocol. Chao's extender with 10% DMSO is rated as the second best as in this extender structural integrity is preserved up to the third stage. The fertility trials in mullets with cryopreserved sperm in these extenders are limited. The confirmation of these extenders as best for mullets can be done only after carrying out fertility trials. However present investigation has succeeded in fulfilling the objective of identifying the best possible extender-cryoprotectant combination (cryodiluent) for the milt of *Liza parsia* and also to pinpoint the strength and weaknesses of various diluent to prevent cryoinjuries at the different stages of cryopreservation. The knowledge gained will be extremely valuable in further cryopreservation studies.

SUMMARY

The present investigation was carried out to find out a suitable cryodiluent for the cryopreservation of *Liza parsia* sperms, taking morphological changes as a clue.

1) Oozing specimens of *Liza parsia* were collected from the wild and milt collected by stripping. Motility of sperms were checked in freshwater and seawater for assessing their quality and to select them for cryopreservation.
2) For identifying a suitable extender for cryopreservation the motility of sperms in extenders checked and the extenders which were capable of keeping the sperms in live but inactive condition were selected as the best. Thus 4 extenders were selected.
3) Using these 4 extenders along with 10% DMSO, the sperms treated in 4 steps of cryopreservation protocol to identify the morphological changes in each step.
4) Morphological changes were studied using Transmission Electron Microscopy.
5) Based on the observations from morphological alterations 2 of the 4 cryodiluents are rated as the best. V2E prepared with seawater as base solution and Chao's extender both with 10% DMSO are the selected cryodiluents.

REFERENCES

Amrit Bart, N., Dwight Wolfe, F., Rex Dunham, A., 1998. Cryopreservation of blue cat fish spermatozoa and subsequent fertilisation of channel cat fish eggs. *Trans. Am. Fish. Soc.*, 127: 819-824.

Babiak, I., Glowgoski, J., Luczynski, M. J., Kucharczyk, D. and Luczynski, M., 1994. Cryopreservation of the milt of the northern pike. *J Fish Biol.*, 46:819-828.

Baynes, S. M. and Scott, A. P., 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. Changes in structure and fertilizing ability of marine and fresh water fish spermatozoa diluted in media of various salinities. *Aquaculture.*, 14:187-198.

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

*Cabrita, E., Alvarez, R., Anel, L., Rana, K. J., Herraez, M. P., 1998. Sublethal damage during cryopreservation of rainbow trout sperm. *Cryobiology.*, 37:245-253.

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

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

Chao, N. H., Wei – Chang Chao, Kuo – Chun Liu and I – Chiu Liao., 1987. The properties of tilapia sperm and its cryopreservation. *J. Fish Biol.*, 30:107-118.

Ciereszko, A., Dabrowski, K., Lin, F., Christ, S. A., Toth, G. P., 1999. Effects of extenders and time of storage before freezing on motility and fertilization of cryopreserved Muskellunge spermatozoa. *Trans. Am. Fish. Soc.*, 128:542-548.

Conget, P., Mireya Fernandez, Guillermo Herrera, Jose Minguell, J., 1996. Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) spermatozoa using programmable freezing. *Aquaculture.*, 143:319-329.

Diwan, A. D. and Nandakumar, A., 1992. A stepping stone in developing marine fish gene bank. *MFIS.*,118:1-3.

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

*Drokin, S., Stein, H., Bartscherer, H., 1998. Effects of cryopreservation on the fine structure of spermatozoa of rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta F. fario). Cryobiology.*, 37:263-270.

Elizabeth Joseph., 1987. Studies on the histological and biochemical changes during spermatogenesis in *Mugil cephalus* Linnaeus and related species. Ph. D. Thesis, Cochin Univ. Sci. Technol., pp.191.

George Brown, G. and Steven Mims, D., 1999. Cryopreservation of paddle fish, *Polyodon spathula* milt. *Journal of the World Aquaculture Society.*, 30:245-259.

Glogowski, J., Babiak, I., Kucharczyk, D., Luczynski, M. and Piros, B., 1999. Some properties of bream, *Abramis brama* sperm and its cryopreservation. *Aquaculture research.*, 30:765-772.

Gopalakrishnan, A., Thakur, K. L., Ponniah, A. G., Kumar, K. and Dayal, R., 1999. Cryopreservation of brown trout (*Salmo trutta fario*) sperm: The influence of extender composition and fertilization procedure. *J. Fishery Technol.*, 36(2):104-109.

Gopalakrishnan, A., Ponniah, A. G. and Kuldeep Lal, K., 2000. Fine structural changes of rohu (*Labeo rohita*) sperm after dilution with cryoprotectants. *Indian J. Fish.*, 47(1):21-27.

Gwo, J. C., Krik Strawn, Michael Longnecker, T. and Connie Arnold, R., 1991. Cryopreservation of Atlantic croaker spermatozoa. *Aquacuture.*, 94:355-375.

Gwo, J. C., 1995. Ultrastructural study of osmolality effect on sperm of three marine teleosts. Proceedings of the fifth International Symposium on the Reproductive Physiology of Fish. Austin. pp.119.

Harvey, B., 1982. Cryobiology and storage of teleost gametes. Proceedings of the International Symposium on the Reproductive Physiology of Fish. Austin. pp.123-127.

Hirano, Masaktsu Tomita and Masan Iwahashi., 1984. Cryopreservation of carp sperm. *Aquaculture.*, 37:267-273.

*Howard Horton, F. and Alvin Ott, G., 1976. Cryopreservation of fish spermatozoa and ova. *J. Fish. Res. Board* Can., 33:995-1000.

Jamieson, G. M., 1991. Fish evolution and systematics: Evidence from spermatozoa. Cambridge university press, 167pp.

Kuldeep Lal, K., Peyush Punia, and Ponniah, A. G., 1999. Extender composition for cryopreservation Tenualosa ilisha spermatozoa with specific reference to inhibition of motility. *Indian J. Fish.*, 46(4):337-343.

*Kurokura, H., Reijiro Howard Horton, F. and Alvin Ott, G., 1976. Cryopreservation of fish spermatozoa and ova. *J. Fish. Res. Board* Can., 33:995-1000.

Kurokura, H., Hirano, R., Tomita, M. and Iwahashi, M., 1984. Cryopreservation of carp sperm. *Aquaculture.*, 37:267-273.

Labbe, C. and Maisse, G., 1996. Influence of rainbow trout thermal acclimation and sperm cryopreservation: relation to change in the lipid composition of the plasma membrane. *Aquaculture.*, 145:281-294.

Lahnsteiner, F., Berger, B., Weismann, T. and Patzner, R. A., 1995. Evaluation of semen fitness of the rainbow trout (*Oncorhychus mykiss*) for cryopreservation by physiological and biochemical parameters. Proceedings of the fifth International Symposium on the Reproductive Physiology of Fish. Austin. pp.125.

Lahnsteiner, F., Weismann, T., Patzner, R., 1996. Cryopreservation of semen of the grayling (*Thymallus thymallus*) and the Danube salmon (*Hucho hucho*). *Aquaculture.*, 144:265-274.

*Linhart, O., Cosson, J., 1997. Cryopreservation of carp (*Cyprinus carpio*) spermatozoa: the influence of external K super (+) and Na super (+) on post-thaw motility. *Polish Archives of Hydrobiology.*, 44:275-279.

Lubzens, E., Daube, N., Pekarsk, I., Magnus, Y., Cohen, A., Yusefoviech, F., Feigin, P., 1997. Carp (*Cyprinus carpio*) spermatozoa cryobanks – strategies in research and application. *Aquaculture.*, 155:13-30.

Magyary, I., Urbanyi, B., Horvath, L., 1996. Cryopreservation of common carp (*Cyprinus carpio*) sperm – optimum conditions for fertilization. *Journal of Applied Ichthyology.*, 12:117-119.

*Michael Christensen, J. and Terrence Tiersch, R., 1996. Refrigerated storage of channel cat fish sperm. *Journal of the World Aquacultural Society.*, 27:341-346.

Ogier-de-baulny, B., Labbe, C., Maisse, G., 1999. Membrane integrity, Mitochondrial activity, ATP content and motility of the European catfish (*Silurus glanis*) testicular spermatozoa after freezing with different cryoprotectants. *Cryobiology.*, 39:177-184.

Olvido Chereguini, Rosa Maria Cal, Catherine Dreanno, Benedicte Ogier de Baulny, Marc Suquet, and Gerard Maisse., 1997. Short-term storage and cryopreservation of turbot sperm. *Aquat. Living Resour.*, 10:251-255.

Oteme, Z. J., Nunez Rodriguez, J., Kouassi, C. K., Hem, S. and Agnese, J. F., 1996. Testicular structure, spermatogenesis and sperm cryopreservation in the African clariid catfish *Heterobranchus longifilis* (Valenciennes, 1840). *Aquaculture Research.*, 27:805-813.

*Park, H. Y., Yoon, J. M., 1992. Studies on genetics and breeding in rainbow trout (*Onchorhynchus mykiss*). 7. Fertilization of fresh egg with cryopreserved sperm and ultrastructural changes. *Bull – Korean – Fish – Soc.*, 25:79-92.

Paul Wheeler, A. and Gary Thorgaard, H., 1991. Cryopreservation of rainbow trout semen in large straws. *Aquaculture.*, 93:95-100.

Piironen, J., 1993. Cryopreservation of sperm from Brown trout (*Salmo trutta m. lacustris*) and Arctic charr (*Salvellnus alpinus*). *Aquaculture.*, 116:275-285.

Rana, K. J. and Mc Andrew, B. J., 1989. The viability of cryopreserved tilapia spermatozoa. *Aquaculture.*, 76:305-345.

Rexaline Sumathi, S. and Sukumaran, N., 1998. Short term preservation of spermatozoa of tilapia, *Oreochromis mossambicus* (Peters). Fish Genetics and Biodiversity Conservation, NBFGR, 231-341.

Ritar, A. J., 1999. Artificial insemination with cryopreserved semen from striped trumpeter (*Latris lineata*). *Aquaculture.*, 180:177-187.

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

Stoss, J. and Holtz, W., 1981. Cryopreservation of Rainbow trout (*Salmo gairdneri*) sperm: effect of pH and presence of a buffer in the diluent. *Aquaculture.*, 25:217-222.

Subramoniam, T. and Arun, R., 1999. Cryopreservation and aquaculture: A case study with penaeid shrimp larvae. *Current Science.*, 76:361-368.

Sultana, M., Santiago, T. C., Chandra, P. K., Gupta, P. S. P. and Kalaimani, N., 1998. Studies on the fertilizing capacity of cryopreserved milt in the brackishwater fish *Liza macrolepis* (Smith). Fish Genetics and Biodiversity Conservation. NBFGR, 223-229.

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

Terrence Tiersch, 1995. Cryopreservation of fishe sperm: Laboratory, Hatchery and field studies of twenty species. Proceedings of the Fifth International Symposium on Reproductive Physiology of Fish. Austin, pp. 147.

Urbany, B., Horvath, A., Varga, Z. and Horvath, L., 1999. Effect of extenders on sperm cryopreservation of African cat fish, *Clarias gariepinus* (Burcheli). *Aquaculture Research.*, 30: 145-151

Weismann, T., Lahnsteiner, F. and Patzner, R. A., 1995. Cryopreservation of semen of salmonid fishes and its adaption for practical application. Proceedings of the Fifth International Symposium on Reproductive Physiology of Fish. Austin, pp. 90-93

*Xia, L., Lalli, M. F., Ansah, G. A. and Buckland, R. B., (1998). Ultrastructure of fresh and frozen-thawed spermatozoa of high and low fertility lines of chickens. *Poultry Sci.*, 67: 819-825

Yao, Z., Crim, L. W., Richardson, G. F., Emerson, C. J., 1995. Cryopreservation, and motility-ultrastructure of sperm from the ocean Pout (*Macrozoarces americanus*) – an internally fertilizing marine teleost. Proceedings of the Fifth Interanational Symposium on Reproductive Physiology of Fish. Austin, pp. 149

Yao, Z., Crim, L. W., Richardson, G. F., Emerson, C. J., 2000. Motility, fertility and ultrastructural changes of ocean pout sperm after cryopreservation. *Aquaculture.*, 181: 361-375

*Zhang, L., Liu, Xintiang, Chen, S., Lu, D., Guo, F., 1994. Effect of dimethyl sulphoxide on osmotic pressure and survival rate of the sperm of several freshwater species. *Acta – Hydrobiol.*, 18(4): 297-302

* ORIGINAL NOT REFERRED