CRYOPRESERVATION OF SPERMATOZOA OF CRITICALLY ENDANGERED YELLOW CATFISH *HORABAGRUS NIGRICOLLARIS* (Pethiyagoda & Kottelat 1994)

By

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THESIS

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TO

THE INNOVATIVE MINDS OF THE WORLD

&

MY PARENTS

DON'T STARE AT THE THINGS THAT ARE ALREADY MADE –

CREATE NEW THINGS TO SPREAD THE PERFUMES OF THE KNOWLEDGE.....

DECLARATION

I hereby declare that this thesis entitled **CRYOPRESERVATION OF SPERMATOZOA OF CRITICALLY ENDANGERED YELLOW CATFISH HORABAGRUS NIGRICOLLARIS (Pethiyagoda & Kottelat 1994)** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship, or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled **CRYOPRESERVATION OF SPERMATOZOA OF CRITICALLY ENDANGERED YELLOW CATFISH, *HORABAGRUS NIGRICOLLARIS* (Pethiyagoda and Kottelat 1994)** is a research work done independently by Miss. RAJANI VADTHYA under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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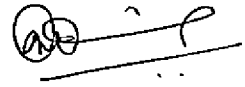

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I love the words you say at times when I move out from home for my Sports or Studies.....

"Where ever you go....
The moving image is yours but
The eyes you have are mine,
That always guard and show you the brightest ways in life" .....

Dear Parents I love you always.

Rajani Vadthya.

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INTRODUCTION

1. INTRODUCTION

Cryopreservation is a branch of cryobiology which relates to the long term preservation and storage of biological material at very low temperatures, usually at −196°C employing liquid nitrogen. At this temperature, cellular viability can be retained in a genetically stable form since the rates of biological processes will be too slow to affect cell survival (Lakra, 1993).

History of cryopreservation dates back to 1776 when Spallanzani, an Italian priest cooled human sperm on ice (Spallanzani, 1776). During late 1930's and early 1940's many observers found that living cells can be stored at −130 °C but with limited survival.

It is believed that artificial insemination (AI), which is the ultimate objective of the cryopreservation of gametes, was originated in the early 1300's when an Indian Chief, improved the quality of his horse herd by adopting this technique. However, the first documented case of successful AI was in dogs by Lazaro Spallanzani in 1776 (Spallanzani, *op.cit.*). Since then, AI process has evolved as a common agriculture and animal husbandry tool aiding genetic improvement.

The effect of low temperature on living organisms is complex, in that it affects the physical structures and biophysical processes of organisms in a complex manner. Living cells can be cooled to extreme low temperatures either to destroy them selectively or to store them for longer periods. Thus, cryobiology has two major applications: cryopreservation of biological systems and cryosurgery (Smith *et al.*, 1997).

Cryopreservation of gametes has been adopted from animal husbandry by the aquaculture industry (Lakra, 1993). Sperm cryopreservation protocols are now available for over 200 species of finfish and shell fish (Gregory, 1968; Zell, 1978; Scott and Baynes,1980; Harvey, 1983a; 1983b; Linhart, 1985; Harvey,1987; Mims, 1991; Blackshaw and Palmerarrett, 1993; Lubzens *et al.*, 1993; McAndrew *et al.*, 1993; Billard *et al.*, 1995; Linhart *et al.*, 1995; Labbe and Maisse, 1996; Tsvetkova *et al.*,1996; Lubzens *et al.*,1997; Porter, 1998; Urbányi *et al.*,1999; Linhart *et al.*, 2000; Mims *et al.*, 2000; Lang *et al.*, 2001; Basavaraja *et al.*,2002; Glogowski *et al.*,2002). Studies on cryopreservation of invertebrate eggs, embryos and larvae have also been successful (McAndrew *et al.*, 1993; Chao and Liao, 2001). However, perfect techniques for the cryopreservation of fish eggs/embryos have not been developed so

far (Renard, 1986; Bart, 1998). Cryopreservation protocol varies from species to species (Anger *et al.*, 2003; Rana, 1995a; Bart *et al.*, 1998).

The first report on cryopreservation is of Polge *et al.* (1949) on human and avian spermatozoa using glycerol as the cryoprotectant. In fisheries, it was by Blaxter (1953) on teleost gametes with 80% cellular motility in Atlantic herring. The first report on the same from India was given by Bhowmick and Bagchi (1971) on carp sperm.

As stated earlier, cryopreservation is a technique used to preserve and genetically stabilize cells, at cryogenic temperatures. The cryopreservation process involves complex biophysical responses of cells and tissues which are still not fully understood. The factors that affect the cell survival after cooling and thawing processes include: cooling rate, phase change temperature, holding time at each step, substance transport and ice formation.

However, the two major cellular responses during freezing is water loss of the cell and intracellular ice formation (IIF). During slow cooling, freezing occurs outside the cell before intracellular ice begins to form (Farrant, 1980). During the extracellular ice formation, water gets removed from the extracellular environment resulting an osmotic imbalance across the leading to the water migration out of the cell (Walsh, 1981). This phenomenon is known as the solute concentration effect of water and the increase in solute concentration outside and inside the cell can be detrimental to cell survival.

Rapid cooling minimizes the solute concentration effects as ice forms uniformly, leading to more intracellular ice formation. It is postulated that ice crystal formation and the solution effect both influence cell inactivation, and that an optimum-cooling rate should minimize the effect of both (Mazur *et al.*, 1972).

Storage of living systems in low temperatures has been successfully applied to various mammalian and vertebrate systems including erythrocytes, lymphocytes, gametes, embryos, hepatocytes, bone marrow stem cells, cornea, skin and pancreatic tissues (Wilmut, 1975; Graham, 1978; Mazur, 1984; McGrath, 1985; Graham and Foote, 1987; Hubel *et al.*, 1991; McCaa *et al.*, 1991; Graham, 1996). Cryopreservation technology is currently available for many biological systems such as mammalian oocytes (Bernard and Fuller, 1996; Younis *et al.*, 1996; Zenzes *et al.*, 2001), stem cells (Hubel, 1997), rat and human liver slices (Day *et al.*, 1999) and

engineered tissues (Oegema *et al.*, 1999). Its other applications extend to a variety of fields such as food science, plant and animal cold hardiness, ecology and agriculture.

Cryopreservation protocol usually includes several necessary steps such as :

a) extension and addition of cryoprotective agents (CPAs)

b) extracellular ice nucleation

c) freezing at an optimal cooling rate to −196°C, (Li-N2 temperature)

d) store at liquid nitrogen temperature for a period of time

e) thawing and removal of CPAs.

The range of temperature lethal to cell and tissues is reported to be about −15 to −60°C, (Mazur, 1984). The cells experience the maximum destructive effects during step c. Clearly, minimizing this destructive effect is essential for the cryopreservation protocol. This goal could be achieved by quantitative knowledge of the biophysical response during freezing of biological system.

The importance of storage of viable gametes and embryos of cultured aquatic organisms, especially of teleosts, has already been recognized (Scott and Baynes, 1980; Stoss,1983; Leung, 1987; Jamieson,1991; Rana 1995a; Cherepanov and Kopeika, 1999). Several methods for the successful cryopreservation of spermatozoa have been developed for fresh water and marine teleost species over the past 20 years. However, much work remains to be done before procedures can be optimized for large-scale application to individual fish species (Rana, 1995 a).

Cryopreservation has the obvious benefits of:

a) Synchronization of gamete availability of both sexes: Blaxter (1953) pointed out that in species like *Clupea harengus* in which ovulations are noticed when sperm production declines, this technique will be highly useful.

b) Use of the total volume of available semen: It is reported that this is useful for sperm economy not only in species where semen is difficult to obtain eg. Japanese eel, *Anguilla japonica* but also in species where only low volume of semen can be

stripped in captivity eg. yellowtail flounder, *Pleuronectes ferrugineus* and turbot, *Psetta maxima* (Ohta and Izawa, 1996; Clearwater and Crim,1995; Suquet *et al.*, 1994).

*c) **Simplifying brood stock maintenance:*** Off-season spawning can be induced in most cultured fish species, by the manipulation of photoperiod and temperature cycles (Bromage, 1995). However, the technique is cost intensive. When cryopreserved sperm is available all year round, the manipulation of the spawning season could be restricted to females.

*d) **Transport of gametes:*** Useful when male and female gametes are to be collected from different locations. This also the facilitates the introduction of genes from the wild into hatchery stocks.

*e) **Avoiding aging of sperm:*** Senescence of sperm during the course of the spawning season has been reported for many fish species resulting in the decline of milt quality (Rana, 1995a). Cryopreservation allows the collection of sperm when it has the highest quality.

*f) **Experimental programs:*** For genetic studies, in comparing the breeding performances of successive generations in the same experiment and for experiments where the use of identical sperm samples is necessary over an extended period for example, study of short term storage of ova.

*g) **Conserving genetic variability in domesticated populations:*** It is a fact that the use of limited number of breeders leads to a reduction of heterozygosity. Tabata and Mizuta (1997) used the cryopreserved semen of sex reversed gynogenetic males of hirame, *Paralichthys olivaceus* for producing better quality offspring.

*h) **Gene banks of cryopreserved semen**:* Semen banks can also be used to maintain genetic diversity of fish populations that are endangered besides protecting against inbreeding. In protogynous hermaphrodite species such as black grouper (*Epinephelus malabaricus*), sperm can only be collected from 5 to 10 year old animals (Gwo, 1993) which will be a laborious task. Sperm preservation will be of great help in such situations.

The cryopreservation techniques have been reviewed in detail by Stein and Bayrle (1978); Scott and Baynes (1980); Stoss (1983); Chao (1991); Jamieson (1991);

Tiersch (1995); Rana (1995a & b); Maisse (1996); Jayaprakas and Paul (1998) and Maisse *et al.* (1998). Much of the work has centered on teleosts like salmonids, tilapias and carps (Tomasic, 1973; Kurokura *et al.,* 1984; Kumar, 1988; Cognie *et al.,* 1989). As fish farming expands, there will be an increasing need to apply sperm cryopreservation techniques in the field.

Cryosurgery is another important application of cryobiology, defined as a technique to kill abnormal biological systems by freezing to low temperature. Low temperature was first used for the treatment of malignant tumors by James Arnott in the 1950s. In his experiment, iced saline solutions were applied to ulcerating organs. The first cryosurgical device capable of freezing the tumor region *in vivo* by liquid nitrogen and producing a cryolesion in the liver of cat was developed by Cooper and Lee in 1961. Currently cryosurgery has been performed in a variety of organs such as the rectum, breast, skin, lung, brain, prostate, uterus, oral cavity, pancreas and liver (Gage, 1992; Gage and Baust, 1998; Donnez *et al.,* 2004; Donnez *et al.,* 2005). The objective of cryosurgery is the complete destruction of abnormal cells without injuring normal cells. However, the wide use of cryosurgery is hampered by lack of effective methods to monitor the freezing process in organs (Gage, 1992).

Due to the increasing knowledge on sperm biology, spermatozoa of fish, molluscs, amphibians, corals and mammals have been recently cryopreserved successfully (Curry *et al.,*1995). Cryopreservation emphasizes the high survival and fertilization capacity generally obtained in frozen-thawed spermatozoa of marine fish in comparison to freshwater species. Special attention should be paid to individual variations in sperm quality and on biotests useful to determine the quality of thawed cells.

Of the total 283 species identified from the diverse river systems of Kerala, 73 are potential ornamental species, while 101 species are food fishes. Among 73 ornamental fish species 10 (including *Horabagrus nigricollaris* and *H. brachysoma*) have already been secured their positions in the national and international markets as ornamental fishes. The captive breeding and seed production technology of most of these fishes are yet to be standardized and this was the major bottle-neck in introducing them as ornamentals (Shaji *et al.,* 2000).

Horabagrus nigricollaris (black collared yellow catfish/ imperial white collared catfish) is a freshwater fish inhabiting the fast flowing stretches in the hilly regions of Chalakudy River, which is the fifth longest river of Kerala, India. It can be used as game (because of their habit of catching bait and alertness to sounds) ornamental (due to their yellow shaded body with a white tinged black collar on the neck) and food (due to its excellent taste and consumer preference) fish. Over the years, natural stocks of black collared yellow catfish have depleted owing to anthropogenic activities, and has been declared as a critically endangered species. To conserve and rehabilitate this fish species, no conservation strategies have been devised. The present study helps to frame a strategy for *in situ* conservation of the species under study.

The rehabilitation of endemic fishes through standardization of captive breeding and mass seed production is necessary for their sustainable utilization. *H. nigricollaris* is not only an ornamental fish but also a food fish of endemic origin and is listed as a "critically endangered fish" (Dahanukar *et al.*, 2004).

Among the catfishes, *H. nigricollaris* is selected for this study due to its beautiful colour pattern and its importance in the national and international markets as an ornamental as well as a food fish. This species has a golden yellow body with black collar like band on the branchial region.

H. brachysoma has already been reared in ponds and its captive breeding technique has been standardized by the National Bureau of Fish Genetic Resources (NBFGR, 2001). *H. nigricollaris* is comparatively a new species (Pethiyagoda and Kettelat, 1994) and the information on its feeding, breeding and cryopreservation is scanty (Shaji *et al.*, 2000; Anvar-Ali and Prasad, 2005).

The present study is the first of its kind in this species. Dearth of literature on the species was a major bottleneck during the study.The work was undertaken to develop a viable protocol for the cryopreservation of the critically endangered black collared - yellow catfish *Horabagrus nigricollaris* spermatozoa. The map of study area, habitat of the species under the study and its picture are given in Plate – 1, 2 and 3 respectively.

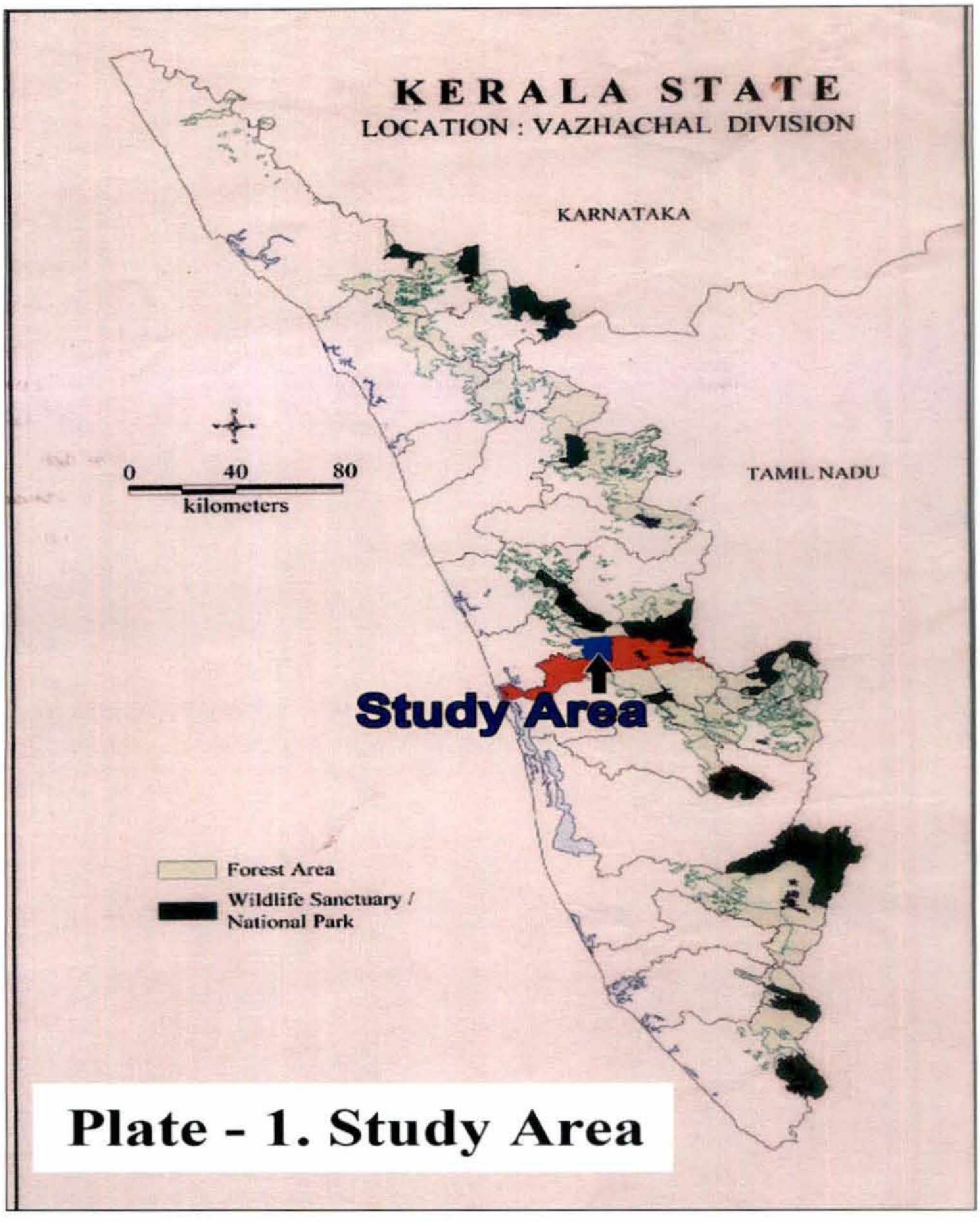


Plate - 1. Study Area

Since the fish under study is identified after HORA and it belongs to the family BAGRIDAE it has been placed under the genus HORABAGRUS. The Species name NIGRICOLLARIS is because of its black collar (Nigri=black, collaris=baring a collar) on the branchial region.

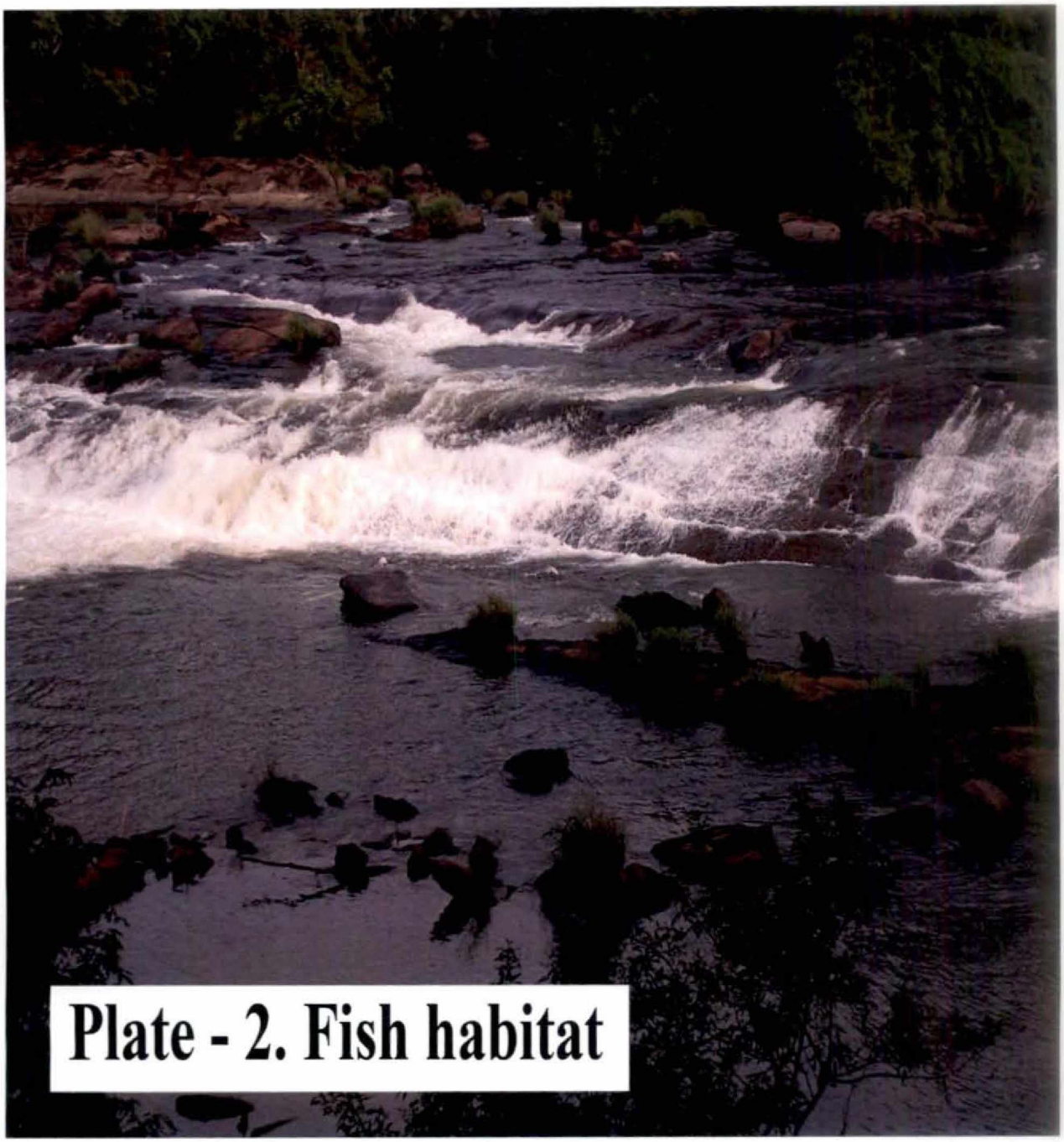


Plate - 2. Fish habitat

Order: Siluriformes, Family: Horabagridae, Genus: *Horabagrus*, Species: *Nigricollaris*.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. CRYOPRESERVATION AND ITS HISTORY

Cryopreservation refers to storage of living organisms or tissues at ultra low temperatures, below −130°C, such that it can be revived and restored to same living state as it was before being stored (Rana, 1995a; Awad and Graham, 2002).

Since the first work of Blaxter (1953) fish sperm cryopreservation has been attempted on many freshwater as well as marine species (Erdahl and Graham, 1980; 1987). Techniques of sperm management have been established in some freshwater fish species such as cyprinids (Billard *et al.,* 1995), siluroids (Legendre *et al.,* 1996) and in salmonids (Scott and Baynes,1980; Billard,1992). Among these techniques, sperm storage and cryopreservation are of special interest.

Cryopreservation of spermatozoa and spermatogic cells for intracytoplasmic sperm injection has been developed primarily to deliver an intact genome. Artificial insemination (AI) has been an important tool to achieve genetic improvement and disease control in important species (Bailey *et al.,* 2000). Sperms from genetically superior males could be used to impregnate multiple females to maximize the distribution of favorable genes. AI also eliminates the need of physical contact between different animals and thus prevents the spread of diseases. This is especially useful in agri-food industry. Thus, the wide use of AI over natural breeding depends on the successful cryopreservation of sperms (Watson, 1995).

Cryopreservation makes it possible to store sperm for a long term and transport over distances. Sperm cryopreservation of agriculturally important species, like cattle, has been an established industry worldwide (Bailey *et al.,* 2000). Genome banking will also benefit those endangered and valuable transgenic species. The application of cryopreservation to bull sperm has been successfully done for quite long time but application to other species is problematic. The major reason for this is our inadequate understanding of the mechanisms at the cellular level, associated injuries and the absence of appropriate cellular models (Wolfe and Bryant, 2001). During freezing, the cells encounter changes in the membrane phase states, ice formation and increased concentration of all other solutes and they must respond to these changes within the finite time allowed by cryopreservation protocol (Mazur and Cole, 1989; Schneider and Mazur, 1984; Formicki, 1997). With all these considerations, an appropriate protocol for maximizing cell survival is required.

Schneider and Mazur (1984) derived some equations to describe the cooling rate and some other variables. However, applications of these concepts are impossible without the understanding of cellular response to solvent and solute movements. Water and solute transport have been studied in various biological systems (Gier, 1989). It is showed that the rate of water transport is affected by composition and phase preference of individual lipids (Lovelock, 1957; Lovelock and Bischof,1959; Gier, 1989; Toner, 1990; 1992) as well as structure of membrane (Mazur, 1970).

Theoretically, sperms should recover fertilizing ability and retain membrane integrity after freezing and thawing. However, ultra-structure damages to plasma membrane, acrosome and flagella of sperm have been observed after cryopreservation (Barthelemy *et al.,* 1990) and the causes are reported to be membrane swelling, acrosome swelling, acrosome loss, and pulling back of the mitochondrial sheath (Royerel *et al.,* 1996). Species differences in female tract anatomy, sperm transport mechanisms, ability to time inseminations and to deliver spermatozoa are also determinants of fertility with cryopreserved spermatozoa (Holt, 2001).

Improvement of this technique depends on a better understanding of the biophysical characteristics of the fish sperm. Cryopreservation has induced many stresses on sperm of some species, like phase transformation of the cell membrane (Purdy and Graham, 2003). Some of these harmful stresses could be decreased by adding lipids to the sperm prior to cooling and thawing. Especially, the phase transformation could be eliminated by adding sufficient quantity of cholesterol to the sample. According to Purdy and Graham (2004), bull sperm treated with proper amount of cholesterol-loaded cyclodetrixn has an increased viability and motility after cryopreservation which may also be applicable to other species.

According to Ashwood-Smith (1980) at 0°C conditions, spermatozoa can be stored for a few hours upto several days, depending on the species while cryopreserved gametes can be theoretically stored between 200 and 32000 years without deleterious effect. The use of cryopreserved spermatozoa can be delayed from the date of collection and adjusted to the moment of ova processing.

Rana (1995a) stated that transport of gametes is useful when male and female gametes are collected from different locations. This enables also the introduction of genes from the wild into hatchery stocks. Senescence of sperm during the course of the spawning season has been reported for many fish species resulting in a decrease of

milt quality which could be tackled by this technique as cryopreservation allows the collection of sperm when it has the highest quality.

Daniels (2003) explained the use of this technique in experimental programs - for genetic studies, in comparing the breeding performances of successive generations in the same experiment and for experiments where the use of identical sperm samples is necessary over an extended period.

Studies conducted by Tabata and Mizuta (1997) revealed the importance of conserving genetic variability in domesticated populations through the technique of cryopreservation. "Gene banks" of cryopreserved semen can also be used to maintain genetic diversity of fish populations that are endangered and protect against inbreeding Gwo (1993). As a consequence, success in breeding is greatly enhanced by the use of frozen sperm.

Most publications on cryopreservation of gametes are devoted to salmonids, tilapias and carps (Guptha, 1992). As ornamental and foodfish farming expands, there is an increasing need to apply sperm cryopreservation techniques. Due to the increasing knowledge in sperm biology also spermatozoa of many fish have been recently cryopreserved successfully. It emphasizes the high survival and fertilization capacity generally obtained in frozen-thawed spermatozoa of marine fish in comparison to fresh water fish. Special attention should be paid on individual variations of sperm quality and on biotests useful to determine the quality of thawed cells (Rana, 1995b).

2.2. SPERM BIOLOGY

Sperm are defined as the male reproductive cells produced by the testicles. They are carried in fluid called semen, capable of fertilizing an egg cell to form a zygote. A zygote can grow into a new organism, such as a fish. Sperm cells contain half of the genetic information needed to create life (Foote, 2002). Generally, the sex of the offspring is determined by the sperm cells, through the chromosomal pair "XX" (for a female) or "XY" (for a male). Sperm cells were first observed by Antoni van Leeuwenhoek in 1679. Spermatozoa are differentiated cells, normally composed of a head, basal body, and tail (Billard *et al.,* 1987). The head contains some cytoplasm and the nuclear material for fertilization. The basal body has a high concentration of mitochondria which provides the energy for sperm motility through the production of ATP. The tail is usually a flagellum served for propulsion. The structure of a Sperm is

shown in Figure 1.

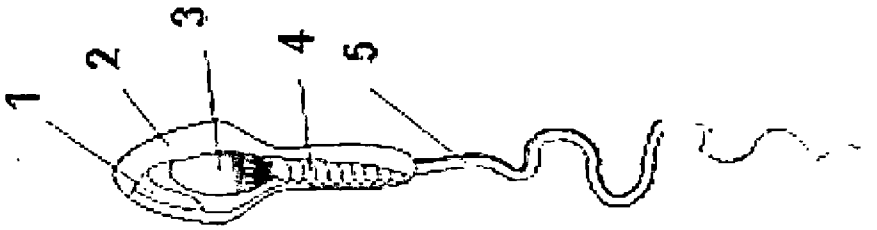


Figure 1. Structure of a Sperm

Parts. 1-5 represent acrosome, cell membrane, nucleus, mitochondria, tail respectively.

2.3. THE ROLE OF TEMPERATURE

Currently only very simplistic organisms and small samples have been successfully recovered after cryopreservation. If the preservation of large and complex systems be accomplished, the benefits to medicine and science would be enormous. But before effectually doing so, it is essential to gain better understanding of the various underlying mechanisms involved, the most important of which is the role of temperature after freezing (Rodriguez-Martinez *et al.,* 1993). It is well known that at temperatures below −196 °C, no changes of biological importance occur, and biological time stops in its tracks; the reason for this being that as temperature decreases below a certain point, the rates of all chemical reactions and biophysical processes will become too slow to affect cell survival. However, in cooling to these stages of 'suspended animation', cells and tissues are exposed to a range of dangerously low temperatures (Toner *et al.,* 1991). This exposure occurs both in cooling to and thawing from storage temperatures. It is in these intermediate phases of cooling and re-warming that the phenomenon of "thermal shock" occurs. This sets up the possibility of unusual mechanical stress due to expansion and contraction of cell membranes thereby contributing towards cell death. There is no exact knowledge of either, when this phenomenon occurs or why it occurs and the effects of the same on the biological system (Mansoori, 1975).

One way of acquiring better knowledge of this phenomenon would be to modulate temperature changes on the cellular level and study the subsequent physico-chemical changes that occur. As a first step towards modulating temperature changes

at cellular level, arrays of individually addressable micro-thermo-electric-coolers (μTECs) were modeled, fabricated and characterized (Devireddy and Bischof, 2003).

The curve of cell survival percentage against cooling rate has a reverse U shape (Thirumala *et al.*, 2003). It is reported that at the maximal point, the injury effect is the least and cooling rate is optimal but the optimal cooling rate is not the same for different cell types. The rate of water loss is found to be affected by cell permeability; more permeable cells are able to tolerate rapid cooling better than less permeable cells (Kedem and Katchalsky, 1958). The optimal cooling rate is also found to be related to the surface area of the membrane and the surface to volume ratio of the cell.

During cryopreservation and cryosurgery, chemicals and additives including cryopreservative agents (CPAs), antifreeze protein (AFP), and cryosurgery adjuvants are often added to improve the survival of cells. With the addition of these chemicals, the composition and properties of the solution will be changed and the phase relationships of the system will be complex (Devireddy *et al.*, 1999a; 1999b; 2000; 2002a; 2002b). Since equilibrium conditions are frequently not attained, the solid-liquid state diagrams are adopted to obtain the thermo-physical properties of solution. Among these additives, the most commonly used one is cryopreservative agents (CPAs) which include sugars, serum, solvents and their combinations (Hovatta *et al.*, 1996).

2.4. CRYOPROTECTIVE AGENTS

During the freezing process, cryoprotective agents can serve several functions: lowering the freezing point, binding with water to prevent it freezing at zero degree, and decreasing membrane damage (Farrant, 1980; Gao *et al.*, 1995). According to their ability to transport through cell membrane, CPAs are classified as penetrating (glycerol and DMSO) and non-penetrating ones (lactose and trehalose). Both types can osmotically induce water egress and cause dehydration of cells, but their ability to enter the cell and reside in cytoplasm and membranes is different (Chandler, 2000).

The choice of cryoprotective agents (CPAs) depends on the characteristics of the cryopreserved substances. Currently, the most commonly used CPAs are glycerol and dimethyl sulphoxide (DMSO) and have proved to be most effective for many

substances (Horváth and Urbányi, 2000a; 200b). However, glycerol with the concentration higher than 3-4 *M* is bio-chemically toxic to human kidney (denature some enzymes) which means it is more critical for the preservation of smaller cells and DMSO is more likely to have a dissolving effect for membrane (Horton and Ott, 1976). Other kinds of CPAs like ethylene glycol and methoxylated compounds are also used in specific cases. For example, human sperm membrane is four times more permeable to ethylene glycol than to glycerol. And the membrane transport of ethylene glycol is less affected by temperature than is glycerol (Gilmore *et al.*, 1997; 1998). Prior to being added to the cell suspension, CPAs should be diluted to the desired concentration in fresh growth media. This procedure will minimize the potentially negative effects of chemical reactions on CPAs and reduce the potential toxic effects. Generally, DMSO and glycerol are used in concentrations ranging from 5-10% (v/v) (Holt, 2000).

2.5. THE MECHANISM OF CELL INJURIES

The mechanism of cell injuries is frequently studied but is not yet clearly understood. According to Wolfe and Bryant (2001) survival of freezing cells involves combination of intracellular ice formation, dehydration, freezing point depression, super-cooling and intracellular nitrification.

Dilution shock is also referred to be crucial in freezing process (Farrant, 1963). This is the damage to cells, induced during the dilution that occurs both as ice melts during re-warming and when any cryo-protective additives are removed after thawing and damage due to high concentrations of solutes and formation of intracellular ice (Mazur, 1963). A useful experimental apparatus, cryo-microscope, can be used to study the freezing and thawing processes in living cells and to determine the conditions requisite for intracellular freezing (Diller *et al.*, 1970; Parkinson and Whitfield, 1987).

2.6. INDIVIDUAL VARIATIONS OF SPERM QUALITY

High individual variations of milt quality are frequently reported (Rana, 1995a). This may be due to genetic variability but also to sampling location (from testes to the genital pore), contamination of sperm samples by urine and aging of spermatozoa during the spawning season.

Similar to the Salmonidae (Morisawa and Morisawa, 1988) the maturation

process of spermatozoa has been reported for several fish species (Morisawa and Suzuki, 1980).

2.6.1. Sampling location

Compared to samples collected by stripping, the percentage of motile spermatozoa of turbot decreases when intratesticular milt is collected. In Japanese eel (*Anguilla japonica)* testicular spermatozoa exhibited only a very slight motility after activation, while spermatozoa of stripped sperm could be successfully activated (Ohta *et al.,* 1997a). Furthermore, testicular spermatozoa of the species, incubated in saline solutions with high concentrations of K^+ and $HCO3^-$, artificially acquire their motility potential and show a similar fertilization capacity as stripped milt (Ohta *et al.,* 1997b).

2.6.2. Contamination with urine

Due to the close vicinity of sperm ducts and urinary ducts, sperm samples are frequently contaminated with urine. This phenomenon is described in freshwater fish species (Rana, 1995a), but more scarcely in marine species. In turbot, a mean contamination rate of 15.3% (urine volume: sperm volume) was reported, based on the determination of urea concentration (Dreanno *et al.,* 1998).

Artificial urine contamination of milt decreased the percentage of motile spermatozoa, sperm velocity, the fertilization capacity and the storage ability. These effects increased with urine concentration and incubation time. Catherization of the ureter prior to sperm collection significantly decreases the urine contamination. In species with high sperm densities such as European sea bass (*Dicentrarchus labrax*), urine contamination is more easily detected because of the lower viscosity and colour changes of portions of sperm samples containing urine (Fauvel *et al.,* 1999).

2.6.3. Aging of sperm during spawning season

Intratesticular aging of sperm has been reported for many fish species and it affects sperm quality at the end of the milting period (Rana, 1995a). In sea bass, the concentration of spermatozoa decreased as the spawning season progressed (Fauvel *et al.*, 1999). Spermatozoa of this species maintained their swimming duration for a longer period at the beginning of the milting season than at the end (Billard *et al.,* 1977; Sorbera *et al.,* 1996). Furthermore, lower motility rates, fertilization rates and reduced short-term storage capacity were recorded at the end of the reproduction

period.

As revealed by electron microscopy, also the structure was changed in aged sea bass sperm (Dreanno *et al.,* 1998). In the Atlantic halibut (*Hippoglossus hippoglossus*), sperm motility was reduced at the end of the reproductive period (Methven and Crim, 1991; Shangguan,1998). The highest motility rates of winter flounder (*Pleuronectes americanus*) sperm were found at the beginning of the milting period (Shangguan and Crim, 1995).

A decline of motility parameters, storage and fertilization capacities as well as alterations of the plasma membrane were reported in turbot as the milting season progressed (Suquet *et al.,* 1998a). As recorded in sea bass and turbot, senescence of spermatozoa can result in a decrease of freezing ability.

Subsequently, when sampling sperm for cryopreservation, one must take care of the three described factors (intra testicular maturation, contamination by urine and aging of sperm) as they can decrease the initial quality of spermatozoa.

2.7. BIOTESTS

Several biological tests are used to improve the cryopreservation techniques and to assess the sperm viability of frozen-thawed spermatozoa. An efficient quality test for frozen-thawed spermatozoa must be correlated with the fertilization capacity (Steyn, 1993). The test must allow objective and sensitive measurements of a large number of cells and give a rapid answer because of the fast deterioration of ova quality immediately after collection (Billard and Cosson, 1992). The presently applied tests describe physiological parameters viz. percentage of motile spermatozoa and fertilization capacity. Complementary biochemical tests (ATP, O_2 consumption etc.) or morphological ones (electron microscopy) precisely localize damages due to the cryopreservation process (Cosson *et al.,* 1997).

2.7.1. Motility assessment

In most teleost species with external fertilization studied so far, the activity of sperm is brief and its intensity, which is maximal immediately after dilution and declines during the period of motion (Lahnsteiner *et al.,*1997; 2000). The beat frequency of the majority of the sperm declines progressively within 20-25 and 80-90 seconds respectively in trout and carp (Saad and Billard, 1987; Billard and Cosson,

1989; Holtz, 1993; Chereguini *et al.*, 1997).

The pattern of motility depends on temperature also. The total duration of the mass progressive movement decreases and beat frequency increases as the temperature is raised from 5 to 25°C in trout (Tomasic, 1973).

Billard and Cosson (1992) reviewed and improved early techniques used to describe fish sperm motility: because of a high sperm concentration in milt, the assessment of sperm motility requires a high dilution rate. A two-step procedure is necessary to initiate simultaneous motility of the maximum number of spermatozoa: sperm is first diluted in a medium that does not initiate motility, then, sperm movement is activated, by mixing spermatozoa directly under the microscope with an activating solution.

Cosson *et al.* (1997) developed the techniques allowing a fine description of motility. This observation is greatly enhanced using dark field optic microscope attached to a camcorder. Spermatozoa can be visualized on a video monitor. Using stroboscopic illumination, the flagellar beat frequency can be measured by reference to the calibrated frequency of the flash illuminator. The percentage of motile cells is estimated using an arbitrary scale, each step representing a wide range of spermatozoa showing progressive movement.

CASA (computer-assisted sperm analysis) system allows the analysis of videotapes using software which set up parameters that are adjusted for the sperm characteristics of the studied species. Straight or curvilinear velocities are rarely assessed using CASA system (turbot, Dreanno *et al.*,1997; sea bass, Fauvel *et al.*, 1998a). Among the criteria used to estimate sperm motility, the percentage of motile cells is significantly correlated with the fertilization capacity of sea bass and turbot spermatozoa. Since no significant correlation was reported between fertilization rate and the velocity of spermatozoa in these species, the success of fertilization was assumed to depend on the number of motile cells rather than on the quality of sperm movement (Dreanno, 1998). However, in cod (*Gadus morhua*), the proportion of spermatozoa exhibiting a progressive movement accounted for only a small percentage of the fertilization success (Trippel and Neilson, 1992) and sometimes spermatozoa showing non-progressive vibrating motion could fertilize ova of this species better.

As a consequence, complementary tests must be used to assess the quality of frozen-thawed spermatozoa.

2.7.2. Assessment of energetic status

The determination of intracellular ATP concentration has proven to be informative in freshwater fish species, because a drastic decrease of nucleotide content has been reported in frozen-thawed spermatozoa compared to fresh sperm (Ogier de, 1997). Furthermore, a significant positive correlation was reported between the ATP content of turbot spermatozoa and the percentage of motile cells. In sea bass, Adenylate Energy Charge (AEC) is correlated with the percentage of motile cells and the fertilization capacity (Dreanno, 1998; Terner and Korsh G., 1963). The measurement of intracellular ATP content at various times of post-activation and the assessment of oxygen consumption allow assessing the degree of cell integrity before and after the cryopreservation (Dreanno *et al.,* 1997).

2.7.3. Chemical composition of sperm and seminal fluid

Sperm

The organic composition of sperm of several cyprinids was described by Belova (1982) who showed that the water content was in the range of 71-82% of fresh weight, where as lipids varied from 2.5 to 3.6 %, phospholipids represented 36-40 % of the total lipid fraction, cholesterol 26 - 32 % and tri-glycerides 0.6 -2.2 %. Glycogen was also found in the toad fish (*Opsanus tau*) sperm but not in rainbow trout (Billard *et al.,* 1995).

Seminal fluid

In cyprinids, the ionic composition range is 94-107 *mM* Na^{+}, 39-78 *mM* K^{+}, 0.02 *mM* Mg^{++}, 0.3-12.5 *mM* Ca^{++} as it may change during the reproductive season (Chutia *et al.,* 1998) .

In rainbow trout (*Oncorhynchus mykiss*) seminal plasma pH and osmolality have been selected to determine the semen suitability for cryopreservation (Malejac *et al.,* 1990; Lahnsteiner *et al.,* 1996a). A high content of 42kd protein in the seminal fluid was also correlated with a low fertilization capacity of frozen-thawed rainbow trout spermatozoa (Maisse *et al.,* 1988). The oxaloacetic transaminase activity of the seminal fluid of thawed rainbow trout spermatozoa was also considered as a quality test (Malejac *et al.,* 1990). The leakage of these different components into the seminal

fluid reflects the alteration of the sperm membrane during the cryopreservation.

The Osmolality of blood plasma of some freshwater fishes are given in Table 1.

Table1. Osmolality of blood plasma of some freshwater fishes

Common name	Species	Osmolality (mOsmol/kg)
Lake trout	*Salvelinus namaycush*	298
Common carp	*Cyprinus carpio*	274
Northern pike	*Esox lucius*	274
Channel catfish	*Ictalurus punctatus*	272

Source: Randall and Hoar (1971).

2.7.4. Swelling tests

Sperm samples of rainbow trout were incubated in hypo-osmotic solutions and their resistance to the osmotic shock correlated with the cryopreservation ability was measured (Malejac *et al.,* 1990). It is reported that this test is difficult to use in turbot, because no lysis of spermatozoa was observed after dilution in freshwater (Dreanno, 1998).

2.7.5. Fertilization capacity

The fertilizing capacity is the most conclusive test of sperm quality. It is currently used in most studies on artificial insemination and sperm preservation. However, it integrates an independent factor, which is the 'quality' of eggs and the interaction between gametes and between seminal and ovarian fluids.

The diluent used must be appropriate for both male and female gametes. For instance in carp (*Cyprinus carpio*) a good diluent for sperm is KCL solution (50m*M*) (Fribourgh 1965; Saad and Billard, 1987). The percentage fertilization, which usually

refers to the percentage of hatched larvae, is also critical, but evaluating the percentage of developing larvae to first feeding may be a better index of gamete quality.

Because of the difficulty to collect good quality ova, the fertilization capacity of frozen-thawed spermatozoa has only been tested in a few studies. However, it represents a major step forward in the management of gametes. A standardized experimental insemination procedure is necessary for reproducible evaluation of the fertilization capacity (Suquet *et al.*, 1995a).

However, correlations between the number of ova and their weight must be calculated for each species and variations could also be due to individual fish conditions or seasonal changes. The fertilization capacity of frozen-thawed spermatozoa has to be tested using discriminating conditions. In rainbow trout, a significant correlation was reported between fertilization rate and motility estimates using a low number of spermatozoa per ovum (<200,000), whereas a supra-optimal ratio did not allow to detect this correlation (Moccia and Munkittrick, 1987).

2.8. MECHANISMS INVOLVED IN THE INITIATION OF MOVEMENT

This aspect was studied in salmonids, where sperm motility is inhibited by high extracellular [K^+] concentration and can be activated by dilution of extracellular [K^+]. Other divalent ions like Ca^{++}, may have an extracellular effect by masking the charges contributing to the hyperpolarization of the membrane (Boitano and Omoto, 1991).

2.9. MECHANISMS CONTROLLING MOTILITY

In some species, the oxidative capacity of the mitochondria is low and the energy (ATP) required for motility (Boitano and Omoto, 1992) seems to be mainly pre-accumulated in the sperm before its release from the testes. Subsequently, this reserve becomes exhausted during motility (Christen *et al.*, 1987). Respiration is the least necessary for the maintenance of the ATP levels during motility since it relies on endogenous energy stores in succer, Atlantic salmon and cod (Robitaille *et al.*, 1987). The Krebs cycle is obviously involved in providing energy as specific enzymes such as MDH and pyruvate kinase have been identified. Research efforts in this field of energitics at the moment remain limited.

2.10. PHYSIOLOGY OF INTRATESTICULAR SPERM

The sperm from the testes and its quality is highly variable. In some cases there is no contribution of the sperm ducts to the formation of seminal fluid and the physiology of such sperm remains unknown (Bromage, 1995).

2.11. OPTIMAL SPERMATOZOA: EGG RATIO

The knowledge of optimal sperm to ovum ratio is necessary to improve the cryopreservation technique i.e. optimal sperm: ovum ratio (number of ova /ml diluent).

As reported by Abascal *et al.* (2007) the optimal ratio of spermatozoa: egg (15000 : 1) for artificial insemination of African catfish, *Clarias gariepinus* gave fertilization and hatching rates of 80 and 67%, respectively. Below a sperm: ova of 3000 : 1, fertilization success decreased significantly. Excessive sperm (>15 000: 1) partly inhibited fertilization success. Although both fertilization and hatching rates provide equally good indicators of fertilization success, the more rapid fertilization rate test is recommended since it requires only 12 h (Abascal *et al.*, 2007).

Therefore, knowledge of the optimal sperm to ovum ratio is necessary to improve the cryopreservation technique. Optimal sperm to ovum ratios are only reported for few fish species in the literature, some of which are given in Table 2.

Table 2. Optimal sperm to ovum ratio in some teleosts.

Species	Optimal sperm : ovum ratio	number of ova / ml diluent	Reference
Pacific herring	24 : 1	unknown	Hourston and Rosenthal, 1976
Atlantic croaker	1,000 : 1	5,000	Gwo *et al.*, 1991
Turbot	6,000 : 1	2,000	Suquet *et al.*, 1995b
Atlantic halibut	10,000 : 1	unknown	Rana and Gilmour, 1996
Sea bass	66,000 : 1	2,000	Fauvel *et al.*, 1999
Wolffish	200,000 : 1	unknown	Mokness and Pavlov, 1996

2.12. DEVELOPEMENT OF CRYOPRESERVATION PROTOCOL

To establish a cryopreservation protocol, the extender, the cryoprotectant as well as the cooling and thawing conditions must be investigated. This is difficult as all parameters may interact with each other.

2.12.1. Extenders

Pure semen is usually not suitable for freezing, the addition of an extender is necessary. The composition of the most successful extenders used for some fish species is given in Table 3. Except for the ocean pout (*Macrozoarces americanus*) which is an internally fertilizing marine teleost, for which a diluent mimicking seminal plasma is used (Yao *et al.*,1995), most diluents used in fish are saline (concentration 1-10%) or sugar (5-10%) solutions. As motility depends on internally stored ATP which can be resynthesized only at very low rates, the extender must inhibit sperm motility before freezing.

The Mounib extender (Mounib, 1978) is suitable for the cod, sea bass and turbot spermatozoa. The presence of reduced gluthatione prevents deleterious effects of free radicals (Maracine and Segner, 1998) and avoids peroxidation of lipids,

protecting the sperm membranes (Ogier de, 1997). The Mounib diluent was also successfully used for spermatozoa of freshwater fish (Mounib, 1978; Legendre and Billard,1980). But, Mounib's medium was not appropriate for freezing Atlantic cod (*Micropogonias undulatus*) spermatozoa (Gwo *et al.*, 1991).

Dilution ratios of sperm in extender range from 1:1 to 1:20 (volume of semen: volume of diluent). Lower survival of frozen-thawed spermatozoa was recorded for dilution ratios larger than 1:20 in Atlantic croaker (Gwo *et al.*, 1991) and larger than 1: 50 in sea bream (Chambeyron and Zohar, 1990). The motility duration of black grouper spermatozoa decreased from 40 to 2 minutes when increasing the semen dilution ratio from 1:10 to 1:100 (Gwo, 1993). Increasing the dilution rate from 1:1 up to 1:9 did not modify the percentage of motile frozen-thawed turbot spermatozoa (Dreanno *et al.*, 1997). It is suggested that seminal plasma proteins protect sperm viability and higher dilution ratios than 1:10 may reduce this effect.

2.12.2. Cryoprotectants

The multiple roles of cryoprotectants during the cooling process were reviewed by Jamieson (1991). Cryoprotectants are classified in permeating and non-permeating according to their ability to pass through the cell membrane (Chaveiro *et al.*, 2004). Permeating cryoprotectants such as ethylene, propylene glycol, glycerol, DMSO and methanol were tested for cryopreservation of spermatozoa of fish.

DMSO generally gave the best results which are given in Table 4 and its success can be explained by the fast penetration into spermatozoa and by its interaction with the phospholipids of the sperm membrane (Ogier de, 1997). Flow cytometric analysis revealed a high percentage of turbot spermatozoa presenting no cryo-injuries of the plasma membrane and mitochondria in the presence of DMSO (Ogier de, 1997). However, DMSO is toxic at high concentrations: the motility duration of frozen-thawed barramundi (*Lates calcarifer*) spermatozoa was reduced when the DMSO concentration was higher than 5% (Leung, 1987) and also in the black grouper, sperm motility was decreased at a concentration of 30% (Gwo, 1993).

Table 3. Composition of the extenders successfully used for freezing spermatozoa of some teleosts.

Species	Extender composition	Reference
Atlantic croaker	NaCl, Glucose or sucrose	Gwo *et al.*, 1991
Atlantic halibut	NaCl-Glycine-$NaHCO_3$	Bolla *et al.*, 1987
Barramundi	Ringer solution for freshwater fish	Leung, 1987
Black grouper	NaCl	Gwo, 1993
Cod	sucrose, reduced glutathione, $KHCO_3$	Mounib, 1978
Grey mullet	Ringer solution for marine fish	Chao *et al.*, 1975
Grouper	NaCl, $NaHCO_3$, fructose, lecithin, mannitol	Withler and Lim, 1982
Hirame	Ringer for freshwater fish	Tabata and Mizuta, 1997
Mullet	Ringer for marine fish	Joseph and Rao, 1993
Ocean pout	Medium mimicking seminal fluid	Yao *et al.*,1995
Pacific herring	Ringer for marine fish	Pillai *et al.*,1994
Plaice	NaCl	Pullin, 1972
Puffer	Glucose	Gwo *et al.*, 1993
Sea bass	sucrose, reduced glutathione, $KHCO_3$	Fauvel *et al.*, 1998a
Turbot	sucrose, reduced glutathione, $KHCO_3$	Dreanno *et al.*, 1997

Table 4. Optimal DMSO concentrations for cryopreservation of sperm of some fish species.

Species	Concentration (%)	Reference
Atlantic croaker	15	Gwo *et al.*, 1991
Barramundi	5	Leung, 1987
Black grouper	20	Gwo, 1993
Grouper	10	Withler and Lim, 1982
Ocean pout	20	Yao *et al.*, 1995
Pacific herring	15	Pillai *et al.*, 1994
Sea bream	10	Maisse *et al.*, 1998
Turbot	10	Dreanno *et al.*, 1997
Yellowtail flounder	10	Richardson *et al.*, 1995

Methanol has low (barramundi : Leung, 1987; turbot : Dreanno *et al.*, 1997) or no (Atlantic croaker : Gwo *et al.*, 1991; black grouper : Gwo, 1993) cryoprotective efficiency in frozen spermatozoa. Ethylene glycerol and propylene glycerol gave intermediate results in Atlantic croaker (Gwo *et al.*, 1991) and yellow fin sea bream (Gwo, 1994).

In turbot, addition of cryoprotectants such as DMSO increased osmolality up to 1100 mOsmol/kg and induced the movement of spermatozoa for a period of less than 1 minute. This activation before freezing did not affect sperm motion capacity in turbot may be because spermatozoa are able to resynthetise energy during the period of sperm motion (Dreanno *et al.*, 1997).

In striped trumpeter (*Latris lineata*) and in yellow fin bream (*Acanthopagrus australis*), the post-thaw motility of spermatozoa was higher with glycerol than with DMSO (Thorogood and Blackshaw, 1992; Ritar and Campet, 1995). Glycerol gave good protection in yellow fin sea bream (Gwo, 1994) but it provided no protection in black grouper (Gwo, 1993) and low protection in the turbot (Dreanno *et al.*, 1997). Due to the species specific cryopreservation requirements, the suitability of each potential cryoprotectant must be evaluated when developing a method for new fish species.

In freshwater fish, non-permeating cryoprotectants such as proteins bismuth sulphate agar (BSA) or lipoprotein (egg yolk) have been commonly used to prevent

damages to the plasma membrane (Scott and Baynes, 1980). Cabrita *et al.* (1998) suggested that they increase the membrane resistance to osmotic stress and the motility rate of frozen-thawed rainbow trout spermatozoa. In the Pacific herring, addition of 10% hen's egg yolk did not improve the survival of frozen-thawed spermatozoa (Pillai *et al.*, 1994). No significant difference was observed in the motility of frozen-thawed turbot spermatozoa when egg yolk (10%), BSA (10%) or a combination of both (5% + 5%) was used (Dreanno *et al.*, 1997). Furthermore, increasing the egg yolk concentration from 10 to 20% or substituting egg yolk with milk, did not change the survival of frozen-thawed spermatozoa in this species (Chereguini *et al.*, 1997).

Permeating and non-permeating cryoprotectants can interact: in barramundi sperm, the protective ability of skim milk gave better cryoprotection than egg yolk when glycerol or methanol was used instead of DMSO (Leung, 1987). Because spermatozoa are small, the penetration of cryoprotectants is rapid (Jamieson, 1991; Basavaraja and Hegde, 2003) and no equilibration period is required. Therefore the toxic effect of DMSO can be minimized.

2.12.3. Equilibration period

Increasing the equilibration period from 5 to 60 minutes and the DMSO concentration from 10 to 30% lowered the post-thaw motility of yellowfin seabream spermatozoa (Gwo, 1994).

In seabream, the fertilizing ability of frozen-thawed sperm decreased in DMSO extender when the equilibration period exceeded two minutes (Billard, 1978). A similar effect was observed after one hour in grey mullet (Chao *et al.*, 1975). An equilibration time of 10 to 60 minutes had no effect on the fertility of frozen-thawed spermatozoa of hirame (Tabata and Mizuta, 1997).

In rainbow trout, Ogier de (1997) observed that DMSO needed 10 minutes to penetrate into spermatozoa although the fertilization capacity of frozen-thawed spermatozoa was the same with or without equilibration period. This observation suggests that the protective role of DMSO does not depend on its penetration into spermatozoa. With glycerol as cryoprotectant, the motility rate of frozen-thawed spermatozoa of bluefin tuna (*Thunnus thynnus*) was increased at long equilibration periods (30 minutes) compared to short ones (10 minutes), but the opposite was recorded for DMSO (Doi *et al.*, 1982). Since penetration of glycerol is slow, an

equilibration time may be necessary.

2.12.4. Cooling rate

In cryopreservation an optimal cooling rate is used so that cooling occurs slowly enough to avoid intracellular ice formation yet fast enough to avoid cellular hydration. Cell injury is likely to be caused by extensive cellular hydration and exposure to high concentration of electrolytes (Drobnis *et al.*,1993). Clearly, cell volume changes during freezing and thawing is a critical factor determining whether the cells survive.

In response to the changes in environment, the volume of cell varies several times as the function of substances movement across the membrane. The first volume adjustment of cells occurs when CPAs are added to cells in isotonic media (Roy *et al.*, 1998). Associated with osmotically driven egress of intracellular water, the component of cells shrink fast, and then as the penetrating CPAs enters, the volume slowly returns to the original value. The second volume adjustment occurs in the process of extracellular water freezing, water move out of cells in response to high concentrations of extracellular salts. Excellent examples of measured changes in the size of embryos and ova during passage through a freezing and thawing cycle are provided by Schneider (1986). However, a more precise description of the cell volume changes needs a quantitative understanding of the water transport during the freezing process.

According to Devireddy *et al.* (2002b), the sperm cell is assumed as a long cylinder and the parameters of cells in the model system can be obtained by microscopy. The initial cell volume is normally determined based on the composition of the CPA medium. The osmotically inactive cell volume can be predicted by extrapolating the figures of equilibrium volume of water in the cells at different subzero temperatures to infinite osmolalities (Devireddy and Bischof, 1998). With the water transport model, the volume changes of cells during the whole process can be monitored and used for further dynamic analysis of the biophysical process occurring during freezing and thawing.

To achieve the best effect of cryopreservation, the biophysical responses of sperm cell and tissues during freezing should be clearly understood. For this study, the differential scanning calorimeter (DSC) technique (McNaughton and Mortimer, 1975) is used to monitor the volume changes of sperm cells during freezing. The

cellular volume data is then coupled with the water transport model to get the permeability parameters of the cell membranes. These data can be further used to predict the optimal freezing and thawing procedure of spermatozoa or ovarian tissues (Devireddy *et al.*, 1998).

2.12.4.1. The Differential Scanning Calorimetry

DSC is a thermo-analytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference are measured as a function of temperature (Gray, 1976). A differential scanning calorimeter consists of two sealed pans: a sample pan and a reference pan (an empty sample pan). These pans are often composed of aluminum, which has a very high thermal conductivity and acts as a radiation shield. Cell suspension with weight from 0.1 to 100 mg is added to the sample pan. The two pans are then heated, or cooled, uniformly while the heat flow difference between the two is monitored in a temperature scanning process. The temperature scanning can be done isothermally or by changing the temperature at a constant rate (Scheiwe and Korber, 1984). The differences in the heat flow between the sample and reference during this process is sent to a computer, and a plot of the differential heat flow between the reference and sample cell as a function of temperature is produced by the computer. A process without thermodynamic chemical reactions will result in a flat or very shallow base line on the plot. However, an exothermic or endothermic process within the sample results in a significant deviation in the difference between the two heat flows and thus there is a peak in the DSC curve. Exothermic processes will show as positive peaks (above the baseline) while peaks resulting from endothermic processes are negative (below the baseline) Devireddy *et al.* (2004) With the above relationship and heat release data, the experiment data of DSC experiments could be converted to the volume change of cells during freezing and thawing process. Coupled with the water transport model, the volume change history of cells can be used to further study the cellular biophysical response including water and solute transport through the membrane (Devireddy *et al.*, 2001).

For freezing of fish semen, generally a two step procedure is applied: milt is cooled in nitrogen vapour on a floating tray or in the neck of a container and then the straws are plunged into liquid nitrogen. The cooling rate is determined by the height of the tray or the depth at which canisters are placed (Scheiwe and Korber, 1984).

The cooling rates most frequently used in fish species are shown in Table 5. In Atlantic halibut (Bolla *et al.*, 1987) freezing has also been carried out by pelleting diluted semen onto dry ice. This approximately corresponds to a cooling rate of 35°C.min^{-1} (Stoss and Donaldson, 1983).

Motility of thawed barramundi spermatozoa was not affected by a freezing rate between 1 and 30°C.minute^{-1}, suggesting high freezing tolerance. On the other hand, a lower freezing tolerance was recorded in seabream as it was optimal only at 10 was successfully applied, whereas 1°C per minute resulted in low post-thaw motility (Mounib, 1978).

Freezing conditions for turbot were optimal at a cooling rate of 99°C.minute^{-1} compared to 1, 5, 20, 50 and 100 °C.minute^{-1} (Billard, 1978). As a consequence, the optimal cooling rate is highly dependent on the fish species.

Table 5. Freezing rates used for cryopreservation of spermatozoa of some fish species.

Species	Freezing rate (°C.min^{-1})	Reference
Barramundi	31	Leung, 1987
Cod	5	Mounib *et al.*, 1968
Hirame	8	Tabata and Mizuta, 1997
Sea bass	10	Villani and Catena, 1991
Sea bass	65	Fauvel *et al.*, 1998a
Turbot	99	Dreanno *et al.*, 1997

2.12.5. Thawing rate

Rapid thawing is necessary to avoid re-crystallisation. Thawing rates used in some species are shown in Table 6 and are lower for marine fish than those reported for freshwater fish (30-80°C: Rana, 1995a). In striped trumpeter, there was no difference in the percentage of motile frozen-thawed spermatozoa using thawing temperatures between 10 and 30°C (Ritar and Campet, 1995). In turbot, thawing temperature between 20 and 40°C did not affect post-thaw sperm motility (Dreanno *et al.*, 1997). But, an increase in the thawing temperature from 1 to 30°C reduced the post-thaw motility of ocean pout spermatozoa (Yao *et al.*, 1995).

When Atlantic croaker spermatozoa were thawed at 0°C, the fertilization rate was significantly lower than at 25 and 50°C (Gwo *et al.*, 1991). In the Atlantic halibut, the optimal thawing temperature was 10°C for straws and 40°C for pellets (Bolla *et al.*, 1987).

Thawed spermatozoa must be rapidly used: 60 minutes after thawing, the percentage of motile turbot spermatozoa stored on crushed ice decreased by 35% (Dreanno *et al.*, 1997). This indicates that cryopreservation induced damages in spermatozoa. Diluting frozen-thawed spermatozoa of this species in a medium mimicking the seminal fluid improved their short term storage capacity. Also, in halibut, short term storage ability of thawed sperm was lower than for fresh sperm (Billard *et al.*, 1993).

Table 6. Thawing rates used in some teleost species

Species	Thawing rate ($°C.min^{-1}$)	Reference
Atlantic halibut	10-40	Bolla *et al.*, 1987
Barramundi	30	Leung, 1987
Bluefin tuna	40	Doi *et al.*, 1982
Cod	38	Mounib, 1978
Grouper	25	Withler and Lim, 1982
Hirame	20	Tabata and Mizuta, 1997
Ocean pout	1	Yao *et al.*, 1995
Puffer	25	Gwo *et al.*, 1993
Sea bass	35	Fauvel *et al.*, 1998a
Turbot	30	Dreanno *et al.*, 1997
Yellowfin bream	20	Thorogood and Blackshaw, 1992
Yellowtail flounder	30	Richardson *et al.*, 1995

2.13. THE QUALITY OF FROZEN THAWED SPERMATOZOA

Generally, the motility rate of frozen-thawed spermatozoa is very high in marine fish species when compared to freshwater species: 21% in rainbow trout (Lahnsteiner *et al.*, 1996b), 25% in carp (Babiak *et al.*, 1997), and from 40 to 85% in tilapias (Chao *et al.*, 1987). In marine species, the high motility rates of frozen-thawed

sperm result also in high fertilization rates as recorded in sea bass (fertilization expressed as a percentage of fresh semen control : 65%; Fauvel *et al.*, 1998a) and turbot (83%; Dreanno *et al.*, 1997), using limited quantities of sperm. The decrease in the fertilization capacity of frozen-thawed semen probably reflects the changes in motility rates observed after freezing and thawing. Other parameters describing the movement of thawed spermatozoa are rarely assessed.

In sea bass, the flagellar beat frequency of swimming spermatozoa was not affected by the cryopreservation process, but straight line and curvilinear swimming velocities were significantly decreased (Fauvel *et al.*, 1998b). The straight line velocity of frozen-thawed turbot spermatozoa did not significantly differ from that of fresh sperm (Dreanno *et al.*, 1997). The mean motility recovery of frozen-thawed spermatozoa of some fish species is given in Table 7.

Table 7. Mean motility recovery of frozen-thawed spermatozoa of some fish species

Species	Motility recovery (%)	Reference
Barramundi	100	Leung, 1987
Black grouper	100	Gwo, 1993
Bluefin tuna	100	Doi *et al.*, 1982
Cod	39	Mounib, 1978
Halibut	65	Billard *et al.*, 1993
Ocean pout	50	Yao *et al.*, 1995
Sea bream	85	Maisse *et al.*, 1998
Striped trumpeter	49	Ritar and Campet, 1995
Turbot	70	Dreanno *et al.*, 1997

Motility rate of frozen-thawed semen expressed as percentage of fresh semen motility rate. Cryo-injuries have been reported for thawed spermatozoa of many freshwater fish species (Rana, 1995a). The fine structure of the head region of 90% of the thawed black grouper spermatozoa was similar to untreated ones (Gwo, 1993). Also in the puffer, 80% of the frozen-thawed spermatozoa had similar ultra-structure as untreated ones (Gwo *et al.*, 1993). Shrinkage of the plasma membrane of the mid-piece was reported for frozen-thawed spermatozoa of the ocean pout (Yao *et al.*, 1995). In frozen-thawed Atlantic croaker spermatozoa, the cristae of mitochondria were disrupted, plasma membrane was swollen or disrupted and the axoneme coiled

(Gwo and Arnold, 1992). Flow cytometric analysis of frozen-thawed turbot spermatozoa revealed a high percentage (up to 93%) of intact plasma membranes and mitochondria (close to 80%; Ogier de, 1997) and the mitochondrial respiratory activity was not altered (Dreanno *et al.*, 1997).

Compared to fresh water fish, the high quality of cryopreserved spermatozoa of marine fish species has been emphasized by Scott and Baynes (1980). Ogier de, (1997) found a significant correlation between the percentage of motile frozen-thawed turbot spermatozoa and the membrane integrity as determined by flow cytometric analysis. Furthermore, it is recorded that up to 93% of frozen-thawed spermatozoa had intact plasma membranes in the turbot, 45% in the trout, 80% in tilapia and 90% in catfish. Drokin (1993) proposed that the cryoresistance of marine fish spermatozoa could be due to the lipid composition of sperm membranes, mainly to the molar ratio of cholesterol to phospholipids which is 2-3 times higher than in freshwater fish. Contrary in rainbow trout spermatozoa, a lower cholesterol content of the plasma membrane was correlated with a higher fertilizing capacity of frozen-thawed sperm (Labbé and Maisse, 1996) and the ratio of cholesterol to phospholipids was not higher in turbot than in trout (Ogier de, 1997).

According to Drokin (1993), phosphatidylcholine may increase the cryoresistance of the sperm membrane and in sperm of marine fish this component has higher levels than in freshwater fish. Its protective role against osmotic and cold stress has been also reported by Simpson *et al.* (1986).

The process of cryopreservation reduces the decrease of intracellular ATP content of turbot spermatozoa for 20-40% (Dreanno *et al.*, 1997; Ogier de, 1997). This decline is lower than in trout (50-90%) and similar to catfish (25%; Ogier de, 1997). This low rate of ATP consumption could explain the higher motility percentages observed in frozen-thawed spermatozoa of turbot (70%) and catfish (60%) compared to trout (21%). Furthermore, the significant decrease of cryoresistance of sea bass spermatozoa recorded at the end of the milting season could be explained by the decrease in endogenous ATP (Dreanno *et al.*, 1997). Contrary, ATP content decreased only slightly in Nile tilapia, *Oreochromis niloticus* spermatozoa during cryopreservation (0-25%). Before movement, ATP content of frozen-thawed trout spermatozoa is about 1.85×10^{-2} m*M* $/10^9$ spermatozoa.

According to Saudrais *et al.* (1998), the demembranated trout spermatozoa move also at much lower concentration of ATP (0.2 m *M* / l). Therefore, the lower

losses of intracellular ATP levels cannot explain the higher survival of frozen-thawed turbot spermatozoa compared to trout spermatozoa. Complementary studies on the cryoprotectant toxicity and on thermal and osmotic stress should be carried out in fish species to understand their capacity to tolerate the cryopreservation.

2.14. EFFECT OF CRYOPRESERVATION ON SPERM PROTEINS

Watson and Plummer (1985) reported that the reduction in motility observed in boar and human spermatozoa following cryopreservation was associated with a decrease in heat shock protein-90 during cooling. Similarly, the loss of P25b (a protein associated with the plasma membrane covering the acrosome) may be responsible, at least in part, for the decrease in fertility following the freezing-thawing procedure of bull semen (Watson, 2000). Few data are available on fish protein pattern by two-dimensional polyacrylamide gel electrophoresis (2-DE), and no information addresses the effect of the freezing-thawing procedure on sperm protein expression. Researchers used the 2-DE and matrix-associated laser desorption/ionization time-of-flight (MALDITOF) mass spectrometry to verify whether the cryopreservation procedure, applied to sea bass milt, affected the expression of proteins involved in the control of sperm functions. Their work shows that the use of the cryopreservation procedure causes the degradation of sperm proteins, and could be responsible (at least partially) for the observed decrease in sperm motility duration and the lower hatching rate of eggs fertilized with cryopreserved sperm. Limited research has explored the use of magnetism in attempts to sort red blood cells (Owen and Sykes, 1984), as well as attempts to trap sperm (Fuhr *et al.*, 1998).

2.15. FERTILITY STUDIES WITH CRYOPRESERVED MILT

The development of fish produced with frozen-thawed sperm was not frequently investigated. Fertilizing ova with cryopreserved sperm did not affect the hatching percentages in cod. Furthermore, the morphology of larvae produced with frozen-thawed spermatozoa was similar to those produced with fresh sperm (Mounib, 1978). Also, the rate of malformed hirame larvae was not significantly different when using fresh or frozen-thawed spermatozoa (Tabata and Mizuta, 1997). In the yellowtail flounder, the hatching rate and the percentage of malformed larvae were not affected by the cryopreservation process (Richardson *et al.*, 1995) and also 29 days old barramundi larvae, produced with frozen-thawed spermatozoa showed no malformations. The hatching rate, the larval survival rate and the larval weight of 10

days old turbot were not significantly different when ova were inseminated with fresh compared to frozen-thawed sperm (Suquet *et al.*, 1998b). But, the hatching rates of eggs inseminated with frozen-thawed sea bass sperm (69%) were significantly lower than those obtained with fresh sperm (81%) (Fauvel *et al.*, 1998b).

2.16. ENVIRONMENTAL CAUSES CONTRIBUTING TO FERTILIZATION FAILURE IN CAPTIVITY

In some cases, inadequate uptake of water post fertilization leads to soft eggs that are susceptible to mechanical injury. Studies by Kinlay (2004) tested how providing mineral ions in the hardening medium would affect the amount of water that was taken up by the egg. Increasing the ionic strength of hardening water decreased the amount of water that is taken up by the egg, resulting in a softer egg in water with higher ionic strength. There was no apparent difference in the water uptake of eggs that were exposed to sodium chloride, calcium chloride, sodium bicarbonate or a sea salt mixture that contained a great variety of ionic species. It was concluded that the occurrence of "soft" eggs is not a simple ionic deficiency in the egg. The Capilano coho salmon is an example, which shows a tendency to produce "soft" eggs - eggs that do not absorb sufficient water after fertilization to make them turgid and resistant to mechanical shock. Capilano Hatchery source water is known to have extremely low ionic content (<6 mg/L total dissolved solids: Muller *et al.*, 1992), since it is derived mainly from snowmelt and rainwater in the mountain temperate rain forest of the 31Capilano River watershed. Since water hardening is caused by a hydrophilic gel located in the perivitelline space inside the egg shell, it was hypothesized that the eggs may derive some of the mineral ions needed in the gel from the environment during the hardening process. Reduction in "soft" egg problems have been accomplished in hatcheries (Billard, 1978) by adding small amounts of salts to the water used for hardening the eggs. It was also hypothesized that there might be a difference between the performance of salt solutions that contained sodium, calcium, bicarbonates or a combination of salts. There is some variation in the individual weights of the eggs; the increase in weight can be noticed after hardening.

2.17. NEW APPROACHES TO THE CRYOPRESERVATION OF FISH OOCYTES AND EMBRYOS

The dramatic decline in fish populations necessitates urgent action to enable gametes and embryo cryopreservation as an aid to conservation. However, high yolk

content and low membrane permeabilities have frustrated their successful cryopreservation, by limiting water removal and cryoprotectant penetration. New approaches should be taken to overcome these barriers to their cryopreservation.

The collapse of both fresh water and marine fish populations is causing great concern, and there is an increasing loss of fish species from many habitats. Retention of the genetic resource and the opportunity to ensure species survival and possible restocking depends on our ability to conserve relevant material. Ideally, such a conservation programme would involve the cryobanking of eggs, sperm and early embryos. Whilst the cryopreservation of fish sperm has, in the main, been relatively successful, fish oocytes and embryos have not been successfully cryopreserved.

There appear to be two main barriers to their cryopreservation, (i) low membrane permeability, makes the removal of water from the material and the penetration by cryoprotective agents difficult; and (ii) the large yolk mass of the oocyte and early embryo represents a compartment that is particularly difficult to reduce water activity. Both these features result in ice crystal formation during the freezing process. In addition the oocytes and embryos are prone to chilling injury unrelated to ice crystal damage (Kopeika *et al.*, 2004).

The first attempts to freeze ovarian tissue were conducted with rodents (Parrott, 1960). Since then, numerous studies of ovarian tissue of many species have been performed, including those of mice (Carroll *et al.*, 1990; Carroll and Gosden, 1993 ; Harp *et al.*, 1994), rats (Yin *et al.*, 2003), sheep (Gosden *et al.*, 1994 ; Baird *et al.*, 1999), monkeys (Schnorr *et al.*, 2002), cattle (Paynter *et al.*, 1999), and humans (Chen, 1986; Newton *et al.*, 1996; Picton *et al.*, 2002; Kim *et al.*, 2002; Donnez *et al.*, 2004; Lee *et al.*, 2004; Gosden, 2000; Oktay *et al.*, 2004; Silber *et al.*, 2005; Lobo, 2005; Donnez *et al.*, 2005). When ice forms within vascular spaces of tissue during freezing, water flows out of the cells of the tissue, i.e. the cells undergo dehydration. If the cells do not dehydrate sufficiently, then at some subzero temperature, intracellular ice forms within the cells themselves. These biophysical responses of cell dehydration and intracellular ice formation (IIF) are directly coupled to cellular injury as described by Mazur's two factor hypothesis (Mazur *et al.*, 1972). According to this hypothesis, the following factors operate.

Factor 1: At low freezing rates, cells may be injured because they are exposed to solutions the properties of which have been drastically altered by the decreasing unfrozen fraction of the vascular/extracellular space.

Factor 2: At high freezing rates, injury may result from IIF. But since the words "low" and "high" are relative terms, the specific freezing rates are characteristic of a given cell type and need to be experimentally determined. These biophysical events during freezing were first elucidated in single cells by cryomicroscopy (Molisch, 1897; Leibo *et al.,* 1978; McGrath *et al.,* 1975; Cosman *et al.*, 1989; Toner, 1993; Smith *et al.*, 1998; Diller, 1982).

Recently, analogous experimental data in whole tissues are emerging. Water transport studies in tissue sections at suprazero temperatures were performed by Newton *et al.* (1998) and Devireddy *et al.* (2004) and measurements of water transport at subzero temperatures have been performed on rat liver (Pazhayannur and Bischof, 1997 ; Devireddy and Bischof, 1998), on rat prostate tumor (Devireddy *et al.,* 1999a) and on uterine fibroid tumor (Devireddy *et al.*, 2001). Subzero permeability measurements have been made using a combination of low temperature microscopy and calorimetric techniques (Bischof, 2000). In fisheries, these studies are still in the stage of infancy.

In the past 10 years, some information on the cryopreservation of fish eggs and embryos, with limited success, was reported for some species: carp (Zhang *et al.,* 1987), multicolour fin rainbow fish, olive flounder, red sea bream (Sasaki *et al.,* 1988), rainbow trout (Stoss and Donaldson, 1983) and other salmonids (Harvey *et al.,* 1983). In general the survival rate after cryopreservation of embryos was far lower than that of sperm. The size of the embryos with a large amount of yolk are larger than sperm, and have more complicated structure as they develop could be the major reasons for difficulty of preservation. Besides, other factors such as different developmental stages must be considered in order to obtain a consistent and manageable working procedure.

2.18. CONCLUSION

Fish losses in the world waters have reached an alarming stage due to habitat loss, environmental damage, pollution and lack of defense against introduced predators and over fishing. Traditionally, aquaculture (popularly known as "fish farming") has endeavored to keep up with fish losses through selective breeding of fish stocks. The efficiency of producing more farmed fish or saving endangered fish species could be enhanced by freezing their spermatozoa and embryos. The freezing or "cryopreservation" of embryos involves exchanging the water in an embryo with antifreeze. However, unlike mammalian embryos (for example, those of a cow or

tiger), fish embryos cannot currently be frozen due to the difficulty of extracting water from the embryo, and getting an antifreeze solution into its yolk. So the ease of cryopreservation of spermatozoa to conserve the valuable gene pools has been adopted in several studies.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

Definitions:

EXTENDER: An extender is a chemical solution, which reduces the harmful deleterious effects of the cryoprotectants to the preserved biological materials.

CRYOPROTECTANT: It is a chemical solution that protects the cell from cryoinjuries.

3.1. EXPERIMENTAL ANIMALS

Horabagrus nigricollaris - black collared yellow catfish of variable sizes were used for the study.

3.1.1. Collection of fishes

Horabagrus nigricollaris were collected using cast net from upstreams of the River Chalakudy, Kerala, Western Ghats during night hours.

3.1.2. Transportation of experimental animals

The fishes were packed in oxygenated bags filled with river water and transported to the College of Fisheries, Panangad.

3.1.3. Acclimatization and stocking

Fishes were acclimatized to the available water and environmental conditions of the college. For that, the oxygenated packs were kept floating on the surface of 10 ton FRP tanks filled with well water of College of Fisheries, Panangad for 15 minutes so as to bring the temperature equilibrium between both media. Then the pack was slowly untied, well water allowed to slowly enter the oxygenated pack and the fishes swam slowly into the well water. Then the tanks were provided with floating aquatic weeds such as *Eichhornia, Pistia* and covered with coconut leaves as the fish likes to inhabit the shadow areas. These fishes normally move in groups.

3.1.4. Rearing and feeding the fish in tanks

Since it is an omnivore, diets like processed clam meat, earthworms, de-headed and frozen prawns etc. were provided as food. In all, it showed a preference for earth worms. When a worm is introduced it catches the worm in a very active and jerky movement. It indicates that this fish can be used for angling if it is cultured in bulk (because it may grow to larger sizes representing more than 1 kg fish). So the earthworm culture was taken up by utilizing the area where waste water is available.

3.1.5. Earthworm culture

This was carried out employing a crude method in earthen pits using the waste- water, cow dung slurry and dry leaves.

3.2. MAINTAINING THE QUALITY OF REARING MEDIUM

The water quality parameters such as water temperature, pH, dissolved oxygen, alkalinity and ammonia were monitored during the rearing period according to A.P.H.A. (1998). Periodic water exchange was done whenever required.

3.3. SEXUAL MATURITY AND GONADOSOMATIC INDEX

Fishes were observed for their gonadal maturity by stripping after each collection.

The Gonado somatic index was calculated by using the following formula:

$$GSI = \frac{\text{Weight of gonads (g)}}{\text{Weight of fish (g)}} \times 100$$

3.4. SEMEN COLLECTION

Semen samples were collected from a few fishes without hypophysation and by applying the gloved hand pressure technique for observing the sperm motility, morphology and its density before cryopreservation.

3.4.1. SOP- Standard Operation Procedures

3.4.1.1. SOP-1. Collection of sperm

Materials required

1. Tricaine methane sulphonate (MS-222) (methyl-m-aminobenzoate)
2. Scalpel
3. Scissors
4. Tweezers
5. Sterile plastic boxes
6. Extenders
7. 100-mL glass bottle
8. Nylon bolt filter
9. 50-mL plastic centrifuge tube

Procedure

1. Select male from which sperm flows from the urinogenital pore when gentle pressure is applied to the abdomen.
2. Kill the fish with an overdose (greater than 5 mg/L) of MS-222.
3. Record total length (mm) and fish weight (g).
4. Open the abdominal cavity using the scalpel and scissors as needed.
5. Remove testis by clamping the tweezers on the base of the testis. Avoid contamination with blood, faeces or urine.
6. Record testis weight (g).
7. Place testis in sterile plastic box with 1-2 ml of selected extender.
8. Crush testis in glass bottle.
9. Pass testis and sperm suspension through filter to remove tissue debris.
10. Put sperm solution into a labeled 50-mL centrifuge tube.
11. Dilute sperm to 1 g testis to 20 ml extender.
12. Estimate motility.
13. Keep at 4 °C on ice.

3.4.1.2. SOP-2. Estimation of sperm motility

Materials required

1. Microscope
2. Glass slide
3. Sperm
4. 10-µL pipette (set at 2 µL)
5. Tap water
6. 40-µL pipette (set at 20µL)

Procedure

1. Set microscope on dark field and use the 10X objective (100X magnification)
2. Pipette 2 µL of sperm onto glass slide.
3. Adjust light and focus to view sperm.
4. Pipette 20 µL tap water onto sperm solution and thoroughly mix with pipette tip.
5. View sperm immediately after mixing with deionized water (within 2-3 sec).
6. Estimate the percentage of motile sperm. Motile sperm actively swim forward; sperm vibrating in place are not considered to be motile.
7. Record motility estimate.

3.4.1.3. SOP-3. Cryopreservation of sperm in cryocans

Materials required

1. Sperm suspension (SOP-1)
2. Extender
3. 50-ml centrifuge tubes (Corning, Inc. Corning, New York)
4. Cryoprotectants
5. PVA (poly vinyl alcohol) powder
6. French straws (IMV International, Minneapolis Minnesota)
7. Ice
8. Cryocans (Model –BA-11, BA-20 of IBP Company Ltd)

Procedure

1. Schedule cryopreservation at least 1 week prior to intended date of cryopreservation.
2. Calculate the volume of sperm and cryoprotectant needed to fill appropriate number of straws (1 straw = 0.5ml).
3. Calculate the volume of sperm and cryoprotectant needed for each treatment.
4. Dilute cryoprotectants 1:1 with extender.
5. Put sperm for each treatment in a 50-ml centrifuge tube.
6. Put cryoprotectant for each treatment in a 50-ml centrifuge tube.
7. Put sperm in a cooler with packing material and ice packs or simply on ice.
8. Fill cryocans with liquid nitrogen.
9. Take straws, sperm, cryoprotectants and cryocans.
10. Label straws
11. Cryopreservation is standardized to use 0.5-ml French straws filled with whole milt extract.
12. Determine how many straws will be frozen (volume of sperm and cryoprotectant used divided by 0.5 ml per straw).
13. Set up appropriate number of French straws.
14. Add cryoprotectant dilution to sperm and record time.
15. Fill straws by placing the intake side in the centrifuge tube holding the sperm and cryoprotectant on sucking, then immediately cease the straws with PVA powder.
16. Place all straws on ice and record equilibration time i.e. for 10 min.
17. Freeze samples by placing on vapor phase liquid nitrogen for 10min.
18. Plunge samples in liquid nitrogen for storage in the shipping dewar/cryocans for a specific period.
19. Thaw one straw per treatment at 27°C for 7 seconds, 2days after cryopreservation.
20. Estimate motility (SOP-2)
21. Thaw straws at 27°C for 7 seconds as needed for fertilization after the storage.

3.4.1.4. SOP-4. Labeling straws for cryopreservation

Materials required

0.5-mL French straws

Procedure

1. Take straws (Figure 2)
2. Label straws with lot number, unit name, species name, cryoprotectant, cryoprotectant concentration, extender, extender osmolality and fish code.

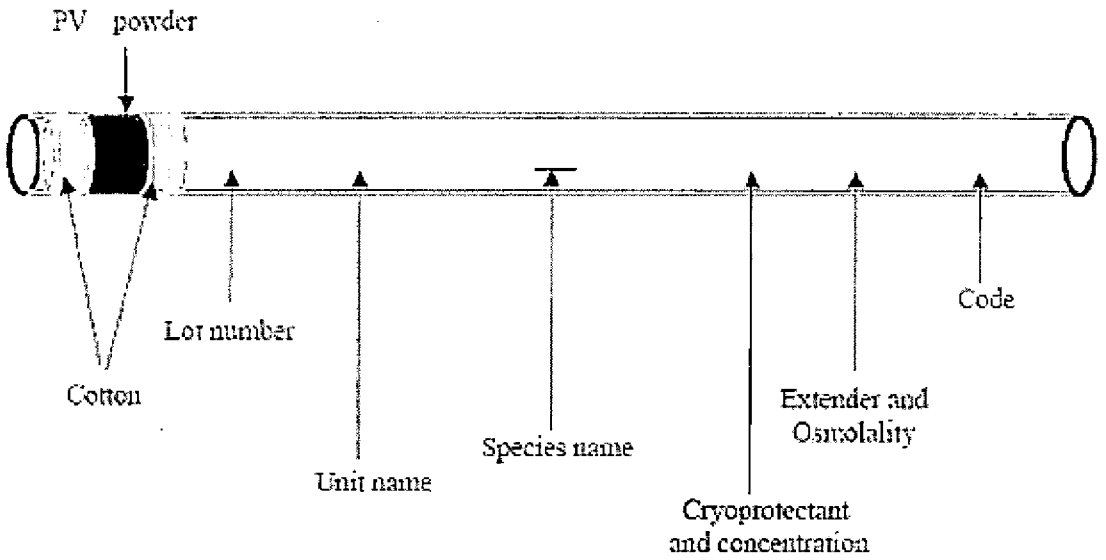


Figure 2. French straw label

The lot number is for record-keeping purpose. This straw represents sperm from *H. nigricollaris* cryopreserved in liquid nitrogen. Sperm were suspended in extender containing 10% DMSO. The sample code is HN16: the 16th male *H. nigricollaris* used in experiments in the year 2006.

The schematic representation of the cryopreservation process used in this study is given in Figure 3.

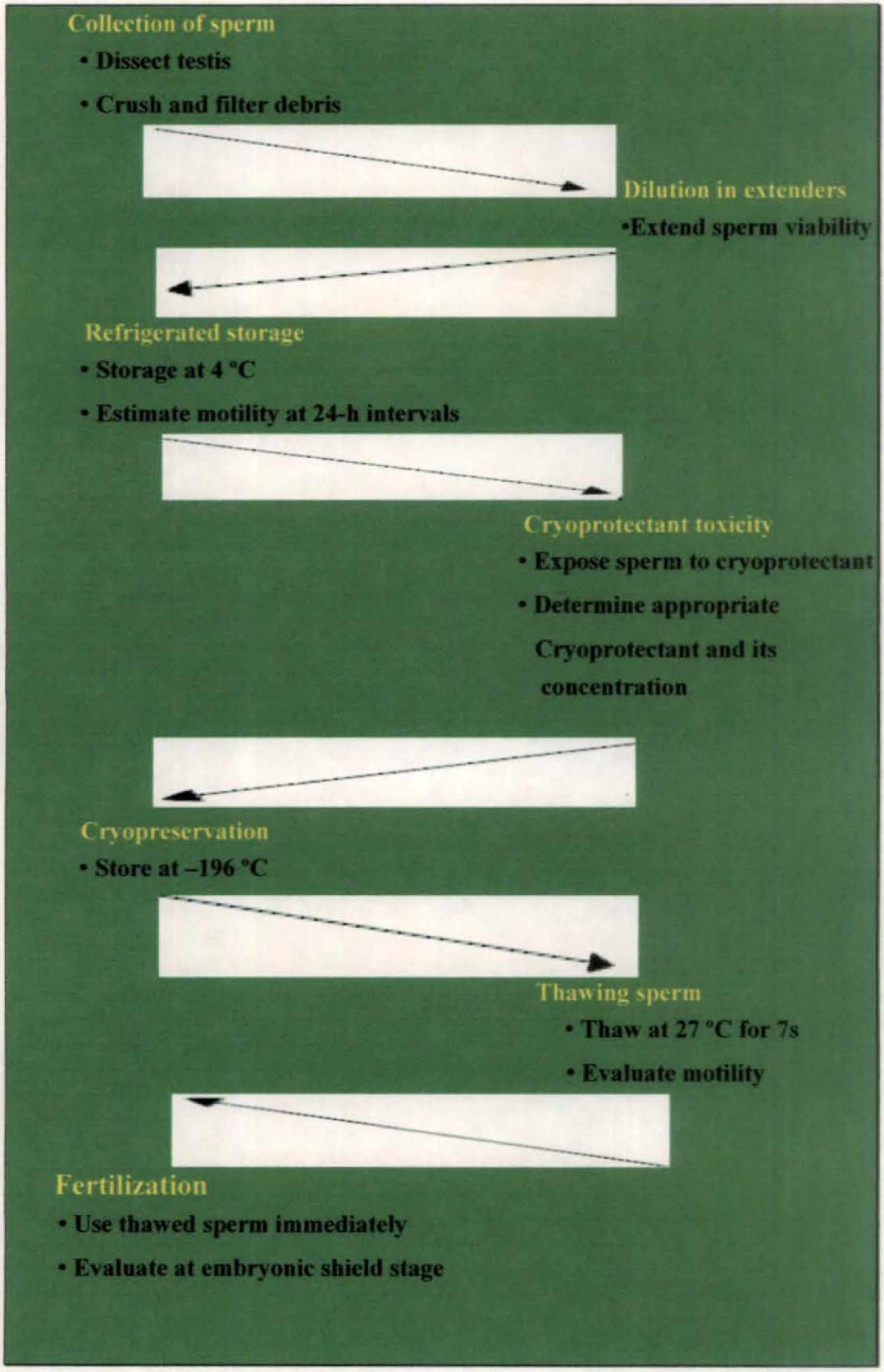


Figure 3. Schematic representation of the cryopreservation process

3.5. MOTILITY TEST

Motility estimates and fertilization are the most common assessments of sperm quality (Wayman and Tiersch, 2000). Estimation of sperm motility is a common assessment procedure because it is quick and relatively simple. Motility estimates in this study were obtained by adding activation solution to sperm. Motility was recorded as the estimated percentage of actively forwardswimming sperm viewed with a microscope. Using the Microcell slide a total of one hundred sperm were counted under the 100X power. The number of moving sperm per one hundred gave the percent motility.

Using the Microcell slide a total of one hundred sperm were counted under the 100X power. The number of moving sperm per one hundred gave the percent motility. However, methods for the estimation of sperm motility are not standardized and sperm motility is not always a good indicator of fertilizing ability (Gwo *et al.*, 1991; Kerby, 1983).

3.6. TESTING FORWARD PROGRESSION OF SPERM

Forward progression of the motile sperm was evaluated using the technique reported by Howard *et al.* (1986) which is a five-point scale as follows: 5+ sperm moving rapidly in a forward direction across microscope viewing field (in approximately one second or less), 4+ sperm moving steady but slower in a forward direction across the microscope field, 3+ in a rapid side-to-side motion with slow forward progression, 2+ sperm moving in a side-to-side direction with no forward motion or in a circular or irregular pattern, 1+ sperm moving slightly side-to-side or in place with slight tail movement, and 0 no movement detected.

3.7. pH DETERMINATION OF THE SEMEN

The pH of the fresh milt was obtained by using pH meter.

3.8. SPERM DENSITY (CONCENTRATION), PCV (PACKED CELL VOLUME) AND SPERMATOCRIT % (DURING PEAK BREEDING SEASON)

To calculate the sperm density (concentration), PCV and spermatocrit percentage, 5 males were injected with Ovaprim @ 0.5ml / Kg body weight. These males were not used earlier for stripping. Milt was collected after 8 hrs from

males were not used earlier for stripping. Milt was collected after 8 hrs from individual fishes in clean, dry plastic milt boxes. Sperm activity tested rapidly under microscope and used to determine the above parameters.

A slide designed to hold a measured volume of semen was used along with an eyepiece micrometer to determine the concentration. When viewed under the microscope at 100X magnification, the number of sperm in 5 blocks (4 corners and a middle block) of the micrometer (selected at random) is used to calculate the number of sperm per milliliter of an un-extended sample. The concentration was recorded in number of sperm per milliliter of semen sample. The sperm density and individual sperm revealed by electron microscopy is shown in Figure 4.

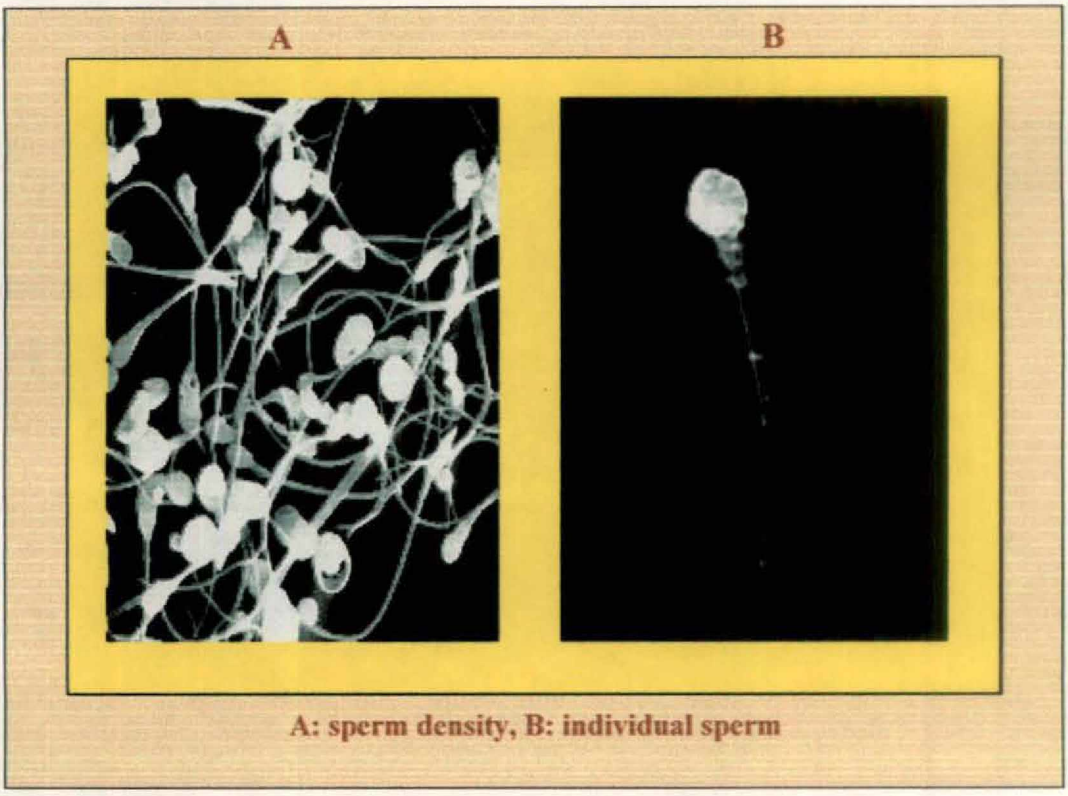


Figure 4. Sperm density and individual sperm revealed by electron microscopy

3.8.1. Procedure for calculating sperm concentration

The concentration of sperm in the milt is often used to characterize semen. An accurate way is to count the number of sperm with haemocytometer or by spectrophotometry. Spermatocrit (volume of sperm: volume of milt) is established after semen centrifugation in microtubes at 10,000 rpm for at least 5-10 min (Bouck

Step 1: Take 0.1ml of individual sample of milt (mean while keep the haemocytometer ready which is slightly moist in extender; 40x10)

Step 2: Dilute1:4 (5 times dilution) with 0.9%Nacl / any other extender in a microfuge tube

Step 3: Mix well and add another 0.5ml of Alcoholic eosin with bit of formaline (now dilution becomes 10 times). This will kill the sperm and then sperm settle down.

Step 4: Mix well and draw the sample up to 0.5 mark of RBC pipette (Red bead)

Step 5: Draw the extender up to 101 mark. Mix thoroughly by hand & shaking the pipette carefully (here dilution becomes 200times; final dilution is made upto 2000 times)

Step 6: Touch the tip of the pipette to haemocytometer at 45° angle. (As spz concentration was high WBC pipette was not used; where dilution is only 20 times)

Step 7: The fluid gets in by capillary action

Step 8: Wait for 3 minutes for the spermatozoa to settle

Step 9: Spermatozoa are counted in the central small squares (4 corner and a central; each one having 16 smallest squares; total smallest squares counted = 16 x 5 = 80)

The apparatus used for sperm cell counting is shown in Figure 5.

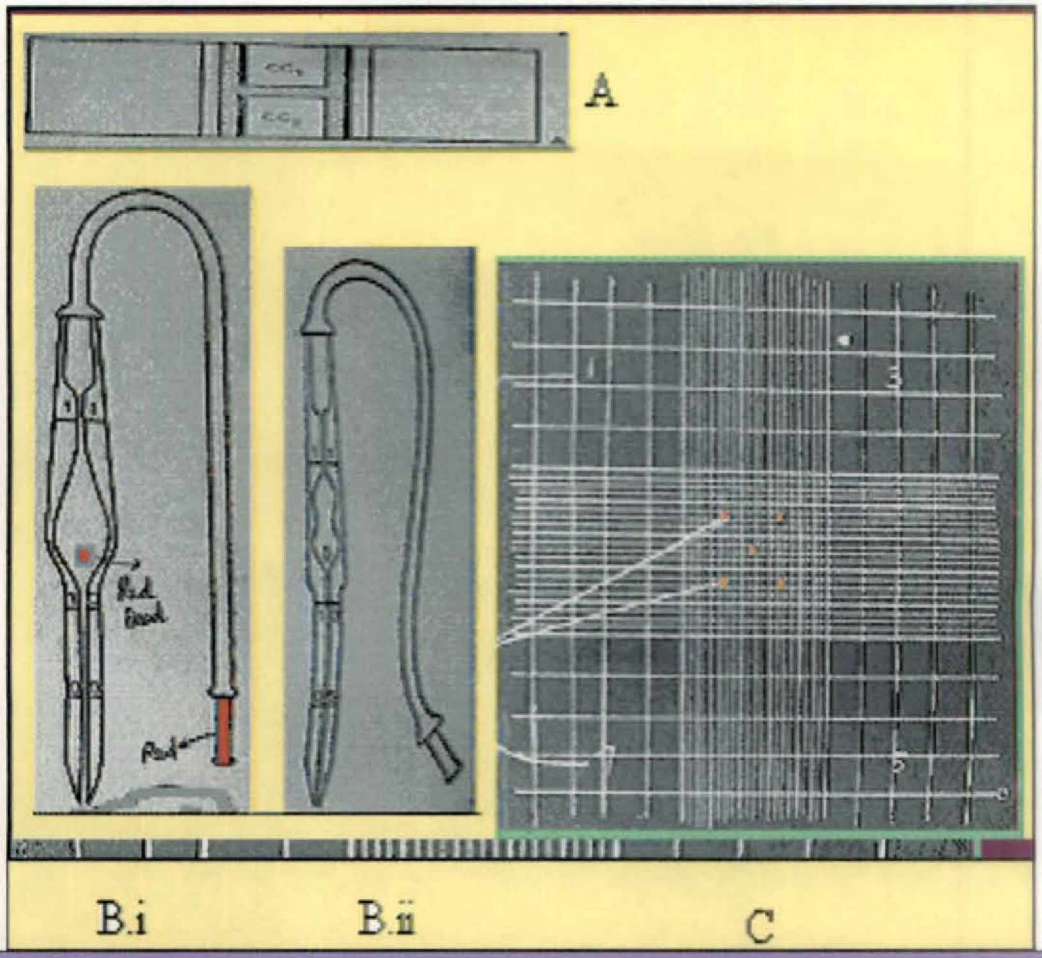


Figure 5. Apparatus used for sperm cell counting

Calculation of spermatozoa concentration:

$$\frac{\text{Total no. of spz in 80 smallest columns}}{80} \times (\text{vol. of each smallest square}) \times 400 \times 10 \times 2000 \text{ (dilution)} = \text{Ж}$$

3.8.2. Spermatocrit %

3.8.2.1. Loading of raw milt in microhaematocrit (capillary tubes)

At first fill 1 to 5 milt samples in 5 tubes, followed by sealing the tubes with wax; note the total height of samples in tubes; then spin the tubes at 10,000 rpm for 10 minutes.

3.9. SPERM MORPHOLOGY

Knowledge of the morphology of sperm has progressed considerably since the development of the technique of electron microscopy. Pioneering work was carried out by Geiger (1955).

An acrosome is present in agnathans (hagfishes and lampreys) and in all groups of fish except the teleosts. However in some group temporary acrosome-like structures have been reported for *e.g.* in *Lepadogaster lepadogaster* (Mattei and Mattei, 1974; Mattei, 1988), rainbow trout *Oncorhynchus mykiss* (Billard, 1992), in *Gambusia affinis* and others (Jamieson, 1991).

As often noticed, the acrosome may not be necessary for fertilization in teleosts because of the presence of a micropyle, but one would expect specialized structures on the plasma membrane at the top of the sperm head to allow cell fusion during fertilization. The shape of the nucleus is highly variable and is related to the complexity of spermatogenesis and especially spermiogenesis (Geiger, 1955). The nucleus is highly polymorphic, filiform, spherical or blade like (Jamieson, 1991).

The mid - piece is well developed in the guppy and much reduced in size in salmonid, cyprinid, mullet and turbot. It is usually located at the posterior part of the

nucleus but it is some times found in the anterior part (elopomorph). More or less complex structures for attachment can be seen (Billard, 1969). These determine the solidity of anchorage of the flagellum. Accessory fibres and structures, somites attached to the axoneme and lateral expansions of the plasma membrane, are commonly found.

The production of sperm has been quantified in several species. In fish species having a seasonal reproduction the total production is found in the testes just before the onset of spermiation. Testes size is a good indicator of the efficiency of spermatogenesis and is quite variable according to the species with sizes varying from 0.2 to 10% of the body weight (Billard 1986, 1988). In practice only a part this production can be collected and, especially in the case of oligospermic species, care should be taken to conserve and save sperm in order to manage properly the production available during the reproductive season. The number of sperm per ml of semen is highly variable from 2×10^{6} to 6.5×10^{10} in various species (Leung and Jamieson, 1991).

3.10. COMPOSITION OF SEMINAL FLUID

According to Linhart *et al.* (1992) the range of ionic composition of seminal plasma of cyprinids is 94-107 *mM* Na^{+}, 39-78 *mM* K^{+}, 0.02 *mM* Mg^{++}, 0.3-12.5 *mM* Ca^{++} as it may change during the reproductive season. This information formed the basis for the preparation of cryopreservation protocol in this study.

3.11. THE TECHNIQUE OF CRYOPRESERVATION

3.11.1. Components of milt cryopreservation

3.11.1.1. Pre-cooling stage

It represents the storage time of semen at low temperature before keeping in the extreme low temperature of liquid nitrogen. Care should be taken in the processes starting from procuring the animals to the testes dissection. This stage needs utmost care in handling the fishes and stress to the animal should be avoided.

3.11.1.2. Milt quality

It can be determined by making the observations of pH, colour, motility, density and spermatocrit% of the semen sample.

3.11.1.3. Diluents (extenders)

To minimize the demand on dissolved O2 the sperm cell density can be reduced by the addition of the diluents. Ideally these diluents should be isotonic to the seminal plasma, maintain the spermatozoa in an immotile state and be able to sustain all the metabolic activities and needs of the cells (Billard, 1983).

3.11.1.4. Cryoprotection

Cryoprotection to the cell can be provided by using the cryoprotective agents such as DMSO and Glyserol.

3.11.1.5. Equilibration time

For effective protection during cooling sufficient time must be allowed to facilitate the penetration of cryoprotectants into cells (Grout and Morris, 1987). The rate of uptake may depend on the size of biological material and molecular-size of the cryoprotectant. Small molecules such as methanol will enter cells at a faster rate than larger compounds such as glycerol (Harvey and Ashwood-Smith, 1982). Equilibration time may be influenced by diluents used.

3.11.1.6. Equilibration temperature

Cooling and thawing rates plays a major role in cryopreserved cell survival. These are the temperatures used in cryopreservation for particular periods of time.

3.11.1.7. Insemination with thawed cryopreserved milt

The injuries sustained during the process of cooling and thawing are difficult to isolate. Cryo-injuries can result in spontaneous activation of spermatozoa and reduce the proportion of activated spermatozoa upon thawing. In addition, the speed and duration of motility can be reduced when compared with fresh unfrozen spermatozoa (Stoss, 1983). In view of such duration, post-thaw milt should be used

immediately after thawing.

To prolong the duration of motility, minimize osmotic shock during fertilization and disperse the sperm cells around the ova, inseminating solutions are advocated. The composition of such solutions should be similar to ovarian fluid (Scott and Baynes, 1980) or have a salinity of 5% and 20% for fresh and sea water species respectively (Stoss, 1983).

3.12. PRESERVATION CONDITIONS

3.12.1. Storage temperature

Storage temperature is a major factor affecting the viability of gametes during *in vitro* storage. Viability can be prolonged by maintaining gametes at near zero temperatures to reduce their metabolic burden. The ability of gametes to tolerate low temperature may vary between temperate and tropical species (Leung and Jamieson, 1991).

The temperature and duration for the storage of ova of sub-tropical and tropical species such as carp (Zlabek and Linhart, 1987) and tilapia (Harvey and Kelly, 1984) are higher and shorter respectively than those reported for salmonids. The sensitivity of eggs to *in vitro* storage may also vary between species, individuals and spawning pattern. In comparison, the viability of milt stored at chilled temperatures can be maintained for longer periods.

3.12.2. Oxygen enriched environments

Maintaining sperm cells in an aerobic environment is a prerequisite for *in vitro* preservation (Scott and Baynes, 1980; Stoss, 1983; Billard, 1988). Studies on rainbow trout suggest that the fertility of spermatozoa can be prolonged when preserved under oxygen compared with air (Stoss and Holtz, 1983). By combining this technique with the use of antibiotics and lowering the storage temperature to 0°C rainbow trout milt has been successfully stored for 34 days (Stoss and Holtz, 1983).

The use of perfluorocarbon emulsions (PFC) such as flusol and FC-77, which were originally used for respiratory gas transport in human medicine and cell culture (King *et al.*, 1990; Lowe, 1991) has increased the longevity of poultry semen under

chilled conditions (Rogoff, 1985). The use of such inert gas carriers, which have a very high affinity for oxygen, to prolong the viability of fish milt was recently reported in rainbow trout (McNiven *et al.*, 1993).

The depth of milt in the storage container, and hence gaseous diffusion, is also reported to influence the milt fertility after storage (Stoss *et al.*, 1987).

3.12.3. Dilution media

To minimize the demand on dissolved O_2 the sperm cell density can be reduced by the addition of the diluents. Ideally these diluents should be isotonic to the seminal plasma, maintain the spermatozoa in an immotile state and be able to sustain all the metabolic activities and needs of the cells (Billard *et al.*, 1999).

The list of diluents used in this study is given in Table 8.

Table 8. List of extenders (diluents)

Composition of extenders used in the experiment				
Chemical	**Ext-A**	**Ext-B**	**Ext-C**	**Ext-D**
NaCl (g/l)	8.00	8.00	8.00	8.00
KCl (g/l)	0.40	0.04	0.40	0.40
$CaCl_2$ (g/l)	0.14	0.16	0.15	0.20
$NaHCO_3$ (g/l)	0.17	0.35	0.20	0.50
KH_2PO_4 (g/l)	0.03	0.06	0.05	0.10
$MgSO_4.7H_2O$ (g/l)	0.20	0.20	0.20	0.20
Na_2HPO_4 (g/l)	0.06	0.12	0.08	0.15
Glucose (g/l)	1.00	1.00	1.00	1.00

3.13. FISH HANDLING AND GAMETE COLLECTION FOR CRYOPRESERVATION

Horabagrus nigricollaris individuals were kept at the department of Aquaculture in 10 ton FRP tanks of College of fisheries, Panangad. Four repeats of the experiment were carried out in June to September 2006. The brood stock (five males and five females for each repeat, 20–20 individuals in total) was collected from the river Chalakudy, Kerala 48 hrs before the planned stripping and transported to the College for acclimatization. Male fishes were injected with Ovaprim @ 0.4ml/Kg

body weight at pectoral region of the fish 12 hours before the planned stripping (at water temperature of 23 °C). Matured females were injected @ 1ml/Kg body weight 24 hours before stripping. Before stripping fishes of both sexes removed from the water, their genital apertures were wiped dry and approximately 1.3 ml of milt was collected from each fish into dry plastic bowls, while eggs were stripped into dry plastic boxes. Eggs were stored at room temperature till fertilization, 5-10 min following stripping. Sperm was collected approximately 10 minutes after the stripping of eggs and was stored at 4 °C until being used for fertilization trial on control i.e., between fresh ova versus fresh milt.

3.14. MILT EVALUATION AND CRYOPRESERVATION

Fresh milt was collected from matured males after stripping. Fishes were injected with Ovaprim @ 0.4 ml/Kg body weight and after 12 hours stripping was done.

Sperm motility Score on the basis of 0-5 point scale was observed under a light microscope by adding tap water as an activator, where 0 = 0% and 5 = 100%. Motility time was also observed.

3.14.1. Cryopreservation trials

Males injected with Ovaprim @ 0.4 ml/Kg body weight were stripped after 12 hours. As the available quantity of milt was less, the fishes were dissected and the testes were taken out surgically. Then the testes were mashed with extenders and sieved through a bolting silk net. The testes extract thus collected were observed for motility score and motility time.

DMSO @ 10% was added just before filling up the straw. Milt: extender ratio was kept as 1: 4. French straws of 0.5 ml capacity were used for filling the milt. The filled straws were sealed with PVA (poly vinyl alcohol) powder. Different coloured straws were used for the different extenders. Then the straws were kept on ice (0°C) for 10 minutes. After 10 minutes straws were kept on liquid nitrogen vapour phase for another 10 minutes. Then the straws were plunged into liquid nitrogen. These straws were then taken in a cannister and kept in cryocan containing liquid nitrogen.

To achieve the best effect of cryoproservation, the biophysical responses of sperm cell and tissues during freezing should be clearly understood. For this study,

the Differential scanning Colorimeter (DSC) technique is used to monitor the volume changes of sperm cells during freezing. The cellular volume data is then coupled with the water transport model to get the permeability parameters of the cell membranes. These data can be further used to predict the optimal freezing and thawing procedure of spermatozoa or ovarian tissues.

Differential scanning colorimetry (DSC) is a thermo-analytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference are measured as a function of temperature. The temperature scanning can be done isothermally or by changing the temperature at a constant rate. The differences in the heat flow between the sample and reference during this process is sent to a computer, and a plot of the differential heat flow between the reference and sample cell as a function of temperature is produced by the computer. A process without thermodynamic chemical reactions will result in a flat or very shallow base line on the plot. However, an exothermic or endothermic process within the sample results in a significant deviation in the difference between the two heat flows and thus there is a peak in the DSC curve. Exothermic processes will show as positive peaks (above the baseline) while peaks resulting from endothermic processes are negative (below the baseline). A typical example of the DSC heat flow curve is shown in Figure 6.

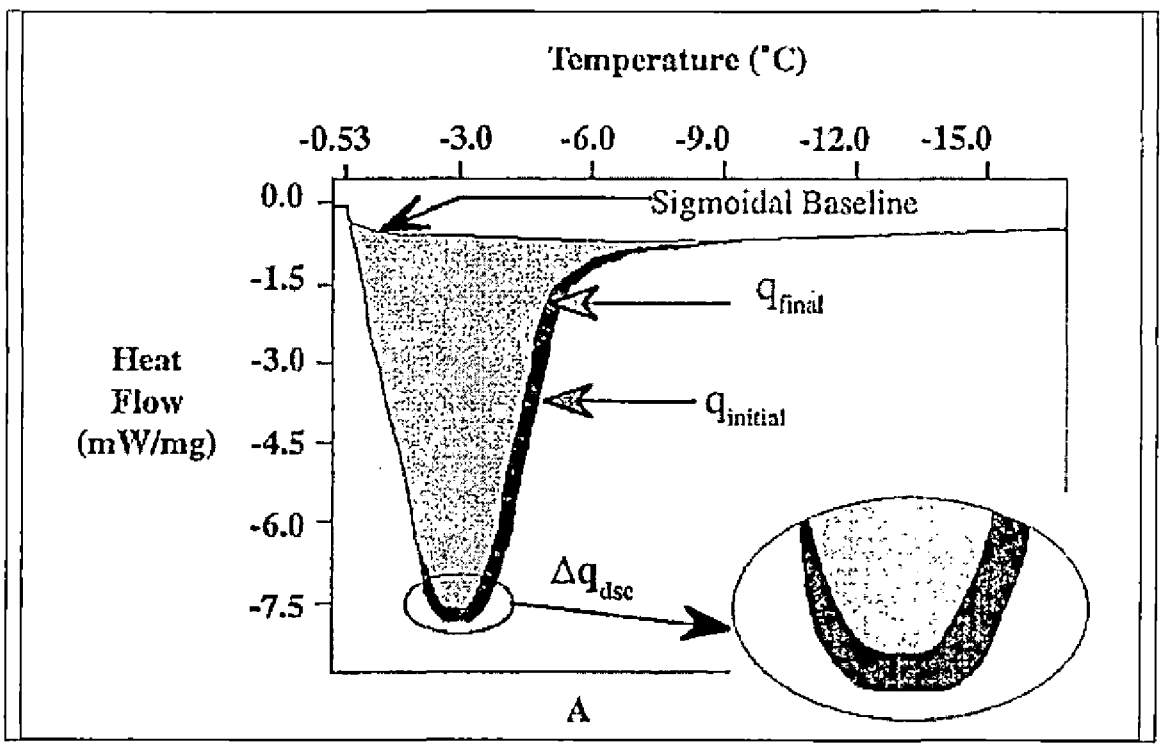


Figure 6. Heat Flow curve of differential scanning calorimeter

3.14.2. Major physico-chemical objectives during cryopreservation:

Cryopreservation of gametes encompasses a set of complex physical and chemical events, with 'cryosuccess' depending on a number of interrelated factors (Franks, 1985). The overall objective during cooling and thawing is to prevent or minimize the formation of damaging intracellular ice crystals (Franks, 1985).

When heat is removed from a cell suspension, its temperature decreases past its freezing point and undercools before ice formation or freezing is initiated usually in the extracellular medium. The freezing process, which is accompanied by ice formation and accumulation, results in an increase in solute concentration and a rise in temperature to the freezing point. Further cooling past the eutectic point, the freezing point of the solutes, results in the solidification of the solution and solutes.

The rise in extracellular solute concentration of the unfrozen fraction during freezing causes cellular water to be removed and cells to de-hydrate. The rate and extent of dehydration is dependent on the size of the organism being frozen and the cooling rate (Grout and Morris, 1987).

3.14.3. Standard operation procedure (SOP)

3.14.3.1. SOP-5. Collection and fertilization of H. nigricollaris eggs

Materials required

1. Synthetic hormone - Ovaprim
2. Distilled water
3. Bacteriostatic filter (0.22μm) (Corning, Inc., Corning, New York)
4. Blood collection tubes
5. 1-ml syringes
6. 19 gauge needles
7. Extenders
8. 100-mL glass bowls
9. Sperm (SOP-1, SOP-3)
10. μm filter
11. Tank water
12. Incubator (Model 1535, VWR Scientific, San Francisco, California)
13. Dissecting microscope (Nikon model SMZ-U Japan)

Procedure

1. Mix 0.5ml Ovaprim with 10 µl of deionized water filtered through a 0.22-µm filter.
2. Place hormone solution in 2 labeled 10-ml blood collection tubes
3. Take *Horabagrus nigricollaris* female fish
4. Weigh fish.
5. Inject the fish with prepared solution of Ovaprim (0.5ml per Kg body weight of female).
6. Place fish in covered tank.
7. Check for eggs 24 hours later.
8. Strip eggs by applying gentle pressure on both sides of the abdomen towards the genital pore.
9. Strip eggs into room-temperature.
10. Separate eggs into appropriate treatment groups in 1500-ml plastic tubs.
11. Add 0.5 mL sperm with a pipette.
12. Add filtered water (10 times volume of sperm) and gently swirl.
13. Wait 5 minutes and fill bowl with filtered tank water.
14. Place fertilized eggs in incubator at 28 ^{o}C.
15. View eggs after 10 h with dissecting microscope for fertilization. Record fertilization as the percentage of eggs that progress to the embryonic shield stage (Kim and Park, 1987).
16. Change the water about every 12 h.
17. Record hatch as the percentage of eggs that hatched into live yolksac fry after 28 – 36hours.

3.15. EVALUATION OF SPERM QUALITY AFTER CRYOPRESERVATION

3.15.1. Motility

It is the most commonly used parameter to evaluate sperm quality. To test motility, semen is diluted in an appropriate diluent and examined under a microscope. Evaluation of motility requires some care. A relatively high dilution is necessary (at least 1:1000) to initiate synchronously the motility of all the sperm. At lower dilution the sperm are not all activated and initiation occurs progressively over a few minutes after dilution. It is therefore difficult to assess accurately the intensity and duration of sperm motility. The duration of motility refers to the total duration of the motility, including agitation of the flagellum without displacement or to the duration of forward motion and the survival time of 50% of the sperm Hines and Yashow (1971).

3.15.2. Medium for sperm activation

Osmotic pressure, ionic composition and pH are the most important factors determining the activation of sperm. Motility occurs in a wider range of osmotic pressures in marine fish than in fresh water species. Changes in osmotic pressure are the factor most commonly known to trigger the initiation of sperm motility, rising in comparison with seminal plasma in marine fish or decreasing in fresh water fish (Chambeyron and Zohar, 1990). pH has been reported as a major sperm activation factor in species such as mullet (Hines and Yashow, 1971). In this study normal tap water was used as an activator of sperm motility.

The motility time of the sperm samples before and after cryopreservation was recorded.

3.15.3. Survival rate of sperm

The curve of cell survival percentage against cooling rate has a reverse U shape as shown in Figure 7 (Thirumala *et al.*, 2005). At the maximal point, the injury effect is the least and cooling rate is optimal.

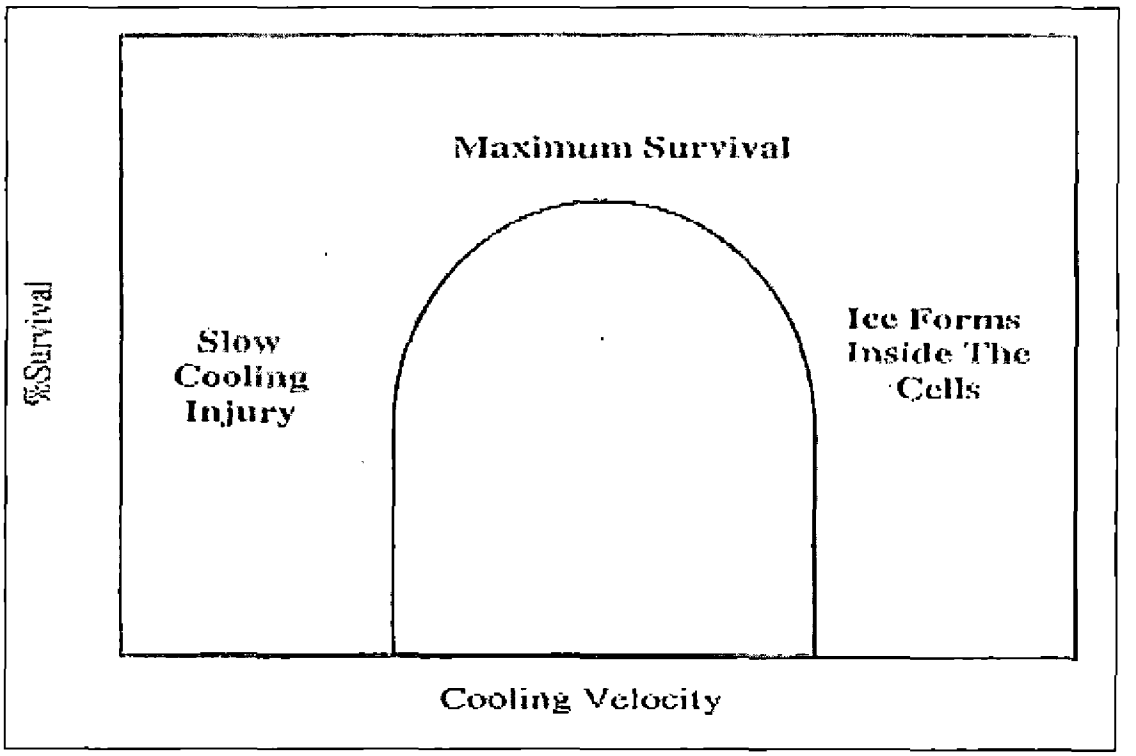


Figure 7. Cell survival percentage against cooling rate

3.15.3.1. Differential scanning calorimetry (DSC)

An exothermic or endothermic process within the sample results in a significant deviation in the difference between the two heat flows and thus there is a peak in the DSC curve. Exothermic processes will show as positive peaks (above the baseline) while peaks resulting from endothermic processes are negative (below the baseline). A typical example of the DSC heat flow curve is shown in Figure 8.

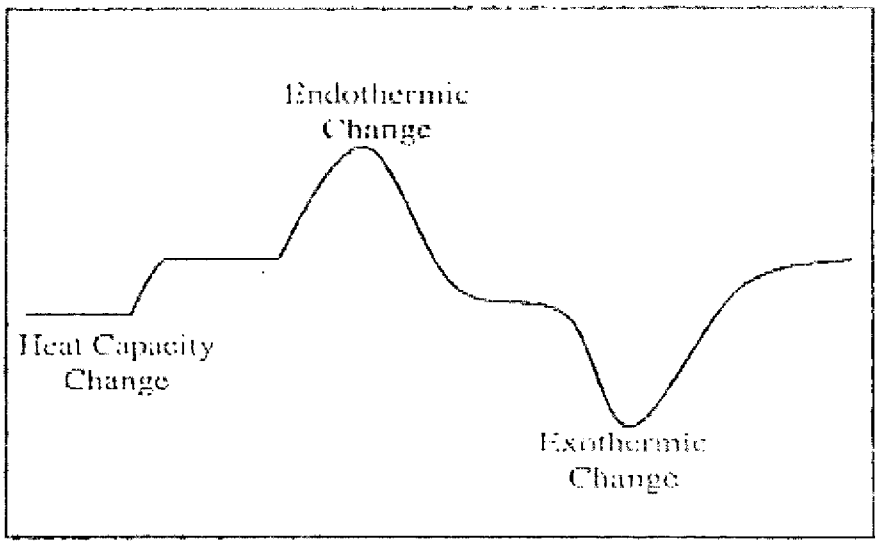


Figure 8. Typical heat flux curve of differential scanning calorimeter

3.16. FERTILIZATION AND INCUBATION OF EGGS

Matured female fishes were given Ovaprim injection @ 1ml/Kg body weight. Fishes were stripped after 24 hours of injection and eggs were collected in a dry plastic box. Approximately 200 eggs were taken in a plastic tray and fertilized with thawed milt. Tap water used to activate the sperm. Eggs were mixed with the milt solution well and excess solutions were cleaned with water.

After six hours of fertilization, dead eggs were removed and from this observation percent fertility was calculated. After 24 hours hatching was observed and after 30 hours free hatchlings were observed by taking number of hatchling and number fertilized eggs into consideration.

Breeding success in terms of % fertilization and hatching rate was determined.

3.17. FERTILIZING CAPACITY

The fertilizing capacity is the most conclusive test of sperm quality. It is currently used in most studies on artificial insemination and sperm preservation. However, it integrates an independent factor which is the 'quality' of eggs and the interaction between gametes and between seminal and ovarian fluids. The diluent used must be appropriate for both male and female gametes.

3.18. STATISTICAL ANALYSIS OF THE DATA

The experiment was carried out using CRD. Statistical analysis of the data on fertilization and hatching percentages were transformed using Angular transformation and analysed using one - way analysis of variance (Zar, 1999) followed by Tukey's Multiple Comparisons test.

3.19. SPECIAL OBSERVATIONS

As this study is for the first of its kind on H. nigricollaris, observations on its behavior, feeding and adaptability to the environmental changes were also were also undertaken.

3.19.1. Gut content analysis

Dead fish samples collected from the river were subjected to gut content analysis to study the food and feeding habits of the fish. The specimens were carefully dissected to expose their gastro intestinal tract and were fixed in 5% formalin for detailed analysis.

3.19.2. Feeding

While keeping the fishes in FRP tanks for further cryopreservation trials, they were fed with various diets to observe on its feeding adaptability in captivity.

3.19.3. Male and female identification

The fishes were visually examined to identify their sex.

3.19.4. Fish behavior and adaptability to the environmental changes

The experimental animals were carefully examined for their behavioral responses for feeding, movement, and also to monitor their health in captivity.

The experimental set up, facilities used, the various stages involved and important observations are displayed from Plate-4 to 26.

Plate - 4. Tank used for keeping the fishes

Plate - 5. Specimens for biological studies

Plate - 6. Examining the gut

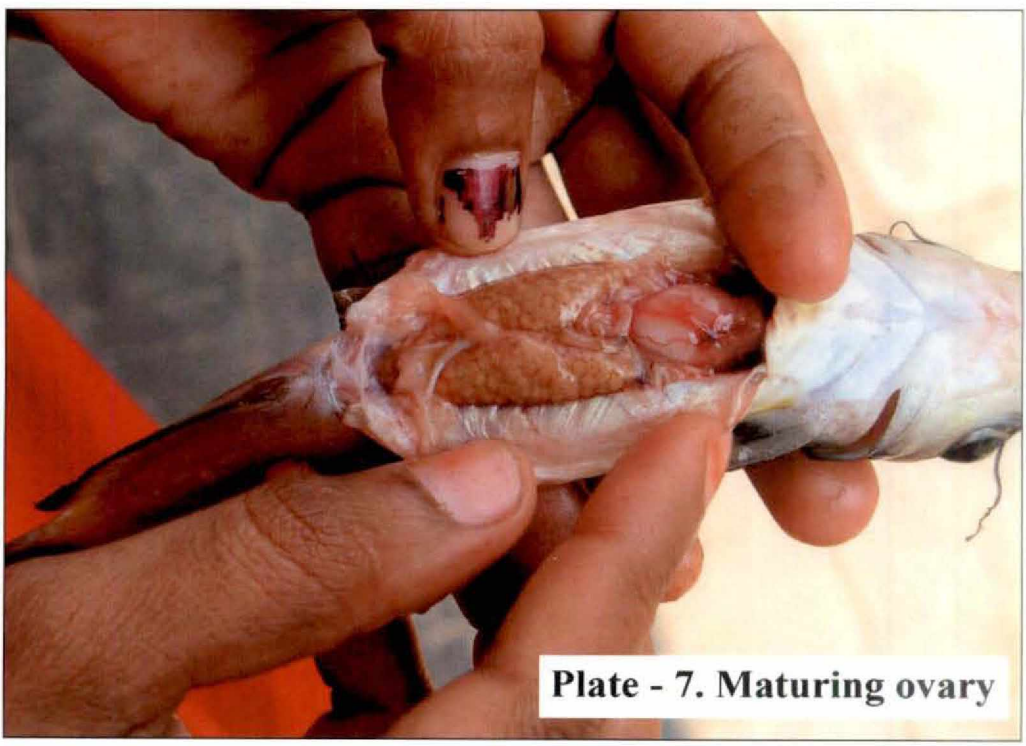


Plate - 7. Maturing ovary

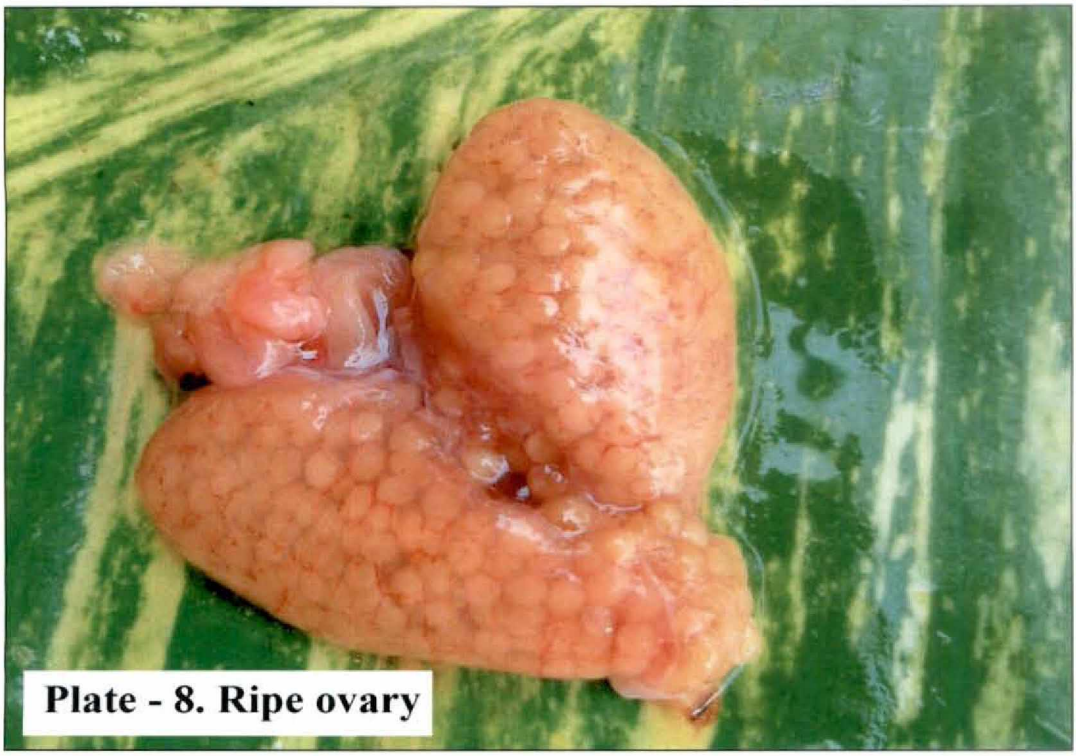


Plate - 8. Ripe ovary

Plate - 9. Examininig the gonadal condition

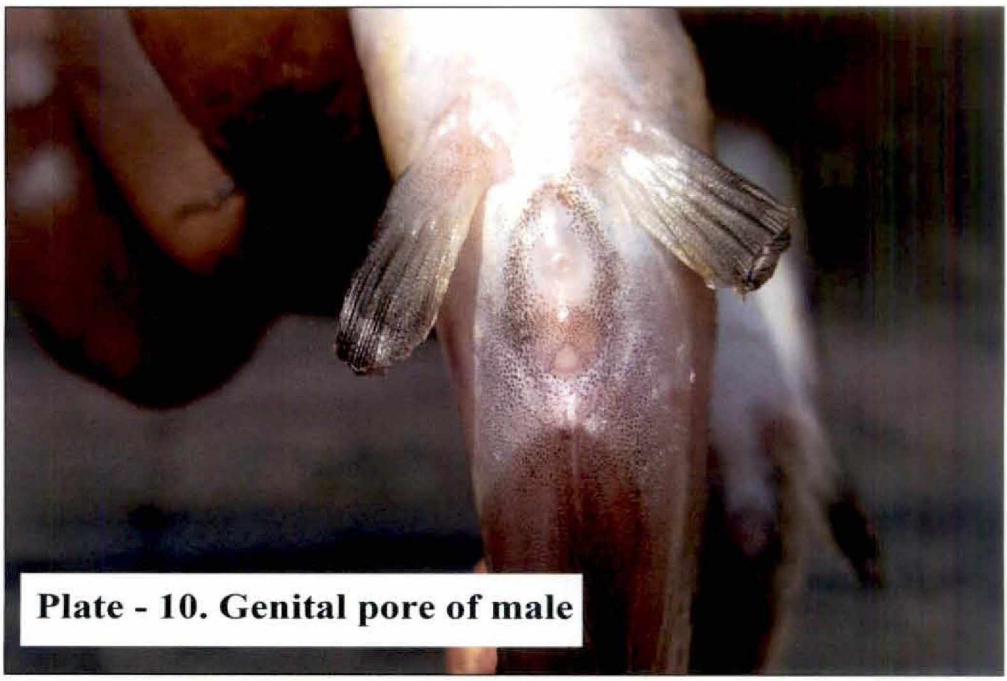


Plate - 10. Genital pore of male

Plate - 11 .Genital pore in females

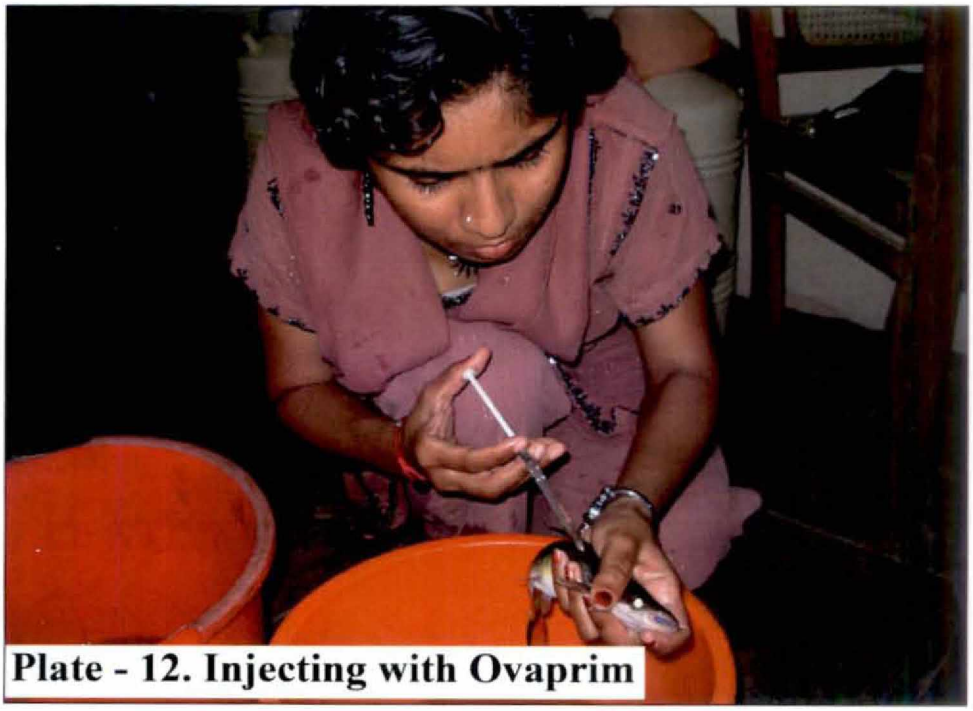


Plate - 12. Injecting with Ovaprim

Plate - 13. Tank used for keeping fishes after ovaprim injection

Plate - 18. Filling the straws

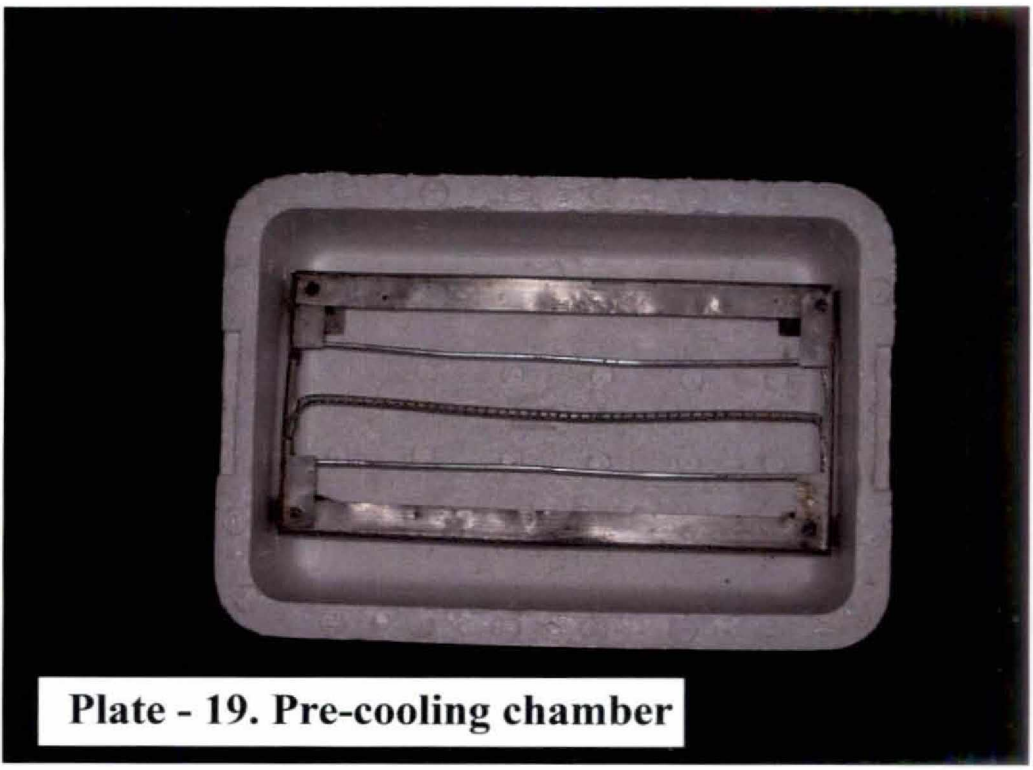


Plate - 19. Pre-cooling chamber

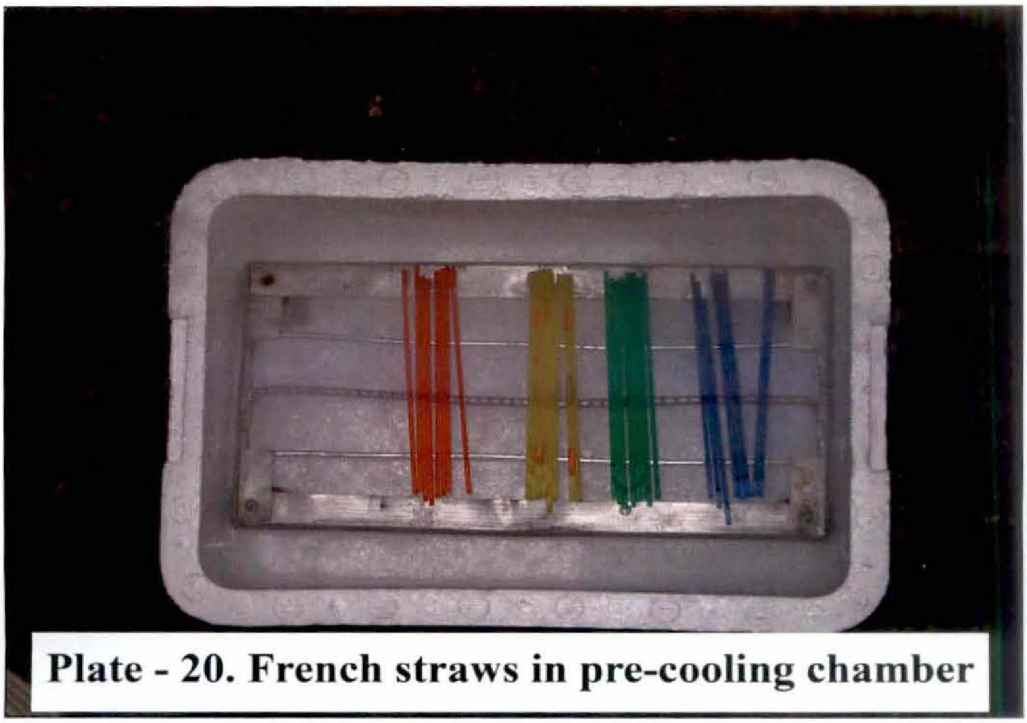


Plate - 20. French straws in pre-cooling chamber

Plate - 21. Transferring Li-N2 for pre-cooling

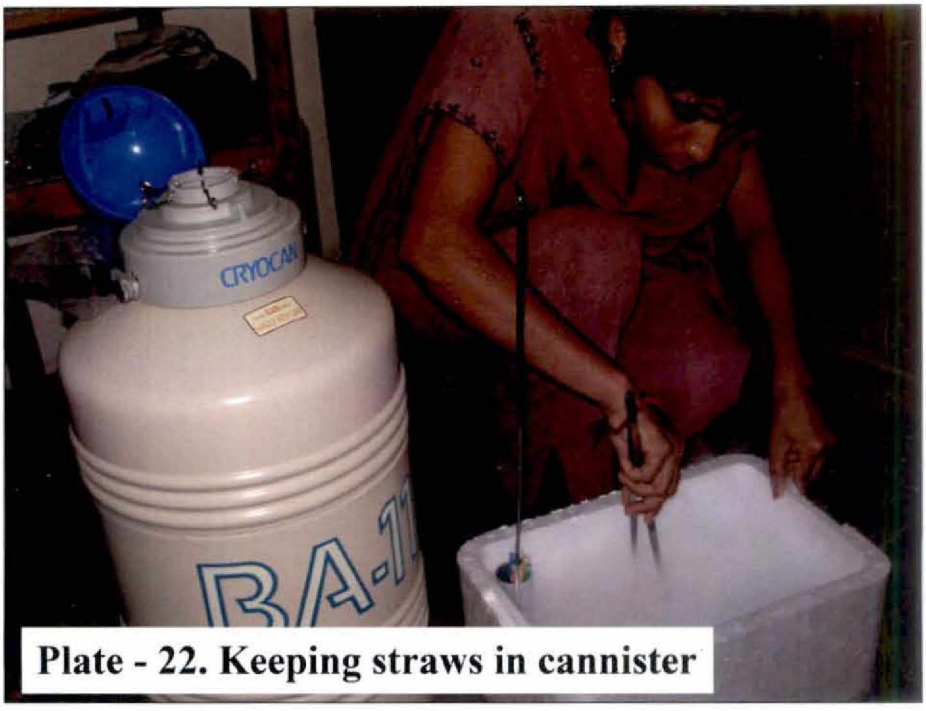


Plate - 22. Keeping straws in cannister

Plate - 23. Troughs for fertilization trial

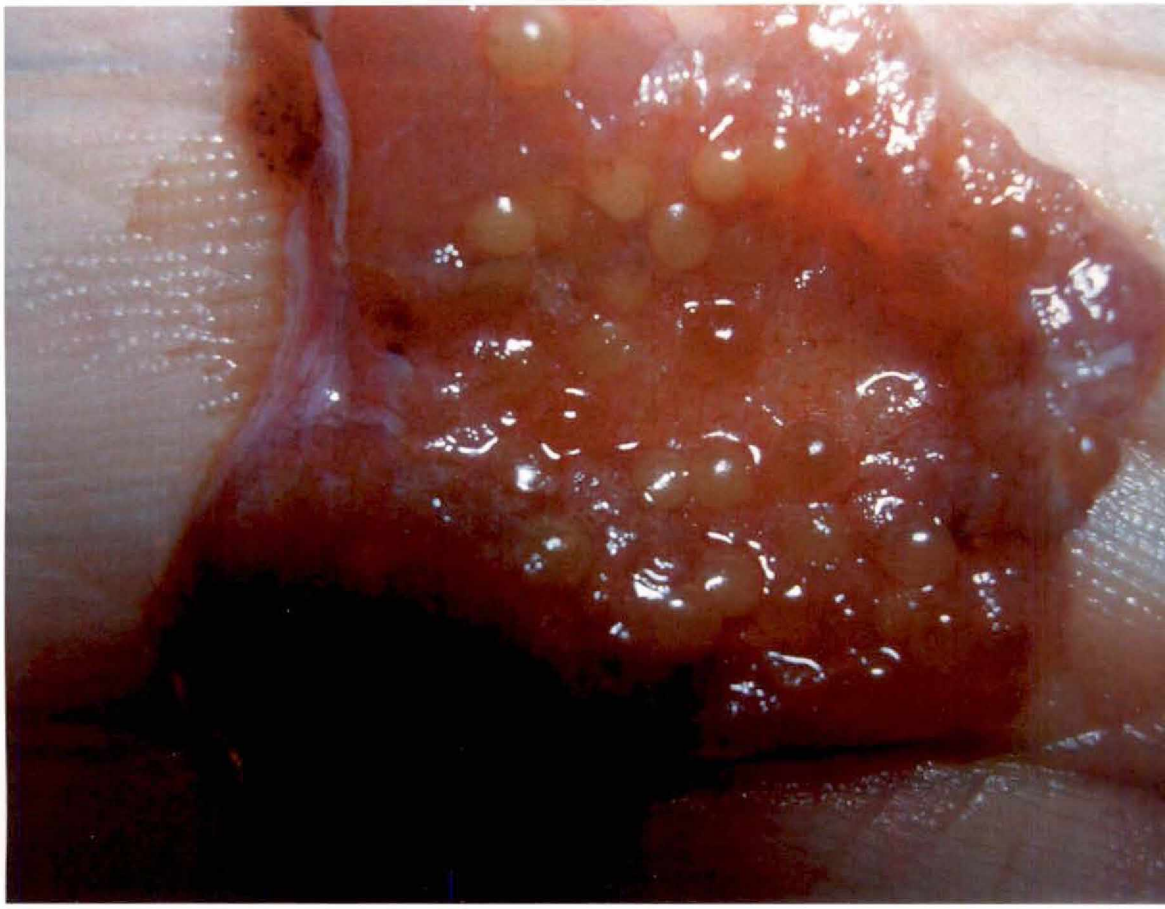
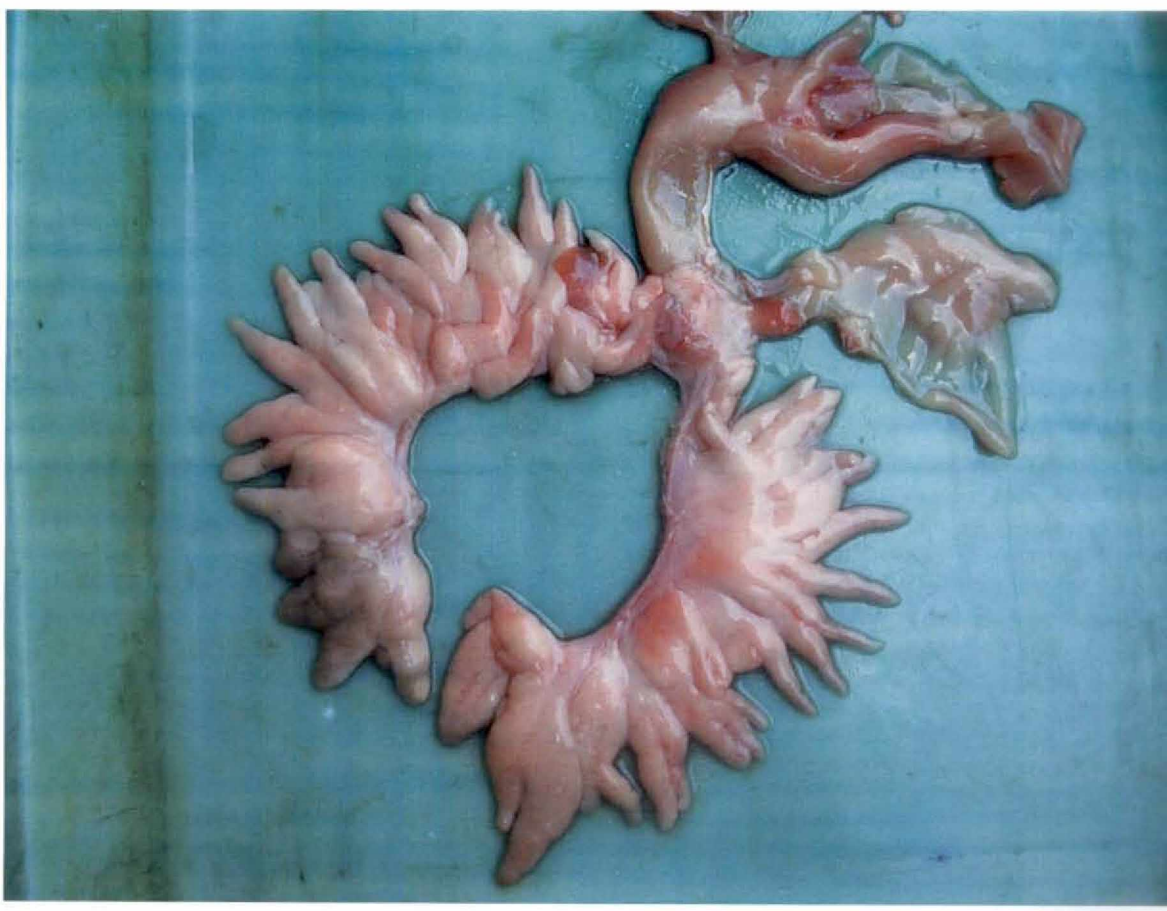


Plate: 27. Matured Ova



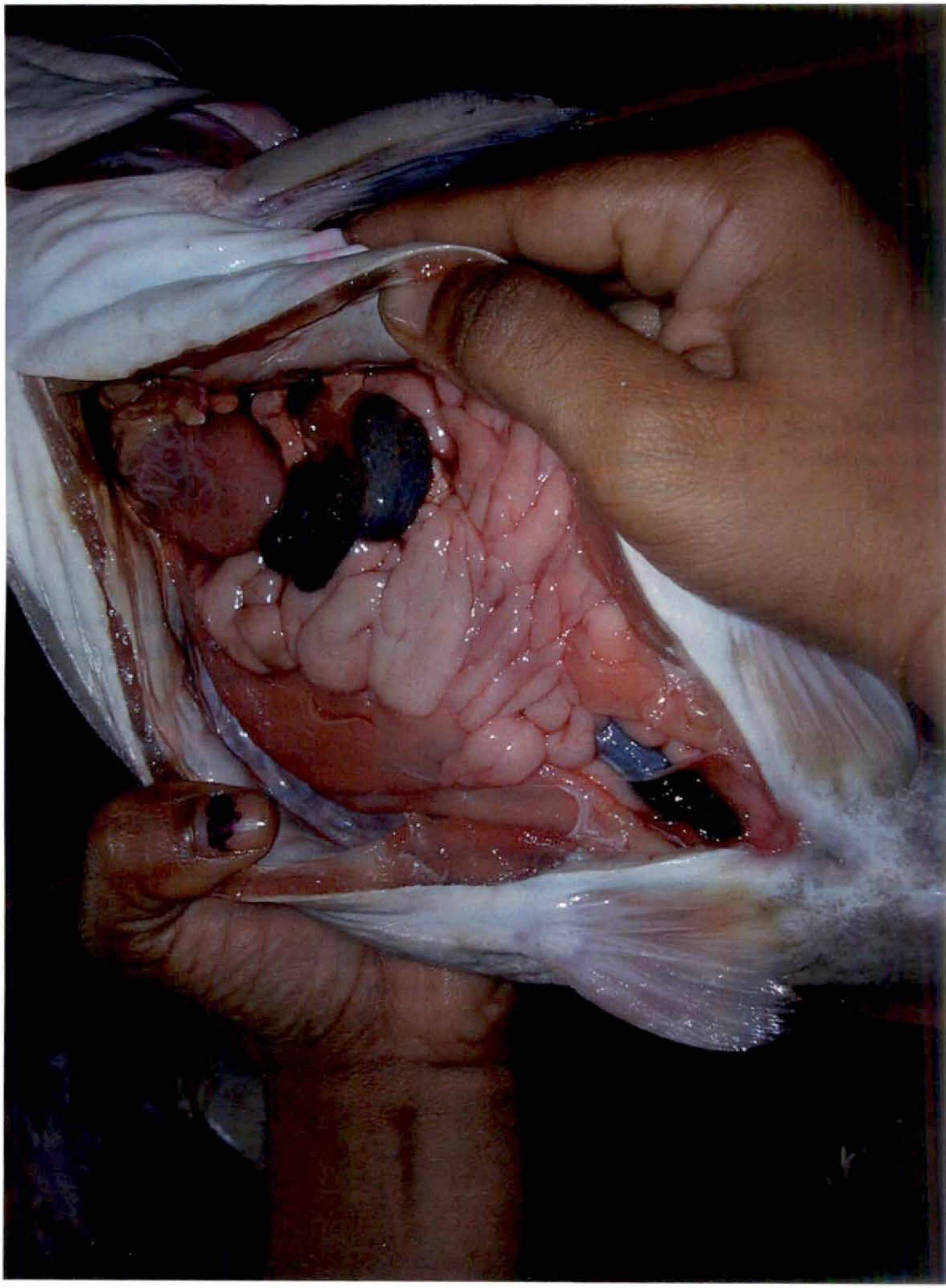


Plate: 29. Male reproductive system exposed from a 2.5Kg specimen of *H. nigricollaris*

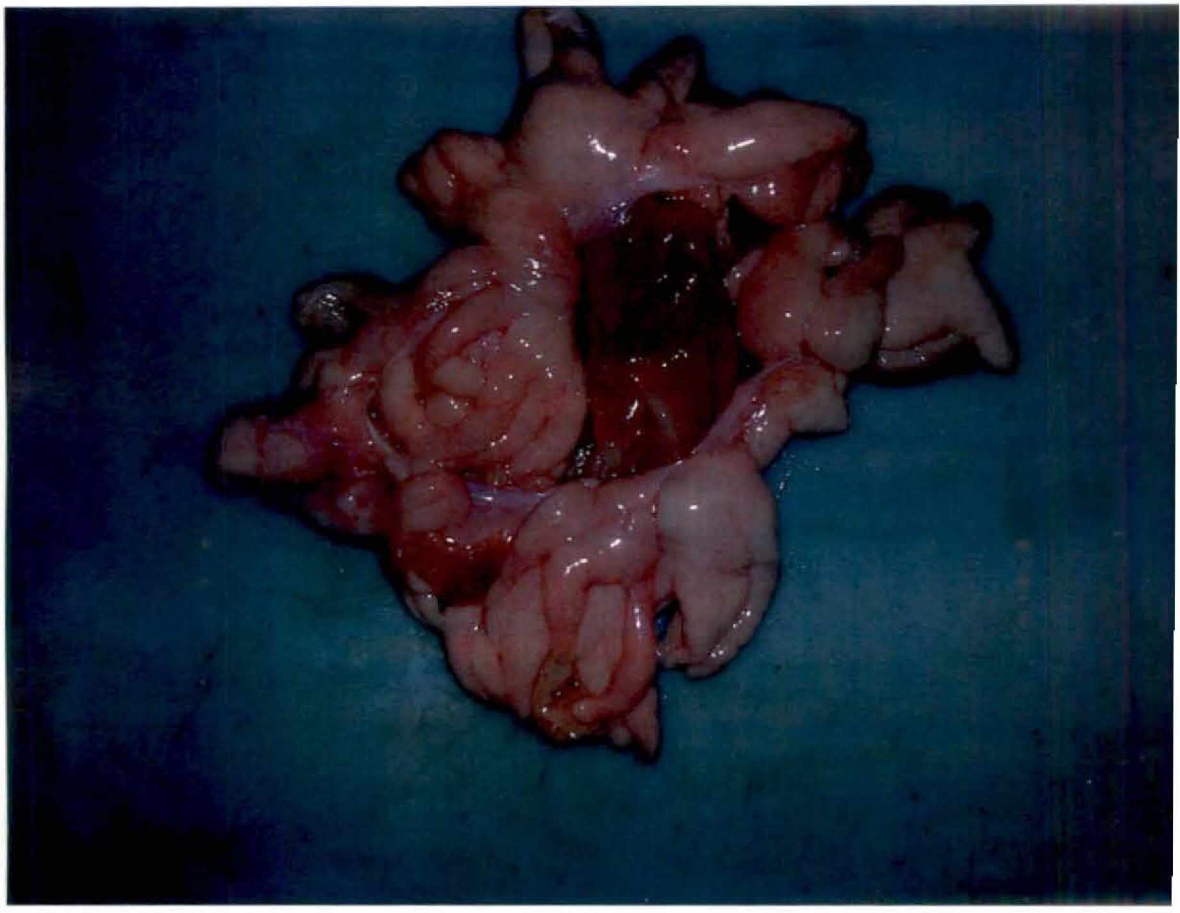
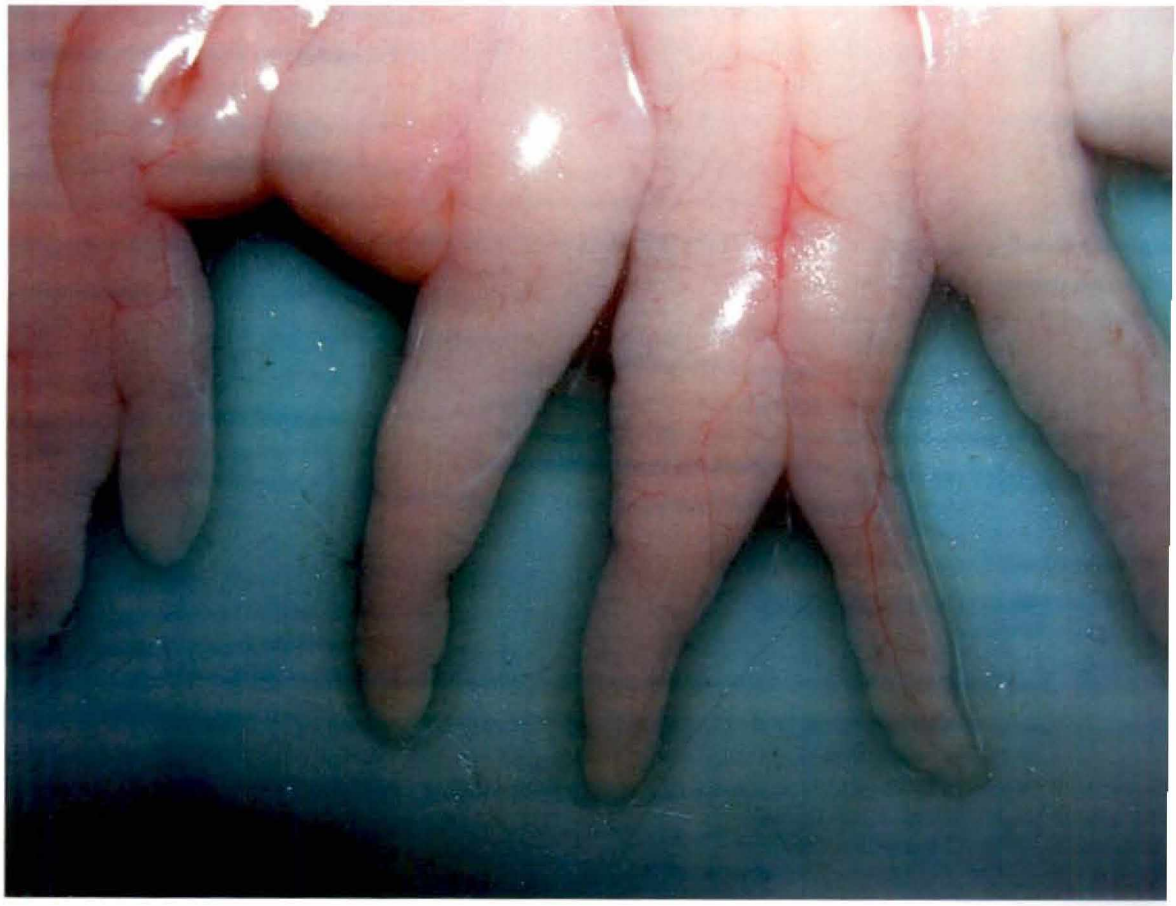
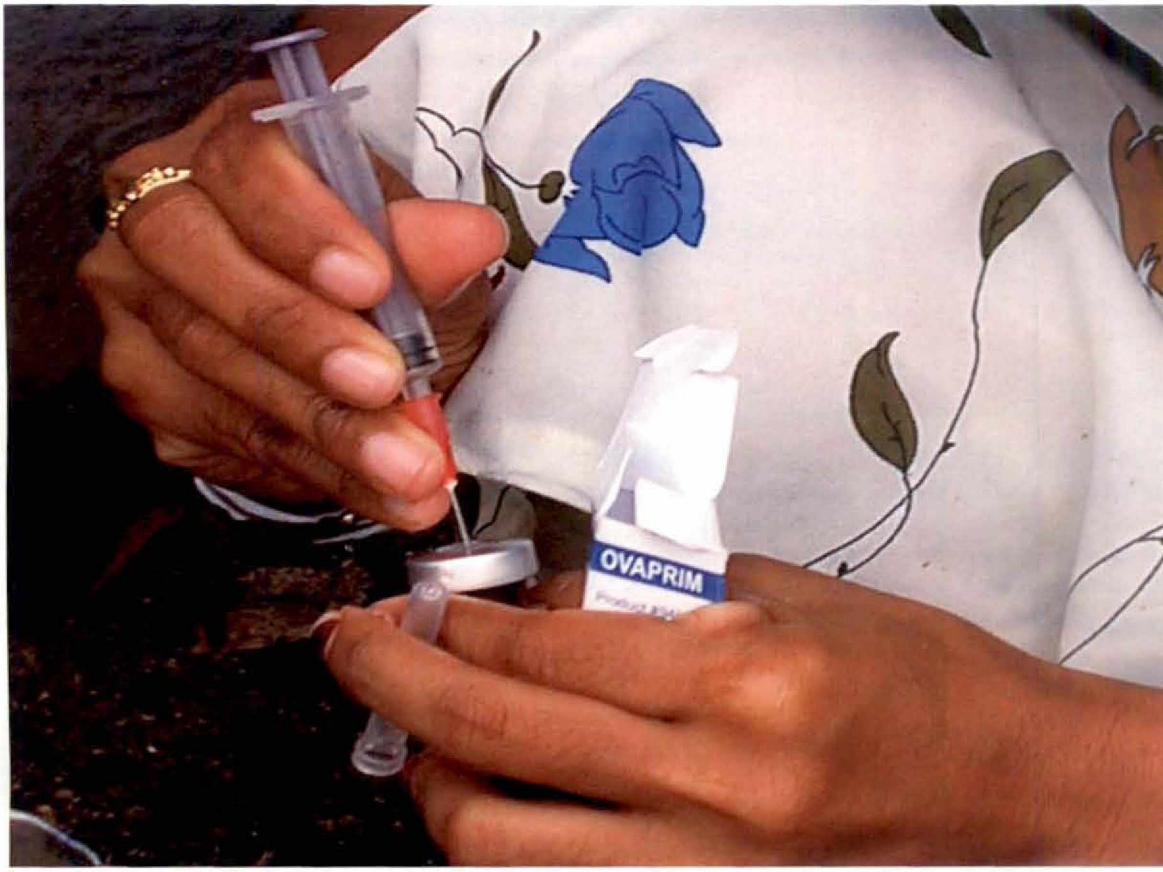
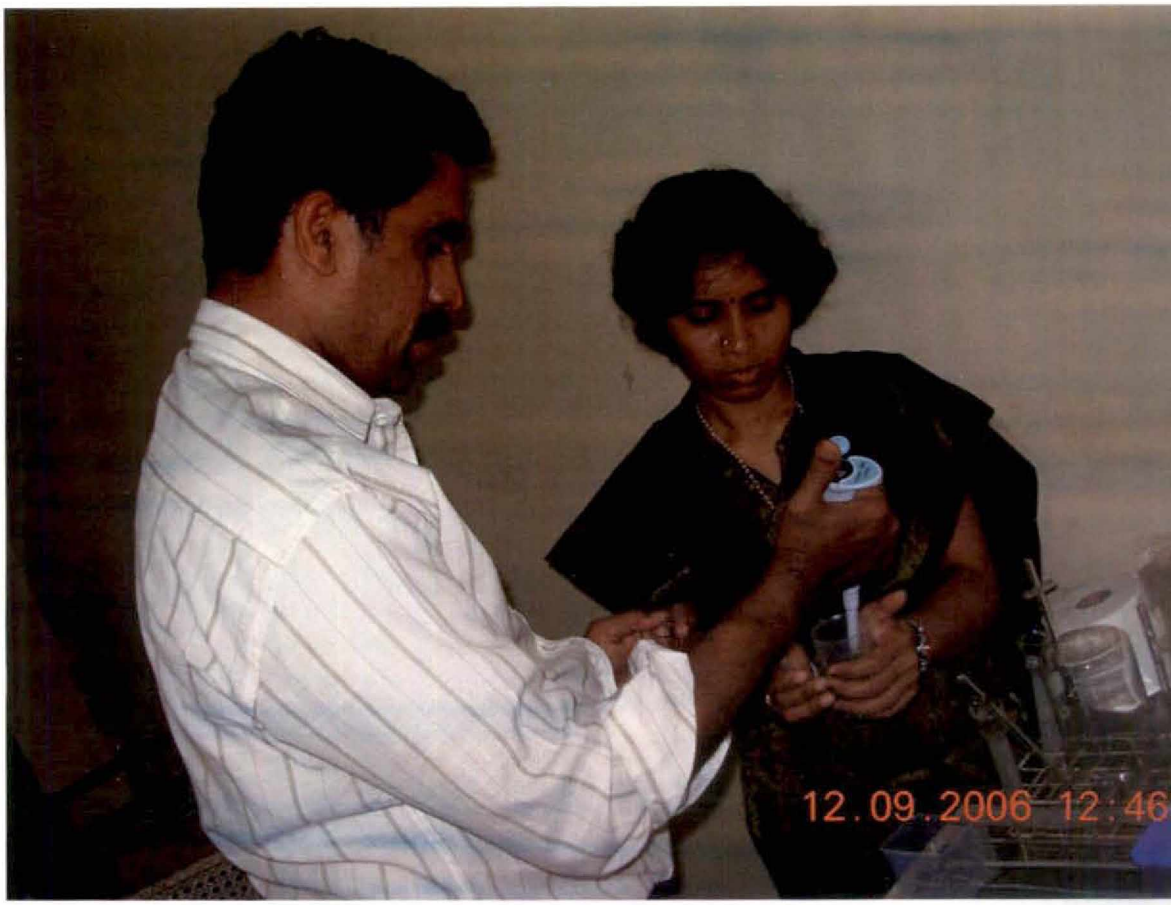


Plate: 30. Male reproductive system



Fertilization trails at a glance





Fertilization studies at a glance

RESULTS

4. RESULTS

4.1. WATER QUALITY PARAMETERS

Water quality parameters of the rearing tanks were monitored as per stand methods (A.P.H.A., 1998) and the ranges of water quality parameters are given in the Table 9.

Table 9. Water quality parameters

Parameter	Range
Water temperature	22 – 28 °C
pH	7.6 – 8.4
DO_2	5.7 – 6.2 mg/L
Alkalinity	50 – 100 mg/L
Ammonia	0.006 mg / L

4.2. GONADOSOMATIC INDEX

The Gonadosomatic index of the female fishes collected was in the range of 7.29 to 9.14% during the study period. The GSI data is given in the Table 10.

Table 10. Gonadosomatic Index

Fish weight (g) (A)	Ovary weight (g) (C)	Egg count (B)	GSI=C/Ax100 (D)
33	2.51	1682	7.6
50	3.82	2820	7.64
42	3.21	2367	7.64
75	5.65	4170	7.53
138	10.5	7665	7.6
48	3.64	2679	7.58
63	4.78	3520	7.58
76	5.78	4230	7.6
150	11.4	8311	7.6
83	6.14	4477	7.39
47	3.43	2508	7.29
45	3.31	1799	7.35
35	3.2	2150	9.14

4.3.MILT CHARACTERISTICS

The semen of *Horabagrus nigricollaris* appeared white in color. The semen acquired a different shade of yellow after spermiation and after being held by the fish for longer than a day. Measured values for semen characteristics are given in Table 11 and 12. The volume of semen collected ranged from 0.5 to 1.3 ml. The spermatocrit ranged between 57.8 – 59.8 %.

4.4. FRESH MILT QUALITY

a) All showed 5+ (100%) motile spermatozoa (spz)
b) white in colour
c) not too watery nor viscous
d) pH ~7.5.
e) Average volume of milt released per stripping is approximately 1.3ml.

Table 11. Sperm density, PCV (packed cell volume) and Spermatocrit

Milt sample	Height of Milt (ml)	PCV	Spermatocrit(%) $\frac{\text{PCV}}{\text{Total milt vol.}} \times 100$
1	8.2	2.55	31.1
2	8.2	2.75	33.54
3	7.5	2.40	32.0
4	8.2	2.44	29.76
5	7.7	2.05	26.62

The sperm concentration in the seminal plasma varied between individuals and ranged from 16.5 x 109 to 20.9 x 109spz/ml of semen. The viscosity of the semen appeared to increase with sperm number. The semen pH ranged from 7.3 to 7.5. In the milt, the spermatozoa were immobile and initiated progressive motility upon dilution with tap water. No differences in sperm morphology or movement were apparent among the various water types.

Table 12. Sperm density, spermatocrit and pH of raw milt

Sample No.	Sperm density (spz*/ml)	Spermatocrit (%)	pH of fresh milt
1	20.7×10^9	58.8	7.3
2	16.5×10^9	57.1	7.4
3	18.1×10^9	57.8	7.5
4	18.9×10^9	58.0	7.4
5	20.9×10^9	59.8	7.4

*spermatozoa in the table

Motility of the sperm was observed under a microscope using tap water as activator and almost all the samples showed more than 80% motility and few samples showed near 100% activity (Table 13 & 14). Motility time was in the range of 73 to 90 seconds in different samples. The duration of sperm motility (mean ± SD) was 78 ± 9 sec and a few sperm cells remained motile for over 2min. Motility score of the cryopreserved and thawed milt was in the range of 60 to 80%. As compared to fresh milt motility score reduced roughly 20% in thawed milt. The motility time also reduced in thawed milt and it was in the range of 30 to 70 seconds.

Table 13. Motility score & time of fresh milt and thawed milt

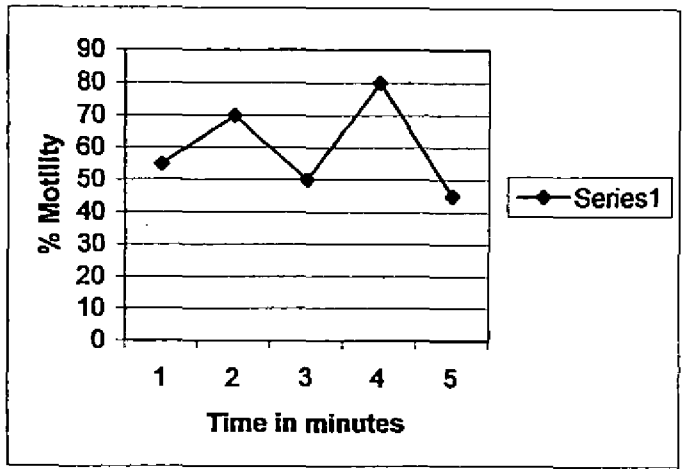
	Fresh milt		Thawed milt	
Sample No.	Motility score	Time	Motility score	Time
1	5	80	4	70
2	4	76	4	67
3	5	82	3	45
4	5	80	3	30
5	4	78	3	32
6	5	76	4	30
7	4	75	3	30
8	5	73	3	34
9	4	90	3	32
10	4	78	4	41

Note: The motility score taken on '0 to 5' scale which means that 0 = 0% and 5 = 100%.

Table 14. Motility estimates of *H. nigricollaris*

Trail-I Sample No.	Fresh milt		Testes extract		Cryopreserved milt	
	Motility score	Motility Time	Score	Time	Score	Time
1	4+	80	4+	78	3+	70
2	5+	70	4+	61	4+	65
3	5+	81	5+	78	4+	71
4	4+	58	4+	42	4+	45
5	4+	62	5+	39	4+	39
Trail-II Sample No.	**Fresh milt**		**Testes extract**		**Cryopreserved milt**	
	Motility score	**Motility Time**	**Score**	**Time**	**Score**	**Time**
1	5+	78	4+	76	4+	63
2	5+	69	5+	72	3+	57
3	4+	82	5+	59	3+	48
4	4+	54	4+	82	4+	43
5	5+	73	4+	56	4+	57
Trail-III Sample No.	**Fresh milt**		**Testes extract**		**Cryopreserved milt**	
	Motility score	**Motility Time**	**Score**	**Time**	**Score**	**Time**
1	5+	69	4+	60	4+	57
2	5+	72	4+	64	4+	50
3	4+	83	5+	69	3+	49
4	4+	65	5+	58	3+	36
5	4+	72	4+	70	5+	58

Motility recovery of sperm after Exposure to DMSO is given in Figure 9.



Note : on X- axis time scale 1minute=2units

Figure 9. Motility recovery of sperm after Exposure to DMSO

The Figure 9 says that cell exposure to DMSO <10minutes and >8 minutes gave good motility%.

4.5. SPERM MORPHOLOGY

Sperm are defined as the male reproductive cells produced by the testicles. They are carried in fluid called semen, capable of fertilizing an egg cell to form a zygote. A zygote can grow into a new organism. Sperm cells contain half of the genetic information needed to create life. Spermatozoa are differentiated cells, normally composed of a head, basal body, and tail. The head contains some cytoplasm and the nuclear material for fertilization. The microscopic observation did not show any deformity of the sperm and showed uniform size and shape.

4.6. FERTILITY TRIAL

Two fertility trails were carried out using cryopreserved milt and the results are given in Table 15 and 16. In control percent fertility was 36.0±0.4 in trial I and 35.8±0.5 in trial II. In control the hatching percentage in trail I was 37.8±1.7 and in trial II 45.5±1.2. In both the trails extender A showed closer to control (81.7% in trial I and 85.9% in trial II). Analysis of variance (Tables 17 and 18) showed that extender-A showed better result compared to control in terms of fertility and hatching. Extender - A showed 81.7% hatching as that of control, followed by extender - C, B

and D in trail I. In trial II also extender A showed better hatching (85.9%) closer to the control.

Table 15. Percentage fertilization and percentage hatching of *Horabagrus nigricollaris* using cryopreserved milt (Trail I)

	Total Eggs	Live	Dead	% fertilisation	Mean ±SD	No. of hatch.	% Hatching	Mean ±SD	%hatching as that of control
A1	198	69	129	34.8		22	31.9		
A2	207	57	150	27.5		18	31.6	30.9±1.5	81.7
A3	215	65	150	30.2	30.8±3.7	19	29.2		
B1	217	48	169	22.1		14	29.2		
B2	194	44	150	22.7		13	29.5	27.3±3.6	72.2
B3	196	52	144	26.5	23.8±2.4	12	23.1		
C1	224	40	184	17.9		12	30.0		
C2	214	38	176	17.8		10	26.3	29.1±2.5	77.0
C3	212	42	170	19.8	18.5±1.1	13	31.0		
D1	210	52	158	24.8		14	26.9		
D2	212	50	162	23.6		13	26.0	24.6±3.3	65.1
D3	206	48	158	23.3	23.9±0.8	10	20.8		
Cntrl. 1	226	82	144	36.3		32	39.0		
Cntrl. 2	216	78	138	36.1		30	38.5	37.8±1.7	100.0
Cntrl. 3	220	78	142	35.5	36±0.4	28	35.9		

Table 16. Percentage fertilization and percentage hatching of *Horabagrus nigricollaris* using cryopreserved milt (Trail II)

	Total Eggs	Live	Dead	% fert.	Mean ±SD	No. of hatch.	% Hatch	Mean +SD	%hatching as that of control
A1	224	73	151	32.6		28	38.4		
A2	212	61	151	28.8		25	41.0	39.1±1.7	85.9
A3	208	58	150	27.9	29.8±2.5	22	37.9		
B1	232	51	181	22		16	31.4		
B2	222	42	180	18.9		14	33.3	28.5±6.7	62.6
B3	224	48	176	21.4	20.8±1.6	10	20.8		
C1	208	38	170	18.3		15	39.5		
C2	196	35	161	17.9		11	31.4	31.1±8.5	68.4
C3	195	40	155	20.5	18.9±1.4	9	22.5		
D1	220	48	172	21.8		12	25.0		
D2	215	46	169	21.4		10	21.7	25.1±3.5	55.2
D3	210	42	168	20	21.1±0.9	12	28.6		
Cntrl. 1	206	74	132	35.9		33	44.6		
Cntrl. 2	218	77	141	35.3		36	46.8	45.5±1.2	100.0
Cntrl. 3	221	80	141	36.2	35.8±0.5	36	45.0		

Table 17. Summary of the percentage fertility of *Horabagrus nigricollaris* using different extenders

Sl.No.	A	B	C	D	Control
1	34.8	22.1	17.9	24.8	36.3
2	27.5	22.7	17.8	23.6	36.1
3	30.2	26.5	19.8	23.3	35.5
4	32.6	22	18.3	21.8	35.9
5	28.8	18.9	17.9	21.4	35.3
6	27.9	21.4	20.5	20	36.2
Mean ±SD	30.3±2.8[a]	22.26±2.4[b]	18.7±1.16[c]	22.4±1.7[b]	35.8±0.40

Statistical analysis of fertility trial (Percentage fertility) for Table 17

Table 17.1. ANOVA table

Source	SS	df	MS	F
Between groups	494.137	4	123.534	75.21*
Within groups	41.062	25	1.642	
Total	535.199	29		

Note : *statistically significant ($P < 0.01$), (% data subjected to angular transformation).

Table 17.2. Summary statistics

Group	No. of observations	Mean	Variance
Control	6	35.88	0.16
Extender A	6	30.30	8.28
Extender B	6	22.27[a]	6.06
Extender C	6	18.70	1.34
Extender D	6	22.48[a]	3.02

Note : Means bearing common superscript are statistically on par.

Table 17.3. Homogeneous subsets*

Group	No. of observations	Subsets at 5% Significance level			
		I	II	III	IV
Control	6	35.88			
Extender A	6		30.30		
Extender D	6			22.48	
Extender B	6			22.27	
Extender C	6				18.70

- Multiple comparisons as per Tukey's test.
- Mean fertility percentages in homogeneous - subsets are displayed.

Table 18. Summary of the percentage hatching of *Horabagrus nigricollaris* using different extenders

A	B	C	D	Control
31.9	29.2	30.0	26.9	39.0
31.6	29.5	26.3	26.0	38.5
29.2	23.1	31.0	20.8	35.9
38.4	31.4	39.5	25.0	44.6
41.0	33.3	31.4	21.7	46.8
37.9	20.8	22.5	28.6	45.0
35.0±4.7	**27.9±4.9**	**30.1±5.7**	**24.8±3.0**	**41.6±4.4**

Statistical analysis of fertility trial (Percent Hatching) for Table 18

Table 18.1. ANOVA table

Source	SS	df	MS	F
Between groups	392.40	4	98.10	11.84*
Within groups	207.19	25	8.28	
Total	599.59	29		

Note : *statistically significant ($P < 0.01$) (% data subjected to angular transformation).

Table 18.2. Summary statistics

Group	No. of observations	Mean	Variance
Control	6	41.63 [a]	19.29
Extender A	6	35.00 [ab]	22.16
Extender B	6	27.88 [bc]	23.82
Extender C	6	30.12 [bc]	32.61
Extender D	6	24.83 [c]	9.18

Note : Means bearing common superscript are statistically on par.

Table 18.3: Homogeneous subsets*

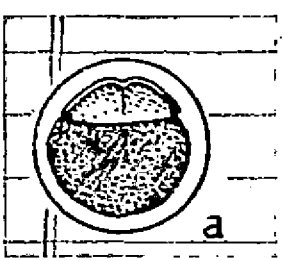
Group	No. of observations	Subsets at 5% Significance level		
		I	II	III
Extender D	6	24.83		
Extender B	6	27.88	27.88	
Extender C	6	30.12	30.12	
Extender A	6		35.0	35.0
Control	6			41.63

- Multiple comparisons as per Tukey's test.
- Mean hatch percentages in homogeneous - subsets are displayed.

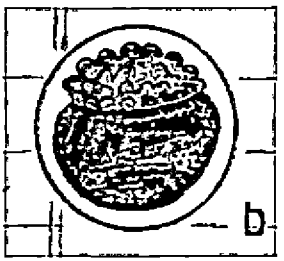
4.7. DEVELOPMENT OF EMBRYO

Embryo development was observed under an ordinary microscope. Early morula stage was observed after 6 hours of fertilization. Late morula stage was observed in 14 hours after fertilization and embryo formation was observed after 18 hours of fertilization. Hatching out of the embryo was observed after 28 hours of fertilization. Complete hatching out was observed after 32 hours. The temperature of medium was in the range 25 - 27 °C. The pH of the medium was 7.5.

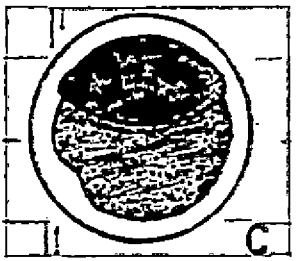
The embryonic developmental stages of *Horabagrus nigricollaris* is shown in Figure 10.



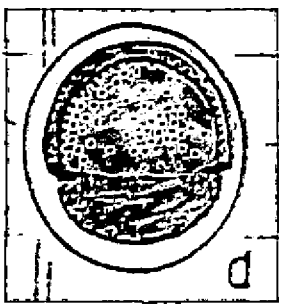
Single cell stage



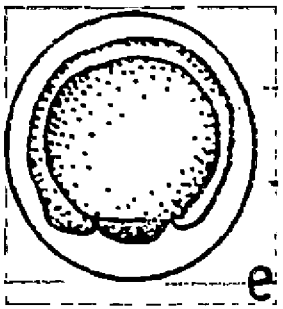
Early morula



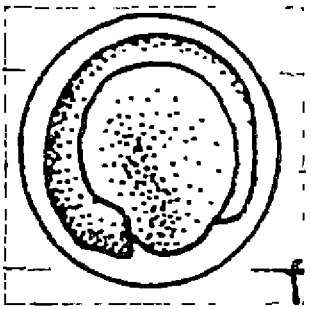
Morula



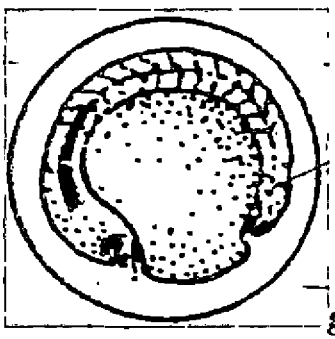
Late morula stage



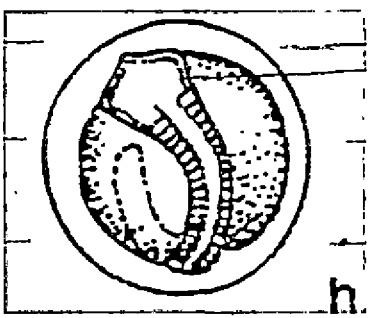
Embryo formation



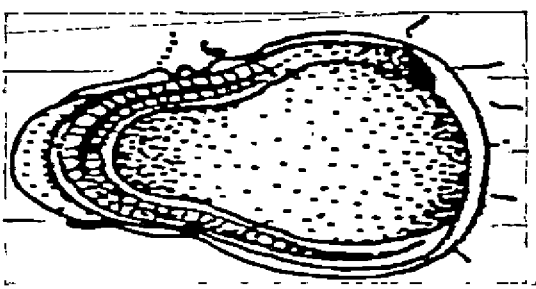
Tailbud early stage



Tail later stage with 12 somites



Formation of optic-cups



Hatching process

Continued in p72

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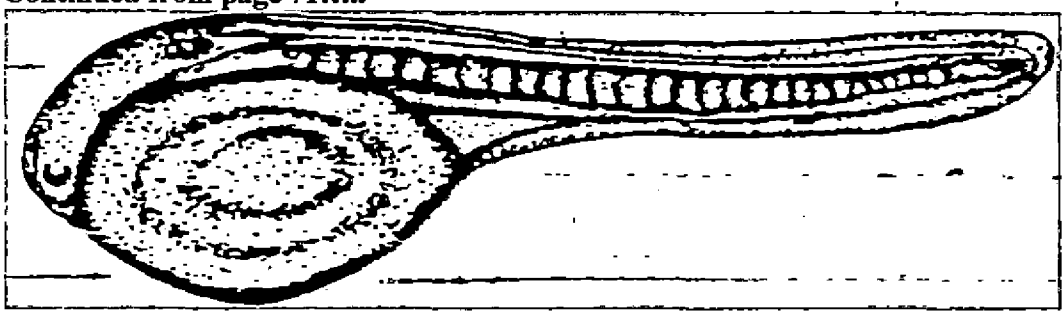
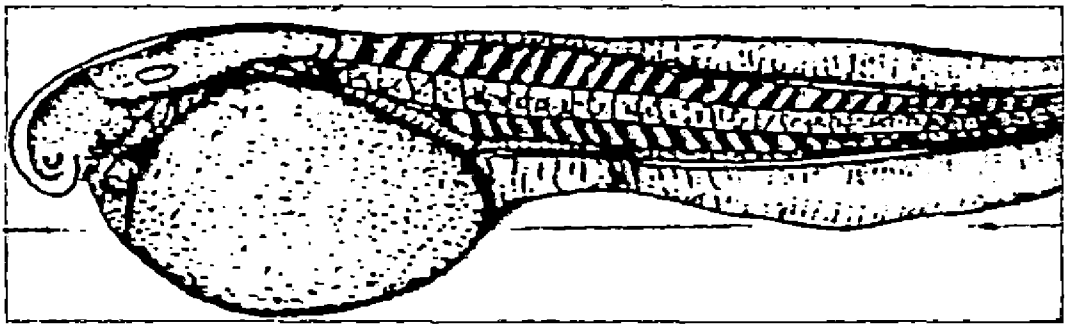
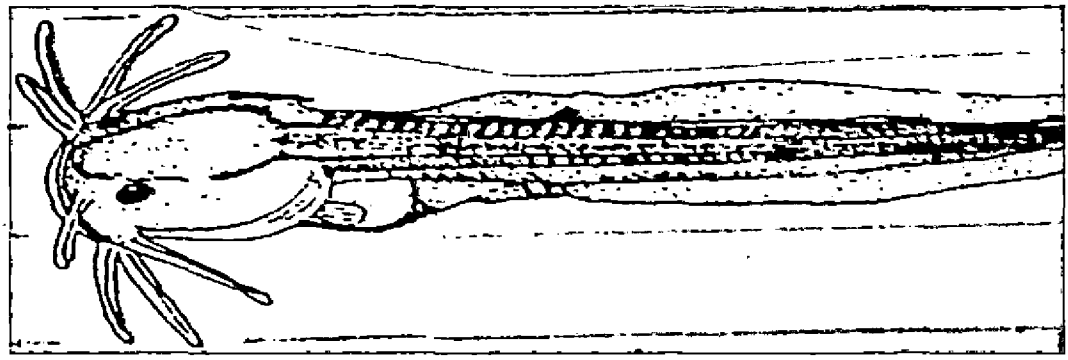


Larvae newly hatched



10 – 12 hours old larva



10 days old post larva

Figure 10. Embryonic developmental stages of *Horabagrus nigricollaris*

4.8. RESULTS OF SPECIAL OBSERVATIONS

4.8.1. Gut content analysis

Observations revealed that *H. nigricollaris* feeds mainly on algae belonging to various groups, leaves of hydrophytes, decayed organic matters, sand granules, small fishes and remainings of crustacean and molluscan shells. This indicates an

omnivorous feeding habit of the fish.

4.8.2. Food and feeding

This fish is very hardy in nature. While maintaining the fishes in tanks when several diets are provided, they did not take any feed at first 15 to 18 days even then the fishes were survived but observations on gonads of these fishes showed adsopted stage. Then the fishes slowly adapted to the feeds provided including frozen prawns, fish choppings, formulated pellets and earthworms. The fish showed a higher preferential index to the earthworm diet, while it catches the worms in a very active and jerky manner. Most portion of their diet will be consumed during the night hours.

4.8.3. Male and female identification

The males of *Horabagrus nigricollaris* have a slender body with genital opening covered with a thin flap and the pectorals are rough and with sharp edges at its inner margin. In case of females, genital-flap is abscent and the pectorals are comparatively smooth. Females have bulged abdomen with reddened genital openings bearing a shape more or less similar to "8".

4.8.4. Fish behaviour and adaptability to the environmental change in captivity

The examination on this aspect shows that the fish is very active in movement. This fish have a grouping behaviour moving always in a friendly manner to their own community. When some fishes like *Puntius denisonii* were introduced along with the experimental fishes but have not found any deleterious affects on either. That means this fish is suitable for community tanks with other herbivorous fishes. When you catch the fish by holding at its pectoral (it's a special holding practice to keep you free from the accidental injection of their very strong pectoral spine) it makes an intresting sound may be producing because of the movement of the bones at its cephalic region. When I mimicked the same sound at the fishes held in separate cement tans, the fishes were very much alertive to that particular sound and move very actively. They always try to hide under dark areas, which are cool. Rainy season under heavy flood and cool temperature were the best index for their spawning in nature. But in captivity the environment was not that much effective to allow their free spawning activity may be due to lack of the above conditions coupled with Physico-chemical and zoo-

geographic changes in the enironment. The extensive study is further needed for its captive breeding to get better results.

DISCUSSION

5. DISCUSSION

Cryopreservation is the freezing of cells in a manner that preserves their biological activity for longer period for future use. Theoretically, cells stored in liquid nitrogen (–196 °C) could be held indefinitely (Leung, 1991). Cryoprotectants allow cells to survive freezing and thawing events (Horton and Ott, 1976).

Recently considerable progress has been achieved in preserving the spermatozoa of fish. Extensive cryopreservation studies have been conducted in salmonids, rainbow trout, carps, catfish and herrings. The first successful attempt to cryopreserve African catfish (*Clarias gariepinus*) sperm was achieved by Steyn and Van-Vuren (1987). Hodgin and Ridgway (1964) attempted to freeze spermatozoa of chinook salmon and pink salmon by using DMSO citrate – dextrose sodium chloride as freezing solution and reported spermatozoa motility after freezing and thawing. In the present study DMSO with the combination of the extenders containing NaCl, KCl, KH_2PO_4, $NaHCO_3$, $CaCl_2$, $MgSO_4.7H_2O$, $Na_2HP\ O_4$ and glucose was evaluated.

Thawing can damage cryopreserved sperm when recrystallization of water causes intracellular ice formation (Leung, 1991) so that the cryopreserved spermatozoa were thawed rapidly at 28°C ± 4°C for 7 seconds to avoid thawing injury. Thawing at 30 °C for 7 seconds has resulted in a better motility percentage. The aim of rapid thawing was to minimize damage to the cell due to ice formation (Horton and Ott, 1976), and crystallization of intracellular ice. Because of this, previous studies have recommended that cryopreserved fish sperm be thawed in the shortest time possible (Leung, 1991).

5.1. MILT CHARACTERISTICS

The quality of the milt plays an important role in cryopreservation technology. Unlike the cyprinids, in catfishes the milt is collected by surgically removing the testes and mascerating. Mongkonpunya and Chairak (1995) collected milt from Mekong giant catfish, *Pangasius gigas,* by taking the testes and mascerating.

However, NBFGR (2001) reported oozing out of milt from *H. brachysoma* after injection of Ovaprim @ 0.4 ml/kg body weight. Linhart *et al.* (1993) got the milt from European catfish, *Silurus glanis,* by direct stripping after injecting with pituitary extract @ 5mg/kg body weight. In *H. nigricollaris* also, milt was found to be oozing after injecting with Ovaprim @ 0.4 ml/kg body weight and stripping in peak breeding season. However, for cryopreservation, due to unavailability of sufficient quantity of milt, testes were surgically taken and mascerated.

5.1.1. Milt colour

In catfishes, the colour and consistency of the milt vary from species to species. In the present study, the fresh milt of *Horabagrus nigricollaris* was appeared to be white in color and its consistency was slightly thicker. While Lakra and Krishna (1997) observed the milt of *Clarias batrachus*, it was found to be watery in colour and thin in consistency. NBFGR (2001) reported that the milt of *Horabagrus brachysoma* is white in colour and thick in consistency. The similarity in quality of milt of *H. brachysoma* and *H. nigricollaris* is may be due to the closeness among these species.

5.1.2. Spermatocrit percentage

It was observed in the present study that the spermatocrit ranged between 57.8 - 59.8% in *Horabagrus nigricollaris*. Sanchez-Rodriques *et al.* (1978) showed that the spermatocrit remains constant throughout the spawning period in salmonids. This suggests that number of sperm per ml of semen for an individual fish must stay fairly constant. While the specimens were analyzed during various months the spermatocrit values remained in the same range.

5.1.3. Sperm concentration

The sperm concentration in the seminal plasma varied between individuals and ranged from 16.5 x 10^9 to 20.9 x 10^9spz/ml of semen in the present study. Mongkonpunya and Chairak (1995) obtained the sperm density in the range of 20 x 10^9 to 40 x 10^9 spz/ml in Mekong giant catfish, *Pangasisu gigas.* Several estimates of the number of sperm per ml for rainbow trout and Antlantic salmon vary in the range

of 10-26 x 10^9 spz/ml. There is a great variation between individual fish.

In the present study, the viscosity of the semen appeared to increase with sperm number. The semen pH ranged from 7.3 to 7.5. In the milt, the spermatozoa were immobile and initiated progressive motility upon dilution with tap water. No differences in sperm morphology or movement were apparent among various samples.

5.1.4. Sperm motility

The quality of spermatozoa is usually assessed by the intensity of motility (Goryezko and Tomassik, 1975), based on the percentage of motile spermatozoa (Terner, 1986). The ability to fertilize eggs is influenced by spermatozoan motility (Aas *et al.*, 1991). The motility of the sperm observed under the present study was 80% on an average. Lakra and Krishna (1997) could only realise motility percentage in the range of 35-40 in *Clarias batrachus*.

Motility time in the present study was in the range of 73 to 90 seconds in different samples. The duration of sperm motility was 78 ± 9 seconds and a few sperm cells remained motile for over 2 minitues. Terner (1986) found that duration of spermatozoan motility of salmonids was very short. The trout sperms are motile only upto 30 seconds while in Atlantic salmon (*Salmo salar*) motility lasts for 1-2 minutes.

The sperm were used for fertilization immediately after activation, the sperm motility of most freshwater fishes ceases after 15 seconds after activation (Stoss, 1983). When fish are artificially spawned, sperm are added to the eggs, activation solution and excess water are added, and the fertilized eggs are incubated (Leung and Jamieson, 1991). The time recommended between activation and incubation is 2 min. (Horton and Ott, 1976) to 5 min. (Wayman and Tiersch, 2000) to allow sperm motility to cease.

5.1.5. Motility score

Motility score of the cryopreserved and thawed milt was in the range of 60 to 80% in the present study. As compared to fresh milt, motility score reduced roughly 20% in thawed milt. The motility time also reduced in thawed milt and it was in the range of 30 to 70 seconds.

5.2. MILT CRYOPRESERVATION

The preservation of gamete was carried out in liquid nitrogen at – 196°C for three months. Liquid nitrogen allows the long term preservation of fish spermatozoa carried out at much lower temperature (-20 to -196°C) and ideally maintains viable gametes for periods of several years. Most techniques of cryopreservation presently in use involve rapid cooling and storage in liquid nitrogen (-196°C) which was also used in this study.

5.3. EXTENDERS

Horabagrus nigricollaris sperm has been cryopreserved using four extenders. The extender-A with 10% DMSO was as good as the control for preserving the spermatozoa in liquid nitrogen. Conget *et al.* (1996) also cryopreserved spermatozoa of rainbow trout using a mixture of permiating (DMSO) and non-permiating (sucrose) agents. Erdahl (1986) reported that addition of sugar (1%) to the extender system may lengthen the motility period of the spermatozoa. The sugar was simply acting to delay the activation and hence gave the impression of increasing the motility period. Yamano *et al.* (1990) obtained 85.2% fertilization in masu salmon (*O. masou*) by using an extender containing 10 % DMSO. The simple glucose - based extenders provided better protection for the preserved spermatozoa of white fish, brown trout, charr and land locked salmon (Piironen, 1994).

5.4. CRYOPROTECTANT

Permeating cryoprotectants provide better protection at slow cooling rates. So, mnay observers used DMSO as a permiating cryoprotectant at various levels of concentration. The present study utilized the same @10% level of concentration. The various results obtained were already discussed.

5.5. SPERM ACTIVATION

Cryopreserved spermatozoa activated by distilled water resulted in an average

of 45.2% of hatch rate compared to 70.6% with the intact sperm in European catfish (Linhart *et al.*, 1993). In the present study normal well water was used to activate the sperm which resulted in good motiliy percentages for both frozen and non-frozen spermatozoa.

5.6. FERTILIZATION

Spermatozoa preserved with extender- A was resulted in 30.3 % fertility suceess with a standard deviation of ±2.8 against 35.8± 0.40 with the control. Mongkonpunya and Chairak (1995) observed 21.4% success rate in *Clarias macrocephalus* uisng cryopreserved milt. Mounib *et al.* (1968) successfully preserved the milt from the Atlantic cod (*Gadus morhua*) for 60 days at -196°C in liquid nitrogen and obtained the mean fertilization of 36%.

In the present study, the mean fertilization rate obtained was 35% for cryopreseved milt in contrast to 41.6% for fresh milt. Pullin (1972) observed a fertilization rate of 39% after 315 days of cryopreservation of spermatozoa against 41% for fresh sperm in *Pleuronectes platessa*. In grey mullets, the cryopreserved spermatozoa retained 15.6% fertility after 36 days of preservation and only 2.7% fertility after one year and four days of storage (Chao *et al.*, 1975). The cryogenic preservation of stripped bass (*Morone sexatilis*) was investigated by Kerby (1983) and reported that samples preserved with dimethyl sulfoxide as the cryoprotective agent resulted in variable fertility (0-55.9%). Marian and Krasznai (1987) have reported the fertilization percentage of 40-95 in European catfish, *Silurus glanis* while used a very high spermatozoa/egg ratio. Linhart *et al.* (1993) reported fertilization rate of 10-48% with frozen and thawed spermatozoa against 14-71% with intact sperm in the same species.

Fertilisation rates of 3.5±3% for frozen sperm in common carp using 10% DMSO compared to higher fertilisaion rate for control, 93±5% (Magyary *et al.*, 1996). When 9% DMSO was used for preservation of Mekong giant catfish spermatozoa, the fertilization rate obtained was 65-66% against 73-74% for the control (Mongkonpunya and Chairak, 1995).

High fertilization rate is obtained for salmonid spermatozoa preserved in dimethyl sulfoxide (DMSO) in distilled water (Stoss, 1987).

5.7. HATCHABILITY

Cloud *et al.* (1990) obtained 23% hatchability in the spermatozoa of rainbow trout frozen in liquid nitrogen for 11 months using 6.25% DMSO. In the present study, spermatozoa frozen in liquid nitrogen for three months using 10% DMSO resulted in 81.7% and 85.9 % hatchability success for trial I and II respectively while fresh ova were used.

Gupta and Rath (1991) successfully preserved the milt of *Labeo rohita* at –196°C for one year and were able to fertilize 40 - 50% of the eggs. Thorogood and Blackshaw (1992) reported the short and long - term cryopreservation of the yellowfin bream, *Acanthopagrus australis* using10% DMSO and obtained 66% fertility after 291 days of storage. Spermatozoa of summer whiting, (*Sillago ciliata*) were successfully cryopreserved by Young *et al.* (1992) using DMSO as cryoprotectant.

5.8. COMPONENTS OF CRYOPRESERVATION – SUGGESTIONS

5.8.1. Pre-cooling stage

Before subjecting the cell to the extreme low temperature of liquid nitrogen, the cooling temperature was maintained at 0 ± 4°C on ice followed by –80 to –100°C on vapours of liquid nitrogen for 10min respectively.

5.8.2. Milt quality

It can be determined by making the observations of pH, colour, motility, density and spermatocrit% of the semen sample. Normally semen of this fish appears in milk white colour. The change in colour shows its deteriaoration. So, care should be taken to presetrve the sperm at 4°C within 1.5 to 3min. after collection.

5.8.3. Diluents

It is evident that to minimize the demand on dissolved O_2 the sperm cell density has to be reduced by the addition of the diluents. Ideally these diluents should be isotonic to the seminal plasma to maintain the spermatozoa in an immotile state to sustain all the metabolic activities required by the cells (Billard *et al.*, 1983). Since there are no studies available on the spoken subject for this fish, four different compositions of diluents (extenders) were used.

The added volume provided by an extender solution allows establishment of an optimum ratio of semen to eggs for fertilization, and also permits a large number of females to be mated to a given male. Our objective was to develop an extender for the storage of semen from *Horabagrus nigricollaris*. In the wild, ripe females and males are collected at different times, and their condition quickly deteriorates after capture. Also, in captive stocks, it is very difficult to synchronize the ripening of individual fish. The use of an artificial semen extender will greatly facilitate hatchery operations and improve the likelihood for a successful seed production.

5.8.4. Cryoprotection

Cryoprotection to the cells was provided by using the cryoprotective agent DMSO. Addition of 10% hen's egg yolk to improve the survival of frozen-thawed spermatozoa was also tried.

5.8.5. Equilibration time

For effective protection during cooling sufficient time was allowed to facilitate the penetration of cryoprotectants into cells as done by Grout and Morris (1987). The rate of uptake may depend on the size of biological material and molecular-size of the cryoprotectant. Equilibration time may be influenced by diluents used.

5.8.6. Cooling and thawing

Cooling and thawing rates play a major role in cryopreserved cell survival. These are the temperatures used in cryopreservation for particular periods of time. The rates for cooling and thawing used for the present study were -196°C of liquid nitrogen and 27°C per 7 seconds respectively.

5.8.7. Insemination of cryopreserved - thawed milt

The injuries sustained during the process of cooling and thawing are difficult to isolate. Cryo-injuries can result in spontaneous activation of spermatozoa and reduce the proportion of activated spermatozoa upon thawing. In addition, the speed and duration of motility can be reduced when compared with fresh unfrozen spermatozoa (Stoss, 1983, Billard *et al.,* 1999). In view of such duration, post-thaw milt should be used immediately after thawing..

To prolong the duration of motility, minimize osmotic shock during fertilization and disperse the sperm cells around the ova, inseminating solutions are advocated. The composition of such solutions should be similar to ovarian fluid (Scott and Baynes, 1980) or have a salinity of 5% and 20% for fresh and sea water species respectively (Stoss, 1983). The present study also followed the same package of practices.

5.8.8. Preservation conditions

Preservation conditions such as storage temperature, depth of storage and dilution media were taken care, during the present study.

5.8.8.1. Storage temperature

Since the storage temperature is a major factor affecting the viability of gametes during *in vitro* storage. Viability can be prolonged by maintaining gametes at near zero temperatures to reduce their metabolic burden. The ability of gametes to tolerate low temperature may vary between temperate and tropical species (Leung and Jamieson, 1991). Utmost care was taken to seal the cryocans perperly to avoid

temperature fluctuations.

5.8.8.2. Depth of storage

The depth of milt in the storage container, and hence gaseous diffusion, is also reported to influence the milt fertility after storage (Stoss *et al.*, 1987). So all the straws were kept at the same depth in the cans.

5.9. FERTILIZING CAPACITY

The fertilizing capacity of the milt was taken as the most conclusive test of sperm quality in the present study.

5.10. CONCLUSION

An increasing number of wild fish species including the one under study, are in danger of extinction, often as a result of human activities. The cryopreservation of gametes and embryos has great potential for maintaining and restoring threatened species. The conservation of both paternal and maternal genetic information is essential. However, although this technique has been successfully applied to the spermatozoa of many fish species, reliable methods are lacking for the long-term preservation of fish eggs and embryos. This study describes a protocol for preserving the spermatozoa of *Horabagrus nigricollaris*. This technique represents a promising tool in efforts to save the fish under study. Moreover, this approach has significant potential for maintaining domesticated fish strains carrying commercially valuable traits for aquaculture purposes.

SUMMARY

6. SUMMARY

1. The objective of the study is to develop a protocol for the cryopreservation of spermatozoa of the black collared yellow catfish, *Horabagrus nigricollaris*. This species is unique in the Western Ghats as far its endemism, ornamental and food values concerned.

2. It's distributed only in a stretch of less than 15 km in the River Chalakudy down the *Athirappilly waterfalls*. Since these fishes are indiscriminately exploited owing to its high market demand, the species is vulnerable to extermination.

3. The samples were collected by cast netting during the night hours from the study area, brought to the college hatchery and were maintained in fibre glass tanks of 12t capacity.

4. The dead fishes were used for analysing the gut content to understand the food and feeding habits.

5. The GSI was calculated during various months. The breeding season extends from May to September.

6. The sex identification of the species under study is reported for the first time.

7. Experimental animals were mainly fed with earth worms cultured in the college premises.

8. Analysis of the fresh milt was carried out for finding out its texture, colour and pH.

9. The milt of the species is comparatively thick, white in colour and pH ranged between 7.2 to 7.4.

10. The spermatocrit values ranged from 57.8 to 59.8%.

11. Sperm density was 16.5×10^9 to 20.9×10^9 spermatozoa/ml of milt.

12. Milt was cryopreserved in liquid nitrogen using four extenders and 10% DMSO as the cryoprotectant for a period of three months.

13. After the cryopreservation, the milt was checked for its motility and potency.

14. Fresh ova collected by stripping were used for fertilization trials. Rates of fertilization and hatching were calculated.

15. Average fertilization % obtained was 30.3 ± 2.8 for extender- A against 35.8 ± 0.4 for the control.

16. Mean hatching % obtained was 35.0 ± 4.7 for extender - A and 41.6 ± 4.4 for the control. No significant difference in fertilization success was found between cryopreserved sperm and untreated sperm from the same milt samples.

17. Viable hatchlings were produced from milt that had been cryopreserved for three months indicating the feasibility of establishing a cryopreservation protocol to bank the genes and popularize the aquaculture of this critically endangered species.

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CRYOPRESERVATION OF SPERMATOZOA OF CRITICALLY ENDANGERED YELLOW CATFISH *HORABAGRUS NIGRICOLLARIS* (Pethiyagoda & Kottelat 1994)

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ABSTRACT

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In order to develop gene banking techniques aimed at conserving the critically endangered black collared yellow catfish of the Western Ghats, *Horabagrus nigricollaris* and popularize this species in the aquaculture scenario a study on cryopreservation of spermatozoa was undertaken. Freshly collected milt was observed for its characteristics. Well water was used to activate the milt in various stages of the experiment. There was no significant difference in motility of spermatozoa or percentage hatching from fresh and milt cryopreserved using 10% dimethyl sulfoxide (DMSO). Screening of four extenders (A, B, C and D) containing NaCl, KCl, $CaCl_2$, $NaHCO_3$, KH_2PO_4, $MgSO_4.7H_2O$, $Na_2H\ PO_4$ and Glucose at various proportions clearly indicated that the extender composition had significant effect on the percentage of motility, fertilization and hatching. Selected milt samples were preserved under cryogenic condition and utilized for experimental spawn production. The quality of milt samples was analyzed for the spermatocrit value, sperm density, pH, motility score and time: values obtained ranged from 57.8 to 59.8%; 16.5 x 10^9 to 20.9 x 10^9 spermatozoa/ ml of milt; 7.2 to 7.4; 4+ to 5+ (i.e. 80-100%) and 30 to 70 seconds respectively. The most promising combination was extender - A with 10% DMSO as the cryoprotectant. The ratio of milt and diluent was 1:4. Samples were equilibrated at 0°C ± 4°C on ice and vapourised over liquid nitrogen fumes for 10 minutes and finally stored in liquid nitrogen for three months. Stored milt samples were thawed and utilized to fertilize the eggs. Quick thawing procedure (27°C ± 2°C for 7 seconds) was followed. A French straw containing 0.5ml milt was found to be sufficient to fertilize 200 eggs approximately. Average fertilization % obtained was 30.3 ± 2.8 for extender- A against 35.8± 0.4 for the control. Mean hatching % obtained was 35.0 ± 4.7 for extender - A and 41.6± 4.4 for the control. No significant difference in fertilization success was found between cryopreserved sperm and untreated sperm from the same milt samples. Viable hatchlings were produced from milt that had been cryopreserved for three months indicating the feasibility of establishing a cryopreservation protocol to bank the genes and popularize the aquaculture of this critically endangered species. With the rapid global expansion of aquaculture, there is a need for year-round availability of larvae that could, to some extent, be met through cryopreservation of gametes.