CHAPTER 1

Ram semen extenders and freezing

1.1 Introduction

The literature reviewed in this chapter deals mainly with the freezing of ram semen, the extenders used, and the freezing process. Also included are physiological details associated with low temperature effects on the spermatozoal cells. Emphasis will be placed on practical aspects of semen extenders, with coverage of the most important biochemical details of the freezing process in cells and cell membranes included to provide a theoretical basis for the various processes.

1.2 Extenders for freezing semen

Our understanding of cell membrane structure is based largely on the model proposed by *Singer & Nicholson (1972)*. In this "fluid mosaic model", the proteins that are integral to the membrane, are a heterogeneous set of globular molecules, each arranged in an 'amphipathic' structure, with the ionic and highly polar groups protruding from the membrane into the aqueous phase, and the nonpolar groups largely buried in the hydrophobic interior of the membrane. These globular molecules are partially embedded in a matrix of phospholipid. The bulk of phospholipid is

organised as a discontinuous, fluid bilayer, although a small fraction of the lipid may interact specifically with the membrane proteins. Therefore, the fluid mosaic structure is formally analogous to a two-dimensional oriented solution of integral proteins or lipoproteins in various phospholipid bilayer solvent. Recent reports suggest that this structure is more complicated, with asymmetry of lipid distribution and the interaction of lipid-lipid and lipid-protein which are the basis for ordered domains within the plane of the membrane (*see reviews by Alonso et al., 1988 and Jain, 1988).* These complex structures result in compartmentation of the plasma membrane *(Parks & Graham, 1992)*, and these have been also observed in the plasma membrane of spermatozoa by *Cardullo & Wolf (1990)*, who reported differences in the distribution of both lipids and proteins in the periacrosomal and the postacrosomal regions of the sperm head, mid-piece and in the principal piece of the flagellum.

It has been shown that primary damage to cell membranes (including animal and plant cells) occurs during the freezing and thawing phase between temperatures of -15 and -60°C, but not during storage in liquid nitrogen at -196°C *(Mazur, 1985).* Ice formation during the cooling phase was described by *Parks & Graham (1992)*, with crystals typically forming first in the extracellular compartment. During slow cooling, the plasma membrane acts as a barrier, preventing ice crystal growth from the medium into the cell. Salts are excluded from the water that becomes ice, resulting in an increasing concentration of salts in the remaining unfrozen water. Thus, an increase in the osmotic pressure gradient across the plasma membrane occurs and this causes water to diffuse out of the cell causing dehydration of both

cells and the plasma membrane. As ice crystals continue to grow, cells will be trapped in the space of unfrozen water.

This slow cooling will lead to cellular damage from two sources. Firstly, *Steponkus & Lynch (1989)* suggested that when more than 90% of water is removed osmotically or by so called "solution effects", the plasma membrane will sustain lateral phase separation or lyotropic phase transitions from the "lamellar" to the "hexagonal" phase which result in membrane structural damage. Secondly, it is also possible that if the unfrozen spaces become too small, cells may be deformed and physically damaged *(Amann & Pickett, 1987)*.

If a rapid cooling rate is used intracellular ice formation will occur, with consequent cell damage and death *(Mazur, 1985; Amann & Pickett, 1987)*. The exact mode by which intracellular ice damages the cells is not yet clearly understood *(Park & Graham, 1992)*. While the crystallisation processes which induce large intracellular ice crystals cause death to the cell, intracellular microcrystals (from vitrification) need not do so *(Mazur, 1985; Amann & Pickett, 1987)*. As a result, the strict control of cooling rate may avoid or at least minimise the cellular damage caused by intracellular ice formation.

Hinkovska-Galcheva et al. (1989) reported that freezing (to -196°C) and thawing of ram spermatozoa without the additional of a cryoprotectant resulted in considerable changes in the lipid composition of the cell membrane (as determined by thin-layer chromatography). As shown in Figure 1.1, it can be seen that 99% of diphosphatidylglycerol (DPG) is found in the inner monolayer of control (intact)

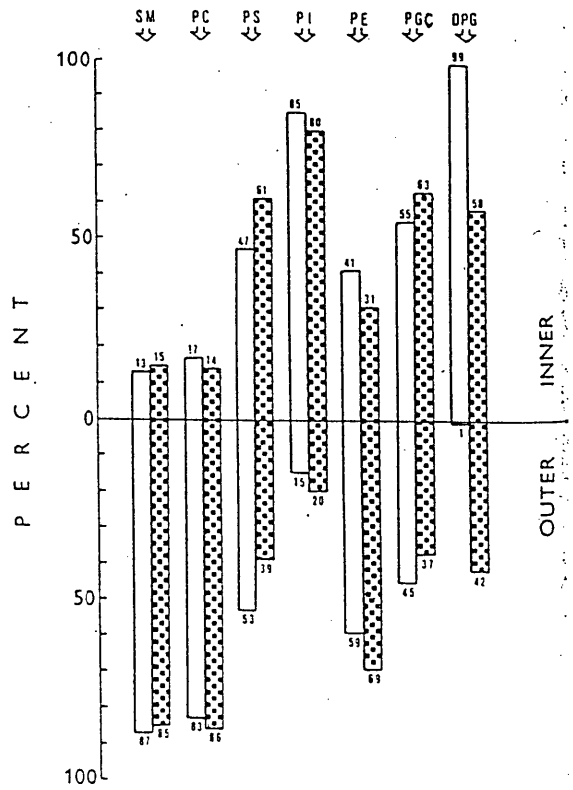

Figure 1.1 The changes in the proportion of ram spermatozoa surface proteins before (cleared column) and after (checked column) freezing *(Hinkovska-Galcheva et al., 1989)*

*SM=sphingomyelin; PC=phosphatidylcholine; PS=phosphatidylserine; PI=phosphatidylinositol; PE=phosphatidylethanolamine; PGC=phosphatidylglycerol; DPG=diphosphatidylglycerol

The basic components of extenders for freezing spermatozoa are: a) water to act as a solvent for seminal and extender components *(Foote, 1964)*; b) dissolved ionic and non-ionic substances to maintain osmolality and to buffer the pH of the medium (commonly zwitterion buffers such as TEST and TES, sodium citrate, or Tris buffer) c) organic materials with the capacity to prevent cold shock (generally egg yolk or skim milk); d) cryoprotectants, usually glycerol; e) a simple sugar as an energy source *(Mann; 1948)* or complex sugars as added cryopreservatives; and f) antibiotics to control microbial growth *(Graham, 1978; Foote; 1980; Evans & Maxwell, 1987)*. The characteristics of an ideal extender for freezing mammalian spermatozoa are summarised in Table 1.1.

Table 1.1 The characteristics of an ideal extender for mammalian semen (from *Graham (1978)* and *Foote (1980)*).

1) Contains nutrients as a source of energy eg. some kind of sugar.
2) Maintains proper osmotic pressure and electrolyte balance to ensure minimal salt effects.
3) Provides a buffer to prevent harmful shifts in pH during cooling and freezing.
4) Contains constituents that provide protection against the harmful effects of cooling (cold shock) and freezing.
5) Be stable, resisting enzymatic and non-enzymatic degradation.
6) Contains antibiotics that inhibit bacterial growth.
7) Substantially increases the volume of semen so that multiple inseminations can be performed.
8) Provides an environment in which the metabolic activities of the sperm can continue.

1.2.1 Energy sources

Simple sugars such as glucose and fructose are included in semen extenders as sources of energy for spermatozoa *(Mann, 1948)*. Only fructose is present in ram semen, but spermatozoa are also able to use glucose and mannose as an energy source when these sugars are included in storage extenders *(White et al., 1954)*. Although no other sugars have been shown to act as energy sources *(Maxwell & Salamon, 1993)*, a wide range of sugars have been examined for their cryoprotective action (to be discussed in section 1.2.4).

1.2.2 The use of buffering systems

During freezing, changes in pH may occur in addition to increases in salt concentration, primarily due to either the formation of ice or the precipitation of salt *(Graham, 1978; Pickett & Berndtson; 1978)*. When an aqueous solution is cooling down below its equilibrium freezing point, chances in ice formation are increased *(Pegg, 1987)*. This in turn will result in an increase in the concentration of solute which involves both changes in pH and the migration of the water from the cell. Thus, cell dehydration will occur during freezing *(Lovelock, 1953)*, and in addition the cells may be damaged by the change in pH *(reviewed by Graham, 1978 and Pegg, 1987)*.

However, measurement of pH at low temperature (after freezing) is difficult and knowledge of pH changes during freezing is limited *(Taylor, 1987)*. To minimise changes in pH due to decreasing temperature, semen extenders should contain buffers such as citrate *(Salamon & Lightfoot, 1969)*, phosphate *(Entwistle & Martin, 1972)*, Tris *(Salamon & Visser, 1972; Johnson et al., 1974)*, or Zwitterion buffers (TES, HEPES, PIPES, TEST) *(Abdelhakeam et al., 1991 a,b; Molinia & Evans; 1991)*.

Salamon & Lightfoot (1969) found that the level of sodium citrate in the extender had a pronounced effect on post-thaw motility, but that this effect interacted with both sugar types and concentrations. It was shown that the optimal level of sugar in an extender fell progressively as the concentrations of sodium citrate increased. Regardless of the sugar type, the optimal result was obtained with sugar and citrate concentrations of 166.5 and 102 mM. respectively. They also found that a hypertonic

solution gave better results than a hypotonic one in terms of post-thaw motility. These results are supported by the later work of *Fiser et al. (1982)* who found that the post-thaw motility and forward progression of ram spermatozoa were increased significantly in hypertonic extenders compared with isotonic extenders, regardless of their composition.

Entwistle & Martin (1972) used a phosphate buffer in a synthetic extenders for freezing ram semen in ampoules and found that while this synthetic extender [247 mM. glucose, 49 mM. NaCl, 5 mM. KCl, 5 mM. phosphate buffer and 7.5% V/V glycerol of pH 7] without egg yolk gave a post-thaw motility percentage of 24%, addition of 6.5% V/V egg yolk significantly improved motility to 37.9%. Unfortunately, the authors did not compare this phosphate buffer with alternatives.

Salamon & Visser (1972) examined the effects of concentration (150, 300 or 450 mM) of Tris (hydroxymethyl) aminomethane "Tris" by measuring the post-thaw motility percentage and found that the optimal concentration, regardless of method of thawing, was 300 mM. *Johnson et al. (1974)* reported different optimal levels of Tris (study range 90 to 409 mM) and glycerol (study range 1 to 13%) in the extender when they used different parameters to assess the results. Thus the post-thaw motility percentage was best when the extender contained 320 mM. of Tris with 5.7% glycerol at a thawing temperature of 69.5 ^{o}C, but the optimal concentration of Tris as assessed by the highest percentage of intact acrosomes was 300 mM with 3.8% of glycerol and a thawing temperature of 45.6 ^{o}C. They concluded that maintenance of the acrosomal

cap requires lower glycerol levels and Tris molality than does maintenance of motility, and that fertility studies are necessary to test this discrepancy.

Evans & Maxwell (1987) suggested the use of synthetic extenders if a one step dilution (ie extend the sample soon after collection to the final concentration) was used to freeze ram and buck semen. The recommended extender consisted of both Tris (hydroxymethyl) amino methane (3.634 g/100 ml.) and citric acid (monohydrate) (1.990 g/100 ml.) as the basic buffering system.

In the case of zwitterion buffers, *Molinia & Evans (1991)* compared a Tris-citrate buffer with six other combinations of zwitterion buffers with Tris or NaOH. The six extenders tested were 1) TES (228 mM.)-NaOH, 2) TES (215 mM.)-Tris, 3) HEPES (236 mM.)-NaOH, 4) HEPES (227 mM.)-Tris, 5) PIPES (119 mM.)-NaOH, and 6) PIPES (105 mM.)-Tris. They found that all the zwitterion buffers gave superior results in terms of post-thaw motility and acrosome integrity compared with Tris-based extenders and concluded that zwitterion buffers may be successfully used in extenders for freezing ram semen. In addition, *Abdelhakeam et al. (1991 a,b)* successfully used the zwitterion buffer TES [Tes (N-tris(hydroxy methyl) methyl-2-aminoethane sulphonic acid) titrated with Tris (hydroxy methyl) aminoethane to a pH of 7.0] in their studies of glycerol effects on fertility.

Overall, it can be concluded that the use of buffering systems including Tris, citrate, phosphate or zwitterion buffers is likely to result in good semen quality in terms of either post-thaw motility or good fertility. These buffers may have to be used in conjunction with other suitable components (ie. egg yolk or milk) and these factors

may have effects on the success rate such that no absolute choices are currently available to ensure optimum fertility after the freezing of ram semen.

1.2.3 Cold shock preventive materials

Cold shock injury, which was first called "temperature shock" *(by Milovanov, 1934)*, is the damage which occurs in cell structure due to a sudden reduction in temperature *(Watson & Morris, 1987)*. Most cells including microorganisms, ova, embryos, blood cells and spermatozoa are sensitive to cold shock *(Morris & Watson, 1984)*. In the case of sperm cells, there are variations in the sensitivity to cold shock when subject to rapid cooling. Among fowl, ram, bull, and boar spermatozoa, boar sperm are the most sensitive and fowl sperm are the most resistant to these effects *(Watson, 1981 a)*.

The studies of *Holt et al. (pers. comm. cited by Watson & Morris , 1987)* indicated that after rapid cooling of ram spermatozoa (10°C min^{-1}) sperm motility decreased rapidly with decreasing temperature. At around 16°C, many spermatozoa began to exhibit a rigid bowing of the mid-piece which resulted in cessation of flagella wave generation and forward cell progression. Below 16°C, progressively more spermatozoa displayed these signs and from 5 to 30 percent of affected spermatozoa sustained a sudden displacement of the head such that it made contact with the midpiece, a movement which resulted in the flagellum becoming deflected through an angle of 180° in the distal region of the mid-piece. This "folding process" occurred between 16 and 8°C. On rewarming, these spermatozoa did not unfold but regained motility with a reverse direction. A number of spermatozoa also showed

signs of localised swelling of the flagellum, mostly in the vicinity of the end-piece but some in the distal region of the mid-piece. Sperm with a swollen flagellum often regained forward motility during rewarming. Staining the acrosome with Giemsa stain showed that following this so-called "cold shock", a high proportion of spermatozoa exhibit damage to the acrosome membrane. Combined, this evidence indicates that after cold shock spermatozoa are unlikely to be able to maintain full fertilising capacity.

To prevent or reduce the effects of cold shock on spermatozoa, some lipid and protein preparations have to be added to extenders *(Parks & Graham, 1992)*; however, the exact protective mechanism of these organic materials remains unknown *(Parks & Graham, 1992)*. In the case of freezing ram spermatozoa, egg yolk or skim milk are most commonly used as cold shock preventive materials *(Memon & Ott, 1981; Evans & Maxwell, 1987)* and the protective mechanisms of these components have been subjected to some investigations as indicated in the following sub-sections.

Milk extenders

The protein -casein- has been identified as the agent in milk responsible for preventing cold shock in spermatozoa *(Choong & Wales, 1962; O'Shea & Wales, 1966)*. *Jones & Martin (1965)* compared the revival rate of thawed spermatozoa (ampoule storage) in different extenders (50% V/V egg-yolk-citrate vs 9% W/V skim milk) and found that there were no differences between the two extenders at a ten fold dilution, but with twenty or forty fold dilutions milk gave better revival rates than at a

ten fold dilution. Spermatozoal revival was depressed at the higher dilution rates when egg-yolk-citrate extender was used.

Jones (1969) examined the influence of extenders and processing time after ejaculation on the survival of deep frozen ram spermatozoa. He found that a reconstituted skim milk extender (9% W/V skim milk) gave a better result in terms of semen motility than a synthetic medium containing lactose (27.5% vs 13.5% of post-thawing motile sperm). *Fiser & Fairfull (1986)* used extenders based on 15% skim milk to freeze ram semen in straws and reported that optimal spermatozoan survival (percentage of motility and rating) occurred when 4 to 6% glycerol was added to the extender and the freezing rate was between 10 and 100 ^{o}C/min. With the addition of 8% glycerol, the optimal freezing rate was between 5 and 30 ^{o}C/min.

However, while skim milk extenders have been successfully used for freezing ram semen, they have a major drawback in that the opaqueness of milk interferes with the measurement of motility and density by optical means (ie. using the light microscope for determination of motility or the spectrophotometer for semen concentration) *(Upreti et al., 1991)*. This may be an important reason for favouring egg yolk extenders for freezing ram spermatozoa, at least in experimental programs.

Egg yolk extenders

Since the discovery that the addition of egg yolk to extenders has a beneficial effect on the fertility of cattle semen *(Phillips & Lardy, 1940)*, it has become widely used as a component of semen extenders. The benefit of egg yolk to the preservation

of ram semen has been illustrated by *Salamon & Robinson (1962 a,b); Jones et al. (1969); Lightfoot & Salamon (1970 a,b) and Jones & Martin (1973).* The protective action of egg yolk on spermatozoa during low temperature preservation is presumed to be due to the presence of low density lipoproteins that bind firmly with the sperm membrane *(Cookson et al., 1984).* The protection afforded to cooled ram spermatozoa by egg yolk includes the maintenance of both motility and fertility, accompanied by evidence of structural integrity *(Robertson & Watson, 1987). Jones & Martin (1973)* found that while cooling of ram spermatozoa from 37 to 5 ^{o}C normally caused swelling of the acrosome with signs of acrosomal vesiculation and/or vacuolation; the additional of egg yolk to the extender prevented only the vesiculation reaction. However, *Jones & Martin (1973)* stated that egg yolk also acted to reduce changes in the organelles of the head and mid-piece of spermatozoa which are of importance for motility and fertility.

Robertson & Watson (1987) found that egg yolk significantly reduced the accumulation of calcium in spermatozoa which resulted from both cold shock and slow cooling, and initially enabled spermatozoa to retain their ability to prevent the influx of calcium ions. Other reports indicated that treatments which resulted in a sustained increase in intracellular calcium level in liver cells induced cytotoxicity *(Orrenius, 1985)* and abnormally high intracellular calcium levels led to cell death in the case of nerve cells due to blood insufficiency *(Astrup, 1983).* Thus, *Robertson & Watson (1987)* suggested that an imbalance of calcium ions in sperm cells may alter the normal functions of the acrosome and plasma membrane, and thus cause infertility.

Pace & Graham (1974) found that when bull semen was pelleted on dry ice using a TES buffer containing egg yolk and glycerol, neither the buffer alone nor buffer plus glycerol, protected cell motility in the absence of egg yolk. They also found that sperm cells frozen in the egg yolk and buffer without glycerol yielded 24% post-thaw motility compared with only 1 and 3% when frozen with 0 and 4% glycerol (without egg yolk) respectively ($p < 0.05$). They concluded that egg yolk was the main cryoprotective agent; but that there was a synergistic effect between glycerol and egg yolk, with the combination providing the greatest post-thaw survival (40%; $p < 0.05$).

Pace & Graham (1974) also purified the lipoproteins in egg yolk by using ultracentrifugation, Biogel and Sephadex filtration and found that it was the low density lipoprotein fraction which protected motility of the sperm cells during the freezing process with glycerol-free extenders. *Foulkes (1977)* separated the lipoproteins from egg yolk by centrifugation and column chromatography using citrate-based semen extenders and found the lipoprotein fraction which had the smallest lipid:protein ratio, gave the best protection to bull semen during the freezing process. He also demonstrated that the lipoprotein components of egg yolk remained associated with spermatozoa even after extensive washing in an attempt to remove the extender. *Vishwanath et al (1992)* separated a positively charged lipoprotein fraction from egg yolk by sequential extraction and precipitation with amaranth, as well as water soluble, saline soluble, and citrate soluble cationic fractions. The water soluble portion (protein concentration of 8.15 mg/ml.) gave the best results in terms of preventing cold shock. The mode of action of this fraction seems to be in its ability to bind strongly to the sperm membrane which has an overall negative charge.

In a comprehensive study of ram semen preservation, *Lightfoot & Salamon (1970 b)* used an extender based on egg yolk and found that when compared with fresh semen, frozen semen was of lower fertility (49% vs 70%). *Smirnov et al. (1978)* compared the effectiveness of 32 extenders in terms of post-thawing sperm motility and found that while the best extender contained raffinose-sodium citrate-glutamic acid-egg yolk and glycerol, the omission of either egg yolk or glycerol reduced post-thawing motility to only 1-2%.

Watson & Martin (1975) reported that egg yolk was beneficial to ram spermatozoa in terms of post-thaw motility and acrosome integrity not only during chilling but also during freezing. They also found that while the inclusion of glycerol in the extender improved the survival of spermatozoa; the acrosome deteriorated at glycerol concentrations above 7%. Further research by *Watson (1976),* using lipoprotein fractions extracted from the egg yolk, showed that the low density lipoprotein fraction (LDF) protected ram and bull spermatozoa in the same manner as egg yolk during both chilling at 5°C storage, and during freezing and thawing. Fresh LDF at an equivalent concentration to egg yolk in the extender gave only about 50% of the latter's' protection potency during 5°C storage. Freeze-dried LDF provided a lower level of chilling protection than fresh LDF and a greater proportion of the acrosomes of ram spermatozoa were damaged after freezing and thawing in extenders containing LDF than in those containing whole egg yolk.

More recently, *Abdelhakeam et al. (1991 a,b)* have successfully frozen ram semen in straws or pellets using egg yolk extender without other cryoprotectants. The

extender in that case, comprised zwitterion buffers (TEST, at 375 mOsm), with 30% egg yolk (V/V) and 10% maltose monohydrate (V/V). This is the first extender that has been shown to have the ability to protect ram sperm from the freezing process without the addition of cryoprotective agents such as glycerol, propylene glycol or DMSO.

1.2.4 Cryoprotectants

Since the discovery of glycerol as a cryoprotective agent for mammalian spermatozoa *(Polge et al., 1949)*, much effort has been devoted to determining its protective characteristics *(Graham, 1978)*. These characteristics include the ability to:

a) Maintain pH during reduction of temperature, and

b) Change the liquid-ice phase (solidification of the aqueous system at low temperature by vitrification rather than by crystallisation).

The increase in intracellular salt concentration due to the removal of water from cells during ice formation is a major cause of decreases in sperm motility due to freezing *(Graham, 1978; Foote, 1980)* and the protective action of glycerol is largely attributed to its salt "buffering" capacity; which minimises electrolytic damage as the water moves out of frozen cells *(Taylor, 1987)*. Glycerol also provides cryopreservation by reducing the amount of ice formed and by increasing the time taken for water to leave the cells in response to the decreased vapour pressure of adjacent ice crystals. Both latter functions occur by increasing the viscosity of extra- and intra-cellular solutions *(Merryman et al., 1977)*.

The actual mechanism by which glycerol protects the cell from damage during freezing is not completely understood *(Parks & Graham, 1992). Amann & Pickett (1987)* suggested that the major beneficial effects occur at the extracellular level (ie. salt "buffering"). However, it also has been clearly shown that glycerol enters and remains in the cell and there is evidence to suggest that glycerol may bind directly to the plasma membranes *(Armitage, 1986; Anchordoguy et al., 1987)*. Large scale rearrangements of membrane structures have been reported after the addition of glycerol *(Rudenko et al., 1984)*. This glycerol can induce interdigitation of the two bilayer leaflets of the cell membrane, and can change membrane fluidity by increasing the order of inferior fatty acyl chains *(Boggs & Rangaraj, 1985)*. It is also able to alter the polymerisation and depolymerisation of microtubules which may affect the plasma membrane indirectly *(Keates, 1980)*. We can reasonably assume that these effects of glycerol will also happen with the sperm cell membrane.

There are conflicting reports as to the most appropriate temperatures and concentrations of glycerol to be used when freezing ram semen. Workers such as *Lightfoot & Salamon (1970 a,b), Salamon & Lightfoot (1969)* and *Colas (1975)* added glycerol by combining it with extenders at 30 to 37 ^{o}C (before cooling), while others *(Healey, 1969, and Watson & Martin, 1975)* have added it later in the cooling process at 5 ^{o}C. According to the latter studies, it is acceptable to add glycerol at 4 to 5 ^{o}C, since it prevents freezing damage during the crystallisation phase which occurs below that temperature. On the other hand, it is more convenient in practice to add it to the extenders before initial dilution at 30-37°C.

The concentration of glycerol is usually 3 to 6% for extended ram semen *(Lightfoot & Salamon, 1970 a,b; Fugui & Roberts, 1977; Abdelhakeam et al., 1991 a). Colas (1975)* found that there were no significant differences in post-thaw-motility at glycerol concentrations of 2 or 4%, but fertility was significantly higher in the 4% glycerol treatment. *Fiser & Fairfull (1989)* studied the effect of glycerol-related osmotic changes on post-thaw motility and acrosomal integrity of ram spermatozoa by varied methods of addition of glycerol as follows:

Method 1: addition of 4% glycerol (V/V) to the diluted semen prior to freezing by a one step addition at 30 ^{o}C; or

Method 2: by first cooling the diluted semen to 5 ^{o}C and then adding the glycerol gradually over 15 to 30 min; or

Method 3: by a one step addition of glycerol prior to equilibration for 2 h. or;

Method 4: by cooling diluted semen to 5 ^{o}C, followed by a holding period of 2 h. at 5 ^{o}C, and the one step addition of glycerol just prior to freezing.

They found that the average post-thaw percentage of motile spermatozoa (39.9%) was significantly lower after using Method 1 than the other three methods. The average percentage of intact acrosomes (61.2%) was highest in semen in which glycerol was added by Method 2. Nevertheless, when averaged over the methods of glycerolation, the post-thaw motility (range, 43.7 - 44.2%) and the percentage of intact acrosomes (range, 56.8 - 59.5%) did not differ significantly for semen where

glycerol concentration and extender osmolality were gradually decreased over 15 to 30 minutes (Method 2); or by a one-step process with 10 fold dilution (Methods 3 and 4). These results indicate that while post-thaw motility can be influenced by the way in which glycerol is added prior to freezing, both motility and acrosomal integrity can be maintained even after a rapid decrease in glycerol concentration such as that which accompanies insemination or dilution of semen for assessment of motility.

Slavik (1987) reported that prolonged exposure of ram semen (30 to 90 minutes) to a concentration of 10% glycerol had an adverse effect on the acrosomal reaction. This was demonstrated by the stimulation of spermatozoa penetration though zona-free hamster eggs (indicating an acceleration of the acrosome reaction), a change that could result in reduced fertility. Thus, it would seem advisable to seek alternative cryoprotectants for freezing ram semen.

Jones (1965 b) reported that ram semen frozen in ampoules with reconstituted skim milk extenders containing 3, 6 and 9% dimethyl sulfoxide (DMSO) or glycerol or a combination of both had very poor post-thaw motility (2.1, 7.4, and 3.8%, respectively). Post-thaw motility was improved (to 7.6, 6.1, and 8.3%) by adding 2% (W/V) fructose to the respective extenders and shortening the equilibration time before freezing to 10 minutes. *Jones* suggested that DMSO operates mainly via protection of the sperm cell membranes, as indicated by the percentage of cells penetrated by Congo Red stain after thawing. In earlier studies, *Jones (1965 a)* found that during incubation at 5 ^{o}C, DMSO was much more toxic to spermatozoa than

glycerol, so that the protective action of DMSO during freezing could be masked by its toxicity.

It has been shown that various sugars also have cryoprotective properties for bovine spermatozoa *(Nagase et al., 1964 a,b; 1968).* Factors known to influence the degree of cryoprotection promoted by sugars in bovine semen include their molecular weight, the rate of freezing, and the presence of other cryoprotectants *(Nagase et al., 1964 b; Nagase et al., 1968; Gibson & Graham, 1969).* Thus greater survival of spermatozoa was achieved as the molecular weight of the sugar increased *(Nagase et al., 1964 b)* and *Nagase et al. (1964 b)* reported that in the absence of glycerol, the post-thaw survival rate was 26.6% in yolk-glucose, 29.4% in yolk-maltose, 33.4% in yolk-sucrose, 35.5% in yolk-raffinose and 37.2% in yolk-lactose. However, glucose was the only sugar of small molecular weight that had greater protective action than fructose or galactose in the same monosaccharide - " hexose " class.

In the case of ram semen, *Marinov et al.(1980)* stated that sucrose added to an extender protected acrosome integrity and the flagellar apparatus (ie. motility) of frozen ram spermatozoa. However, *Abdelhakeam et al. (1991 b)* reported that adding sugars such as monosaccharides (fructose, glucose, mannose), disaccharides (lactose, maltose, sucrose), trisaccharides (raffinose), or sugar alcohol (erythritol, sorbitol, and inositol) did not significantly improve the survival of chilled ram spermatozoa. Maltose gave more cryoprotection during freezing than the other sugars, and extenders containing it gave the best post-thawing motility (60.4%) when semen was cooled to 5 ^{o}C for 3 h. before the addition of the extender.

Glycerol may be successfully used for freezing ram semen in terms of post-thaw motility or membrane integrity; however, it also has some adverse effects on the fertility of frozen-thawed semen which will be discussed later in section 2.1.

1.3 Freezing processes

1.3.1 Extension rate

It is a common practice to partially extend the semen at 37 ^{o}C immediately after collection. This is followed by slow cooling to 5 ^{o}C *(Graham, 1978; Pickett & Brendtson, 1978)*. For fresh ram semen, *Tewari et al. (1968)* found that the motility of spermatozoa was best maintained at a dilution rate of 1:10 and was worst at 1:1 when stored for 6 to 30 h. at a temperature of 5 to 7 ^{o}C.

To freeze ram semen, *Lightfoot & Salamon (1970 a)* added extender immediately after collection at 37 ^{o}C and found no significant differences in the number of sperm in the uteri of ewes inseminated with either fresh semen or that diluted 1:1. Ram semen frozen at an extension rate of 1:4 had better fertility than semen frozen at 1:2 *(Visser & Salamon, 1974)*. Optimal results in terms of post-thaw survival rate of pelleted semen were obtained when semen was diluted four to sixfold *(Lightfoot & Salamon, 1969)*, whereas *Pontbriand et al. (1989)* found that a dilution rate of 1 in 3 yielded the same results in terms of post-thaw motility and acrosomal integrity at a dilution rate of 1 in 6.

As a refinement to the above results, *Colas (1975)* found that ram semen diluted to a constant concentration gave superior post-thaw motility to that diluted on a volume/volume basis. *Colas (1975)* diluted semen to 900 x 10^6 cells/ml. and found that there was no difference using this concentration as compared to a constant rate of dilution of 1:4 (lambing rate of 34 vs 25%). *Mathur et al. (1991)* compared concentrations of 1,000 and 2,000 x 10^6 spermatozoa/ml. for pelleted semen and found that the former yielded superior results in terms of post-thaw motility.

The volume/volume dilution rate (ie. 1:4; semen:extender) appears to be more convenient than diluting to a constant sperm concentration. This may be the reason for many researchers having chosen the former method over the latter. The number of inseminated spermatozoa can also be adjusted to the required number prior to AI either by adjust volume of frozen-thawed semen or dilute it with the extender in case of volume/volume dilution rate.

1.3.2 Cooling time

Graham (1978) stated that a rapid decrease in semen temperature from 37 ^oC to 5 ^oC caused "cold shock" through damage to sperm as a result of changes in gas solubility, metabolism, fluid viscosity, and chemistry. The major effects of cold shock are a reduction in number of motile spermatozoa, the release of enzymes, ion movement across the cell membrane and lipid loss from spermatozoa *(Weitz & Petzoldt, 1992)*. However, prolonged exposure of semen to higher (ie. 20 °C-37 °C) temperatures maintains greater metabolic activity and induces earlier death of spermatozoa due to the toxicity of the metabolic waste products generated *(Pickett &*

Brendtson, 1978). Ram spermatozoa are subject to cold shock *(Graham, 1978)*; thus, the optimal cooling rate is one that prevents cold shock but is fast enough to minimise metabolism activity and thus maintain healthy, viable sperm cells. *Gomes (1977)* and *Foote (1980)* suggested that an optimal cooling rate is approximately 0.5 ^{o}C/min from 37 to 5 ^{o}C.

Holding sperm prior to dilution permits seminal plasma constituents to bind to the sperm membrane *(Pavelko & Crabo, 1976; Abdelhakeam et al., 1988, 1991 a). Roberts & Haulaban (1961)* reported a higher pregnancy rate in ewes inseminated with undiluted ram semen stored at 5 ^{o}C for 24 h. compared to stored diluted semen. On the other hand, cooling ram spermatozoa to 5 ^{o}C and storing it at this temperature for longer than 24 to 72 h. may have detrimental effect on sperm including a significant increase in the incidence of swollen acrosomes and in the proportion of cells which are vesiculated or vacuolated *(Jones & Martin, 1973)*. They stated that it was very difficult to conclude whether the acrosomal changes were degeneration or whether they represented a phase in the final maturation of the spermatozoa *(Jones, 1973)*. However, if the maturation processes has been accelerated by dilution and chilling, this in itself may affect later fertilising capacity.

Salamon (1970) cooled semen from 30 to 5 ^{o}C in 1.5 h. and stored the semen at this temperature for between 1 and 4 h. before pellet freezing. He found that there were no significant differences in post-thaw motility between the 1 and 4 h. storage periods. However, in the studies of *Abdelhakeam et al. (1991 a,b)*, where glycerol was not used in the extender, the best dilution method was to cool raw semen from 37 to 5

^{o}C and then store for 3 h. before the addition of the extender, followed by maintenance of this temperature for another 1 h. prior to freezing. These benefits were attributed to prolonged exposure to seminal plasma as described above.

As a general conclusion it can be said that ram semen should be cooled immediately after collection with the cooling rate slow enough to prevent cold shock but fast enough to avoid accumulation of excessive levels of metabolic wastes.

1.3.3 Equilibration time

Graham et al. (1957) and *Martin (1963 a,b)* have demonstrated that post-thaw motility and fertility of bovine spermatozoa are both enhanced by extending the interval between adding glycerol and the beginning of freezing (equilibration time).

In the case of ram spermatozoa, *Blackshaw (1960)* found better recovery rates when spermatozoa were equilibrated for more than six hours and *Colas (1975)* reported that post-thaw motility was better after equilibration for 150 rather than 20 min. *Colas (1975)* explained that glycerol penetration into the ram spermatozoa is not as rapid as has been shown in bovine semen. Unfortunately, in his fertility trials, *Colas* examined only the effects of glycerol concentration on CR., and no data on fertility variation due to equilibration time is thus available. In current normal practice as suggested by *Evans & Maxwell (1987)*, glycerol is already included in semen extenders before mixing with semen, and thus the cooling period of 1.5 h. to 2 h. from 37°C to 5°C before freezing may be considered as the "equilibration time" and there is apparently no need to then "incubate" semen for a longer time.

1.3.4 Freezing rate

Smith & Polge (1950) originally suggested that for the optimal recovery of bull spermatozoa frozen in 1 cm^3 ampoules, an average freezing rate of 1 to 2 °C/min from -5 to -15 °C and of 4 to 5 °C/min from -15 to -79 °C is required. This became the " standard " rate of freezing semen *(Graham, 1978; Memon & Ott, 1981)* but later workers have found other rates also work well for ram semen. Thus *Aamdal & Anderson (1968)* successfully used a rate of 67 °C/min from +5 to -196 °C followed by storage of semen in straws in liquid nitrogen. *Salamon (1970)* reported that post-thaw survival rates were similar after freezing ram semen by either cooling the metal plate with the semen droplets on it from +5 to -140 °C in 11 min, or by pelleting on either dry ice (-79 °C) or on a plate at -140 °C. *Entwistle & Martin (1972)* compared two rates of freezing ram semen in ampoules, a " rapid " with an average rate from 0 to -40 °C at 2 °C/min (liquid nitrogen vapour) or a " slow " in 1 °C/min (dry ice) and found that " rapid " freezing gave lower survival of spermatozoa than the " slow " rate (33% vs 42% of post-thaw motility respectively). *Watson & Martin (1972)* used a freezing rate of 3 °C/min from 5 to -40 °C followed by rapid cooling (plunge into liquid nitrogen at -196 °C) and found bull spermatozoa to be less severely damaged than ram spermatozoa in terms of acrosome integrity during the freeze/thaw processes.

Storage in pellets and straws has allowed semen to be frozen and thawed much faster than in the older ampoules, primarily due to a larger surface to volume ratio *(Graham, 1978; Memon & Ott, 1981)*. The "standard" method for pellet freezing

is to drop "extended" semen onto dry ice *(Nagase & Graham, 1964)*, while straws are suspended on a rack in liquid nitrogen vapour following the method of *Jondet (1964)*.

For the initial stage of freezing ram semen, before entry into liquid nitrogen, *Salamon (1970)* found that freezing pellets at -79 ^{o}C or -120 ^{o}C yielded similar results in terms of post-thaw motility. *Colas (1975)* froze ram semen in straws and reported that initial temperatures between -75 and -125 ^{o}C gave similar recovery rates after thawing but if the temperature was higher than -75 ^{o}C, the recovery rate was lower.

Fiser & Fairfull (1986) studied the effects of cooling velocity and the osmolality of skim milk extenders on the freezing of ram spermatozoa and found the cryosurvival percentage ([freeze-thaw motility percentage x 100] / percentage of motile spermatozoa before freezing) was best within the cooling velocity range from 10 to 100 ^{o}C/min. They suggested that any slow freezing injury is directly related to the " solution effect " rather than to intra-cellular ice formation because the cryosurvival percentage increased continually with increasing cooling velocity in the absence of glycerol and also increased gradually with increasing glycerol concentration. These results agreed with the earlier work of *Mazur et al. (1972)* who observed that slowly cooled cells are killed after relatively long exposure to a high concentration of solutes, to dehydration, to changes in pH, and to the precipitation of solutes. However, *Asahina et al. (1970)*, who studied ice formation in the protoplasm of bacteria during freezing, revealed that an "extremely rapid" cooling rate (100 ^{o}C/min) increased the survival of cells because it produced very small, relatively

innocuous ice crystals (ice formation by vitrification rather than crystallisation). A cooling velocity of the magnitude of 100 °C/min may thus be suitable and sufficient to reduce injury due to the "solution-effect" and intracellular ice formation.

1.3.5 Packaging and storage

Spermatozoa are most commonly stored in liquid nitrogen (at -196 ^{o}C) and packaged in one of three ways *(Graham, 1978; Foote, 1980)*;

a) In glass ampoules, normally containing 0.5 to 1.2 ml. of semen.

b) In polyvinyl chloride straws with a volume of 0.25 to 0.5 ml..

c) In pellets containing about 0.1 - 0.2 ml..

Specifically, ram spermatozoa have been packaged in all manners, glass ampoules *(Entwistle & Martin, 1972; Watson & Martin, 1972)*, pellets *(Nagase & Graham, 1964; Lightfoot & Salamon, 1970 a,b)*, and straws *(Colas, 1975; Abdelhakeam et al., 1991 a)*. Though good results can be obtained with any of these "packages", and the choice between them is largely influenced by personal preference *(Brendtson & Pickett, 1978; Graham, 1978)*, the pellet has been the most "successful" means of storage of ram semen because the technique is easiest and least expensive *(Graham, 1978; Gomes, 1977; Foote, 1980)*. Table 1.2 summarises the advantages and disadvantages of each of the above packages *(Modified from Brendtson & Pickett, 1978; Graham, 1978 and Evans & Maxwell, 1987)*.

Freezing of ram spermatozoa by the pellet method has given acceptable post-thaw survival *(Salamon & Lightfoot, 1970 a,b). Salamon et al. (1985)* reported that ram spermatozoa frozen and stored at -196 ^{o}C in either pellets or straws retained good fertility after storage for at least up to 16 years.

Table 1.2 Advantages & Disadvantages of the frozen semen storage package.

Advantage	Disadvantage
Ampoules	
• Glass ampoules are convenient for flame sealing and are easily labelled.	• The thickness of the glass makes very high cooling or warming rates difficult. • Because of their shape, ampoules are not efficient in terms of storage space.
Straws	
• The use of straws is widespread in the cattle breeding industry. • They can be readily labelled for identification and sealed to exclude contaminants. • If necessary, they can be used with high cooling and warming rates (during freezing and thawing) • More efficient in the use of storage space than ampoules (an important factor for long term storage). • Waste little extended semen because an insemination can be made directly from them.	• They need relatively complicated techniques and equipment for filling, sealing and insemination.

Pellets	
• Easy technique, pellets are formed by placing a drop of extended semen directly onto the surface of dry ice or on a pitted metal tray positioned above the surface of liquid nitrogen.	• Positive identification of each unit is more difficult than with ampoules or straws. The frozen spermatozoa must be placed in suitable containers after freezing.
• The cooling rate is very consistent because the surface of the medium is always at the same temperature and the volume per pellet is relatively small (0.1 - 0.2 ml.).	• Food colouring materials can be added to the pellets for identification, but the colours are not readily recognisable.
	• It is very difficult to vary the cooling rate of pellets, although some cooling rate variation can be achieved by varying the pellet volume.
	• They can become contaminated with organisms present in liquid nitrogen.
	• They can "pick up" foreign spermatozoa (ie. broken pieces of other pellets) in the liquid nitrogen during storage.
	• Organisms from contaminated pellets are shed into liquid nitrogen and may contaminated pellets in different canisters and at later times.
	• Spermatozoa become attached to forceps during handling of pellets.

1.3.6 Thawing

Thawing frozen ram semen in straws at temperatures above body temperature (37 °C) generally improves spermatozoal viability compared to lower thawing temperatures *(Fiser et al., 1986).* Thus, *Aamdal & Anderson (1968)* thawed semen at 75 °C for 12 seconds and at 35 °C for 30 seconds and found that the higher temperature gave considerably better results as assessed by vital (live:dead) staining.

Graham et al. (1978) also found that thawing at 65 ^{o}C was better than at 40 ^{o}C in terms of post-thaw motility and *Graham (1978)* stated that as a general rule, a fast thawing rate is superior to a slow rate. Sperm cells thawed at a greater rate are theoretically exposed to the concentrated solutes and the cryoprotectants for a shorter interval *(Robbins et al., 1976)*.

In contrast, *Pontbriand et al. (1989)* thawed ram semen frozen in straws in a water bath at 60 ^{o}C for 8 seconds or at 37^{o} ^{o}C for 20 seconds and found no significant differences in terms of spermatozoal motility, progressive status rating and acrosomal integrity.

Lightfoot & Salamon (1969) first reported that pellets could be thawed successfully in a warmed, dry test tube without a thawing solution . In the case where a thawing medium is used, thawing of pellets presents dual problems. Since pellets are generally thawed and diluted simultaneously, both the medium (thawing solution) and the rate of thawing are important factors *(Gomes, 1977)*. *Pontbriand et al. (1989)* reported that the dry tube technique yielded a better post-thaw motility than thawing in solution at 37 ^{o}C when assessment took place after subsequent incubation both for 3 and 6 h.

Srivastava & Kalba (1985) found that collective thawing of pellets (0.2 ml.) of frozen ram semen gave superior post-thaw motility compared with thawing a single pellet. The best result was obtained when 3 or 4 pellets were thawed together (58% live), whereas pellets thawed singly gave only 26% live sperm.

Once thawed, sperm cells do not remain viable for as long as cells that have never been frozen *(Graham, 1978; Ott & Memon, 1981)* and regardless of the method of freezing or thawing, it is generally agreed that semen should be used as soon as possible after thawing.

1.4 Conclusion

Ram spermatozoa can be frozen in extenders specially designed for freezing semen. These extenders should comprise at least buffers to reduce the effects of changes in pH, an anti freeze protein (ie: casein in milk or lipoprotein in egg yolk), some kind of sugar as an energy source and with or without glycerol as a cryoprotectant. The dilution rate employed depends on the number of active spermatozoa initially present, but can be as high as 1:4 (semen:extender). The equilibration time for semen to cool from 37°C - 30°C to 5°C should be at least one hour or cold shock may be experienced. Diluted semen can most easily be frozen in pellet or straw form. Frozen pellets of semen are best thawed by the 'dry tube' technique.

There are still a number of problems involved with the freezing of ram semen and in the insemination techniques used, both of which may contribute to low fertility. These issues will be addressed in Chapter 2.

CHAPTER 2

Factors affecting the fertility of deep frozen ram semen

2.1 Introduction

This chapter will discuss the fertility problems that have been associated with the use of deep-frozen ram semen. Of the several factors that have been suggested to influence the fertility of ewes after AI with frozen ram semen, sperm transport in the female genital tract, route of administration, number of spermatozoa and time of insemination have been quite extensively researched. These factors are not the main subject of experimentation in this thesis, but an understanding of them is necessary for a full appreciation of the consequences of deep freezing ram semen, and for the purposes of experimental design. A broad overview of them is thus presented in the last four sub-sections of the review which follows.

Other factors possibly influencing the fertility of frozen-thawed ram semen have received little experimental attention, but two of these, namely acrosome damage caused by glycerol and between-ram variation in response to

freezing/thawing, have been chosen for study here and will be reviewed in as much detail as possible.

2.2 Effects of glycerol on the acrosomal membrane

Glycerol is generally regarded as a successful "cryoprotectant" for use in extenders for freezing spermatozoa *(Graham, 1978; Foote, 1980; Evans & Maxwell, 1987)*. However, there are a number of reports which indicate that the presence of glycerol lowers the quality of unfrozen as well as frozen semen in a number of species including pigs *(Bower et al., 1973, Crabo et al., 1970)*, fish, birds *(Sexton, 1973; Graham et al., 1984; Lake & Ravie, 1984)* and humans *(Tulandi & McInnes, 1984)*.

Ram spermatozoa are also affected by the presence of glycerol in the freezing extender, with good evidence available that glycerol lowers fertilising capacity *(Slavik, 1987; Abdelhakeam et al., 1991 a)*.

The study of *Slavik (1987)* clearly revealed that glycerol adversely effected the acrosomal membrane of ram spermatozoa. By determining the ability of ram spermatozoa to penetrate through zona-free hamster eggs, *Slavik* found that the presence of glycerol in the extender accelerated the induction of the acrosome reaction. With no glycerol the percentage of eggs penetrated by spermatozoa was 52.6% and the average number of spermatozoa per egg was 1.57. This was in contrast to 58.0% and 1.62 for 5% glycerol and 63.4% and 2.02 for 10% glycerol. *Slavik (1987)* also found that the time of incubation with glycerol had a significant effect on the penetration ability of spermatozoa. Incubation for 30 minutes resulted in the highest penetration activity, whereas longer periods of incubation gradually decreased

the activity. In contrast, untreated spermatozoa achieved maximum activity after 6 h. of incubation and were still active after 10 h. of incubation. For glycerol-treated spermatozoa penetration occurred without incubation, and reached a maximum in only 4h, whereas, after incubation for 10 h. spermatozoa showed very low penetration activity. The mechanism whereby this occurred was suggested to be associated with the binding of fatty acids (possibly on the acrosome membrane) to glycerol.

Aonuma et al. (1982) and *Juetten & Bavister (1983)* demonstrated that free fatty acids function as "decapacitators" and after their removal (ie. by binding to albumin), the sperm membrane is destabilised and the acrosome reaction induced. Thus, *Slavik (1987)* assumed that when free fatty acids bind to glycerol the equilibrium of the reaction 'membrane phospholipid → lysophospholipids + free fatty acids' is destroyed. These lysophospholipids, several of which function as factors stimulating the acrosome reaction in the guinea-pig *(Fleming & Yanagimachi, 1981 cited by Slavik, 1987)*, could possibly induce the acrosome reaction in ram spermatozoa as well.

Slavik (1987) also concluded that glycerol may reduce the fertility outcome of vaginal or cervical insemination compared with intrauterine insemination because of the longer passage from the cervix to the fertilisation site. Thus, during this extended transit time, spermatozoa may "use up" the acrosomal enzymes necessary for fertilisation.

Evidence that glycerol depresses the fertility of frozen ram spermatozoa was reported by *Abdelhakeam et al. (1991 a)*, who found that lambing percentages to

cervical AI declined progressively when using fresh semen (maintained at 5 °C for 6 h.) diluted 1:4 (semen:extender) in either a glycerol-free or a 3% glycerol extender and also in frozen ram semen with 3% glycerol extender. The lambing rate from non-glycerolated semen was 83%, while the figure for similar semen treated with 3% glycerol was 41% and for glycerolated frozen semen only 33% ($p < 0.05$). These results were not evaluated via large scale fertility trials but they do support the finding of *Slavik (1987)*, *Salamon & Lightfoot (1967)* and *Armstrong & Evans (1984)*. Thus, it could be concluded that the effect of glycerol on the ram acrosomal membrane is one of the important factors affecting fertility.

2.3 The variation between individual rams

Generally, researchers who have assessed the fertility of deep frozen "ram semen" have in fact used the pooled semen from a group of rams. The major reason for this appears to have been a desire to avoid possible variation between rams which in turn may cause difficulties in the interpretation of main effects. However, such designs have masked individual variability, and there are very few reports which have directly investigated the result of freezing semen from individual rams.

In one such experiment, *Maxwell (1980)* found that the lambing rate after insemination of ewes with frozen semen from Texel rams was lower than for Suffolk rams (29.9 vs 48.9%; $p < 0.005$). He also reported that lambing rate using frozen "pooled" ejaculates was greater than for frozen semen from individual rams (36.3 vs 26.7%; $p < 0.05$). *Maxwell (1986 b)* reported that after using frozen-thawed semen from six individuals rams, the lambing rate and the number of lambs born varied

significantly between individuals with a range from 39.1% to 50.0% in number of ewes lambing and from 52.2% to 91.7% in number of lambs born. *Hunton (1987)* found that overall fertility results after AI using frozen-thawed semen (either pellet or straw frozen) collected individually from three rams were similar between two rams (47.3% vs 46.3%) but significantly different in the third (56.3%, $p < 0.05$). *Hunton* also reported that thawed semen from the third ram had a better survival rate after freezing in straws than the other two but he did not show the actual figures. *Butler & Maxwell (1988)* reported fertility data of frozen stored semen from 14 individual rams after intrauterine insemination on various farms in Western Australia and found that there was a significant 'sire effect' ($p < 0.05$) on all three categories namely; lambs marked per ewe inseminated (LM), lambs born per ewe inseminated (LB) and ewes lambing per ewe inseminated (EL). The mean of LM, LB and EL (averaged from a number of reports per ram) varied from 41 to 86% (overall mean: 65%), 52 to 96% (overall mean: 67%) and 46 to 77% (overall mean: 57%) respectively.

These data suggest that there are differences between rams in the fertilising capacity of frozen-thawed semen and *Maxwell (1986 b)* suggested that to maintain fertility as high as possible, insemination dosage should be adjusted by increasing the number of inseminated spermatozoa when it is impossible to pool ejaculates from different rams before freezing. Because of the limited information available at present, it is not yet possible to identify the factors involved in individual ram variation in frozen-thawed semen quality.

2.4 Sperm transport in the female tract and the importance of semen insemination routes

During natural mating in sheep, semen is deposited in the anterior vagina and the volume of the ejaculate is relatively small (0.25-1.5 ml.) *(Lightfoot & Restall, 1971)*. Transportation of spermatozoa from the cervix to the site of fertilisation (the ampulla of the oviduct) is a straightforward process consisting of the gradual progression of motile spermatozoa aided by smooth muscle contraction and ciliary action in the oviduct (Harper, *1982)*. The cervix is the first major barrier to spermatozoa before entry into the uterus and a relatively small proportion of spermatozoa will normally pass though the cervical canal *(Lightfoot & Salamon, 1970 a)*, which is believed to act as the main reservoir for spermatozoa *(Robinson, 1973)*. After insemination, spermatozoa will move continuously to the oviduct for approximately 8 h. where fertilisation occurs in the ampullae *(Thibault, 1973)*. It has been suggested that the establishment of an adequate cervical reservoir of spermatozoa is a prime requirement for fertilisation following cervical insemination in sheep *(Lightfoot & Salamon, 1970 a; Lightfoot & Restall, 1971)*. Other regions of spermatozoal storage exist in the uterotubal junction and in the folds of the lower isthmus *(Thibault, 1973)*. The later region was identified to be a significant pre-ovatory barrier to upcoming spermatozoa and to act as the main spermatozoa reservoir for fertilisation *(Hunter, 1985; Cummins, 1982)*.

Three methods for the deposition of frozen-thawed ram semen or AI have been developed: vaginal, cervical, and intrauterine insemination *(Evans & Maxwell,*

1987), and these have been shown to significantly affect the fertility outcome *(Gustafsson, 1978; Evans & Maxwell, 1987).*

2.4.1 Vaginal insemination

Vaginal insemination of sheep is generally known in Australia as "shot in the dark" or SID *(*first coined by *Fairnie & Wales, 1982)*. SID requires little skill, less handling and stress to the ewes and the time required for insemination is greatly reduced relative to cervical or intrauterine insemination *(Maxwell & Hewitt, 1986)*, and is most applicable to fresh semen. SID is generally regarded as a non effective insemination technique for frozen ram semen *(Evans & Maxwell, 1987; Evans, 1988)*, but the results are affected by insemination dose of spermatozoa. *Maxwell & Hewitt (1986)* reported pregnancy rates of 9% vs 30% for frozen-thawed and fresh diluted ram semen respectively at an similar insemination dosage of approximately 1.0 x 10^6 sperm/ml. (0.1 ml. inseminated volume). Fertility using the vaginal route did not improve until the insemination dosage increased to 6 times (0.1 vs 0.6 ml.) *(Maxwell & Hewitt, 1986)*. There have also been other reports suggesting that conception rates after vaginal AI are lower than after cervical insemination, even with a relatively high number of ram spermatozoa per insemination dose *(Rival et al., 1984). Rival et al.* used insemination dosages (0.1 ml.) containing 150 or 300 x 10^6 active spermatozoa and obtained a non-return rate 15% lower than that of the cervical route, regardless of the number of sperm used ($p < 0.05$).

2.4.2 Cervical insemination

Because of the reduced sperm dose needed, cervical insemination allows for more economical use of fresh semen; however, fertilisation using frozen-thawed ram semen by this route is still low *(Evans, 1988)* as conception rate appeared to be at least 20% lower than that of fresh semen *(Gustafsson, 1978)*. *Maxwell & Hewitt (1986)* reported that after cervical AI with frozen-thawed ram semen at 1,000 x 10^6 cells/ml. concentration and insemination volumes of either 0.1 or 0.6 ml., fertilisation rate always remained at a low 8%.

Anatomically, the cervix of the ewe does not permit easy penetration of insemination devices and full penetration can be achieved in only approximately 50% of ewes *(Gustufsson, 1978)*. Special forceps together with uniquely designed insemination guns have been developed and have been reported to be effective in terms of conception rate when used with frozen-thawed ram semen *(Fukui & Roberts, 1979)*. *Fukui & Roberts* used a specially designed insemination pipette fitted with a ball-tipped needle, and obtained non-significantly different lambing rates between this method with frozen-thawed semen and cervical AI with fresh semen (50.9% vs 64.0%, respectively). However, the tortuous structural nature of the cervical lumen prevents these methods from being used in practice in large scale field insemination programs.

There have been some reports relating the use of hormones, such as oxytocin, relaxin, and prostaglandins (PGs) which have effects on the ewes' reproductive tract, to either dilate the cervix (and thus facilitate cervical insemination) or to improve

sperm transport. The smooth muscle of the uterus and cervix of ewes can be stimulated by oxytocin in vitro *(Edqvist et al., 1975)*, but when used in an attempt to improve spermatozoal transportation, neither 0.5 nor 5.0 iu of oxytocin (by i.m. injection) was stimulatory, and the 5.0 iu dose actually depressed lambing rate (20.8 vs 37.5% of 0.5 iu; $p< 0.01$) *(Salamon & Lightfoot, 1970)*.

Relaxin causes relaxation of the cervix *(Rosenberger, 1979)* but, at dosages of 100, 500 or 2,500 guinea-pig units, this hormone did not significantly improve the possibility of cervical insemination (as assessed by attempts to pass a catheter into the cervix; *Salamon & Lightfoot, 1970)*.

The smooth muscle of the female genital tract is responsive to either endogenous or exogenous PGs *(Hunter, 1980)*, thus there have been some attempts to use these hormones to stimulate the genital tract in order to improve sperm transport. Adding $PGF_{2\alpha}$ to semen samples before freezing did improve spermatozoa transport from the posterior cervix to the oviduct *(Gustufsson et al., 1977)*. They reported that the total numbers of sperm in the oviduct 16 h. after cervical AI of frozen-thawed ram semen were 3.8, 13.8 and 18.4 x 10^3 sperm for control (no PGs), PGE_1 and $PGF_{2\alpha}$ respectively ($p <0.05$). Others have shown that the addition of $PGF_{2\alpha}$ and PGE to diluted ram semen in amounts equivalent to the PG content of a normal ejaculate improved fertility by around 15% *(Dimov & Georgiev, 1977)*. Thus, it is possible that the reduction of PG concentration by semen dilution before freezing can be compensated for by supplementation with exogenous PG in order to maintain acceptable sperm transport *(Gustufsson, 1978)*.

While some authors have reported that there are no adverse effects on the metabolism or motility of spermatozoa after the addition of various PGs to ram semen *(Edqvist et al., 1975; Marley et al., 1976)*, *Hawk (1973)* suggested that the induction of luteolysis by an intramuscular injection of $PGF_{2\alpha}$ may also result in a reduction in the efficiency of spermatozoa transport to the oviduct at the following oestrus as the number of sperm found in the oviducts at 24 h. after insemination was 5,100 compared with 20,700 for a control. Unfortunately, there was no explanation of these results.

At present the structural barrier of the cervix cannot be consistently removed by hormonal treatments and further studies are necessary before the cervical route of insemination can be routinely used successfully for frozen-thawed ram semen.

2.4.3 Intrauterine insemination

The most significant achievement in improving the fertility of frozen semen is laparoscopic intrauterine insemination, a technique first reported by *Killen & Caffery (1982)*. The reduction in the motility and efficiency of transportation of frozen ram semen can be overcome by intrauterine insemination which places the spermatozoa closer to the oviduct.

Maxwell & Hewitt (1986) reported a considerably higher pregnancy rate after AI using frozen-thawed ram semen by the laparoscopic method compared with SID and cervical insemination. When using a 0.1 ml. insemination dose of 1000 x 10^6 cells/ml. concentration, they obtained 9%, 8% and 25% pregnancy rates for SID, vaginal and intrauterine insemination respectively. After increasing the dosage to 0.6

ml., the pregnancy rates were 19%, 8% and 31% respectively. Thus, regardless of the dosage, SID was inferior to intrauterine insemination. They also concluded that the intrauterine insemination of frozen-thawed ram semen is as effective as the cervical insemination of fresh semen (55.6 vs 60.0 % pregnant respectively) with 0.1 ml. of semen inseminated at the same sperm concentration.

The exact insemination location in the uterus is also important to fertility, since *Maxwell (1986 b)* reported that insemination into the middle of the uterine horn was superior to that into either the tip or the region adjacent to the bifurcation. Furthermore, this middle part of the uterus is easy to handle and visualise by laparoscopic devices *(Evans, 1988)*, so it is the obvious choice for the inseminator. However, *Jabbour & Evans (1991)* found that when frozen ram semen was injected directly into the oviduct, the number of fertilised ova was almost four times greater than that obtained from "middle" uterine horn injection for the same sperm number.

Some workers have reported that insemination with a volume of 0.02 ml. of ram semen per horn (60 x 10^6 total sperm) into both uterine horns was superior to injection into only one horn with 0.04 ml. of semen (also 60 x 10^6 total sperm), in terms of fertility (50% vs 71%, $p < 0.05$) *(Killeen et al. 1982)*, while *Evans et al. (1984)* found that insemination into only one (80 x 10^6 motile sperm) or both horns (40 x 10^6 motile sperm for each horn) gave similar fertilisation rates (40% vs 38%) indicating that spermatozoa are quite able to travel between the uterine horns.

Pontbriand et al. (1989) suggested that the poor fertility from using frozen-thawed ram semen may result, not only from a reduction in sperm motility, but also

from adverse changes in ultrastructural integrity. These comprehensive studies revealed that the viability of spermatozoa after thawing depended on both freezing method and the cryoprotectant. The ultrastructural damage, including morphological changes to the acrosome, resulted mostly from the freezing method, which in turn was markedly influenced by the cryoprotective properties of the extender used. BF5F extender, which was included with the surfactant mixture of sodium and triethanolamine lauryl sulfate, maintained the post-thaw motility of spermatozoa better than TEST extender when incubated for 6 h. However, the most remarkable change which influenced fertility was in acrosome integrity. *Pontbriand et al. (1989)* concluded that a suitable freezing procedure should maintain both the motility and structural integrity of post-thawing ram spermatozoa in order to achieve acceptable fertility.

Since the introduction of laparoscopic AI, there have been considerable efforts to improve and refine the method and equipment used *(Evans, 1988)*. However, other factors such as time of insemination and insemination dosage also play an important role in achieving acceptable levels of fertility, regardless of AI method *(Evans, 1988)*.

2.5 Number of spermatozoa per insemination dose

The number of ram spermatozoa per insemination dose is very important in determining the economical and beneficial aspects of using deep frozen semen. The number of spermatozoa required per insemination dose to achieve acceptable fertility has been shown to depend on the method of insemination and type of stored semen

(Lightfoot & Salamon, 1970 a,b; Armstrong & Evans, 1984; Tervit et al., 1984; Evans & Maxwell; 1987).

Cervical AI with fresh semen will normally result in conception rates of around 50% and also with the use of a large volume and a high concentration of semen success can also be high for frozen-thawed semen *(Colas, 1975)*. However, the high concentration required (400 x 10^6 sperm) makes such a technique uneconomic as it allows only 4 or 5 doses per normal ejaculate.

Table 2.1 Millions of active ram spermatozoa required per "safe" insemination dosage for particular insemination methods and types of semen storage *(Evans & Maxwell, 1987)*.

Insemination route	Type of semen		
	Fresh	Liquid storage	Frozen storage
Vaginal	300	ineffective *	ineffective *
Cervical	100	150	180
Intrauterine via laparoscopy	20	20	20

* not recommended

The viability of frozen-thawed ram spermatozoa was found to be lower than that of fresh, chilled semen *(Lightfoot & Salamon, 1970 a,b; Maxwell et al., 1984; Maxwell & Hewitt, 1986; Evans & Maxwell, 1987)*. The site of semen deposition also affects fertility significantly (discussed in section 2.2). To overcome this problem the number of spermatozoa in a given sample should be adjusted in order to give an acceptable fertility rate *(Armstrong & Evans, 1984; Evans & Maxwell, 1987)*. However, the fertility of frozen-thawed semen of individual rams may not always be

improved by increasing the insemination dose *(Evans, 1988)* and the reasons for this are unknown.

The number of ram spermatozoa needed for good fertilisation (95%) using natural mating seems to be only about 60 x 10^6 cells *(Fulkerson et al., 1982)*. One of the advantages of laparoscopic intrauterine insemination is that it needs only a small number of spermatozoa to achieve fertilisation. While successful AI of sheep using fresh semen or liquid storage of semen can be achieved by vaginal or cervical insemination, laparoscopic intrauterine insemination seems to be the obvious choice when using deep frozen ram semen, as acceptable fertility can be achieved from a spermatozoa number as low as 20 x 10^6 cells *(Evans & Maxwell, 1987)*.

Maxwell & Hewitt (1986) reported pregnancy rates at 50 days after intrauterine AI of 65% and 56% after using 600 x 10^6 and 100 x 10^6 spermatozoa per inseminate respectively. The latter dose was similar to that previously reported by *Maxwell et al. (1984)*. The lowest dose for "successful" intrauterine AI was 0.5 x 10^6 active cells, which resulted in only 29% lambing *(Maxwell, 1986 b)*. However, the lambing rate was improved to 56% when the insemination dose was increased to 25 x 10^6 cells.

The wide variation in fertility even when using a similar insemination dose for intrauterine insemination may be due to factors such as timing of the insemination as well as unidentified "ewes factors" which also influence fertility *(Evans, 1988)*.

2.6 Insemination timing

The appropriate timing of AI relative to oestrus is an important factor influencing fertility regardless of type of semen or the insemination route *(Evans & Maxwell, 1987; Evans, 1988)*. Normally, laparoscopic AI is used at a "fixed time" relative to the synchronisation of oestrus or superovulation. This ensures that all ewes in the given group will be inseminated within a short period of time. As the practical use of deep frozen semen is at present almost entirely dependent on intrauterine AI., the timing of insemination using frozen-thawed ram semen is of prime practical importance.

There have been a limited number studies which have examined the optimum insemination timing in association with the synchronisation method and/or the ovulation time. *Evans et al. (1986)* found that the time of insemination of superovulated ewes was much more important for deep frozen semen than for fresh semen. *Maxwell (1986 a)* reported that the optimum intrauterine insemination time, after synchronisation with a progestragen sponge and 400 iu of PMSG (i.m.) was between 60 and 72 h., whereas the median time of ovulation was between 56 and 60 h., indicating that lambing rate was higher when AI was performed after ovulation. *Maxwell et al. (1984)* also noted that the mortality rate of embryos after intrauterine AI was increased when AI took place relatively early in relation to ovulation. This report is in contrast with that of *Hunter et al. (1980)*, who reported that in naturally mated ewes, spermatozoa will be found in the female genital tract before ovulation. Other workers have also obtained acceptable fertility after performing AI immediately before the expected time of ovulation *(Killeen et al., 1982; Davis et al., 1984)*.

Jabbour & Evans (1991) found that successful fertilisation and development of ova in superovulated ewes are highly dependent on the insemination timing in relation to progestragen sponge withdrawal, especially with frozen-thawed ram semen. They reported the optimum timing for the intrauterine AI of fresh semen was 64 h. after sponge withdrawal, a practice which resulted in fertilisation rates of at least 80%. For frozen semen the corresponding number of fertilised ova was less than 20%. However, if semen was injected directly into the oviduct 94-95% fertilisation was obtained using either fresh or frozen semen. These results clearly indicated that the transportation of frozen ram semen into or within the oviduct is impaired *(Jabour & Evans, 1991).*

2.7 Conclusion

Although, intrauterine insemination seems to be the best technique presently available to improve the fertility of frozen-thawed ram semen, it requires highly trained technicians and expensive laparoscopic equipment.

Those factors which influence the fertility of frozen-thawed ram semen, seem to be very complex and difficult to isolate. The reduction of post-thawing semen quality in terms of the viability, velocity and ultrastructural integrity of spermatozoa in conjunction with the difficulty to perform efficient intrauterine insemination, makes it very difficult at present to improve fertility following the use of deep frozen ram semen. One possible way is to use a cryoprotectant other than glycerol in conjunction with improvement in transcervical insemination. If successful, such

techniques could greatly increase the acceptance and use of deep frozen semen for AI in the sheep industry.

In the experimental chapters which follow, the amidase assay is validated as a means of quantifying damage to the acrosomes of ram spermatozoa, and the technique developed is used to assess the consequences of cryoprotection by egg yolk and its components in the absence of glycerol. The between-ram variation in post-thawing semen quality (motility and amidase levels) will also be investigated.

Part B: Experimental

injection of 60 μg oestradiol-benzoate was given to ensure the maintenance of oestrous behaviour for two days *(Land et al., 1972)*.

At semen collection a teaser ewe was restrained by the head in a service crate . The rams were put into a catching pen, where they were able to see the ewe. After a few minutes individual rams were placed with the teaser ewe and semen was collected by means of an AV, which was maintained at 40 °C . The collections were made on the basis of two collections per ram per day with an interval of at least 5 min between them. Consecutive ejaculates from each ram were pooled. In each experiment, the collections began at 0900 h. on each of two days per week (Tuesday and Thursday) for 3 weeks.

3.3 Semen evaluation techniques

Initial evaluation of each pooled ejaculate was carried out within a few minutes of collection. Ejaculate volume, mass motility and total number of sperm per pooled ejaculate were recorded and all of the ejaculates were then mixed (unless otherwise stated in the relevant chapter) and maintained at 37 °C. After mixing with the extenders, the samples were assessed for progressive motility and acrosomal integrity before freezing. Similar assessments were also used post-thawing.

3.3.1 Enumeration

The volume of the two consecutive ejaculates was measured to the nearest 0.1 ml. in calibrated collection vials. After the volumes were recorded, semen motility (mass activity) was scored immediately. One drop of semen was placed on a warm slide which was maintained at 37 °C by means of a warm - stage, and motility was

scored on the basis of wave - like motion observed under a phase contrast microscope (100 X). The motility scoring index system used has been described by *Evans and Maxwell (1987)* and was as follows:

Score	Class	Description
5	Very good	Dense, very rapidly moving waves. Individual sperm cells cannot be observed. 90% or more of the spermatozoa are active.
4	Good	Vigorous movement, but the waves and eddies are not so rapid as for score 5. About 70-85% of sperm cells are active.
3	Fair	Only small, slow moving waves. Individual spermatozoa can be observed. 45-65% of sperm cells are active.
2	Poor	No waves are formed, but some movement of spermatozoa is visible. Only 20-40% of sperm cells are active, and their motility is poor.
1	Very poor	Very few spermatozoa (about 10%) show any signs of life, with weak movement only.
0	Dead	All spermatozoa are motionless.

Sperm density or the concentration of each ejaculate was obtained by means of a colorimeter (Corning model 252) as described by *Evans & Maxwell (1987).* However, saline *(Evans & Maxwell, 1987)* extender was replaced by one containing 40 g of chloramine-T (ICN Biochemicals # 150115) and 30 g of trisodium citrate (Univar # 467) in 1 litre of distilled water, at the dilution rate of 1:100 (Semen 0.1 ml. : Extender 10 ml.). Colorimeter readings on the diluted sample from each ejaculate

were taken after all collections had been completed. All sperm density measurements are reported as the number of sperm $\times 10^9$/ ml..

3.3.2 Sperm motility assessment

Percentage of progressive movement of spermatozoa was measured by the method described by *Evans & Maxwell (1987).* Three 10 µl drops of diluted semen (Section 3.3.1) were dropped onto a warm (37 °C) microscopic slide, covered by a 22 x 22 mm coverslip, and then examined under 150 x magnification. To improve the visibility of spermatozoa, and to provide a storage facility, the microscope was connected to a VGA monitor and videotape recorder. Two fields of each drop were examined from the centre of each half area of the cover slip (Figure 3.1) and video recorded. Thus, each drop was accessed twice with a total of six records per sample. These records were averaged and used for the record in the experiment.

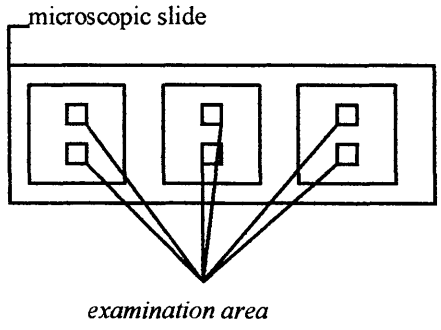

Figure 3.1 The areas (2 under each cover slip) used for motility assesment.

3.3.3 Acrosomal integrity assessment

" Sperm-bound amidase activity " has been used throughout the current experiments as an index of acrosome damage during freezing and thawing. Amidase activity is measured by hydrolysis of the amide bond of BAPNA (N- Benzol - DL - arginine -p - nitroanilide) by acrosin enzyme which is a chromogenic substrate for trypsin - like proteases (restricted to the acrosome in sperm). The optical density index (OD) has been shown to be correlated with acrosome damage in both bull *(Froman et al., 1987)* and ram semen *(Edward et al., 1992).*

Amidase activity was determined as follows:

1. 40 µl of diluted semen was added to 3 ml. of Krebs phosphate solution containing 1.68 mM BAPNA (Sigma chemical # B 4875) in a 3.5 ml. centrifuge tube and thoroughly mixed.

2. A sub sample of 400 µl was taken at time 0 to act as a blank and the remainder was incubated at 30 °C for 30 min (reaction).

3. 2 ml. of 25 mM Benzamidine hydrochloride (Sigma chemical # B 6506)was immediately added to the blank (time 0) to stop BAPNA hydrolysis by acrosin; and the same volume added to the reaction tube after 30 min.

4. The reaction tube was centrifuged for 15 minutes at 500 g, and the absorbance of the supernatant was read against blank at 410 nm (Beckman spectrophotometer model DU® 64).

Preliminary trials indicated variability in the results from the above method (that of *Edward et al., 1992*), variability which was found to result from resuspension (after centrifugation) of the sediment which was interfering with the absorbency reading. To avoid this, an additional step, involving filtration of the supernatant (Whatman qualitative filter paper grade # 6) was added before the final absorbency reading was taken.

The estimated number of intact acrosomes is reported as a percentage of the total number of acrosomes in the sample.

3.4 Semen extenders

Semen samples were diluted at a rate of 1:4 (semen:extender) for the freezing process throughout this thesis. Two types of extender were used: Tris - citrate extender *(Evans & Maxwell, 1987)* and TEST extender (Tes-tris) *(Abdelhakeam et al., 1992 a,b).*

3.4.1 Tris - citrate extender

This buffer, which has been used widely in many studies, was used in experiments 1 and 2 (Chapters 4) as a control. The composition was as follows *(Evans & Maxwell, 1987)*;

Tris (hydroxy methyl amino methane)	3.634 g
(Aldrich # 15456-3)	
Citric acid (monohydrate)	1.990 g
(Sigma chemical # C 7129)	

Glucose (D - glucose)	0.500 g
(Sigma chemical # G - 5146)	
Egg yolk	15 ml.
Glycerol	5 ml.
Penicillin	100,000 iu
(Sigma chemical # P - 3539)	
Streptomycin	100 mgr
(Sigma chemical # P - 3539)	
Glass distilled water to	100 ml.

3.4.2 TEST extender

This extender was used in every experiment as the 'evaluated' extender for freezing semen without cryoprotectants (eg; glycerol). The base composition of the buffer system was as follows *(Abdelhakeam et al., 1992 a,b):*

48.6 g / litre TES (N - tris (hydroxymethyl) methyl - 2 - aminomethane - sulphonic acid) (Aldrich # 22,320 - 4) , titrated with 11 g / litre Tris (hydroxymethyl aminomethane) to pH 7.1 and with the osmotic pressure adjusted to 375 mOsm. In practice, to achieve those levels of pH and osmotic pressure, the following formula was applied:

TES	6.774 g
Tris	1.223 g

Glass double distilled water to 100 ml.

Regardless of the level of egg yolk used (varying, depending on the methodology of each experiment), this initial buffer was then mixed with 10% (V/V) isoosmotic maltose (D-maltose monohydrate; Aldrich # 11,256-9). Egg yolk and/or lipoprotein from egg yolk was then added as described in later chapters.

3.5 Freezing and thawing techniques

Regardless of cooling rate and dilution method (which are described in the relevant chapters), a 0.2 ml. drop of diluted semen was dropped onto a metal plate which had a depression of 0.5 ml. size on its surface. The plate was placed above the vapour of liquid air and maintained at an initial freezing temperature of - 80 °C. Semen drops were frozen and formed pellets which were allowed to remain on the plate for 3 - 4 minutes before being transferred directly to liquid air (- 193 °C) for storage.

Experimental semen pellets were thawed by the dry tube technique *(Evans & Maxwell, 1987).* To achieve this, pellets were placed individually in clean, dry test tubes which were immediately plunged into a water bath at 40 °C and shaken until the pellets had completely thawed (35 - 45 seconds). The samples were then subjected to the tests described in Sections 3.3.2 and 3.3.3.

3.6 Statistical analysis

The general statistical procedures used were Analysis of Variance (one way, two way, factorial and multiple comparison), and Duncan's multiple range test of the

means. These procedures have been described by *Snedecor & Cochran (1967)* and the Systat© for Windows™ Version 5.0 (1992) and Microsoft® Excel Version 5 (1993) statistical computer programs were used to analyse the data.