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ACROSOME REACTION AND CRYOPRESERVATION OF DOG SPERMATOZOA

by

Ömer Uçar

A thesis submitted in partial fulfilment of the requirements of the University of Bristol for degree of Doctor of Philosophy

May 2000
ABSTRACT

The use of AI in dogs has been limited by the lack of effective and reliable means of cryopreservation of semen and by the poor correlation between traditional methods of post-thaw assessment of semen quality and fertility. In order to address these problems, the present study focuses upon methods of cold storage and cryopreservation of dog spermatozoa by undertaking comparative evaluations of post-thaw motility and in vitro induction of acrosome reaction.

Split-ejaculate protocols were used to compare the effect of storage at +4°C and cryopreservation upon (i) the maintenance of spermatozoal motility and (ii) spontaneous or A23187-induced acrosome reactions during incubation at 39°C (in 5% CO₂ in the humidified air) for 60 or 120 min. The assessments of samples were made by Bright-Field, Phase Contrast (PC), Differential Interference Contrast (DIC), Scanning (SEM) and Transmission (TEM) Electron Microscopy.

The interaction between the process of glycerolisation and the presence of seminal plasma is one of the key limitors for success in cryopreservation of dog spermatozoa. Interactions between the effects of removal of seminal plasma (by centrifugation), dilution rate, the temperature at which glycerolisation took place and the concentration of glycerol upon survival of spermatozoa at +4°C were studied in a series of split-ejaculate experiments. Spermatozoa were suspended in Tris-fructose-citric acid extender containing 20% (v/v) egg yolk and 8% (v/v) glycerol at +4°C for 48 h. Survival was assessed as the percentage of spermatozoa displaying progressive motility. Survival of spermatozoa was higher (P<0.05) after glycerolisation at +4°C than at the room temperature. At dilution rates of 1:1 and 1:2 (semen: extender), the survival was higher (P<0.05) in samples that were centrifuged and glycerolised at +4°C than the samples that were neat and glycerolised at the room temperature. While at the dilution rate of 1:16 it was higher (P<0.05) in samples that were neat plus glycerolised at +4°C than all samples that were glycerolised at the room temperature. Concentrations of glycerol that were >2% (v/v) resulted in lower (P<0.05) survival than at lower concentrations.

Following the initial stage of the investigations, the optimisation and validation of a method for in vitro induction of acrosome reactions were required. Suspensions of spermatozoa in TALP medium were incubated in the presence of a logarithmic series of concentrations of the calcium ionophore, A23187. Induction of acrosome reactions was assessed by bright-field (using naphthol yellow S/aniline blue stain, NA) and phase contrast (PC) microscopy. Using these methods, it was determined that incubation in the presence of 1 µM A23187 for a period of 30-45 min was optimal for inducing acrosome reactions in fresh semen. It was also noted that the assessments of acrosome reactions by using NA staining, were highly correlated with PC microscopy. In consequence, the simple procedure of NA staining might be an acceptable alternative to PC microscopy for use in the field.

Subsequently, the effect of chilling and glycerolisation upon in vitro induction of acrosome reactions by A23187 was assessed. Acrosome reactions were studied as these have been described in the literature as providing accurate bioassay of spermatozoal functionality in vivo. Acrosome reactions were assessed by using DIC microscopy. The acrosomal integrity was impaired after chilling, which accelerated the A23187-induced acrosome reaction such that a lower concentration (0.1 µM/l) of A23187 was also effective to induce the reaction within 60 min of incubation. However, the presence of 2% glycerol (v/v, final) in standard Tris extender, containing 20% egg yolk, did not significantly affect the sequence of acrosome reaction.

The optimal freezing regimen (from +4°C to -120°C) was determined by using a programmable biological freezer in a series of experiments, in which various cooling rates were combined in a Latin square designs. Semen was diluted in standard Tris extender containing 20% egg yolk and 2% glycerol (v/v, final) and packed in 0.25 ml French palettes (straws). The optimal cooling regimen was -0.5°C/min from +4°C to -9°C, -40°C/min to -20°C, -100°C/min to -120°C, followed by direct immersion of the straws in liquid nitrogen. Changes in temperatures within an individual straw were continuously measured and these data were found to be highly correlated with the eventual post-thaw motility of frozen-thawed spermatozoa.

Although freezing and thawing resulted in major acrosomal deterioration, there were no significant differences between freezing regimens on the basis of in vitro induction of acrosome reactions, as assessed by DIC microscopy.

Finally, ultrastructural studies, using SEM and TEM, upon chilled (as 'ready to freeze') and frozen-thawed spermatozoa subjected to A23187-induced acrosome reaction demonstrated that freeze-thawing provoked the acrosome reaction such that, with TEM (i) the plasma membrane was usually damaged or missing, (ii) the acrosomal changes (including the loss of acrosomal content, as seen by decondensation and swelling) except vesiculation of the acrosomal membranes, exceeded to the equatorial segment and (iii) a further damage occurred to the post-acrosomal region.

In summary, these results show that semen should be; (i) centrifuged for dilutions of<1:8, (ii) diluted at 1:8 in Tris-fructose-citric acid extender containing 20% egg yolk, (iii) glycerolised at +4°C at a final concentration of 2% glycerol (v/v), (iv) cooled at -0.5°C/min from +4°C to -9°C, at -40°C/min to -20°C and at -100°C/min to -120°C, followed by direct immersion of the straws in liquid nitrogen for cryopreservation and (i) introduced to 1 µM/l A23187 in TALP, (ii) incubated for at least 30-45 min for induction of acrosome reaction in vitro and, thereby, demonstrated that optimisation of cryopreservation and in vitro induction of acrosome reaction of dog spermatozoa are possible.
DEDICATION

To those whom I love

(Sevdiklerime)
ACKNOWLEDGEMENT

I wish to express my sincere thanks to my supervisors, Dr. Susan E. Long and Assoc. Prof. Tim J. Parkinson, for their valuable help in the planning of this investigation, their endless encouragement, great patience, understanding, tolerance and stimulating criticism which they have devoted to my work.

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Finally, but most importantly, I would like to thank all members of my family for their continuous encouragement, patience, advice and great support during my doctoral training.
DECLARATION AND COPYRIGHT

Declaration

I declare that apart from the advice and assistance acknowledged, the work reported in this thesis is my own and has not been submitted for consideration for any other degree of academic qualification.

Ömer Uçan
May 2000

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<td>AC</td>
<td>acrosomal content</td>
</tr>
<tr>
<td>AI</td>
<td>artificial insemination</td>
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<tr>
<td>Amp</td>
<td>amplitude (or magnitude) of the latent heat plateau phase during ice nucleation</td>
</tr>
<tr>
<td>Area</td>
<td>total area of the plateau calculated by sum of the heights (per mm) under the amplitude</td>
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<tr>
<td>AspAT</td>
<td>Aspartate Amino Transferase</td>
</tr>
<tr>
<td>CLONE</td>
<td>Cryogenic Laboratories of New England, Inc.</td>
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<tr>
<td>CR1</td>
<td>first cooling rate</td>
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<tr>
<td>CR2</td>
<td>second cooling rate</td>
</tr>
<tr>
<td>CR3</td>
<td>third cooling rate</td>
</tr>
<tr>
<td>C-RT</td>
<td>centrifuged, glycerolised at room temperature</td>
</tr>
<tr>
<td>C-4°C</td>
<td>centrifuged, glycerolised at +4°C</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>ES</td>
<td>equatorial segment</td>
</tr>
<tr>
<td>I</td>
<td>acrosome-intact</td>
</tr>
<tr>
<td>IAM</td>
<td>inner acrosomal membrane</td>
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<td>N</td>
<td>nucleus</td>
</tr>
<tr>
<td>NA</td>
<td>naphthol yellow S/aniline blue</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide reductase</td>
</tr>
<tr>
<td>NM</td>
<td>nuclear membrane</td>
</tr>
<tr>
<td>N-RT</td>
<td>non-centrifuged, glycerolised at room temperature</td>
</tr>
<tr>
<td>N-4°C</td>
<td>non-centrifuged, glycerolised at +4°C</td>
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<tr>
<td>OAM</td>
<td>outer acrosomal membrane</td>
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<tr>
<td>PAR</td>
<td>post-acrosomal region</td>
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<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>PC</td>
<td>phase contrast</td>
</tr>
<tr>
<td>PR</td>
<td>partially acrosome-reacted</td>
</tr>
<tr>
<td>R</td>
<td>acrosome-reacted</td>
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rate1: initial cooling rate (°C/min) with a sudden fall in temperature before ice nucleation temperature on the freezing curve
rate2: second cooling rate (°C/min) following freezing point depression on the freezing curve
SEM: Scanning Electron Microscopy
TEM: Transmission Electron Microscopy
Ti: intermediate temperature
time: duration (sec) of latent heat plateau phase
T1: ice nucleation temperature (°C)
T2: freezing point depression (°C)
T1-T2: difference (°C) between ice nucleation and freezing point depression temperatures
ZP: zona pellucida
TALP: modified Tyrode's medium
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CHAPTER 1. REVIEW OF LITERATURE

1.1- Introduction
There are three major requirements for the successful artificial insemination (AI) of any species. First, its spermatozoa must be able to survive outside the body. Secondly, it must be possible to inseminate the female as close as possible to the site of fertilisation. Thirdly, it must be possible to detect the fertile period of the female, preferably without recourse to the presence of a male.

The relatively limited uptake of AI in the dog reflects that it, as a species, meets none of these criteria particularly well. Thus, although dog spermatozoa survive reasonably well for short periods in simple diluents, they generally respond poorly to cryopreservation. Similarly, insemination can only be performed into the vagina or, at best, the caudal cervix unless laparascopy or laparotomy is used to allow an intrauterine insemination. The fertile period (i.e. the optimum time for insemination) is not easy to determine accurately due to the idiosyncrasies of the canine oestrous cycle. The consequences of an inability to perform an optimally-timed deep insemination are that conception rates achieved with the relatively fragile cryopreserved spermatozoa are generally less than with natural service or insemination of fresh semen unless the concentration of spermatozoa is very high.

Many of these problems could be alleviated if a reliable method of cryopreservation of dog semen was devised, permitting consistently high levels of survival of functionally-intact spermatozoa which could also withstand the rigours of passage through the female genital tract.

Given the difficulties of undertaking conception rate trials in the dog, it is generally necessary to examine the survival of functional spermatozoa in terms of the characteristics that they display in vitro. Of such parameters of functionality that have been studied in cryopreserved spermatozoa, the motility has received a great deal of attention. Yet it is clear that whilst motility is of importance, it has but a weak relationship with fertility, and that there are far more accurate measures of functional integrity available. Foremost amongst such measures is the functionality of the
acrosome, in terms of both its structural integrity and its ability to undergo the acrosome reaction after cryopreservation. Studies in other species (notably the bull) have indicated that very high levels of correlation exist between parameters of acrosomal integrity and fertility.

It is evident from the literature that most cryopreservation regimens for canine spermatozoa have drawn heavily upon the protocols used for the semen of ruminant species, and in consequence, relatively little attention has been paid to the details of cooling rates that are most appropriate to (and optimal for) the dog. It should be possible to identify the optimum conditions for cryopreservation by examining the losses of motile spermatozoa at each stage of the cooling curve, using the maintenance of a functionally intact acrosome as the main criterion of sperm survival.

It is this hypothesis that is investigated in this thesis.

1.1.1- Background

Until the early 1990's, little AI was undertaken with frozen dog semen due to lower pregnancy rates, lower litter sizes compared with fresh semen and to the regulations of many Kennel Clubs (e.g. American, British, Swedish, Norwegian and French). Therefore, genetic exchange between countries of different continents and storage of frozen semen have been hampered throughout the world.

Nevertheless, there have been a number of reports of successful cryopreservation of dog semen even though the vast majority of workers have used methodologies initially designed for other species (mainly the bull: Foote, 1964a; Seager, 1969; Andersen, 1975). Fertility rates with frozen dog semen have generally been low. This is related to excessive cryogenic damage of spermatozoa during freeze-thawing, including

(i) a decrease in motility (Oettle, 1986b),
(ii) acrosomal deterioration (Ström Holst et al., 1998),
(iii) a loss of proacrosin and acrosin activity (Froman et al., 1984), and
(iv) alterations in the chromatin structure (Rodriguez-Martinez et al., 1993).

Consequently, alterations in capacitation, acrosome reaction or the ability of spermatozoa to recognize the zona pellucida (ZP) may result in poor oocyte penetration by cryopreserved dog spermatozoa (Hay et al., 1997b). The time for which cryopreserved
dog spermatozoa continue to exhibit satisfactory levels of motility (post-thaw spermatozoal thermostability) is relatively brief (Olar, 1984; England and Ponzio, 1996) in comparison with other species (Concannon and Battista, 1989). This may also need to be taken into consideration when the methods of freezing canine semen are being evaluated.

Intrauterine insemination itself is difficult due to the long and narrow vagina and rather tortuous cervix of the bitch although successful transcervical intrauterine inseminations have being performed in Norway since 1975 (Andersen, 1975) and in Sweden for several years (Linde-Forsberg, 1995).

A marked variation exists in both pre-freeze semen quality of fertile individuals (England and Allen, 1989b) and post-thaw motility of semen within and between dogs (Dobrinski et al., 1993; Thomas et al., 1993) and thus the selection of donors may exert a marked effect on the fertility of frozen semen.

Further difficulties arise with AI in dogs in identifying the optimal time for insemination of the bitch. Exact timing of AI is imperative due to the delayed maturation of the ovum (Tsutsui, 1989), although immature ova can also be penetrated by capacitated spermatozoa (Mahi and Yanagimachi, 1976; Hewitt and England 1997). Overall, it has been difficult to achieve good results from AI with frozen semen in the dog under field conditions.

1.1.2- Historical overview of the use of frozen semen in the dog

Surprisingly, given the many difficulties associated with canine AI, the first animals ever reported to become pregnant by artificial means were dogs, which were inseminated with fresh semen by the Italian scientist L. Spallanzani around 1780 (Concannon and Battista, 1989). In the early 1960’s, a few studies were conducted (Martin, 1963a,b; Foote, 1964a,b,c). In 1964, Foote (1964b) found that the long-term preservation of dog semen was possible and so interest in both the freezing of semen (Quinn and White, 1966) and its use in AI increased. The first successful canine pregnancy was achieved by S.W.J. Seager in 1969 (Seager, 1969).

New extenders for freezing semen had also been developed for the semen of other species during the 1960’s. Skimmed-milk based extender, originally developed for use in the bull, was used in early studies of cryopreservation (Martin, 1963a; Takeisi et al., 1976). However, in recent studies, it was widely used for cold storage of dog semen at +4°C
(Rota et al., 1995; England and Ponzio, 1996; Pinto et al., 1999; Brewis et al., 1999). Therefore, many of the workers investigated either constituents (Martin, 1963a; Foote 1964b) or, more widely, comparisons of diluents (Foote, 1964a; Battista et al., 1988; Dobrinski et al., 1993; Ström et al., 1997). However, high pregnancy rates have been achieved by using egg yolk based diluents (e.g. lactose-egg yolk-glycerol, developed by Nagase and Graham (1964): Platz and Seager, 1977; Tris-egg yolk-glycerol, developed by Davis et al. (1963a,b): Andersen, 1975; Triladyl plus 0.5% Equex STM paste: Nöthling and Volkmann, 1993). Eventually, Tris-based extenders became most popular (Linde-Forsberg, 1995). Commercial extenders such as Biociphos (Silva and Verstegen, 1995) or CLONE (Govette et al., 1996) have also proved to be extremely successful. In recent years, there has been increased interest in AI with both fresh and frozen semen (England, 1992; Kabasakal, 1995; Hay, 1996; Rota, 1998).

Investigations have rarely been based upon in vivo fertility in large numbers of bitches (Linde-Forsberg et al., 1999). Because of the lack of information about dog spermatozoa under in vitro and in vivo conditions, thermolability (i.e. poor post-thaw spermatozoal viability at different storage temperatures from 0°C to 39°C) especially at body temperature remains as a major problem in the dog (Olar, 1984; England and Ponzio, 1996; Peña et al., 1999). This is regrettable, since the long-lasting viability, especially at body temperature, is desirable for optimum fertility.

Post-thaw motility of dog spermatozoa, as with other species, is regarded as a reliable indicator of fertility (Nöthling et al., 1997) although its value (as a single criterion) remains controversial, especially when laparoscopic intrauterine insemination is used (Silva and Verstegen, 1995). Recently, Bedford (1998) indicated that spermatozoa of eutherian mammals have evolved a wholly new strategy for zona penetration based on "cutting thrust" (as generated by hyperactivated motility, lateral head displacement and the perforatorium) whilst an acrosome reaction is also a prerequisite for sperm penetration through the zona.

1.2- Cryobiology

1.2.1- General principles of cryopreservation

Cryoinjury to a variety of cell organelles is regarded as being due to two major stresses of cryopreservation, i.e. the change in temperature and the formation and dissolution of ice.
and its consequences (Watson, 1995). During the freezing of a cell suspension, ice forms as the temperature falls below the freezing point of extracellular material. The crystallisation of water out of the solution leaves the remaining solutes at a higher concentration. The intracellular fluid becomes supercooled as the cell plasma membrane prevents the entry of extracellular ice crystals into the cell. This stress alters the osmotic pressure across the plasma membrane, so that the intracellular water tends to move out of the cell. The process of dehydration of the cells which accompanies slow freezing is potentially associated with cell survival, whereas at more fast rates, cell death is more likely (Watson, 1995). Cooling the cells at fast rates limits the ability of intracellular fluid to move out of the cell quickly enough and thus may result in an increased degree of supercooling and intracellular ice formation (Mazur, 1965), usually leading to cell death after thawing.

Although the amount of ice and the size of crystals are important factors in cell survival, the intracellular ice formation, which may be increased by cell-cell contact (Acker and McGann, 1998), does not necessarily cause cell death (Farrant et al., 1977). Rapid freezing may form small ice crystals within the cells, but such cells will survive unless thawing is slow, otherwise recrystallisation (i.e. ice crystal growth during thawing) can occur. Recrystallisation reduces cell survival, and under this condition, osmotic forces play a pivotal role in the resultant cell damage. At freezing rates which are slower than optimal, the loss of cell viability is related to several events; namely, a decrease of intracellular water content leading to increased intracellular solute concentrations, precipitation of these solutes and subsequent disturbances in pH (Van den Berg and Soliman, 1969).

Osmotic gradient is a pivotal factor in cell damage during slow thawing. With a permeating cryoprotectant (e.g. glycerol), too fast thawing may not allow the agent to leave the cell fast enough to maintain osmotic equilibrium. This can lead to cellular swelling and damage through the entry of water (Griffiths et al., 1979).

Therefore, optimal freezing and thawing of cell solutions should allow maintenance of the osmolality, pH and ionic strength. In addition, the solutions should provide a source of energy, prevent cryoinjury and be resistant against bacterial growth.
1.2.2- Cryoprotective agents

Different types of compounds have been used as a cryoprotectant to cells during freezing. These are divided into two groups: penetrating (e.g. glycerol, methanol and dimethyl sulfoxide, (DMSO)) and non-penetrating (e.g. proteins, sugars and polyvinyl pyrrolidone, (PVP)) agents (Watson, 1990). The former group penetrate into cells and possibly bind water, so that the proportion of intracellular water remaining unfrozen at any given temperature is greater in the presence of cryoprotectant (Mazur et al., 1970) and thus freezing at lower temperature occurs. This probably retards dehydration of cells and the resultant harmful "solution effect" (i.e. cell dehydration during freezing). Hence, cells may be cooled sufficiently and slowly to prevent the formation of large intracellular ice crystals.

Non-penetrating cryoprotectants remain extracellular and promote cell dehydration by acting osmotically during freezing (Watson, 1990). They do not modify the "solution effect" within the cell during cooling and hence they are most effective during fast freezing (Nagase et al., 1968).

The optimal concentration of cryoprotectant varies with freezing rate: faster freezing generally requires lower concentrations (Watson, 1990) although this may not be apparent when a relatively narrow range of freezing rates is used (Robbins et al., 1976). Also, the surface area: water volume ratio is a key determinant of freezing rates (Hammerstedt et al., 1990). Cells with a high surface area and volume ratio need fast freezing (or they die of dehydration) while cells with a low ratio need a much slower rate (or they die of ice crystal formation during freezing and/or thawing).

1.3- Assessment criteria for cryopreserved canine spermatozoa

1.3.1- Introduction

Various methods have been used to assess the degree of success in semen freezing techniques in the dog. The most common ones include; (i) post-thawing motility, (ii) acrosome morphology, (iii) enzymatic activity (i.e. acrosin, aspartate amino transferase and β-nicotinamide adenine dinucleotide reductase), (iv) hypoosmotic swelling, (v) oocyte penetration and hemizona assay, and (vi) thermostability. The most reliable test of the functional integrity of spermatozoa is, of course, the capacity to fertilise the ovum and to sustain embryogenesis (Watson, 1979). Therefore, data from post-thaw fertility outcome
give the best assessment of quality of frozen sample. However, with the exception of bovine semen, sufficient inseminations were rarely performed to allow accurate (statistically valid) comparisons of fertilising ability. Hence, fertility prediction of a semen sample is often made by examining simple characteristics of spermatozoa in the laboratory.

The initial quality assessment (numbers of spermatozoa, motility and morphology) of canine semen has been shown to correlate with its fertility (Oettlé, 1993). However, Nöthling and Gerstenberg (1996) considered that the routinely assessed variables of fresh semen quality, with the exception of the incidence of distal and proximal cytoplasmic droplets, have little value in predicting motility after cryopreservation. Semen quality from normal fertile individuals is very variable (England and Allen, 1989b) and so the initial semen quality only provides a very rough guide to the characteristics of semen after cryopreservation.

1.3.2- Post-thaw motility and acrosomal status
The most frequently measured index of post-freezing spermatozoal survival is post-thaw motility, although only in a very few trials have values for motility been reported along with fertility testing results. However, although post-thaw motility has been used to examine the effects of different diluents, equilibration times, freezing formats, freezing and thawing rates, etc., its correlation with fertility has yet not been firmly established in the dog. Indeed, Silva and Verstegen (1995) comparing the effect of different extenders upon fertility found a very diffuse relationship between post-thaw motility and fertility after laparoscopic intrauterine insemination. However, Nöthling et al. (1997) suggested that the post-thaw motility of spermatozoa is a better indicator of fertility than either morphology or acrosomal integrity.

In the boar, Pursel et al. (1972) demonstrated that spermatozoa could be highly motile but non-fertile due to acrosomal damage during in vitro storage. Likewise, in dogs, marked acrosomal deterioration after freezing and thawing has been shown by numerous workers. Oettlé (1986b) demonstrated that acrosomal damage occurs in a significant percentage of spermatozoa during each of the three major processing steps (i.e. dilution, cooling/equilibration, freeze-thawing) when a standard Tris-fructose-citric acid-egg yolk diluent is used. At no stage was the acrosomal damage significantly correlated with
motility. Therefore, the evaluation of acrosomal integrity should be a major part of post-thaw assessment of semen quality.

1.3.3- Enzyme activity

Measurements of enzyme activity have been used to evaluate cryopreserved canine spermatozoa. These include; (i) aspartate amino transferase, AspAT (Kosiniak et al., 1992), (ii) acrosin (Froman et al., 1984; Olar, 1984), and (iii) β-nicotinamide adenine dinucleotide reductase, NADH (Ivanova-Kicheva et al., 1995).

No clear relationship between AspAT activity and post-thaw motility of spermatozoa was found (Kosiniak et al., 1992). However, Froman et al. (1984) considered that the elevated percentages of proacrosin and acrosin activity of cryopreserved spermatozoa to be indicative of cellular damage. In contrast, Olar (1984) observed no difference in acrosin activity of spermatozoa before or after cryopreservation.

Ivanova-Kicheva et al. (1995) observed that NADH activity of spermatozoa, as an indicator of plasma membrane integrity of the midpiece, was well maintained after cryopreservation. Hence, they suggested that maintenance of NADH activity might be related to the proportion of cells possessing sufficient energy reserves to permit progressive motility after thawing.

Nevertheless, the variation between results of different trials means that the value of these measurements of enzyme actions remains, at best, uncertain.

1.3.4- Hypo-osmotic swelling

The use of an hypo-osmotic swelling (HOS) test, i.e. measuring the curling response of the tail membrane in an hypoosmotic solution, has been described for canine spermatozoa by both England and Plummer (1993) and Kumi-Diaka (1993). Spermatozoal swelling was highly repeatable, but there was no correlation with motility, morphology or vital staining (England and Plummer, 1993). Indeed, the correlation between HOS test and acrosome lesions is also unclear (Kumi-Diaka and Badtram, 1994; Rodríguez-Gil et al., 1994). Therefore, only a small number of workers have attempted to use this assay for assessing the post-thaw quality of spermatozoa (Kumi-Diaka, 1993; Rota et al., 1995; Guérin et al., 1996; England and Ponzio, 1996). Of these, England and Ponzio (1996) and Kumi-Diaka (1993) considered that the HOS assay could have predictive value in assessing the quality
of cryopreserved semen whilst the others did not. Therefore, the value of HOS test remains unclear.

1.3.5- Oocyte penetration and Hemizona assay
It is well established that capacitated dog spermatozoa can penetrate homologous immature oocytes *in vitro* (Hay *et al.*, 1997a; Hewitt and England, 1997). In addition, Hay *et al.* (1997b) have shown that the decreased motility and increased acrosomal damage of cryopreserved canine spermatozoa are correlated with a reduced penetration of oocytes. Interestingly, however, they also observed that although spermatozoa appeared to have survived cryopreservation as determined by viability and motility, there was a marked decrease in the number of zona-penetrating spermatozoa. Hence, their conclusion was that oocyte penetration (i) offers a useful alternative as an adjunct to other evaluation methods, (ii) can be used to evaluate semen handling and freezing procedures more effectively, and (iii) can be used to test valuable canine gametes to ensure that stored germ plasm has acceptable fertilising potential. Therefore, an oocyte penetration bioassay could be used as an indicator of spermatozoal performance before (Hewitt and England, 1997) or after cryopreservation (Hay *et al.*, 1997a).

A modification of this test, the hemizona assay (HZA), which utilises halves of the ovum having split zona, has also been used in dogs for prediction of *in vivo* (Mayenco-Aguirre and Pérez-Cortés, 1998) and *in vitro* (Ivanova *et al.*, 1999) fertility of fresh or frozen-thawed spermatozoa, respectively. Mayenco-Aguirre and Pérez-Cortés (1998) found that although zona binding was affected significantly by fertility status, there was no clear difference between fertile and infertile dogs mainly due to a poor zona binding capacity of spermatozoa from some of the fertile individuals. Ivanova *et al.* (1999) found that the binding activity of frozen-thawed spermatozoa was markedly reduced compared to fresh spermatozoa. Furthermore, semen samples (from different dogs) with similar sperm parameters before cryopreservation displayed different sperm zona-binding capacity after thawing. Therefore, they concluded that HZA might be a valuable tool for evaluating the post-thaw fertilising ability of canine spermatozoa. This conclusion may be premature due to the small number of individuals tested.

Hence, it appears that neither bioassay is sufficient as a sole predictor of the ultimate fertility *in vivo*. Additional information could be obtained when they are both considered
alongside other semen evaluation techniques.

1.3.6- Post-thaw thermoresistance

The maintenance of post-thaw motility during incubation either at 0°C (Hay et al., 1997a), +5°C (Foote, 1964b; Mayenco-Aguirre and Gomez-Cuatara Aguilar, 1996), room temperature (Fontbonne and Badinand, 1993b; Nizanski et al., 1997), 32°C (Oettlé, 1982) or body temperature (England and Ponzio, 1996; Peña et al., 1999) has been used in assessing the semen quality. It is well established that the duration of post-thaw survival, restricted to a few hours at body temperature, is a major problem for canine spermatozoa; perhaps more so than for other species (Concannon and Battista, 1989). Empirical studies have shown that although relatively longer survival periods (5-24 h) could be achieved at room temperature (Nizanski et al., 1997; Ström et al. 1997), thermolability is more apparent when body temperature is used for storage (Olar, 1984; Ström et al., 1997). Indeed, Peña et al. (1998c) and Ström et al. (1997), comparing the storage at body and room temperatures, observed a significant decline in post-thaw motility at body temperature. Likewise, Olar (1984), comparing 30 min or 1 h of storage at 37°C, observed that the post-thaw motility was often 0% after 1 h.

Koutsarova et al. (1997) used pentoxifylline, an inhibitor of phosphodiesterase activity, to stimulate the metabolism of spermatozoa for a longer period. Their finding was that pentoxifylline increased the period of survival of spermatozoa by 4-5 h when it was added after thawing.

Undoubtedly, a longer period of survival would increase the number of spermatozoa available for fertilisation and hence it may be a useful criterion of semen quality for optimisation of freezing conditions. Therefore, it is possible that the post-thaw thermoresistance test (especially after storage at body temperature) should be incorporated into semen evaluation techniques in order to allow the development of insemination schedules that are based upon the estimated survival time of spermatozoa in the female reproductive tract.

In conclusion, the suggestion is advanced that multiple semen evaluation methods (before and after freeze-thawing) should be considered, so that reliable criteria of spermatozoal quality can be developed for future studies of cryopreservation and AI as well as
prediction of fertility both in vitro and in vivo.

1.4- Cryopreservation of dog semen (I) Diluents
1.4.1- Dilution rate
Dilution with a protective solution is necessary in order to maintain survival of spermatozoa for longer periods and to cool and freeze semen. When mammalian semen is diluted in a simple medium, spermatozoa respond to dilution by; (i) an initial increase in activity, followed by (ii) a loss of motility and (iii) an increase in the number of dead cells (Mann, 1964).

In AI of cattle and sheep, the semen is diluted to a standard concentration of spermatozoa before freezing. This has an advantage in that it is easier to calculate the number of spermatozoa for each straw although this seems to neglect the effect of dilution on spermatozoal viability and the variation in diluent constituent concentration. Dilution effects occupied the minds of early AI technologists very greatly. It was the realization that "dilution effects" were only a property of early, poorly suited diluents and that with well formulated diluents spermatozoa would tolerate very wide ranges of dilution that allowed AI to develop. In the dog, since the relationship between chemical concentration of the diluent and spermatozoal concentration is unknown, dilutions to standard concentration should use a variety of extenders with constituents of different concentrations (England, 1993).

The survival of ejaculated dog spermatozoa in seminal plasma alone is limited to a few hours (Christiansen, 1984; England, 1993). Different formulae have been used for dilution of samples before freezing, and dilution ratio has varied with semen volume, spermatozoal concentration or packaging system. One method is to dilute the semen at a particular volume ratio; a method which is often used under field conditions in order to allow spermatozoal concentration to be determined later. An other method, which is more widely used in most other species, requires an initial determination of spermatozoal concentration, followed by dilution to a known concentration in order to obtain insemination units of a desired number of spermatozoa. Numerous workers have used a standard dilution ratio in the dog (Hay et al., 1997b; Nöthling et al., 1997), while the dilution to a known concentration of spermatozoa (the latter method) has been the method of choice in other studies (Ström et al., 1997; Peña et al., 1998a).
Considerable data exist about dilution ratios for dog semen. An approximate 1:5 dilution has generally been found to produce best results (Yubi, 1984; England, 1993). In 1969, Seager obtained the first pups using frozen pelleted semen in lactose-based extender with the dilution ratio being 1:3 (Seager, 1969). However, dilution rate varies with spermatozoal concentrations (Seager and Fletcher, 1973; Yubi et al., 1987). This is due to the fact that while improved spermatozoal motility is achieved by using lower dilutions (England, 1993), the leakage of intracellular components and/or dilution of protective agents (such as albumin; Dott et al., 1979) in seminal plasma are responsible for the loss of viability at higher dilutions (Wales and White, 1963). However, involvement of egg yolk in the diluent may partly prevent the damage (especially on spermatozoal membranes) during storage at +5°C and freezing (Watson, 1976; also see below). In conclusion, as a rule, the dilution rate (of an extended semen) in straws is somewhat higher than in pellets, usually ranging from 1:3 to 1:5 (Andersen, 1980) or up to 1:8 (Christiansen, 1984) depending on the initial spermatozoal concentrations used.

1.4.2- Cryoprotective agents

Freezing dog semen in the absence of a cryoprotectant causes a great reduction in the number of motile spermatozoa surviving after thawing. In most species, including the dog, glycerol is the most widely utilised cryoprotectant (Watson, 1990) and apparently gives better results than others such as DMSO (Olar et al., 1989; Kim and Kim, 1995). Olar et al. (1989) reported that the addition of DMSO to either egg yolk-lactose-based or egg yolk-Tris-based extenders, alone or in combination with glycerol, was not beneficial to post-thaw survival, often reducing motility to a level less than observed when no cryopreservative (other than egg yolk) was used.

The optimum concentration of glycerol, which represents a compromise between its cryoprotective and toxic effects (Watson, 1979), varies with the extender, cooling rate and species. The amount of glycerol that is needed has been a matter of interest, since in some species, e.g. poultry, it depresses fertility of spermatozoa unless it is removed before the insemination (Hammerstedt and Graham, 1992).

In the dog, glycerol causes toxic effects even in unfrozen spermatozoa. Hay et al. (1997b), using unfrozen semen, observed that the addition of glycerol (4%, v/v) at +4°C affected neither the acrosomal status nor motility, but resulted in a decline in the number
of spermatozoa bound per oocyte, which might be an indication of a subtle damage to the sperm cells. Similarly, Martin (1963a) reported that post-thaw removal of glycerol was necessary before strong motility was seen although this observation was not subsequently confirmed.

Interestingly, Nöthling and Volkmann (1993) found that the insemination of frozen semen, to which homologous prostatic fluid was added post-thaw, resulted in 100% pregnancy rates although the means by which it exerts its beneficial effect remain to be clarified.

Smith (1984) found that an egg yolk-Pipes diluent containing 9% glycerol was optimal. Similarly, a concentration of 8% glycerol was optimum for maintenance of acrosomal status and motility when a Tris-fructose based diluent was used (Peña et al., 1998b). However, Olar (1984) recommended a lower percentage of glycerol (3-4%) in Tris-glucose based extender. Olar et al. (1989) reported that post-thaw spermatozoal motility was the highest when Tris extender contained 2-4% glycerol. Smith (1986) stated that AI with semen, which had been frozen in extenders containing 4-8% glycerol, resulted in pups. In the preliminary trials of England (1992), acceptable pregnancy rates were achieved following AI with semen that was frozen in a Tes/Tris extender containing 10% egg yolk and glycerol at a final concentration of 2% (v/v). Other workers have found no effect of glycerol concentration on sperm motility (Günzel-Apel et al., 1993; Mayenco-Aguirre and Gomez-Cuéllar, 1996).

Different aspects of glycerolisation such as its concentration and addition temperature have been further investigated before and/or after cryopreservation (England, 1992; Fontbonne and Badinand, 1993b; Peña et al., 1998b). England (1992) compared the toxicity of a wide range of glycerol concentrations (0-10%) simply by incubating fresh semen (diluted in Tes/Tris extender) at 39°C. On the basis of motility, acrosomal morphology and vital staining, the following observations were made:

(i) There was no significant difference between the concentrations of glycerol up to 2%, however concentrations of $\geq 4\%$ were markedly detrimental to spermatozoal motility.

(ii) Deleterious effect upon the acrosome was exerted by only 10% glycerol.

(iii) There was no significant influence of glycerol upon vital staining.
In cryopreservation studies, Fontbonne and Badinand (1993b), who used Tris-fructose-citric acid extender containing egg yolk, found that glycerol concentration could not be reduced below 1.6-3.2% (v/v) without affecting post-thaw motility. However, they also noted that the optimum concentration of glycerol might vary among different extenders. Recently, Peña et al. (1998b), who used various concentrations of glycerol (2-8%) in a similar extender, found that post-thaw motility and acrosomal integrity were superior when 8% glycerol was used. In contrast, Kim and Kim (1995), using Tris extender which contain either fructose or glucose, observed no significant difference between 5 and 10% glycerol concentrations. The cooling rates were not identical in the above studies. Therefore, it can be concluded that a relatively wide range of concentrations of glycerol can be tolerated and that the ideal concentration depends as much upon the other constituents of the diluent and the cooling regimens that are to be subsequently employed, as upon the toxic effects glycerol may have directly upon spermatozoa.

England (1992), using 4% glycerol in Tes/Tris extender, found no significant difference between one- or two-step dilution when examining glycerolisation temperatures. Likewise, Fontbonne and Badinand (1993b), using Tris-fructose based extender, showed that as far as post-thaw motility is concerned, glycerol can be added at room temperature and does not need to be added in several steps. Peña et al. (1998b), who used 8% glycerol in a similar extender for freeze-thawing, observed no difference between one- or two-step dilution, confirming previous findings of Foote (1964b; who also used 8% glycerol in citrate-egg yolk based extender). Overall, these studies suggest that one- or two-step glycerolisation have yielded almost equivalent results. Nevertheless, fertility trials are needed to clarify the actual effect of glycerol, since the ability of spermatozoa to penetrate the ovum in vitro can be impaired by glycerol, even at a relatively low level, i.e. 4% (Hay et al., 1997b).

1.4.3- Buffering capacity

Hydrogen-ion concentration is one of the most important factors influencing the survival of mammalian spermatozoa. The metabolic activity of spermatozoa results in an increase of hydrogen ion concentrations, leading to a decrease in viability and fertility (Smith, 1984). Seminal plasma has a pH of about 7.0 and the optimum pH of spermatozoa in most species is therefore close to neutrality (Watson, 1990). Hence, buffering systems are used
in semen extenders, and most of them are buffered at between pH 6.9 and 7.1 (England, 1993).

The mean pH of the sperm-rich fraction of the dog ejaculate is \(\sim 6.3 \pm 0.3\) (England, 1993; Dobrinski et al., 1993) while prostatic fluid is \(6.8 \pm 0.2\) (Wales and White, 1958). Nevertheless, Wales and White (1958) found that with fresh semen, maximum motility was maintained in the pH range of 7.0-8.5. However, Foote and Leonard (1964) found that pH 6.6 was best for post-thaw survival in citrate buffer compared to pH 5.3 and pH 7.3. Smith (1984) found that the maximum post-thaw motility occurred at pH 7.0 for semen that were frozen in Pipes extender. England (1992) found that the optimal pH was 7.3 using Tes/Tris buffer with a dilution rate of 1:2 (semen: buffer, v/v). This finding was also confirmed in a subsequent study with 1:4 dilution ratio (England, 1993).

Therefore, it is concluded that optimum pH of buffers for freeze-thawing of dog semen is higher than that of the second fraction of the ejaculate, and that it may also vary with the type of buffer used.

1.4.4- Egg yolk

In most species, egg yolk has been utilised extensively as a component of extenders for preservation of spermatozoa at temperatures both above 0°C and in freezing. It is known that the active component in egg yolk is the low-density lipoprotein fraction, which is a high molecular weight component. This protects the cell membranes against cold shock (Watson, 1981). Additionally, egg yolk protects spermatozoa against the toxic action of seminal plasma constituents (Shannon and Curson, 1972). These toxins are antibacterial cationic peptides that are, with time, released from large disaggregating proteins and cause severe membrane damage (Shannon et al., 1987). Vishwanath et al. (1992) demonstrated that the water soluble cationic fraction of egg yolk (in which lipid and proteins and lipid:protein ratio are optimum) may bind firmly with the plasma membrane of spermatozoa because of its charged nature and may also compete with detrimental seminal plasma cationic peptides in binding to the plasma membrane. The improvement of the protective action of the egg yolk by detergents, i.e. sodium dedocyl sulphate (Peña et al., 1998c), Orvus ES (Nizanski et al., 1997) or Orvus WA (Thomas et al., 1992) paste may optimise the close association of the lipid components of egg yolk with the cell membrane, perhaps resulting in a modification of the phase transition events. Therefore, Watson (1995)
concluded that; (i) the protection is due to the lipid in the egg yolk and that (ii) emulsification increases its activity. Interaction with the membrane surface is an essential component of its action.

The ideal concentration of egg yolk may differ according to species although it is commonly used at concentrations of 3-25%, w/v (Watson, 1979). In the preservation of dog semen a range of egg yolk concentrations has been used. In the early studies, using unfrozen semen, it was found that there was a marked interaction between egg yolk level and storage temperature (Foote, 1964a). While 20% egg yolk was optimum at +5°C, 1% was optimum at 25°C, the former of which being markedly better than the latter for survival. Subsequently, a concentration of 20% egg yolk in sodium citrate-glucose extender also proved to be successful in freezing, with a mean post-thaw motility of 41% (Foote, 1964b). Seager (1969) obtained the first pups after insemination of frozen semen that was diluted in lactose extender containing 20% egg yolk. He suggested that egg yolk diluent is the most successful freezing extender (Seager, 1976). A similar concentration of the egg yolk has been utilised by the majority of other workers using a range of diluents (Andersen, 1972a; Rota et al., 1995; Nöthling et al., 1997). By contrast, England (1992) found that 10% egg yolk in Tes/Tris extender was optimum. However, in a subsequent study he used 20% egg yolk in Tris based extender (England and Ponzio, 1996). 15% egg yolk (v/v) in Test extender (Tes/Tris based) was satisfactorily used by Battista et al. (1988).

Unfortunately, the final concentration of egg yolk in diluted semen is often unknown due to the variable dilution rates used. Therefore, it is concluded that the final concentration of egg yolk in a variety of diluents may vary between 10 to 20% (w/v or v/v) although the concentration of 20% (v/v) in the extender, regardless of dilution rates used, usually gives adequate results. Nevertheless, further studies are needed to compare different concentrations of egg yolk with varying extenders, dilution rates as well as detergents to improve the membrane quality of dog spermatozoa before and after freeze-thawing.

1.4.5- Sugars
Sugars have been included in semen extenders as; (i) exogenous energy substrates for promoting motility during incubation, (ii) osmotic components to maintain osmolality of the extender and (iii) cryoprotective agents (Watson, 1979). Glucose, fructose and
mannose are glycolysable by spermatozoa (Mann, 1964), and therefore they are potential energy sources. The group of higher molecular weight of sugars, such as lactose and raffinose have low permeability and are generally considered to be good cryoprotectants especially for fast freezing in pellets (Watson, 1990). For many species (i.e. dog, ram, bull and rabbit), glucose and fructose are equally oxidised by spermatozoa while the rate of lactate oxidation greatly exceeds that of glucose and fructose in dogs and in rabbits (Murdoch and White, 1966).

Lactose extenders, containing egg yolk and glycerol, have been extensively utilised for the pellet method of freezing semen in several species. This method was also successfully used in the dog (Seager, 1969), although Tris-fructose-citrate (Yubi, 1984; Battista et al., 1988) or Tris-glucose-citrate (Thomas et al., 1993) extenders containing egg yolk and glycerol were considered to be more suitable for freezing in plastic straws. In addition, with straw freezing, motility was found better in Tris-glucose-citrate extender than in lactose-glucose (Olar et al., 1989). Some workers have directed their research towards the use of sugars during cryopreservation of dog semen. Commonly, extenders contain either lactose (Seager, 1969; Marks et al., 1994), glucose (Foote, 1964b; Hay et al., 1997a) or, as most widely, fructose (Andersen, 1975) in Tris buffer. Smith (1984) used glucose in Pipes extender, but gave no information involving comparisons with other sugars. Recently, Dobrinski et al. (1993) compared Tris-fructose, Pipes-glucose, Triladyl and IMV Universal extenders and concluded that, with straw freezing, although Triladyl and IMV Universal extenders resulted in more vigorous post-thaw motility, the four extenders were equally suitable for freezing.

It may be concluded that the choice of a particular sugar for cryopreservation of canine semen is not critical, as a number of mono- and disaccharides seem to be equally capable of maintaining post-thaw spermatozoal motility. However, the most widely used sugar, which gives adequate results in a variety of freezing and packaging regimens, is fructose.

1.4.6- Diluents

In the early studies, Krebs-Ringer-phosphate buffer was used for freezing dog semen (Martin, 1963a). Later, it was found that reconstituted skimmed-milk was better than either Ringer-fructose-phosphate or citrate-fructose-phosphate (Martin, 1963b; Takeisi et al., 1976). Recently, for storage of semen at +4°C skimmed-milk diluent was also found
slightly better than Laiciphos and Tris-fructose based extender (Bruyas et al., 1996). However, Rota et al. (1995) found that a Tris-based extender (see below) was markedly superior to skimmed-milk or citrate based extenders. Seager described an 11% lactose extender (Seager, 1969) which, with various subsequent modifications, has since been widely used (Lees and Castleberry, 1977; Marks et al., 1994).

The use of Tris-based buffers subsequently became popular. Foote (1964a) was the first to use a Tris-egg yolk-citrate buffer in the dog. Modifications of this buffer have subsequently been used widely. Indeed, the most commonly used diluent for freezing dog semen is now Tris-fructose-citric acid extender containing egg yolk and glycerol (Andersen, 1975). With this extender, a number of supplements were also included such as (i) methylxanthines, i.e. pentoxifylline (Koutsarova et al., 1997), (ii) amino acids, i.e. proline and glycine betaine (Peña et al., 1998a) and (iii) detergents, i.e. sodium dedocyl sulphate (Peña et al., 1998c) and Orvus WA paste (Thomas et al., 1992). Likewise, incorporation of a detergent such as Orvus ES Paste, with Tris-glucose based diluent, was also beneficial (Nizanski et al., 1997). As a modification of Tris-fructose-citric acid extender, Triladyl either alone (Dobrinski et al., 1993) or together with 0.5% Equex STM paste (Nöthling and Volkmann, 1993) was also used. With the latter diluent to which prostatic fluid was added post-thaw, for the first time in dogs, 100% pregnancy rate was achieved, even with intravaginal insemination (Nöthling and Volkmann, 1993). A similar pregnancy rate has been obtained by Silva and Verstegen (1995), who used Biociphos as a new freezing extender. However the semen was inseminated directly into the uterus by laparascopy.

Numerous studies into freezing dog semen have included comparisons between diluents, although many of the results have been contradictory. Smith (1984) and Smith and Graham (1984) found better post-thaw motility with Pipes-based extender than with Bes, Tes or Tris. Other workers found Pipes gave poor post-thawing fertility (Battista et al., 1988). These workers suggested that lactose extender was superior to Pipes for freezing in pellets and that Tris and Tes/Tris extenders were superior to Pipes or lactose for freezing in 0.5 ml straws for post-thaw survival. Thomas et al. (1993) found that Tris-glucose-citrate extender (compared to Bes-lactose) was the most efficacious combination for freezing either in pellets or in 0.5 ml straws. These findings agree with those of Olar et al.
(1989) and Ivanova et al. (1992). However, Yubi et al. (1987) reported that, when straws was used for packaging, there was no difference in the post-thaw survival in either lactose or Tris-fructose extenders. Ivanova-Kicheva et al. (1995), using Tris-fructose, Tris-glucose and Sucrose-lactose extenders with pellet freezing system, found that the optimum results (motility and survival) were achieved using Sucrose-lactose extender while the results for Tris-glucose were the lowest. However, there were no differences between the extenders in terms of spermatozoal morphology (i.e. the acrosome, midpiece and tail). In another study, Ivanova-Kicheva et al. (1997) compared Tris-fructose, Tris-glucose and lactose based extenders using pellets and aluminium tubes for packaging and showed that the overall results were optimum for lactose while those of Tris-fructose were the lowest. For tube packaging, optimum survival were achieved by using Tris-glucose. Koutsarova et al. (1996) compared Tris-fructose based extender with new extenders, i.e. commercial SGI extender supplied with blood serum, Bis-Tris-fructose, MOPSO-NaOH and MOPSO-KOH all of which contained egg yolk and glycerol, for post-thaw survival of spermatozoa in pellets. Their findings were:

(i) Tris-diluted spermatozoa did not differ from those with SGI and Bis-Tris, although it was better than both MOPSO-NaOH and MOPSO-KOH.

(ii) Bis-Tris was better than SGI at the beginning of storage, but the opposite occurred at the end.

(iii) Bis-Tris was better than MOPSO-NaOH.

(iv) There was no difference between MOPSO-NaOH and MOPSO-KOH.

Dobrinski et al. (1993), comparing Triladyl, Pipes, IMV Universal and Tris-fructose-citric acid extenders, concluded that although Triladyl and IMV Universal resulted in more vigorous post-thaw motility, the four extenders were equally suitable for freezing canine semen in straws. England (1992), comparing a variety of buffers (Bes, Caps, Ches, Hepes, Mes, Pipes, Tricine, Tris and Tes/Tris), found that Tes/Tris was the most successful diluent. Silva and Verstegen (1995), comparing Biociphos, Laiciphos and Tes/Tris extenders, have reported 100% pregnancy rate when Biociphos was used. The use of other extenders also produced pregnancies but only at 60%. In recent studies, Ström et al. (1997) and Peña et al. (1999) compared Tris-fructose based (Andersen, 1975) and a commercial CLONE extender (Govette et al., 1996) using different freeze-thawing
techniques. Whilst Govette et al. (1996) reported that both extenders resulted in high whelping rates, Ström et al. (1997) and Peña et al. (1999) found that sperm thermolability was more closely associated with the CLONE freezing. In all the studies, there were egg yolk and glycerol at a concentration of mostly 20%, v/v and 4-8%, respectively.

In conclusion, it appears that for dog semen;

- Tris-fructose (or glucose) -citric acid extender containing egg yolk and glycerol provides a suitable in vitro medium conditions for both cold storage (at +4°C) and freeze-thawing, not only in straws but also in pellets. Likewise, Triladyl (Tris-fructose based) extender may also be of value, especially when using with additives (e.g. Equex STM Paste).
- Lactose (or Sucrose-lactose) extender containing egg yolk and glycerol may be preferred to any other diluents especially for packaging in pellets or aluminium tubes.
- Additives (i.e. proline, sodium dodecyl sulphate, Equex STM Paste, Orvus Paste, pentoxifylline) should be incorporated in extender constituents for optimising post-thaw semen quality (by improving the spermatozoal membrane integrity and/or motility).
- Even with intravaginal insemination, optimum pregnancy rates (100%) are achievable using frozen semen to which prostatic fluid is added post-thaw.

Future studies of the role of diluents in cryopreservation of canine semen should be directed towards investigating the actual effects of prostatic fluid and additives using a variety of extenders. Also, different constituents of new extenders such as Triladyl and Biociphos should also be compared with Tris based and lactose based diluents and their inter-relationship with freezing rates and glycerol.

1.5- Cryopreservation of dog semen (II) Freezing technology
1.5.1- Semen packaging

The use of straws, pellets or tubes is not only a choice between alternative packaging methods. For the semen in straws, the rate of freezing can be precisely controlled by using a programmable freezer (Rota, 1998). With pellet freezing, there is a little control over the rate of freezing, although it does produce a rapid cooling rate that is associated with high post-thaw motility (Nizanski et al., 1997; Koutsarova et al., 1997). Freezing semen in
straws makes the processes of thawing and insemination of the bitch easier than for pellets. Fougner (1989) considered that medium (0.5 ml) straws were the best and most practical method for AI breeding with frozen semen in the fox. This is also the case in the dog as straw packaging appears to be preferred by the vast majority of workers for cryopreservation of semen.

The various containers used for freezing dog semen are:

- pre-scored glass ampoules (Martin, 1963a; Foote, 1964a)
- aluminium tubes: (i) 1 ml (Kosiniak et al., 1992), (ii) 5 ml (Ivanova-Kicheva et al., 1997)
- pellets (Seager, 1969)
- plastic straws: (i) 0.25 ml (Oettlé, 1986b), (ii) 0.5 ml (Andersen, 1975), (iii) 0.90 ml (Seager et al., 1975a), (iv) 1 ml (Takeisi et al., 1976), (v) 2.5 ml (Thomas et al., 1993).

Pellets and especially ampoules were adopted in many of the early studies of semen freezing. More recently, most semen has been frozen in plastic straws in varying sizes. Although Seager and Fletcher (1973) indicated that the recovery of spermatozoa was similar after freezing in either 0.25 or 0.5 ml straws or pellets, Seager et al. (1975a) subsequently used the pellet method, describing better post-thaw spermatozoal recoveries than following packaging in straws.

Other studies have indicated that the nature of the container is inter-related with both the diluent and the cooling conditions. For example, Battista et al. (1988) suggested that the post-thaw motility is better with pellets than with 0.5 ml straw packaging, especially with lactose diluent, which was superior to Pipes in pellets. However, for straws, Tris and Tes/Tris extenders were better than Pipes or lactose. Olar et al. (1989), using Tris-glucose and lactose based extenders in straws, found that motility was better in Tris. Thomas et al. (1993) compared Bes-lactose and Tris-glucose based extender in pellets and in 0.5 ml and 2.5 ml straws. They found that overall motility results were superior in Tris to Bes-lactose, but that precise results depended upon the freezing and packaging conditions to which the spermatozoa were exposed.

Nizanski et al. (1997) found that Tris-glucose extender was suitable for freezing in straws, minitubes or pellets. In another study, Ivanova-Kicheva et al. (1997) compared three
diluents (Tris-glucose, Tris-fructose and lactose) and two packaging systems (pellets and 5 ml aluminium tubes), again finding that the ideal diluent depended upon the packaging and cooling regimen.

Finally, Kosiniak et al. (1992) compared 0.5 ml straws and 1 ml tubes using Tris-fructose extender and found that straws were superior to tube packaging.

In conclusion, for freezing dog semen;

- Tris-glucose (or fructose) -citric acid extender containing egg yolk and glycerol appears to be superior not only in straws, but also in pellets.
- Lactose extender appears to be considerably better in pellets than in straws.
- Large size aluminium tubes (5 ml) appear to be better than pellets irrespective of widely used extenders (Tris- or lactose based).
- Medium size (0.5 ml) straws are used widely for semen packaging.

1.5.2- Cooling and equilibration before freezing

The spermatozoa of most mammalian species require a period above 0°C in the presence of a cryoprotectant to develop maximal resistance to the effects of freezing. It was originally believed that this was to allow the penetration of glycerol into the cells. However, Watson (1979) argued that this was not the case, as full penetration of glycerol into the sperm cell takes only 3-5 min at 25°C or 5°C (Berndtson and Foote, 1972). Rather, the equilibration period is to allow time for membrane changes and ionic fluxes to occur which render membranes more resistant to cooling (Watson, 1979). Ennen et al. (1976) showed that slowly cooled spermatozoa required a shorter equilibration time in comparison to rapid cooling before freezing. Additionally, the duration of cooling has been correlated with glycerol concentration (Wilmut et al., 1973), with higher motility and fertility being achieved with longer cooling and/or equilibration times (Martin, 1965; Martig and Almquist, 1966). Therefore, it appears that the acquired resistance to cooling above 0°C may provide extra resistance to freeze-thawing (Paquignon and Courot, 1976).

Optimum results have usually been obtained by cooling semen in a storage temperature of +4°C as soon as possible after collection to reduce metabolism of spermatozoa.

Few studies have fully evaluated either cooling rates or equilibration times before freezing in the dog. Seager (1969), who froze semen in pellets 3 h after collection, suggested that a
long equilibration period is not necessary with canine semen. Yubi (1984) concluded that between 2 h and 3 h should be allowed for equilibration at +7°C before freezing semen in either pellets or straws. In contrast, Olar (1984) suggested that, for storage in straws, there should either be 1 h cooling plus 1 h equilibration or 2 h cooling plus 2 h equilibration periods before freezing. Subsequently, Olar et al. (1989) demonstrated that spermatozoa withstood a range of cooling and equilibration times with no detrimental effect on motility prior to freezing, but that, after cryopreservation, there was an interaction between cooling time and equilibration time in determining post-thaw motility. In this context, Peña et al. (1998b) found no difference in post-thaw semen quality after 1 and 2 h equilibration. Bouchard et al. (1990) suggested that the semen should be cooled at medium (-0.3°C/min) or fast (-1.0°C/min) rates to a storage temperature of +4°C, although they did not study the effect of such cooling rates on subsequent post-thaw survival. A similar study was undertaken by Bruyas et al. (1996) with similar conclusions. A more precisely controlled cooling regimen was used by Dobrinski et al. (1993), who cooled semen at -0.5°C/min to 12°C, then at -0.6°C/min to +3°C and finally allowed it to equilibrate for 30 min. They achieved a moderate post-thaw survival.

England (1992) calculated cooling rates as follows:
For each of several cooling regimens, the mean rate of temperature change from 39°C to +5°C was calculated. Exponential curves were fitted to the mean cooling rates using the formula;

\[ t_{1/2} \text{(Half rate of cooling)} = A^{(k)} + C \]

Where
A= Initial temperature (39), C= Final temperature (5) and K= Fractional cooling per minute.

t_{1/2} and K were calculated and the curves were assessed using the correlation coefficient (r). He demonstrated that (i) a half cooling rate of 9.1 min or slower gave improved recoveries and (ii) the optimum equilibration time was 4 h. Similar values were also used later by England and Ponzio (1996).

Hay et al. (1997b) studied the effects of cooling rate upon oocyte penetration, acrosomal integrity and motility of spermatozoa. They showed that, after 3 h, cells that had been cooled quickly exhibited lower numbers of spermatozoa per ovum than in fresh or slowly
cooled samples. The cells that had been cooled quickly also exhibited a reduction in motility and acrosomal integrity. It was therefore concluded that slow cooling of canine spermatozoa to 0°C did not adversely affect their ability to penetrate oocytes. Nevertheless, although the slow cooling and long equilibration appear to be most beneficial, many workers have used arbitrary periods of cooling and equilibration before freeze-thawing.

In summary, it may be concluded that semen quality and fertility may be maximised by slow cooling (at a rate of between -0.1 to -1°C/min) to ~ +4°C (from +7 to 0°C) with equilibration for 2-4 h before freezing. However, few fertility data are available to substantiate the foregoing, so it would probably be valuable if such results were also supported by fertility trials.

1.5.3- Freezing
1.5.3.1- Principles
The rate of freezing is one of the most significant determinants of post-thaw survival and fertility of spermatozoa. On the one hand, a freezing rate that is too rapid causes the formation of intracellular ice and damages the cell by disrupting membranes of intracellular organelles. On the other hand, excessively slow freezing causes cell damage by permitting abnormal osmotic gradients to develop across the membranes during the formation of extracellular ice with cell membrane damage mainly occurring during the cell shrinkage and the resultant changes in osmotic pressure. The optimal freezing rate, which is mainly determined by surface area: water volume ratio of cells may vary with the extender, cryoprotectant concentration, species and thawing procedure. However, it has been suggested that spermatozoa are less sensitive to the cooling rate than most other cell types. The optimal cooling rates of spermatozoa lie between -10 to -170°C/min (Watson, 1979).

Freezing rate is also affected by sample volume and surrounding temperature. With pellets, there is little control over the rapidity of freezing, since it is a function of pellet size. The rate of freezing of semen in straws can be crudely controlled by placing the freezing rack in a higher or lower position in liquid nitrogen (LN2) vapour above the surface of the LN2 or it can be more precisely controlled by the use of programmable
biochemical freezers.

Five distinct processing steps can be recognized from cryopreservation to insemination. These are; (i) extension and cooling, (ii) penetrating cryoprotectant addition and packaging, (iii) freezing, (iv) storage, and (v) thawing and insemination. Each step has its own special relationship with membrane structure-function and cell metabolism altering the physical properties of cell membranes leading to cell volume excursions (Hammerstedt et al., 1990):

- The first volume adjustment of cells in the process cycle occurs in response to the addition of cryoprotectant in isotonic media. Its components are an initial fast shrinkage stage associated with osmotically driven egress of intracellular water, followed by a slower return to the original volume as the penetrating cryoprotectant enters.

- The second volume adjustment occurs when the extracellular water freezes. It reflects the outward movement of water in response to high concentrations of extracellular salts resulting from freezing of extracellular water.

Thawing yields analogous, but opposite volume changes. Since the organisation of membrane components can be changed by the processing cycle, the kinetics of water transport may not be equivalent throughout. The plasma membrane structure is not unique for internal, membrane-bound compartments of the spermatozoa such as the head, tail and intracellular membranes of the acrosome, nucleus and mitochondria (Eddy and O'Brien, 1994). All these membranes can be damaged, to a greater or lesser extent, by the freezing process, which allows breakdown of compartments whose separation is necessary for the cell to remain both functional and viable.

1.5.3.2- Freezing regimens

In some reports of cryopreservation of dog semen, rapid freezing rates were achieved by placing pellets of semen on a solid carbon dioxide (dry ice) block (Seager, 1969; England, 1992). In other reports, semen samples were frozen in ampoules (Foote, 1964a; Seager et al., 1975a) or in straws (Andersen, 1975) in LN2 vapour. As a slight modification of the latter freezing technique, an alcohol bath at -80°C was used to freeze the semen in straws before further freezing in LN2 vapour (England, 1992) or immediately plunging in LN2
Recently, aluminium tubes of different sizes (1 and 5 ml) were also used as a novel packaging technique for dog semen (Kosiniak et al., 1992; Ivanova-Kicheva et al., 1997). Ivanova-Kicheva et al. (1997), comparing 5 ml aluminium tubes and pellet methods, achieved a better quality of semen packed in aluminium tubes, but Kosiniak et al. (1992) showed that semen frozen in straws had considerably higher post-thaw motility than those in 1 ml aluminium tubes.

The effect of freezing rate upon dog spermatozoa has not been systematically evaluated (England, 1993). Nevertheless, attempts have been made to control the freezing rate by placing straws at different heights above LN2 (Dobrinski et al., 1993; Govette et al., 1996), on dry ice (Yubi et al., 1987; England, 1992), and either directly placing them in forced vapour freezers (Hay et al., 1997a; Rota, 1998) or by using cryomicroscopy (England, 1992). In the comparative investigations of freezing rates, most have simply compared various but undefined regimens (England, 1992; Dobrinski et al., 1993; Hay et al., 1997a,b; Ström et al., 1997). The most noteworthy of these trials was that of England (1992), who used a wide range of techniques to develop a practical freezing method, although he only compared three cooling regimens.

In the early studies, Foote (1964a) compared the effects of cooling rates of either -0.8 or -3 °C/min between +5°C and -15°C. Storage temperatures were either on solid carbon dioxide (at -79°C) or in LN2 (at -196°C) and the samples were thawed in an ice water bath. Although the faster rate led to apparently greater survival, no direct comparison between the freezing rates can be made since different storage temperatures and diluents as well as glycerol concentrations were used. In a later study from the same laboratory, Gill et al. (1970) used a slight modification of Foote’s together with fertility trials. Although 40-50% of spermatozoa had vigorous motility upon thawing, conceptions did not occur. Even the removal of glycerol after thawing was of no help. The failure might be due to intravaginal insemination and its timing in that male acceptance was the main criterion.

Subsequently, Andersen (1975), Takeisi et al. (1976) and Christiansen and Schmidt (1980) also froze dog semen in LN2 vapour, although the freezing rates were not given. Olar (1984), who used Tris-citric acid-glucose extender, which contained egg yolk and 3% glycerol in 0.5 ml French straws and different thawing rates, compared three rates for freezing. He found that a moderate rate (-5°C/min from +5 to -15°C, followed by -20°C/
min to -100°C) was markedly superior to either a fast (-75°C/min to -100°C) or a slow (-2°C/min from +5 to -15°C, followed by -10°C/min to -100°C) rate and its effect upon motility was apparent when fast thawing (at 75°C for 12 sec) was used. Smith and Graham (1984), using Pipes/KOH-citrate-glucose extender, which contained egg yolk and 9% glycerol, and thawing in a water bath at 37°C for 50 sec, found that the best freezing rate for dog semen packaged in 0.5 ml French straws was -1.89°C/min from +5°C to -100°C, followed by immersion of the straws in LN2.

The study of England (1992), who used Tes/Tris extender, which contained 10% egg yolk and 2% glycerol, and together with cooling rates of -10, -20, -40 and -60°C/min between +5 and -50°C, is worthy of further comment. In this trial, it was observed that the maximum post-thaw motility occurred at -20°C/min. While using the same extender in 0.5 ml plastic straws, he further compared the following regimens:

- In LN2 vapour 4 cm above LN2 (8 cm deep) for 10 min (Protocol A; -4.35°C/min from +5 to -10°C; -15.03°C/min to -50°C)
- On the surface of crushed/powdered dry ice covered immediately with powdered dry ice for 45 sec and then placed 4 cm above LN2 (8 cm deep) for 10 min (Protocol B; -15.98°C/min from +5 to -10°C; -20.31°C/min to -50°C)
- In a -80°C alcohol bath for 5 sec then placed 4 cm above LN2 (8 cm deep) for 10 min (Protocol C; -24.28°C/min from +5 to -10°C; -14.45°C/min to -50°C).

All samples were immersed into LN2 before thawing in a water bath at 39°C for 2 min. The finding was that although there was no difference in post-thaw spermatozoal motility between Protocol A and B, the values were significantly higher in Protocol C. This avoided the latent heat plateau (i.e. an exothermic reaction of a sample in liquid form as water moves to a solid state during supercooling below 0°C). Even minimising the latent heat plateau was shown to be beneficial in spermatozoa of other species, e.g. bulls (Parkinson and Whitfield, 1987; Liu et al., 1998). In another study, Dobrinski et al. (1993), using different extenders (i.e. Tris-fructose (with 8.8% glycerol), Pipes/KOH (with 9% glycerol), commercial Triladyl and IMV Universal (with 8% egg yolk) both with unknown concentrations of glycerol), studied three freezing regimens for cryopreservation of dog semen:
A slow freezing rate was generated by placing 0.5 ml French straws 20 cm above LN₂ for 30 min, with an average freezing rate of -5.1°C/min between +3 and -157°C. (-27°C/min between +3 and -135°C; -2.8°C/min to -150°C; -0.35°C/min to -157°C).

An intermediate freezing rate was resulted from placing the straws 12 cm above LN₂ for 20 min, with an average freezing rate of -8°C/min between +3 and -164°C (-29.6°C/min between +3 and -145°C; -2.9°C/min to -160°C; -0.4°C/min to -164°C).

A fast freezing rate was achieved by placing the straws 4 cm above LN₂ for 10 min, with an average freezing rate of -18.8°C/min between +3 and -191°C (-36.4°C/min between +3 and -179°C; -2.5°C/min to -191°C).

The straws were then immersed and stored in LN₂ before thawing in a water bath at 37°C for 2 min. The findings were:

(i) The slow, intermediate and fast freezing rates resulted in highest, intermediate and lowest motility values, respectively.

(ii) There was no difference between the extenders with regard to progressive motility.

(iii) All extenders were equally suitable for freezing canine semen at a slow freezing rate.

Since the start of the experiments reported in this thesis there have been a number of other studies (Ström et al., 1997; Hay et al., 1997a,b; Rota, 1998; Peña et al., 1999). These studies, using similar methods, were conducted in parallel with this thesis at roughly the same time and drew conclusions that are not dissimilar to those of the present studies. Therefore, detailed consideration will be given to them in the discussion of the relevant experimental chapters.

1.5.3.3- Thawing methods

It has been suggested that the warming phase of the freeze-thaw process is as important to cell survival as the cooling phase (Mazur, 1985). The effects of warming rate depend on the prior cooling and on the cell type (Watson et al., 1992a). Although the detrimental effects of rapid cooling have been recognized for many years, until recently rapid warming has been regarded as benign (Watson, 1995). Acrosomal damage that results from rapid warming has been described in the bull (Senger et al., 1976), buffalo (Narasimha Rao et al., 1986) and boar (Bamba and Cran, 1985) spermatozoa. However, fast thawing of semen in straws, i.e. at 70°C for 8 sec (Rota, 1998), 75°C for 6.5 sec (Andersen, 1975) or
12 sec (Aamdal and Andersen, 1968, in bulls), was also found to be successful.

Straws of frozen semen are frequently thawed by immersion in cold or warm water, whereas pellets are commonly thawed in physiological saline or citrate solutions. In dogs, several thawing procedures have been reported to be satisfactory for post-thaw motility. For pellets of semen in a lactose-egg yolk extender, thawing solutions of 2.5 ml of 37°C saline or citrate (Seager, 1969) or in Tris-fructose extender, 0.5 ml of saline have been used with success (Ivanova et al., 1999). Semen frozen in straws has been successfully thawed in a 75°C water bath for 6.5 sec (Andersen, 1975) or in a 37-40°C water bath for 1 min (Morton, 1988). Yubi (1984), comparing three thawing rates (i.e. 75°C for 6 sec; 37°C for 2 min; 7°C for 3 min), found that the most rapid thawing produced a decline in the percentage motility and live spermatozoa as compared to slower rates. Smith (1984) compared different thawing temperatures (5, 20, 37, 45 and 60°C) as well as thawing techniques (pelleted semen: on an aluminium block; semen in straws: in a water bath or in the air). She found that the optimum motility occurred with a straw that was thawed in a water bath at 37°C for approximately 50 sec. In contrast, Olar (1984), comparing three thawing rates (i.e. 75°C for 12 sec, 35°C for 30 sec, 1°C for 120 sec), obtained the best results after thawing at 75°C for 12 sec. An improved motility has also been reported after thawing at 70°C (Battista et al., 1988; Farstad and Andersen Berg, 1989; Rota, 1998). Ivanova-Kicheva et al. (1995) compared two thawing rates (37°C for 8 sec and 55°C for 5 sec) and achieved better results with the latter. In contrast, Kim and Kim (1995) found no difference between 5°C for 30 min, 37°C for 30 sec and 75°C for 10 sec. Finally, for pelleted semen, a novel thawing technique (also see Smith, 1984), which consisted of an aluminium funnel warmed to 40°C (without using a thawing solution), was also shown to be satisfactory (Nizanski et al., 1997).

It was suggested that for dog semen frozen in straws, post-thaw motility was better after fast thawing than the slow (Concannon, 1991). However, this may be due to the fact that no systematic studies of freezing rates have been undertaken for dog spermatozoa (England, 1993). Cryoprotectant concentration and the freezing rate can significantly affect the success of the thawing rates as faster freezing rates require fast thawing and vice versa. Watson (1995), considering the data in many species, indicated that care must be exercised with rapid thawing from the cryopreserved state. However, it is expected that
as the slower freezing rates become available for each species, slower thawing rates (e.g. at body temperature) could be employed more widely.

1.5.3.4- Influence of individual dog and breed
In all species, there is a considerable variation in the ability of semen from different individuals to withstand freezing and thawing (Watson, 1979). Indeed, on the basis of the freezability of semen, individual to individual variation has been demonstrated in human (McLaughlin et al., 1992), boar (Larsson et al., 1976; Medrano et al., 1998), stallion (Cochran et al., 1983), bull (Parkinson and Whitfield, 1987) and fox (Farstad et al., 1992). In the dog, early studies led to the conclusion that semen from certain sires freezes better than from others (Seager and Fletcher, 1973), and similar effects have also been shown from a number of later investigations (Dobrinski et al., 1993; Thomas et al., 1993; Rota, 1998). However, Nöthling et al. (1997), using frozen semen, found no significant effect of individual dogs on the fertility. It is important to recognize the considerable variation in pre-freeze quality (morphology, live/dead ratio) of spermatozoa within individual dogs (England and Allen, 1989b) when choosing a stud dog as a donor for cryopreservation of semen. It may be that freezing conditions could be optimised in individual animals to improve spermatozoal survival, as is the case in bulls (Parkinson and Whitfield, 1987).

Although breed influences have been noted in the post-thaw fertility of boar spermatozoa (Johnson et al., 1981), no such relationship has been established in the dog (England, 1993). This is probably due to the lack of systematic studies rather than the lack of any such effects.

1.6- Method of insemination
1.6.1- Technique of insemination
The artificial insemination of bitches with fresh or frozen semen resulted in varying success that remains the subject of controversy. Although it is generally accepted that the deposition of fresh semen into the vagina or uterus gives results that are as high as natural mating, artificial insemination with frozen semen has been less successful. Furthermore, the results of insemination of frozen semen have varied greatly from one study to another. According to Silva et al. (1996), this variation may be due to:
   i. the large variations in the protocols used,
   ii. the route of insemination (vaginal or uterine),
iii. the quality of semen before freezing and after thawing,
iv. the semen volume or concentration,
v. the number and timing of the inseminations, and
vi. the method for freezing and thawing the semen.

It is known that the unfrozen (fresh) spermatozoa may survive in vivo for long periods up to 268 h (Doak et al., 1967) and remain fertile an average of 5 to 7 days in the uterus of the oestrous bitch (Concannon et al., 1983; Tsutsui, 1989). Ellington et al. (1995), using the isthmus portion of the uterus, suggested that dog spermatozoa could remain viable in vitro for approximately 7 days whilst in contact with cells of the female reproductive tract.

When dog spermatozoa are thawed from the frozen state they have a comparatively short period (i.e. 1 to 8 h) of motility in vitro, especially at body temperature (Olar, 1984; England and Ponzio, 1996). The period of in vivo survival was considered to be approximately one day in the uterus of the oestrous bitch (Badinand et al., 1993). Andersen (1972a,b) noted that when frozen-thawed semen was introduced into the uterus, there was a good fertility, but no pregnancies occurred following vaginal insemination. There is no data available regarding survival of spermatozoa in the vagina of the bitch. Some reports exist of good pregnancy rates following vaginal insemination of semen that was frozen in pellets (Seager et al., 1975b), but high pregnancy rates have not generally been produced using vaginal insemination of semen frozen in straws (Olar et al., 1989; Kabasakal, 1995; Linde-Forsberg et al., 1999). An exception to this was the results of Nöthling and Volkmann (1993). They suggested that when dog semen was frozen in Triladyl extender, containing 0.5% Equex STM paste, the post-thaw addition of autologous prostatic fluid offers an easy and repeatable method for obtaining optimum fertility (100% pregnancy rate) after intravaginal insemination. These findings were later confirmed (Nöthling et al., 1997) and therefore their technique may be of practical value.

Despite the limited success of transvaginal AI, more acceptable pregnancy rates have usually been achieved when either transcervical intrauterine- or intracervical insemination of the bitches has been employed (Fontbonne and Badinand, 1993a; Linde-Forsberg et al., 1999). Silva et al. (1996) suggested that both vaginal and intrauterine
techniques might be of value for AI of the bitches with fresh or frozen semen, provided that the timing of the insemination is accurately determined and the semen is from fertile dogs. However, their results from intravaginal insemination were not comparable to those achieved by using freshly inseminated semen. In the canine, transcervical intrauterine deposition of semen by AI is difficult due to the long and narrow vagina and, long and rather tortuous cervical canal of the bitch. Developments in endoscopic catheterization of the cervix of the bitch have, however, resulted in high pregnancy rates (83%) using frozen semen in Tris based extender (Wilson, 1993). With this insemination technique, an acceptable pregnancy rate (75%) also achieved by England (1992), using frozen semen in Tes/Tris extender. Linde-Forsberg et al. (1999), using frozen semen in CLONE extender (Govette et al., 1996), obtained a lower whelping rate (58%) which could have been due either to insufficient training in fiberoptic endoscopy, post-thaw thermolability of spermatozoa or the extender used. In a previous study by Battista et al. (1988), using a similar technique but different extender (Pipes), the pregnancy rates were also relatively low (25%). They considered that differences with other studies might reflect differences in semen freezability of the stud dog(s) used and extender formulation or freezing rate.

In some of the earlier studies, frozen-thawed semen had also been inseminated surgically directly into the uterus of a bitch, producing live pups (Andersen, 1972a,b). Smith and Graham (1984) and Olar (1985), using a similar technique, only produced 57 and 60% pregnancy rates, respectively. This might have been due to improper insemination timing and/or poor quality of frozen-thawed semen. Recently, a more sophisticated surgical technique (laparoscopic intrauterine insemination coupled with an accurate measurement of blood hormone levels in bitches) has been described (Silva et al., 1995). With this technique, inseminations with fresh semen resulted in 100% pregnancy rate and, the litter size was equal to those of naturally mated bitches. Subsequently, Silva and Verstegen (1995) achieved similar results with frozen semen in Biociphos extender while semen that was frozen in either Laiiciphos or Tes/Tris extender yielded lower but acceptable pregnancy rates (at 60% both). However, the use of surgery to accomplish intrauterine semen deposition is considered by many to be unethical, unacceptably stressful and risky for the bitch, and it is time-consuming and
expensive (Linde-Forsberg et al., 1999). Nevertheless, surgical intrauterine insemination may be used in breeding management programs, in cases of apparent infertility (Brittain et al., 1995) or insemination of frozen-thawed semen in certain breeds (Vaughan, 1998).

A similar laparoscopic technique for intrauterine insemination of frozen semen that was obtained from the testicles of a male dog just after its death has also resulted in the delivery of a live pup (Marks et al., 1994).

Because of the difficulties in determining optimum insemination time, a number of recent studies have focused on ovulation time as an indicator of the ‘fertile window’ in the bitch. Badinand et al. (1993) considered that ovulation is likely to be between 4 and 8 days after the surge of plasma luteinising hormone, LH. As the LH surge is followed by a rise in progesterone level, they suggested that AI with frozen semen should be performed 1.5-4.5 days after the progesterone concentration is >5 ng/ml. Their findings also confirmed that day 0 (the day of onset of cytological diestrus) usually occurs 7-8 days after the LH peak. Nöthling et al. (1997) suggested that; (i) fertility is highest if inseminations are performed on day -3 and day -2 and (ii) it is essential that frozen-thawed semen is inseminated on day -2. LH peak is a brief event that can be used as a marker of the oestrus cycle and is the most reliable reference for estimating the date of ovulation (Guérin et al., 1997).

However, the indirect observation of the time of peak circulating LH concentration is possible by the detection of increasing plasma concentrations of progesterone (> ~8 ng/ml), which appears to be the most reliable method in predicting the onset of optimal mating time of the bitch (Jeffcoate and England, 1997). Consequently, the fertile period can be detected using plasma measurements of progesterone (as the most accurate method; Jeffcoate and England, 1997) and LH (Jeffcoate and Lindsay, 1989; Guérin et al., 1997) or vaginal endoscopy (Lindsay, 1983) and vaginal cytology (Wright, 1990; Jeffcoate and England, 1997). Other techniques such as using LH in the urine (Jeffcoate and England, 1997), ferning, i.e. crystallisation patterns (England and Allen, 1989a) or electrical resistance ( Günzel et al., 1986) of cervicovaginal mucus, and ovarian ultrasonography (Wallace et al., 1992) may also be of value.

Thus, the consensus from the literature is that high fertility in the bitch can mostly be obtained from the intrauterine insemination of frozen semen, although intravaginal insemination has also proved successful in a few cases. In consequence, the main factors
which affect fertility are likely to be; (i) the facility with which a viable population of spermatozoa can be established within the uterus, (ii) the duration of viability of frozen-thawed spermatozoa that is less than that of freshly ejaculated spermatozoa, and (iii) the timing of insemination in relation to ovulation.

1.6.2- Numbers of spermatozoa

There are a number of aspects of the reproductive physiology and anatomy of the bitch, which constrain the numbers of spermatozoa required for successful insemination. The constraints imposed by the anatomy of the genital tract of the bitch have already been mentioned. The oestrus period of the bitch is long, 3-21 days (Linde-Forsberg, 1991) and the ova may require 1-3 days to mature and to cast off the polar bodies following ovulation (Tsutsui, 1989; Feldman and Nelson, 1996). Hence, the fertile life of matured ova may be an additional 2-4 days. Unless the timing of ovulation is known, spermatozoa need to survive for at least 5 to 7 days in the female genital tract (Concannon et al., 1983; Tsutsui, 1989).

Therefore, successful inseminations have usually used large numbers of spermatozoa (Seager et al., 1975a; Platz and Seager, 1977) and/or frequent inseminations (Silva et al., 1996; Nöthling et al., 1997; Pinto et al., 1999). Seager et al. (1975b) suggested that an 85% conception rate could be achieved with an intravaginal insemination of 50 x10^6 spermatozoa in frozen-thawed semen. However, subsequent studies from this group used a 200-800 x10^6 spermatozoa for an average of four inseminations per bitch (Platz and Seager, 1977). With fresh semen, such insemination doses (at least 200 x10^6 viable spermatozoa) were used up to four times to achieve normal conception rate with intravaginal insemination (Tsutsui et al., 1988). They also observed that there was a marked difference between conception rates of bitches that were inseminated with 100 and 200 x10^6 spermatozoa. In contrast, Nöthling and Volkmann (1993) achieved 100% pregnancy rates using a daily insemination of 100x10^6 progressively motile spermatozoa. Nöthling et al. (1997) suggested that a single intravaginal insemination dose of 100-110 x10^6 progressively motile spermatozoa, or twice (24 h apart) is adequate to achieve a mean implantation rate of 75% or higher. However, they also concluded that it is likely that a higher number of spermatozoa per insemination will yield higher fertility (especially after intravaginal insemination) up to a threshold. Indeed, Linde-Forsberg et al. (1999) reported
considerably higher whelping rates as the concentrations of total spermatozoa were increased from $\leq 100 \times 10^6$ to $400 \times 10^6$ for each intravaginal insemination, but this was not the case when the intrauterine insemination was used.

Generally, intrauterine insemination requires fewer spermatozoa per insemination than the intravaginal technique (Linde-Forsberg et al., 1999). Whilst $150-200 \times 10^6$ normal motile spermatozoa inseminated daily on two or three occasions is usually adequate in the bitch (Morton and Bruce, 1989), two intrauterine insemination doses of $30-35 \times 10^6$ (as performed 48 h apart; Wilson, 1993) or $75 \times 10^6$ (as performed once; Fougner, 1989) live spermatozoa have also been successful in the bitch and in the fox, respectively. Since, it has been shown that a proportion of spermatozoa could be alive but non-motile after thawing (Soosula et al., 1975), investigations into this proportion may also be relevant to considerations of the number of spermatozoa that are required for insemination.

1.7- Summary
1.7.1- Summary and conclusions

In the last four decades, there have been great improvements in the methodology and success of cryopreservation of dog semen. However, there are a number of major problems remaining. These include;

i. variability of both pre- and post-freezing quality of semen from fertile individuals,
ii. poor viability, that is associated with substantial acrosomal damage post-thaw,
iii. variation in the ability of semen between and within individuals to withstand cryopreservation,
iv. determining the minimum number of spermatozoa that are required to ensure optimum conception rates and litter size,
v. identifying the time of optimal fertility in the bitch, and
vi. achieving intrauterine insemination.

A greater understanding of the normal reproductive physiology of the bitch (Concannon et al., 1977) has allowed more exact timing of insemination, although the day of maximum fertility has yet to be fully established for insemination with frozen semen. Significant improvements have also been made with techniques of AI, especially in the development of non-surgical methods of intrauterine insemination (Linde-Forsberg,
1991; Wilson, 1993; England, 1998). Even so, very reasonable pregnancy rates (i.e. 87-100%) have been achieved with intravaginal insemination of fresh and chilled (Pinto *et al.*, 1999) or frozen (Nöthling *et al.*, 1995, 1997) semen.

Although some advances have been made with extenders and freezing technologies, these have generally lagged behind those made in other species. New packaging systems such as large size aluminium tubes may result in a better spermatozoal survival (Ivanova-Kicheva *et al.*, 1997). Likewise, slow cooling rates above 0°C may improve the spermatozoal membrane quality and oocyte penetration capability (Hay *et al.*, 1997b). Some advances have been made in understanding the requirements of diluents for successful cryopreservation.

It is likely that poor spermatozoal viability after thawing (thermolability) is the main cause of low pregnancy rates, especially after intravaginal insemination. So far, little attention has been paid to improving post-thaw survival of spermatozoa through the precise control of freezing conditions. Yet, it could be postulated that it is in the area of controlling freezing rate that the greatest potential for improving survival of spermatozoa lies. Indeed, the acrosome, as the critical effector of spermatozoal functionality, is highly vulnerable to both of the main stressors of cryopreservation *viz.* ice crystal damage and osmotic damage (Oettlé, 1986b).

1.7.2- Hypotheses and experimental plan

The major objective of this thesis is to maximise the survival of functionally intact spermatozoa after cryopreservation. It is postulated that this objective can be achieved by a detailed examination of the losses of spermatozoa at each stage of the freezing curve, using a variety of cooling regimens. It is further postulated that the key identifier of success is the presence of large numbers of motile sperm that possess functional acrosomes.

In order to investigate these hypotheses, it was first necessary to:

- establish repeatable regimens for dilution, glycerolisation and equilibration of spermatozoa and, in parallel,
- develop a method for *in vitro* assessment of the functional integrity of the acrosome (in fresh and chilled spermatozoa).
Having achieved these objectives, it was proposed to:

♦ investigate the effects of freezing regimen upon survival of spermatozoa over the segment of the freezing curve that is known, in other species, to be responsible for the majority of losses of spermatozoa, namely from +4 to -35°C, and

♦ re-examine the inter-relationships between the losses over this part of the freezing curve with those that occur subsequently during cooling to -120°C.

The general method that was proposed to achieve this objective was to:

□ undertake initial examinations of the effects of the foregoing regimens upon spermatozoal survival by using the simple, but subjective measure of post-treatment motility assessment, so that the parameters that lead consistently to maximal survival of spermatozoa can be established, and

□ re-examine these regimens in terms of their effect upon the objective measure of spermatozoal functionality that is afforded by determination of acrosomal integrity (during the acrosome reaction in vitro).
CHAPTER 2. PRELIMINARY STUDIES

The main hypothesis of this thesis was that freezing conditions of canine semen can be optimised and that motile sperm with functional acrosomes can identify successful cryopreservation.

In order to examine this hypothesis, a number of preliminary investigations were required. Methodologies had to be established for the assessment of acrosomal integrity. It was also necessary to establish diluent and dilutions as a prelude to any attempts to optimise freezing conditions for spermatozoa.

2.1- Experiment 1: Storage of Tris-extended semen at +4°C

Hypothesis:

Different dilutions of Tris extender, glycerolisation (its concentration and addition temperature) and presence of seminal plasma will affect sperm survival at +4°C.

2.1.1- Introduction

In the course of the literature review, it was concluded that the diluents which have been most widely and successfully used for the cryopreservation of dog semen are those based upon Tris-egg yolk extenders (see Linde-Forsberg, 1995 for detail). It was decided to use an extender (Tris) that was closely based upon that described by Rota et al. (1995).

As discussed in Section 1.2.1, diluents have to provide protection to spermatozoa during a number of phases of the cryopreservation process.

The basic model of the biological membrane is of a phospholipid bilayer with integral proteins and surface-attached glycoproteins and glycolipids, which are arranged in a fluid mosaic (Singer and Nicolson, 1972). This structure is true of spermatozoal membranes (Watson, 1995), although the particular combination of lipids, phospholipids and cholesterol are unique to spermatozoa (Eddy and O'Brien, 1994). White (1993), who reviewed the role of lipids and calcium uptake in relation to cold shock of spermatozoa, concluded that the susceptibility of spermatozoa to cold shock is linked with a high ratio of unsaturated: saturated fatty acids in the phospholipids and a
low cholesterol content. These two factors would give a more open, less stable, and cohesive membrane structure to the spermatozoa than in many other cells. Watson (1995) considered that bilayer asymmetry may also be an important function of spermatozoal plasma membranes, since breakdown in asymmetry is characteristic of capacitated spermatozoa (Yanagimachi, 1994; Fraser, 1998). Apparently, such membranes become fusogenic and more permeable and labile, which can lead to a shortened life of the cell. Moreover, it was considered that since enzymes are temperature dependent, a disruption of bilayer asymmetry might be associated with cooling and rewarming (Watson, 1995). It is perhaps surprising, therefore, that in the face of such complex changes to membrane structures during the development of cold shock, that such relatively simple means as the addition of skimmed milk and/or egg yolk can protect the membranes from many of the lesions that are associated with cooling. Nevertheless, although little is understood of the details of the mechanisms, by which such simple sources of lipids protect spermatozoa against cold shock, the inclusion of such substances in diluents is an effective means of doing so.

Amongst the many other variables which contribute towards the success rate in cryopreservation of semen is the potential toxicity of cryoprotective agents such as glycerol. Glycerol is the most commonly used cryoprotectant, since this role was first discovered by Polge and his co-workers (Polge et al., 1949) in bovine spermatozoa. However, as discussed in Section 1.4.2, there is a compromise between its protective and toxic effects (Watson, 1979) and, in general, it is used at a concentration of 4-9% (v/v) depending on the cooling rate and species (Watson, 1990). The mechanisms by which glycerol acts as a cryoprotectant were considered in Section 1.2.2. However, some consideration also needs to be given to the means by which it exerts a toxic effect.

The toxic role of glycerol was reviewed by Hammerstedt and Graham (1992), who considered there to be three main effects: (i) osmotic stress associated with the introduction and removal of molar concentrations from the cell interior, (ii) modification of the lipid bilayer by the insertion of glycerol among membrane phospholipids, and (iii) potential of glycerol to become involved in intermediary metabolic pathways. It has often been assumed that adverse effects of glycerol on spermatozoa might be diminished as the addition temperature is lowered, and hence it is
frequently added at around +5°C (Watson, 1990). On the other hand, glycerol penetrates human spermatozoa readily at ambient temperature, but its permeability declines rapidly with lowering temperatures (Watson et al., 1992a).

Following addition of glycerol to spermatozoal suspensions, the cells undergo a transient reduction in volume, in response to anisosmotic conditions. It has therefore been further suggested that glycerol should be added in such a manner as to minimise harmful excessive cell shrinkage by introducing it slowly (Watson et al., 1992a). Consequently, Watson et al. (1992a) considered that since glycerol permeability decreases so markedly with lowering temperature, its addition temperature becomes a relevant consideration. At present, however, there is still controversy on this subject, since some workers have found no detrimental effects of a one-step dilution (containing glycerol) at 30°C (Salamon and Ritar, 1982) while others considered it better to undertake a two-step glycerolisation at +5°C (Graham et al., 1978).

The significance of seminal plasma as a potential source of damage to spermatozoa during cooling and/or cryopreservation has been debated for many species. For the dog, England and Allen (1992b) demonstrated that the first and the third fractions of the ejaculate might adversely affect semen quality with prolonged (up to 6 h) storage at 37°C. Furthermore, Platz and Seager (1977) demonstrated that centrifugation to concentrate canine semen does not interfere either with spermatozoal quality or with fertility. Removal of seminal plasma and the use of a smaller amount of diluent results in concentration of the spermatozoa in a small volume, which can help to prevent harmful effects of higher dilutions (Wales and White, 1963). Moreover, there are a number of reports from other species of toxic interactions between components of the seminal plasma and components of diluents (chiefly egg yolk and/or milk). Taken together, these data indicate that there is a necessity to re-examine the role of seminal plasma in cooling and cold shock in canine semen as a prerequisite for successfully undertaking freezing trials.

Finally, there has been much debate over the extent to which semen can be diluted during cooling or cryopreservation. The debate has centred on whether there is a "dilution effect" that causes spermatozoa to die, simply as a consequence of dilution, or whether the losses of spermatozoa that occur during dilution occur irrespectively of the
degree to which the cells have been diluted. \textit{A priori}, it might be considered that if the diluents were perfect and there was no interaction between the components of seminal plasma, diluent and cells, the survival of cells would not be affected by the degree of dilution. Moreover, in an ejaculate, the concentration of spermatozoa can vary greatly between collections, between animals and within animals. Nevertheless, as spermatozoa need to be diluted for cryopreservation, the optimum dilution rate and the debate over the presence or absence of a "dilution effect" is of a major concern. This is particularly so as, for ruminants (especially bull), it has been clearly established that in a high quality (i.e. near ideal) diluent, no dilution effect exists on fertility (Foote, 1970). It is known that, for dog semen (i.e. for spermatozoa plus seminal plasma), the dilution rate may vary between 1:1 to 1:5 (Andersen, 1980) or to 1:8 (Christiansen, 1984) depending on the initial concentration of spermatozoa. Likewise, although England (1992), using Tes/Tris buffer, found that the optimum dilution rate is 1:4 (semen: extender, v/v), the interactions of diluents with either the presence/absence of seminal plasma or with glycerolisation (concentration and temperature of addition) are not known.

Therefore, the objectives of this preliminary investigation were to determine, for canine semen diluted in a Tris-egg yolk diluent:

- optimal dilution rates and glycerol concentrations
- whether interactions exist between glycerolisation temperature, dilution rate and the presence or absence of seminal plasma.

It was decided, for this study, that the evaluation of spermatozoal survival would be based on the maintenance of motility at +4°C for up to 48 h. The use of motility, rather than acrosome reactions was preferred as; (i) it was necessary to undertake these studies before a method of acrosome assessment could be validated and (ii) acrosomal assessments are likely to be too laborious to be justified in such a straightforward preliminary experiment, whose conclusions would also be subjected to assessments of acrosomal integrity in later experiments.

\textbf{2.1.2- Materials and Methods}

Two experiments were undertaken:

- Experiment 1a) Investigation of the effects of removal of seminal plasma by centrifugation (i.e. 275 g for 5 min), temperature at which glycerol (8\%, v/v) was
added (i.e. room temperature against +4°C) and dilution rate (i.e. 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32), and
- Experiment 1b) Similar effects were further investigated over a narrower range of dilution rates (i.e. 1:2, 1:4 and 1:8) and at a range of concentrations of glycerol (i.e. 0, 0.5, 1, 2, 4 and 6%).

2.1.2.1- Experiment 1a

Two ejaculates were collected from each of three dogs (Dogs A, B and C; see Appendix 2.1 for detail), as described in Section 2.1.2.3.

Following initial evaluation (see Sections 2.1.2.4 and 2.1.2.6), each ejaculate was divided into two aliquots. One of these was centrifuged at 275 g for 5 min to remove the seminal plasma. After discarding the supernatant, the pellet was resuspended in a Tris-fructose-citric acid extender containing 20% egg yolk (see Appendix 2.2) to the pre-centrifugation volume. The second aliquot was kept at room temperature during this period.

Each aliquot was then subdivided into two and a series of doubling dilutions in Tris extender was prepared to produce dilutions of 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 for each aliquot. Samples were then glycerolised, by the addition of 8% glycerol (v/v) at room temperature or +4°C as follows:
- Centrifuged, glycerolised at room temperature (C-RT, Aliquot 1)
- Centrifuged, glycerolised at +4°C (C-4°C, Aliquot 2)
- Non-centrifuged, glycerolised at room temperature (N-RT, Aliquot 3)
- Non-centrifuged, glycerolised at +4°C (N-4°C, Aliquot 4).

Spermatozoal motilities were estimated (see Section 2.1.2.6) in all aliquots immediately after the addition of glycerol into Aliquots 1 and 3 at room temperature. This was counted as time zero h. After cooling at +4°C for 1 h, the same concentration of glycerol was added into Aliquots 2 and 4 at +4°C. All samples were then further equilibrated at +4°C for 1 h. Spermatozoal motilities were evaluated again as time 2 h and continued at 4, 6, 8, 24 and 48 h.

A summary of the experimental procedures is given in Figure 2.1:
Figure 2.1. Experiment 1a: Summary of procedures
2.1.2.2- Experiment 1b

Two ejaculates were collected, as in Experiment 1a, from each of the same three dogs (see Appendix 2.1 for detail), as described in Section 2.1.2.1. Following initial evaluation, the seminal plasma was removed. After discarding the supernatant, the pellet was resuspended in Tris-fructose-citric acid extender containing 20% egg yolk to the pre-centrifugation volume. A series of doubling dilutions was prepared to produce dilutions of 1:2 (Aliquot 1), 1:4 (Aliquot 2) and 1:8 (Aliquot 3). Thereafter, each aliquot was divided into six subsamples for glycerol. Following the assessment of motility (counted as time zero h), all samples were cooled at +4°C for 1 h. The samples were then glycerolised at concentration of 0, 0.5, 1, 2, 4 and 6% (v/v) at +4°C. Finally, all samples were further equilibrated at +4°C for 1 h before motility was assessed. This assessment was counted as time 2 h and further assessments were made at 4, 6, 8, 24 and 48 h.

A summary of the experimental procedures is given in Figure 2.2:
SEMEN SAMPLE
(3 Individuals x 2 ejaculates)

Initial assessment
(volume, colour, motility, concentration & morphology)

Removal of seminal plasma
(Centrifugation at 275 g for 5 min) &
Resuspension in Tris (to pre-centrifugation volume)

"Aliquot 1"
Dilution at 1:2
(6 tubes)

"Aliquot 2"
Dilution at 1:4
(6 tubes)

"Aliquot 3"
Dilution at 1:8
(6 tubes)

Assessing motility (as time 0 h)

Cooling (at +4°C for 1 h)

Glycerolisation
(0, 0.5, 1, 2, 4, 6%; v/v, final conc.)

Equilibration (at +4°C for 1 h)

Assessing motility
(as times 2, 4, 6, 8, 24, 48 h)
with a routine lab. microscope
(Magn. 200x, dark ground illum.)

Figure 2.2. Experiment 1b: Summary of procedures
2.1.2.3- Semen collection

Dog semen contains three fractions (Seager, 1986) as follows: The first fraction is clear (as it contains very few spermatozoa), has a volume of 1-2 ml in a medium size dog, and it is released during the first 1-2 min of coitus. The second, sperm-rich, fraction is normally white in colour. The colour varies between cloudy, milky or creamy, depending on the concentration of spermatozoa, which varies from 200 to 1,200 x10^6/ml (Farstad, 1998). The volume of the second fraction is between 0.5 and 1.0 ml. Ejaculation of this fraction occurs over a few seconds, 1 or 2 min after the start of coitus or semen collection, respectively. The third fraction (which contains prostatic fluid) is clear. Its volume is very variable, from 2 to 40 ml, and it is released during the final 5-25 min of ejaculation.

The technique of semen collection was that described by Allen (1992). No teaser bitch was used. This simple method was chosen as it avoids exposure of the semen to an artificial vagina. The lubricant jelly (Froman and Amann, 1983), or contact with vinyl or latex gloves (Althouse et al., 1991) and the rubber of the artificial vagina (England and Allen, 1992a) all have deleterious effects upon spermatozoal motility and morphology.

The collection procedure is summarised as follows:

- The penis was massaged through the wall of the prepuce and the *bulbus glandis* began to engorge. The penis was then grasped (through the prepuce) behind the *bulbus glandis* so that a full erection could be obtained.
- Continued pressure or rhythmic squeezing of the base of the *bulbus glandis* stimulated further engorgement of the penis until the full erection was gained.
- After a brief period of quiescence (as was the case with most of the collections), the dog then started thrusting movements, which ceased just before the second fraction was ejaculated (Christiansen, 1984). The second, sperm-rich fraction is released in 4-10 urethral contractions (Allen, 1992).
- Once the ejaculation of sperm-rich fraction was completed, the clear third fraction appeared almost immediately. Collection stopped once between 0.5 and 1.0 ml of the third fraction had been collected.
• The ejaculate was collected in a warm, 400 ml plastic pot and it was then transferred into a warmed 5 ml bijou for transport to the laboratory at body temperature.

Seager (1986) recommended that the dog should have a minimum of 4 days of sexual rest before semen evaluation. Subsequently, however, England (1999), studying the effect of a short-interval (at a mean of 63 min) until the second ejaculation upon the semen quality, found that there were no differences in the percentage of motility or the percentage of morphologically normal live spermatozoa. Furthermore, there was no strictly timed collection scheme during the course of the present study due to limitations such as availability of the dog, the duration of both the experiments and the assessment procedures. Therefore, in the present experiments at least three days interval was allowed between successive collections from each dog.

2.1.2.4- Initial evaluation of semen
Following collection, semen was transferred to the laboratory and kept at 35°C during initial evaluation. The colour and the volume of each sample were determined, and spermatozoal concentrations were measured with an improved Neubauer haemocytometer. Estimation of spermatozoal motilities were undertaken (see Section 2.1.2.6) on a microscope (Leitz, Wetzlar, Germany) under dark ground illumination. Slides were prepared on a warm stage for assessment of spermatozoal morphology (Barth and Oko, 1989) after staining with nigrosin/eosin (George T. Gurr Ltd., London, England).

Before preparation of morphology smears, slides and nigrosin/eosin stain were kept at 35°C. Smears were prepared by mixing one drop of semen with two drops of stain in a warmed tube, smearing onto a warmed slide and drying the smear at room temperature. The ratio of live to dead spermatozoa and the proportion of morphologically normal cells were evaluated on the stained smears (Barth and Oko, 1989), using a routine laboratory microscope (Leitz, Wetzlar, Germany) under immersion oil at a total magnification of 1,000x. One hundred spermatozoa were counted from each slide. Any semen sample with less than 60% morphologically normal spermatozoa was excluded from the study (Oettlé, 1993).

2.1.2.5- Diluent
Semen was diluted in a Tris-fructose-citric acid diluent containing 20% egg yolk (v/v). Full details of the diluent are given in Appendix 2.2.
The pH of the basic Tris-fructose-citric acid diluent was adjusted to 6.70. Batches of diluent were kept at -20°C until needed. After thawing, 20% egg yolk was added to the diluent to produce the final extender. Following the addition of egg yolk, the final extender (referred here to as ‘Tris extender’) was stored at +4°C up to five days.

2.1.2.6- Evaluation of spermatozoal motility

Motility of spermatozoa was assessed as part of the initial examination of the ejaculate and to determine the effects of the treatment regimens used in the above experiments. An identical method was used in both assessments. One drop of semen or of diluted spermatozoa was diluted with one or two drops of 2.9% (w/v) sodium citrate (trisodium salt, Na₃C₆H₅O₃.2H₂O; BDH Chemicals Ltd., Poole, UK) at 35°C. A drop of this dilution was placed on a warmed slide (35°C) under a cover slip and examined by a routine laboratory microscope (Leitz, Wetzlar, Germany) under dark ground illumination at 200x magnification on a warm (35°C) microscope stage. Motility was estimated by examining at least two different microscopic fields for the presence of spermatozoa that exhibited directional motility. Spermatozoa, which either moved without progression or showed no sign of movement at all, were considered to be non-motile. Slides were examined in a blind manner (Barth and Oko, 1989) to avoid bias. Semen samples with less than 75% initial progressive motility were not included in the study (Yubi et al., 1987).

It was recognized that such assessments of motility were subjective, and consequently, their accuracy depends largely upon the experience and skill of the observer. Objective measurements of motility can be made by photographic recording of the movement of spermatozoa (Elliot, 1978; Hafez, 1993), or from computer-aided or laser measurement of swimming speed. However, neither of these methods was routinely available to this project, so assessments had to be made subjectively. Additionally, Rota (1998), in her thesis, reported that although a computer assisted sperm analyser was available it was not used owing to the inaccuracy observed when the samples were diluted in some of the extenders (e.g. skimmed-milk or cream). The particulate matter was confused with spermatozoa. Furthermore, given the large number of publications available in the literature that are based upon such subjective assessments, this was considered to be a valid method of assessment. An attempt to validate more thoroughly the subjective motility scores against the laser measurement of swimming speed was unsuccessful as it
was not possible to assess the samples by the two methods at the same temperature. Additionally, the presence of many dead spermatozoa (especially after freeze-thawing) might have interfered with the results of computer analysis (W.C.L. Ford, personal communication). Hence, in the preliminary observations of motility, the author worked alongside the experienced observers until coincident scores were routinely obtained. Thereafter, parallel observations were periodically made between the author and another experienced observer (results not given) throughout Experiments 1a and 1b to ensure that motility estimations remained consistent.

2.1.2.7- Statistical analysis
Data from Experiment 1a, which were normally distributed, were subjected to analysis of variance with respect to treatment (i.e. seminal plasma removed, glycerolised at room temperature; seminal plasma removed, glycerolised at +4°C; seminal plasma present, glycerolised at room temperature; seminal plasma present, glycerolised at +4°C), dilution rate and time, and with respect to individual dogs.

Likewise, data from Experiment 1b, which were also normally distributed, were subjected to analysis of variance with respect to the concentration of glycerol, dilution rate and time, and with respect to individual dogs.

Effects were considered to be statistically significant at P<0.05, and where such effects were found, comparison between individual means was made by calculating least significant differences (Snedecor and Cochran, 1967).

2.1.3- Results
2.1.3.1- Characteristics of ejaculates
Results of the initial semen examination of each ejaculate used in Experiments 1a and 1b are given in Table 2.1:
Table 2.1. The characteristics of ejaculates used in Experiments 1a and 1b

<table>
<thead>
<tr>
<th>Dog</th>
<th>Ejaculate No.</th>
<th>colour</th>
<th>volume (ml)</th>
<th>progressive motility (%)</th>
<th>concentration (x10^6/ml)</th>
<th>morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>normal live</td>
</tr>
<tr>
<td><strong>Dog A:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solo</td>
<td>I</td>
<td>cloudy</td>
<td>2.75</td>
<td>85</td>
<td>300.0</td>
<td>89 8</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>milky</td>
<td>1.80</td>
<td>85</td>
<td>480.0</td>
<td>86 6</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>creamy</td>
<td>1.60</td>
<td>90</td>
<td>650.0</td>
<td>84 7</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>light milky</td>
<td>1.15</td>
<td>92</td>
<td>400.0</td>
<td>89 6</td>
</tr>
<tr>
<td><strong>Dog B:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gillie</td>
<td>I</td>
<td>milky</td>
<td>2.45</td>
<td>90</td>
<td>340.0</td>
<td>95 1</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>milky</td>
<td>2.50</td>
<td>85</td>
<td>600.0</td>
<td>86 4</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>milky</td>
<td>2.30</td>
<td>84</td>
<td>580.0</td>
<td>81 2</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>light milky</td>
<td>2.03</td>
<td>89</td>
<td>325.0</td>
<td>86 3</td>
</tr>
<tr>
<td><strong>Dog C:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hands</td>
<td>I</td>
<td>milky</td>
<td>3.50</td>
<td>90</td>
<td>180.0</td>
<td>82 0</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>milky</td>
<td>2.70</td>
<td>85</td>
<td>120.0</td>
<td>89 4</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>light cloudy</td>
<td>2.55</td>
<td>85</td>
<td>210.0</td>
<td>82 5</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>milky</td>
<td>1.45</td>
<td>90</td>
<td>350.0</td>
<td>81 5</td>
</tr>
</tbody>
</table>
2.1.3.2- Experiment 1a

The results of analysis of variance of the data are summarised in Table 2.2:

Table 2.2. Analysis of variance table (Experiment 1a)

<table>
<thead>
<tr>
<th>Term</th>
<th>df</th>
<th>F-ratio</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog (n=3)</td>
<td>2</td>
<td>152.9</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Storage time</td>
<td>6</td>
<td>11.27</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Dilution rate</td>
<td>5</td>
<td>150.73</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>3</td>
<td>19.17</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Storage time x Dilution rate</td>
<td>30</td>
<td>0.90</td>
<td>NS</td>
</tr>
<tr>
<td>Dilution rate x Treatment</td>
<td>15</td>
<td>1.96</td>
<td>P≤0.01</td>
</tr>
<tr>
<td>Storage time x Treatment</td>
<td>18</td>
<td>2.10</td>
<td>P≤0.005</td>
</tr>
<tr>
<td>Storage time x Treatment x Dilution rate</td>
<td>90</td>
<td>0.43</td>
<td>NS</td>
</tr>
</tbody>
</table>

Thus, the changes of motility with time were significantly (P≤0.005) related to treatment (i.e. glycerolisation temperature and the presence/absence of seminal plasma) and the effects of dilution rate upon motility were also significantly (P≤0.01) related to treatment.

There was a significant interaction (P≤0.01) between the effects of treatment and dilution rate based on the maintenance of motility (Figure 2.3). At 1:1 and 1:2 dilutions, there were significant (p<0.05) differences between the samples that were centrifuged and glycerolised at +4°C and the samples that were neat and glycerolised at room temperature (1:1: C-4°C: 52 ± 3% versus N-RT: 40 ± 3%; 1:2: C-4°C: 56 ± 3% versus N-RT: 44 ± 3%). However, at 1:16 dilution, the motility was significantly (p<0.05) higher in samples that were neat and glycerolised at +4°C compared to those centrifuged or neat and glycerolised at room temperature (N-4°C: 55 ± 3% versus C-RT: 47 ± 3%; N-RT: 45 ± 3%).

There was also a significant interaction (P≤0.005) between the effects of treatment and the storage period based on the maintenance of motility (Figure 2.4). All treatment groups exhibited a substantial and statistically significant (P<0.05) decrease in
percentage motile spermatozoa between 0 h and 2 h (C-RT: 71 ± 1% versus 50 ± 3%; C-4°C: 84 ± 1% versus 55 ± 3%; N-4°C: 78 ± 1% versus 56 ± 3%; N-RT: 69 ± 2% versus 49 ± 3%, respectively). Thereafter, slower rates of decrease occurred. However, there were also a number of marked effects of treatments upon the motility at different time points. At time zero (T0) h, the motility was significantly (p<0.05) higher in samples that were glycerolised at +4°C than those glycerolised at room temperature, irrespective of the presence of seminal plasma (C-4°C: 84 ± 1%; N-4°C: 78 ± 1% versus C-RT: 71 ± 1%; N-RT: 69 ± 2%). Likewise, at time 4 h, in the presence of seminal plasma, the motility was significantly (p<0.05) higher in samples that were glycerolised at +4°C than those glycerolised at room temperature (N-4°C: 53 ± 2%; N-RT: 47 ± 3%). However, at 24 h, the motility was significantly higher in samples that were centrifuged and glycerolised at +4°C than the samples that were neat and glycerolised at room temperature (C-4°C: 47 ± 3%; N-RT: 37 ± 3%). Finally, at 48 h, motility values were significantly (P<0.05) higher in samples from which the seminal plasma had been removed than from those in which it was still present (C-RT: 38 ± 3%; C-4°C: 38 ± 3% versus N-4°C: 31 ± 3%; N-RT: 24 ± 3%).

There was a statistically significant (P<0.001) difference in motility values between dogs following storage at +4°C. This was due to the spermatozoa from one individual, which had significantly lower (P<0.05) maintenance of motility than others (Table 2.3).

Finally, to summarise the residual main effects: The percentages of motile spermatozoa were significantly (P<0.05) lower at dilution rates of 1:1 and 1:32 (45 ± 2% and 45 ± 2%, respectively) than at intermediate dilutions. There were no significant differences between dilutions of 1:2, 1:4, 1:8 and 1:16 (51 ± 1%, 52 ± 1%, 53 ± 1% and 49 ± 2%, respectively). Motility of spermatozoa declined significantly (P<0.05) during the first 2 h of cooling (T0: 76 ± 1%; 2 h: 52 ± 1%). The decrease in motility that occurred by 4 h (50 ± 1%) was not significant, although there was a further significant (P<0.05) decline by 6 h (46 ± 1%). As expected, there was also a significant (P<0.05) decrease in motility between 24 h (41 ± 1%) and 48 h (33 ± 2%). Finally, overall motility was significantly (P<0.05) higher in samples glycerolised at +4°C (N-4°C: 51 ± 1%; C-4°C: 53 ± 1%) than in samples glycerolised at room temperature (N-RT: 45 ± 1%; C-RT: 48 ± 1%).
Figure 2.3. The effect of treatment (removal of seminal plasma and temperature of glycerolisation) and dilution rate upon the maintenance of motility of spermatozoa in Tris extender stored at +4°C for 48 h (P<0.01)

Means (± SEM) sharing a common superscript are not significantly different from each other (P<0.05). (n=6, Experiment 1a)

Legends:
Centr.+RT : centrifuged, glycerolised at room temperature
Centr.+4°C : centrifuged, glycerolised at +4°C
Neat+4°C : non-centrifuged, glycerolised at +4°C
Neat+RT : non-centrifuged, glycerolised at room temperature
Figure 2.4. The effect of treatment (removal of seminal plasma and
glycerolisation temperature) and storage time (up to 48 h) at +4°C upon
the maintenance of motility of spermatozoa in Tris extender (P<0.005)

**Legends:**
- Centr.+RT: centrifuged, glycerolised at room temperature
- Centr.+4°C: centrifuged, glycerolised at +4°C
- Neat+4°C: non-centrifuged, glycerolised at +4°C
- Neat+RT: non-centrifuged, glycerolised at room temperature

*a-g* Means (± SEM) sharing a common superscript are not significantly different from each
other (P<0.05). (n=6, Experiment 1a)
Table 2.3. The effect of individual dogs upon the maintenance of motility of spermatozoa in Tris extender stored at +4°C (Experiment 1a)

<table>
<thead>
<tr>
<th>Dog</th>
<th>% motility (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog A</td>
<td>54.75b ± 0.88</td>
</tr>
<tr>
<td>Dog B</td>
<td>38.92a ± 1.21</td>
</tr>
<tr>
<td>Dog C</td>
<td>53.55b ± 0.88</td>
</tr>
</tbody>
</table>

Significance: P<0.001

a,b Means sharing a common superscript are not significantly different from each other (P<0.05). (n=6)
2.1.3.3- Experiment 1b

The results of analysis of variance of the data are summarised in Table 2.4:

Table 2.4. Analysis of variance table (Experiment 1b)

<table>
<thead>
<tr>
<th>Term</th>
<th>df</th>
<th>F-ratio</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog (n=3)</td>
<td>2</td>
<td>9.67</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Storage time</td>
<td>6</td>
<td>30.69</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Dilution rate</td>
<td>2</td>
<td>116.76</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Glycerol concentration</td>
<td>5</td>
<td>6.09</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Storage time x Dilution rate</td>
<td>12</td>
<td>0.85</td>
<td>NS</td>
</tr>
<tr>
<td>Dilution rate x Glycerol concentration</td>
<td>10</td>
<td>0.42</td>
<td>NS</td>
</tr>
<tr>
<td>Storage time x Glycerol concentration</td>
<td>30</td>
<td>0.82</td>
<td>NS</td>
</tr>
<tr>
<td>Storage time x Glycerol concentration x Dilution rate</td>
<td>60</td>
<td>0.33</td>
<td>NS</td>
</tr>
</tbody>
</table>

In this experiment, only the main effects were statistically significant (P<0.001). None of the higher order interaction terms were significant, so the presentation of the results will be in terms of main effects only.

As in Experiment 1a, there was a significant difference (P<0.001) in overall motility values between dogs (Table 2.5). The motility of spermatozoa from one individual (Dog B) was significantly (P<0.05) lower than others (in Dogs A and C).

There was a significant (P<0.001) effect of dilution rate (1:2, 1:4 and 1:8) upon the motility. The decrease in motility was significantly (P<0.05) less with more dilute samples (Figure 2.5). Thus, there was significantly (P<0.05) lower motility at 1:2 than at 1:4 (73 ± 1% and 76 ± 1%, respectively). Likewise, the motility was significantly (P<0.05) higher at 1:8 (78 ± 1%).

There was also a significant (P<0.001) effect of storage time upon motility. Spermatozoal survival declined over the storage period (Figure 2.6). The most substantial (P<0.05) decline occurred between the initial dilution (T0) and 2 h (89 ± 0% to 77 ± 1%, respectively). Thereafter, motility gradually declined significantly (P<0.05) between 2 h
and 6 h (77 ± 1% and 75 ± 1%, respectively), and between 4 h and 8 h (76 ± 1% and 74 ± 1%, respectively). As expected, motility values were also significantly (P<0.05) decreased by 24 h (71 ± 1%) and 48 h (67± 1%).

The concentration of glycerol in the extender had a significant effect (P<0.001) upon the survival. The motility decreased as glycerol concentrations increased (Figure 2.7). The spermatozoa were unaffected by glycerol concentrations up to 2%. There were no significant differences between the concentrations up to 2% (0%: 76 ± 1%; 0.5%: 77 ± 1%; 1%: 76 ± 1%; 2%: 76 ± 1%). However, motility values were significantly (P<0.05) lower at 4% (74 ± 1%).
Table 2.5. The effect of individual dogs upon the maintenance of motility of spermatozoa in Tris extender stored at +4°C (Experiment 1b)

<table>
<thead>
<tr>
<th>Dog</th>
<th>% motility (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=3)</td>
<td></td>
</tr>
<tr>
<td>Dog A</td>
<td>76.34(^b) ± 0.58</td>
</tr>
<tr>
<td>Dog B</td>
<td>74.06(^a) ± 0.58</td>
</tr>
<tr>
<td>Dog C</td>
<td>76.21(^b) ± 0.57</td>
</tr>
</tbody>
</table>

Significance: \( P<0.001 \)

\(^{a,b}\) Means having different superscripts are significantly different from each other \((P<0.05)\). (n=6)
Figure 2.5. The effect of final dilution rate upon the maintenance of motility of spermatozoa in Tris extender stored at +4°C for 48 h (P<0.001)

Means (± SEM) having different superscripts are significantly different from each other (P<0.05). (n=6, Experiment 1b)
Figure 2.6. The effect of storage time upon motility of spermatozoa stored at +4°C (P<0.001)

% motility

Time (hour)

Means (± SEM) sharing a common superscript are not significantly different from each other (P<0.05). (n=6, Experiment 1b)
Figure 2.7. The effect of glycerol concentration upon motility of spermatozoa stored at +4°C (P<0.001)

Means (± SEM) sharing a common superscript are not significantly different from each other (P<0.05). (n=6, Experiment 1b)
2.2- *In vitro* induction of acrosome reactions

**Hypothesis:**

It is possible to induce *in vitro* acrosome reaction using modified TALP medium and calcium ionophore.

2.2.1- Introduction

The difficulties of predicting the fertility of male animals have been one of the main problems confronting reproductive research, the artificial insemination industry and breeders around the world. The only reliable test of the functional integrity of a semen sample is its capacity to fertilise the ovum and to sustain embryogenesis (Watson, 1979). However, the assessment of fertilising capacity *in vivo* is costly, time-consuming and laborious. Therefore, alternative methods of assessing semen have been sought, particularly *in vitro* methods of analysing the functionality of spermatozoa.

The value of routine semen analysis in the prediction of fertility has been discussed (Section 1.3.2), with the conclusion that it is both subjective and only poorly predicts fertility (Linford *et al.*, 1976; Rodriguez-Martinez *et al.*, 1997). Similarly, evaluation of spermatozoal function in the dog by traditional methods has not been well correlated with actual fertility (Mayenco-Aguirre and Pérez-Cortés, 1998), even though they are widely used (Hewitt and England, 1997).

By contrast, the induction of acrosome reaction *in vitro* has been found to be predictive for bovine fertility both *in vitro* (Lenz *et al.*, 1983) and *in vivo* (Whitfield and Parkinson, 1992, 1995). Furthermore, apart from its predictive power for assessing *in vivo* fertility in the bull (Whitfield and Parkinson, 1995), A23187-induced acrosome reactions have also been used as an *in vitro* tool to detect subfertile men (Liu and Baker, 1998).

The hemizona binding assay (HZA) is also considered to be an effective and very promising test for predicting the fertilising potential of spermatozoa *in vitro* (Ivanova *et al.*, 1999). Nevertheless, even by using this assay, it was found that while zona binding was significantly affected by fertility status, it did not permit clear differentiation between fertile and infertile dogs due mainly to poor zona binding capacity of spermatozoa from some of the fertile individuals (Mayenco-Aguirre and Pérez-Cortés, 1998).
2.2.1.1- Physiology of the acrosome

The acrosome is a membrane-bound, cap-like structure, covering the anterior portion of the sperm nucleus. It originates from the Golgi complex in the spermatid (de Kretser and Kerr, 1994), varies in shape from species to species (Yanagimachi, 1994), and contains a large number of powerful hydrolysing enzymes (Tulsiani et al., 1998) which are essential for fertilisation. In the presence of extracellular calcium ion, stimulatory messengers such as ZP proteins (McLeskey et al., 1997), progesterone (Brewis et al., 1999) and calcium ionophore (A23187; Tesarik, 1985) increase intracellular concentrations of calcium, which may modify the local charge aggregation of the membranes (Purohit et al., 1998) and hydrogen ion concentration (Fraser, 1998) during the acrosome reaction. Eventually, these lead to fusion of the outer acrosomal and plasma membranes (Bedford, 1970), thus permitting vesiculation of the membranes and release of the acrosomal enzymes. The eggs of many animals, including those of the bitch, are surrounded by glycoprotein coats, i.e. the ZP (Wassarman and Albertini, 1994) through which spermatozoa must pass before reaching the egg plasma membrane (Yanagimachi, 1994; McLeskey et al., 1997). Therefore, the acrosome reaction has at least two functions: it renders spermatozoa capable of (i) penetrating through the zona and (ii) fusing with the egg plasma membrane (Yanagimachi, 1994).

Mammalian spermatozoa leaving the testis are neither able to survive in the female reproductive tract nor to fertilise oocytes (Florman and Babcock, 1991; Amann et al., 1993). The molecular structures related to the plasma membrane and acrosome are functionally immature but gradually become mature during epididymal transit (Toshimori, 1998). Therefore, the spermatozoa become capable of undergoing the acrosome reaction as they pass through the epididymis (Yeung et al., 1998). In addition, upon leaving the epididymis, the mature spermatozoa are capable of actively moving (Amann et al., 1993; Jones and Murdoch, 1996).

After insemination, spermatozoa need a species-dependent period of residence in the female genital tract before they are able to penetrate freshly ovulated eggs (Austin, 1951; Chang, 1951). This priming process for fertilisation is referred to as 'capacitation' (Austin, 1952). Jaiswal et al. (1998) have recently demonstrated that capacitation is a prerequisite for spermatozoa to be able to undergo the acrosome reactions. During capacitation, spermatozoa undergo a variety of changes, the majority of which appear to involve
alterations in the plasma membrane structure and function (Cohen-Dayag and Eisenbach, 1994; Yanagimachi, 1994). Such changes include the removal or alteration of coating material and antigens, the release of glycosilated proteins, changes in the lectin-binding ability, modifications to both intramembraneous particles and the fluidity of membrane and lipids, and the removal of cholesterol (Yanagimachi, 1994). After receiving proper external stimuli (see below), these changes to the plasma membrane, which allow the exchange of ions between intra- and extracellular environments, will modify the intracellular concentrations of ions which include calcium and hydrogen ions (Fraser, 1998). Harrison (1996) suggested that capacitation is a series of positive destabilising events, within which spermatozoa display hyperactivated motility and can undergo a zona-induced acrosome reaction that eventually lead to cell death. Additionally, Bedford (1998) suggested that lateral head displacement and the perforatorium of sperm are other crucial factors to generate "cutting thrust" through the oocyte vestments during the acrosome reaction. Therefore, it can be assumed that the plasma membrane of a spermatozoon may be considered to be "biologically frozen" when the spermatozoon leaves the male's body and its "defrosting" represents capacitation (Yanagimachi, 1994).

Generally, mammalian spermatozoa require extracellular calcium ions in order to complete capacitation (Fraser, 1998) and fertilise oocytes. Indeed, Yanagimachi and Usui (1974), who demonstrated a calcium dependency for activation and acrosome reaction of guinea pig spermatozoa, observed that when calcium ions were made available after spermatozoa have become capacitated, they immediately underwent hyperactivation and acrosome reaction. Subsequently, Mahi and Yanagimachi (1978) showed that canine spermatozoa did not undergo the acrosome reaction when calcium was deleted from the incubation medium. Therefore, they concluded that the presence of calcium ion is essential for viability and the acrosome reaction. This was confirmed by Rota (1998). Furthermore, Bailey et al. (1994) investigated the correlation between motility, in vitro calcium ion fluxes and in vivo fertility of bovine spermatozoa, and suggested that calcium ion regulation affects both spermatozoal motility and fertility. Finally, Fraser (1995) indicated that the pivotal factor controlling capacitation and acrosomal exocytosis is modulation of the intracellular calcium ion concentration of mammalian sperm cells. She also concluded, from published works, that treating sperm cells with calcium ionophore such as A23187, in
the presence of extracellular calcium ion, causes a rapid influx of calcium ion and promotes acrosomal exocytosis within a relatively short time.

**2.2.1.2- In vitro assessment of acrosome reactions**

Assessment of the acrosomal morphology and of the progress of the acrosomal reaction, a modified form of exocytosis, is required mainly for the following reasons:

- The spermatozoa must undergo the acrosome reaction to fertilise the ovum via penetrating the ZP and fusing with the egg plasma membrane *in vivo* and *in vitro* (Yanagimachi, 1994). This gives a good indication of fertility potential of a male (Whitfield and Parkinson, 1992, 1995). This was also used in the evaluation of human male subfertility *in vitro* (Liu and Baker, 1998).

- The acrosomal exocytosis is an indirect indicator of the maturation of spermatozoal populations, which may allow monitoring of the development of fertility (Cardullo and Florman, 1993).

- Since freshly ejaculated spermatozoa need to be capacitated before undergoing the acrosome reaction (Harrison, 1996), it can also be used as a marker of this physiological stage (Cross and Meizel, 1989).

- Spermatozoa may also lose their acrosomes when they die (false or degenerative acrosome reaction). Therefore, assessment of this type of acrosomal loss is widely used to evaluate damage to the spermatozoal head (Cross and Meizel, 1989).

- It can also be used to determine the potential of physiological or non-physiological stimulants in provoking capacitation as well as the acrosome reaction *in vivo* and *in vitro*, which may allow refinement of the culture conditions for *in vitro* fertilisation.

In mammalian spermatozoa, both capacitation and the acrosome reaction can be achieved in the absence of oocytes under *in vitro* conditions which mimic the physiological environment of the female reproductive tract (Mahi-Brown, 1991; Yanagimachi, 1994). Indeed, it has been reported that capacitation and spontaneous acrosome reactions in dog spermatozoa could occur *in vitro* in defined media, either in the presence or absence of oocytes and corona cells (Mahi and Yanagimachi, 1976, 1978). They also found that canine spermatozoa, as with other species, require capacitation before they are able to penetrate the zona.
Since then, there have been a considerable number of studies upon the in vitro capacitation and/or acrosome reaction of canine spermatozoa (Szász et al., 1997; Kawakami et al., 1998b; Hewitt and England, 1998). In these studies, a wide range of incubation media and assessment techniques have been used. The techniques used to determine that the acrosome reaction had occurred include phase contrast (PC) microscopy (Mahi and Yanagimachi, 1976). Stains used were; (i) triple stain (Kawakami et al., 1993a), (ii) naphthol yellow S/erythrosin B stain (Kumi-Diaka and Harris, 1994), (iii) fluoresceinated Pisum sativum lectin stain alone (Kawakami et al., 1993b) or together with monoclonal antibodies (Geussovä et al., 1997), (iv) fluoresceinated Peanut agglutinin lectin stain (Szász et al., 1997), (v) chlortetracycline stain alone (Hewitt and England, 1997; Rota, 1998) or in comparison with Spermac stain (Guérin et al., 1999), and (vi) indirect immunofluorescence by using a specific (not stated) monoclonal antibody (Brewis et al., 1999).

However, whilst these studies provided a great deal of information about the process of spontaneous acrosome reaction in vitro, only a few trials have investigated the means by which acrosome reactions could be actively induced. These include the works of Kawakami et al. (1993b), who induced acrosome reactions by using homologous ZP, and Metzler (cited by Mahi-Brown, 1991), who used A23187. However, at the start of the study reported in this thesis as Experiment 2, no data could be found in the literature of optimal conditions for the in vitro induction and assessment of the canine acrosome reaction. Since the start of this experiment, there have been five trials using a very similar experimental paradigm to investigate these same questions by Szász et al. (1997), Geussovä et al. (1997), Hewitt and England (1998), Brewis et al. (1999) and Guérin et al. (1999) all of whom induced acrosome reactions with A23187 alone or together with other inducers such as progesterone and solubilised ZP proteins (Brewis et al., 1999). The findings of these authors will be considered in regard to the results of the present investigations in Section 2.3.

2.2.1.3- Experimental design

The methods used to induce acrosome reactions were based on those described for the bull by Whitfield and Parkinson (1992, 1995).

Several possible methods exist for assessment of the acrosome. Throughout the literature, two light microscopy methods are regarded as providing unequivocal information about
the status of the acrosome; namely direct examination of the acrosome under either phase contrast microscopy (Mahi and Yanagimachi, 1978) or differential interference contrast microscopy (Cardullo and Florman, 1993; Christensen et al., 1994). These two methods are regarded as “gold standards”, against which any other methods should be compared. They do, however, require an element of experience, so it might be desirable to use a staining method as a simpler alternative. In the dog, dual staining of spermatozoa by aniline blue/eosin B stain (Casarett, 1953; Kawakami et al., 1988) or naphthol yellow S/erythrosin B stain (Kumi-Diaka and Harris, 1994) have been employed (before or after the acrosome reaction, respectively) while Christensen et al. (1994) used naphthol yellow S/aniline blue (NA) stain to assess A23187-induced acrosome reactions in the bull.

Therefore, the objectives of this study were:

- to determine whether canine spermatozoa would undergo the acrosome reaction in vitro in modified TALP medium, as described by Christensen et al. (1994) for cattle,
- to determine the minimum effective concentration of A23187 for induction of acrosome reactions and to describe the time course of such induced acrosome reactions,
- to compare a staining method (NA) with an established standard method (phase contrast microscopy) of assessment of acrosome reactions, in order to determine whether staining would provide a simpler alternative method of evaluation than phase contrast microscopy, and
- to determine whether glycerol affects the in vitro induction of acrosome reactions by A23187.

2.2.2- Experiment 2a. In vitro induction of acrosome reactions in fresh semen

The objectives of this experiment were to investigate:

- whether acrosome reactions can be induced in canine spermatozoa in vitro by the use of a calcium ionophore (A23187), and
- whether NA staining can be used as an alternative to the established method of PC microscopy for the assessment of acrosomal status.

The procedures used in the experiment are summarised in Figure 2.8.
2.2.2.1- Semen collection and initial evaluation

Two ejaculates were collected from each of three dogs (Dogs A, B and C; see Appendix 2.1 for detail) by manual stimulation (see Section 2.1.2.3).

Following collection, the volume of each ejaculate was recorded and the concentration of spermatozoa was determined with an improved Neubauer haemocytometer. Spermatozoal motilities were estimated (see Section 2.1.2.6). Slides were prepared using nigrosin/eosin staining (see Section 2.1.2.4). These evaluations were undertaken to ensure that the semen attained a minimum threshold value (i.e. >60% morphologically normal and >75% progressively motile spermatozoa: Oettle, 1993 and Yubi et al., 1987, respectively) for use in the experiment.

2.2.2.2- Semen processing

The seminal plasma was removed by layering semen onto an equal volume of Percoll solution, containing 45% Percoll (Sigma Chemical Co., Poole, UK) and 55% TALP (see Appendix 2.3a,b). After centrifuging at 275 g for 5 min, supernatant was separated and pellet was resuspended to pre-centrifugation volume in incubation medium (TALP). The suspension was then further diluted in TALP to a final concentration of 10x10⁶ sperm/ml.

2.2.2.3- Induction of acrosome reaction

In order to ascertain the conditions under which A23187 could be used to induce acrosome reactions in canine spermatozoa, a 2x2 factorial experiment was designed to investigate the effects of concentration of ionophore and time on the proportion of acrosome reacted spermatozoa (see Figure 2.8). Some sperm were cryopreserved (see Experiment 6) and their characteristics after NA staining were compared with the present findings.

2.2.2.4- Evaluation of acrosomes

The acrosome reaction was assessed by staining the spermatozoa with naphthol yellow S/aniline blue, as described below and by their direct visualisation under PC microscopy.

The slides, which were used for PC microscopy, were uncoated. After placing 1-2 drops of suspension of spermatozoa on the slides, they were quickly covered with a coverslip. Then, DPX mounting was applied around the coverslips to ensure the samples remained wet at room temperature until they were examined by PC microscopy (Leitz, Wetzlar, Germany) at a total of 7,000x magnification, without a filter.
Samples that were to be stained were smeared onto slides that were precoated with poly-l-lysine (Sigma Chemical Co, Poole, UK) to adhere spermatozoa onto the slide. After smearing, the slides were dried on a warm stage. Naphthol yellow S/aniline blue (NA) staining was performed according to method of Christensen et al. (1994). Slides were placed in a 2.8 mM/l solution of naphthol yellow S (Aldrich Chemical Co. Ltd., Dorset, England) for 15 min. After rinsing in 0.167 mM/l aqueous acetic acid for 10 to 15 sec they were blotted dry and then dried in air. The slides were further stained for 10 min in an aqueous solution of 5.6 mM/l naphthol yellow S/ 1.25 mM/l aniline blue (water soluble; Raymond A. Lamb Waxes & Gnl. Lab. Suppl., Middx, UK), whose pH was adjusted to 3.5 with acetic acid. After rinsing in distilled water for 5 to 10 sec they were dried in air. No further mounting was performed before the evaluation of acrosome reaction was carried out on a Bright-Field microscope (Leitz Laborlux K, Wetzlar, Germany) at a magnification of 1,000x with a filter.

A major aim of attempting NA staining of spermatozoa was to facilitate the evaluation of the acrosome reaction in cryopreserved cells. Thus, spermatozoa from Experiment 6, which had undergone cryopreservation, were stained by NA, using an identical protocol to the above. Results were compared with those obtained by PC.

All spermatozoa which exhibited the acrosomal region fully, in a single microscope field, were evaluated for the stage of acrosome reaction (Cardullo and Florman, 1993). For wet samples (PC microscopy), spermatozoa were often not found in one single focal plane. Therefore, where the acrosome could be evaluated by altering the focus up and down slightly on the cell (Barth and Oko, 1989), the sperm was included in the assessment. However, where further focusing was of no value in determining the status of the acrosome, the spermatozoon was excluded.

The criteria used for the assessment of the acrosome reaction by PC microscopy were as follows:

- The acrosome was considered to be intact where the entire acrosomal cap (particularly the apical ridge) had uniform, dark background with a smooth, clear outline (Cross and Meizel, 1989).
- All other spermatozoa were considered to be either partially or completely acrosome reacted, as determined by the degree of changes on the acrosome. Thus, cells that
exhibited decondensation, swelling, fuzzy appearance, or an irregular outline were considered to be partially reacted while the spermatozoa in which the acrosome was completely lacking, were considered to have undergone complete acrosome reaction.

2.2.2.5- Statistical Methods

For the purposes of statistical analysis, all spermatozoa that exhibited any degree of acrosome reaction (i.e. those classified as partially or completely reacted) were counted as acrosome reacted while only those cells that exhibited no changes whatsoever of the acrosome were counted as acrosome intact (unreacted).

These data, which were normally distributed, were subjected to analysis of variance with respect to treatment (i.e. concentration of A23187) and time, and with respect to individual dogs. Where statistically significant effects were noted, comparisons between individual pairs of means were quantified by calculating least significant differences. Data from the NA assessment of acrosomes were compared with that for PC by correlation and linear regression analysis.
Figure 2.8. Experiment 2a: Summary of procedures
2.2.3- Experiment 2b. *In vitro* induction of acrosome reactions in extended, glycerolised semen

**Hypothesis:**
The addition of 2% glycerol will not affect the induction of the acrosome reaction *in vitro* in chilled spermatozoa.

The objectives of this study were to investigate the effect of A23187 concentration (i.e. 0, 0.1 and 1 μM/l) in chilled semen in the presence of 2% (v/v) glycerol upon *in vitro* acrosome reaction of dog spermatozoa during incubation at 39°C for 60 min.

**2.2.3.1- Semen collection and initial evaluation**
Single ejaculates were collected from three dogs (Dogs A, B and C; see Appendix 2.1 for detail) by manual stimulation (Section 2.1.2.3).

Semen was kept at 35°C during initial evaluation, which included an estimation of volume, colour, and spermatozoal concentration. The assessment of spermatozoal motilities and morphologies (Sections 2.1.2.6 and 2.2.2.4, respectively) were also undertaken. This initial evaluation was used to determine that semen attained the minimum threshold values of motility and morphology (Section 2.2.2.1) prior to further processing.

**2.2.3.2- Semen processing**
This was as shown in Figure 2.9.

Some cryopreserved sperm were processed (see Sections 3.5.3.2 and 3.5.3.3 for detail) and the results were compared by chilled sperm using DIC microscopy.

**2.2.3.3- Induction of acrosome reaction**
Both the unglycerolised and glycerolised semen were divided into two aliquots for assessment of the response to calcium ionophore (A23187, see Appendix 2.4) as follows:

- Unglycerolised control (Aliquot 1)
- Unglycerolised, A23187-induced (Aliquot 2)
- Glycerolised control (Aliquot 3)
- Glycerolised, A23187-induced (Aliquot 4).
Semen suspension (100 µl) was added to TALP (900 µl), which contained either 0 (Aliquots 1 and 3) or 0.1 and 1 µM/l (Aliquots 2 and 4) A23187. The semen was then incubated at 39°C in 5% CO₂ in humidified air for 60 min. Samples were withdrawn at 0, 30 and 60 min, fixed in 10% Formaldehydrate, and placed on a slide under a coverslip. DPX mounting medium was placed around the coverslip to ensure that preparations remained wet until they were examined.

2.2.3.4- Evaluation of acrosome reactions

The slides were examined for the assessment of acrosome reactions by using differential interference contrast (DIC) microscopy at 1,000x magnification. The criteria that were used to evaluate the acrosome were identical to those described for PC microscopy in Section 2.2.2.4. DIC was used in preference to PC as it is regarded in the literature as the "gold standard" light microscopy technique for evaluating acrosomes (Cardullo and Florman, 1993), against which other methods are judged. As DIC optical systems had become available to the author at this stage of the work, it was used in preference to PC as being both an easier and more accurate method of assessing the acrosome.
Figure 2.9. Experiment 2b: Summary of procedures
2.2.4- Experiment 2a: Results

The characteristics of the ejaculates used in these experiments are given in Table 2.6:
Table 2.6. The characteristics of ejaculates used for evaluation of the acrosome reaction by Bright-Field and phase contrast microscopy (Experiment 2a)

<table>
<thead>
<tr>
<th>Dog</th>
<th>Ejaculate</th>
<th>colour</th>
<th>volume (ml)</th>
<th>progressive motility (%)</th>
<th>concentration (x10⁶/ml)</th>
<th>morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>normal live</td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>dead live</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>dead abnormal</td>
</tr>
<tr>
<td><strong>Dog A:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Solo)</td>
<td>I</td>
<td>milky</td>
<td>2.50</td>
<td>83</td>
<td>188.0</td>
<td>83 live</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>milky</td>
<td>1.15</td>
<td>85</td>
<td>453.0</td>
<td>93 live 3 dead</td>
</tr>
<tr>
<td><strong>Dog B:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Gillie)</td>
<td>I</td>
<td>cloudy</td>
<td>1.00</td>
<td>75</td>
<td>94.0</td>
<td>80 live 1 dead</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>milky</td>
<td>0.30</td>
<td>78</td>
<td>156.0</td>
<td>84 live 5 dead</td>
</tr>
<tr>
<td><strong>Dog C:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Callum)</td>
<td>I</td>
<td>milky</td>
<td>0.80</td>
<td>85</td>
<td>313.0</td>
<td>82 live 2 dead</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>milky</td>
<td>0.77</td>
<td>85</td>
<td>422.0</td>
<td>87 live 10 dead</td>
</tr>
</tbody>
</table>
2.2.4.1- Comparison of NA staining with PC standard

In NA stained slides, intact acrosomes (Plate -1a) appeared light blue or blue, with a slight but nevertheless distinct, dark blue acrosomal ridge. The equatorial segment and midpiece were pale yellow, and the post-acrosomal region was light blue.

Loss of the clear outline of the acrosomal ridge (Plate -1c) was considered to be indicative of the acrosome reaction (Cross and Meizel, 1989). Complete loss of the acrosome (Plate -1e) revealed a yellow-stained nucleus (i.e. the loss of the overlying blue-stained acrosome revealed the underlying yellow stained nucleus).

PC microscopy also yielded the results expected from the widespread reports of the use of the method in the literature (Yanagimachi, 1994). Thus, with PC microscopy, intact acrosomes (Plate -2a) appeared as a dark shadow, having a regular and smooth outline, together with a clear equatorial segment and post-acrosomal region. Any degree of changes within the acrosome, i.e. decondensation and swelling (Plates -2b,c,d,e,f) or irregular (fuzzy) outlines (Plates -2g,h) was indicative of the partial acrosome reaction that results in the completion of acrosome reaction. Of particular note was that, during the first 15 min of incubation in the presence of A23187, initiation of the acrosome reaction could be seen as changes which started at or just above the border between the acrosome and equatorial segment (Plates -2b,c) and developed towards the apex of head of spermatozoa (Plates -2d,e,f). It was considered that these changes represented restricted decondensation (which was followed by swelling at this point: Plate -2b), and thus represented an early sign of the initiation of the acrosome reaction. All such spermatozoa were classified as partially reacted.

Finally, with PC microscopy, lack of the acrosome (Plate -2i) was revealed by a light sperm nucleus with a distinctly dark equatorial segment. Such spermatozoa were classified as being completely acrosome reacted.

While the PC slides had to be evaluated shortly after the preparation, those of the NA-stained slides remained readable up to 2-3 months. In addition, the solution of NA stain appeared to be stable for up to 2 months when stored at room temperature.

Comparison of the results from NA with those obtained from PC (Figure 2.10) demonstrated that there was a significant (P<0.0005) correlation (r=0.95) between the two methods:
NA (%) = (0.902 ± 0.021) x PC + (4.13 ± 0.91).

Where PC is the proportion of spermatozoa assessed as being acrosome reacted (i.e. partial plus complete reaction) by PC, and NA the proportion similarly assessed after staining.

This indicated that evaluation after NA staining produced comparable estimates of acrosomal status to that achieved by using a well-established method (PC). Both the slope of the regression line between the two methods was significantly different from 1 (P<0.0005) and the intercept was significantly (P<0.0005) higher than zero, indicating that NA staining underestimates the results (4.13 ± 0.91% lower) obtained by PC microscopy.

Therefore, we have shown that NA staining can be used to assess acrosome reaction. When NA staining of frozen-thawed sperm was evaluated, it was found that the results were less distinctive than with fresh semen (see Plates -1b,d,f). It was not always possible to differentiate precisely whether acrosome reaction had taken place.
Figure 2.10. Regression plot for the relationship between evaluation methods (PC microscopy and NA staining) based upon the percentage of reacted spermatozoa (P<0.0005)

\[(n=6, \text{Experiment 2a})\]
2.2.4.2- Induction of acrosome reactions with A23187

The results of analysis of variance from the data of assessment of acrosome reactions by PC are summarised in Table 2.7:

Table 2.7. Analysis of variance table (Experiment 2a: Assessment of acrosomes by PC microscopy)

<table>
<thead>
<tr>
<th>Term</th>
<th>df</th>
<th>F-ratio</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog (n=3)</td>
<td>2</td>
<td>2.37</td>
<td>0.10&gt;P&gt;0.05</td>
</tr>
<tr>
<td>A23187 concentration</td>
<td>4</td>
<td>228.78</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Incubation time</td>
<td>6</td>
<td>12.35</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>A23187 concentrations x Incubation time</td>
<td>24</td>
<td>1.11</td>
<td>P&gt;0.05</td>
</tr>
</tbody>
</table>

The results of analysis of variance from the data of assessment of acrosome reactions by NA are summarised in Table 2.8:

Table 2.8. Analysis of variance table (Experiment 2a: Assessment of acrosomes after staining with NA)

<table>
<thead>
<tr>
<th>Term</th>
<th>df</th>
<th>F-ratio</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog (n=3)</td>
<td>2</td>
<td>3.18</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>A23187 concentration</td>
<td>4</td>
<td>254.94</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Incubation time</td>
<td>6</td>
<td>8.62</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>A23187 concentrations x Incubation time</td>
<td>24</td>
<td>1.13</td>
<td>P&gt;0.05</td>
</tr>
</tbody>
</table>

Tables 2.7 and 2.8 show that there were statistically (P<0.05) significant differences in the numbers of reacted acrosomes between dogs as assessed by NA but not by PC (P=0.096). When assessed by NA, there was a significant (P<0.05) difference between Dog A and B (31.0 ± 3.5% and 35.6 ± 3.5%, respectively). Spermatozoa from Dog C occupied an intermediate position (34.8 ± 3.1%).
It should be noted that, after assessment by either PC or NA staining, only concentration of A23187 (P<0.001) and incubation time (P<0.001) were statistically significant. With neither PC nor NA assessments was the concentration x time interaction statistically significant.

The relationship between concentration of A23187 and percentage of acrosome reacted spermatozoa, as assessed by PC and NA, is illustrated in Figure 2.11. The percentage of reacted spermatozoa was significantly (P<0.05) greater (PC: 84.6 ± 3.1%; NA: 83.9 ± 2.8%) when incubated in the presence of 1 µM/l A23187. Percentages of acrosome reacted spermatozoa did not significantly differ between the lower concentrations of A23187. It is important to notice that a proportion of spermatozoa underwent a spontaneous acrosome reaction in the absence of A23187 (PC: 21.1 ± 2.0%; NA: 21.3 ± 1.7%), and that this proportion was not significantly different (P>0.05) from the overall mean values occurring with the three lower concentrations of A23187.

The relationship between incubation time and percentage of acrosome reacted spermatozoa is illustrated in Figure 2.12. As the incubation period continued the percentage of reacted spermatozoa gradually increased over time as follows:

- At the beginning of incubation (<5 min), a proportion of spermatozoa (PC: 20.4 ± 4.5%; NA: 22.8 ± 4.3%) had already undergone acrosome reaction.
- By 15 min, there was significant (P<0.05) increase in the percentage of reacted spermatozoa, as assessed by both technique (PC: 27.5 ± 5.5%; NA: 29.9 ± 5.0%).
- At both 30 and 45 min, there were further but non-significant increases in reacted spermatozoa (PC: 31.8 ± 5.7%; NA: 36.1 ± 5.4%).
- By 60 min, values for both PC and NA were significantly (P<0.05) higher than at 15 min (PC: 37.3 ± 5.4%; NA: 37.2 ± 5.2%).
- By 90 min, values were significantly (P<0.05) higher than at 15 or 30 min (PC: 38.9 ± 5.4%; NA: 38.1 ± 5.1%).
- By the end of incubation (120 min), the values for PC (44.4 ± 5.2%) were significantly (P<0.05) higher than at 30, 45 and 60 min. The values for NA (40.8 ± 5.3%) were significantly (P<0.05) higher than at 15 and 30 min.
Figure 2.11. Relationship between concentration of A23187 and percentage of acrosome-reacted spermatozoa, as assessed by phase contrast microscopy and naphthol yellow S/aniline blue staining (P<0.001)

![Graph showing the relationship between A23187 concentration and percentage of acrosome-reacted spermatozoa.](image_url)

Means (± SEM) sharing a common superscript within each assessment technique are not significantly different from each other (P<0.05). (n=6, Experiment 2a)
Figure 2.12. Relationship between incubation time and percentage of acrosome-reacted spermatozoa, as assessed by phase contrast microscopy and naphthol yellow S/aniline blue staining (P<0.001).

Means (± SEM) sharing a common superscript within each assessment technique are not significantly different from each other (P<0.05). (n=6, Experiment 2a)
2.2.5- Experiment 2b: Results

The characteristics of the ejaculates used in Experiment 2b are given in Table 2.9:
Table 2.9. The characteristics of ejaculates used for evaluation of the acrosome reaction of glycerolised spermatozoa (Experiment 2b)

<table>
<thead>
<tr>
<th>Dog</th>
<th>Ejaculate</th>
<th>colour</th>
<th>volume (ml)</th>
<th>progressive motility (%)</th>
<th>concentration (x10⁶/ml)</th>
<th>morphology (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=3)</td>
<td>No.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog A:</td>
<td>I</td>
<td>light milky</td>
<td>1.40</td>
<td>93</td>
<td>350.0</td>
<td>88 8</td>
<td></td>
</tr>
<tr>
<td>(Solo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog B:</td>
<td>I</td>
<td>milky</td>
<td>1.40</td>
<td>93</td>
<td>175.0</td>
<td>85 2</td>
<td></td>
</tr>
<tr>
<td>(Gillie)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog C:</td>
<td>I</td>
<td>light milky</td>
<td>0.90</td>
<td>95</td>
<td>687.5</td>
<td>67 3</td>
<td></td>
</tr>
<tr>
<td>(Robi)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.5.1- Morphology of the acrosome

Using DIC microscopy, intact acrosomes (Plate -3a) appeared as pale-dark shadow with a slightly distinct, dark acrosomal ridge while the equatorial segment and post-acrosomal region revealed pale-dark or dark shadow. Loss of clear outline of the acrosomal ridge revealed the acrosome as a brighter shadow with irregular outlines, which was considered as having undergone the partial acrosome reaction (Plate -3c). Complete loss of the acrosome (Plate -3e) revealed the nucleus as a pale shadow (lacking acrosome), although the equatorial segment and post acrosomal region were still intact. Thus, as expected, evaluation of the acrosome was straightforward using DIC microscopy.

Samples of cryopreserved semen were also examined by DIC. Again, as expected, it proved a straightforward method of visualising changes in the acrosome (Plates -3b,d,f). There were no obvious differences between cooled and frozen-thawed spermatozoa in terms of their acrosomal status. However, the entire head of frozen-thawed spermatozoa usually had a slightly uneven surface together with a less dense contrast.

2.2.5.2- Induction of acrosome reactions with A23187

The results of analysis of variance of the data are summarised in Table 2.10:

Table 2.10. Analysis of variance table (Experiment 2b)

<table>
<thead>
<tr>
<th>Term</th>
<th>df</th>
<th>F-ratio</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog (n=3)</td>
<td>2</td>
<td>23.19</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>A23187 concentration</td>
<td>2</td>
<td>44.12</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Incubation time</td>
<td>2</td>
<td>30.23</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>0.44</td>
<td>NS</td>
</tr>
<tr>
<td>A23187 concentration x Incubation time</td>
<td>4</td>
<td>9.24</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>A23187 concentration x Treatment</td>
<td>2</td>
<td>0.92</td>
<td>NS</td>
</tr>
<tr>
<td>Incubation time x Treatment</td>
<td>2</td>
<td>0.40</td>
<td>NS</td>
</tr>
<tr>
<td>A23187 concentration x Incubation time x Treatment</td>
<td>4</td>
<td>0.08</td>
<td>NS</td>
</tr>
</tbody>
</table>
There was a statistically significant difference (P<0.001) in acrosome reaction between dogs, in that the individuals significantly (P<0.05) differed from each other (Table 2.11).

Neither the presence of glycerol nor any other interactions between glycerol, storage times and A23187 concentration had any significant effect upon the percentage of acrosome reacted spermatozoa.

The interaction between different concentrations of A23187 and different times was, however, statistically significant (P<0.001) based upon the percentage acrosome reaction (Figure 2.13):

- At the beginning of incubation (T0), values in all samples were similar. Although there was no significant difference, there was a trend (0.10>P>0.05) for more acrosome reacted spermatozoa in the presence of 1 µM/l A23187 (45.0 ± 4.9%) or 0.1 µM/l A23187 (41.5 ± 4.3%) than in control samples (40.8 ± 4.3%).

- By 30 min, there was a significant (P<0.05) increase in the percentage of acrosome reacted spermatozoa in the presence of 1 µM/l A23187 (89.3 ± 2.3%). Although there were slight increases in the presence of 0.1 µM/l A23187 and in control samples (50.2 ± 6.1% and 48.3 ± 7.1%, respectively), these were not statistically different from T0 (time zero) values.

- By 60 min, although there was no further significant increase in reacted spermatozoa at 1 µM/l A23187 (90.3 ± 4.1%), there was a significant (P<0.05) increase in reacted spermatozoa in the presence of 0.1 µM/l A23187 (64.0 ± 9.2%), whereas values in control samples remained unaltered (45.7 ± 5.7%). Also, at this time, the percentages of spermatozoa at both of A23187-treated groups were significantly (P<0.05) higher than in control samples.

Other points of interest from the main effects included a significant (P<0.001) effect of A23187 concentrations upon the percentage of reacted spermatozoa, in that there was significantly (P<0.05) higher percentage of reaction at the highest concentration (1 µM/l) than the lower one (0.1 µM/l) (74.9 ± 5.6% and 51.9 ± 4.3%, respectively). Even although there was a high level of spontaneous acrosome reaction (42.4 ± 3.2%) in chilled control samples (without A23187), the percentage of reacted spermatozoa was significantly (P<0.05) lower than in A23187-treated samples.
Likewise, there was a significant ($P<0.001$) main effect of duration of incubation upon the percentage reacted spermatozoa, in that there was a significant ($P<0.05$) increase in percentage of spermatozoa which had undergone acrosome reaction by 30 min (from $42.4 \pm 2.3\%$ to $62.6 \pm 5.5\%$). However, there was no significant difference upon the results with further incubation from 30 to 60 min ($62.6 \pm 5.5\%$ and $66.7 \pm 5.7\%$, respectively).
Table 2.11. The effect of individual dogs upon the acrosome reaction of chilled spermatozoa (Experiment 2b)

<table>
<thead>
<tr>
<th>Dog</th>
<th>% acrosome reaction (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog A</td>
<td>56.28b ± 5.87</td>
</tr>
<tr>
<td>Dog B</td>
<td>69.06c ± 3.97</td>
</tr>
<tr>
<td>Dog C</td>
<td>46.39a ± 4.78</td>
</tr>
</tbody>
</table>

Significance: P<0.001

*a-c Means having different superscripts are significantly different from each other (P<0.05). (n=3)
Figure 2.13. The effect of incubation time and concentration of A23187 (µM/l) upon the acrosome reaction of chilled spermatozoa (P<0.001)

\[ \text{% reacted spermatozoa} \]

Time (min)

\[ \text{0} \quad \begin{array}{c} a \ a \ a \\ \text{30} \quad \begin{array}{c} a \ a \ c \\ \text{60} \quad \begin{array}{c} a \ b \ c \\ \end{array} \\ \end{array} \\ \end{array} \]

\(0\) \quad \begin{array}{c} \text{0.1} \\ \text{1} \\ \end{array} \)

\(a-c\) Means (± SEM) having different superscripts are significantly different from each other (P<0.05). (n=3, Experiment 2b)
2.3- Discussion

2.3.1- Experiments 1a and 1b

The major parameters of glycerol concentration, dilution rate and the effects of removal of seminal plasma that were examined in Experiments 1a and 1b were largely as predicted from the literature. Therefore, they provide the basis for diluent conditions for use in the remainder of this thesis.

2.3.1.1- Dilution rate and removal of seminal plasma

The results of Experiment 1a indicate that, across a wide range of diluents, there is little effect of dilution rate. When the data are examined with respect to main effects, it appears that the lowest (1:1) and highest (1:32) dilutions were associated with significant reductions in motility. In both cases there was a marked decrease of motility after storage for 48 h. For 1:1 and 1:2 dilutions, there was a significantly (p<0.05) greater reduction in motility when seminal plasma was not removed. However, for 1:16 dilution, there was a significantly (p<0.05) higher motility when the semen was glycerolised at +4°C in the presence of seminal plasma.

In Experiment 1b, which examined a narrower range of dilutions (1:2 to 1:8), there was a significant main effect of dilution rate, but whereas in Experiment 1a, the range of overall mean motilities was relatively wide (45% at both 1:1 and 1:32 versus 53% at 1:8), in Experiment 1b, the range was much narrower (73% at 1:2 versus 78% at 1:8). Whether the statistically significant differences of Experiment 1b reflect useable biological differences is, given this narrow range of response, debatable.

A number of other experiments have recorded similar optimal dilution rates to those of the current work. These include the early work of Freiberg (1935), suggesting that dilution of 1:8 was optimal for dog semen. Martin (1963a,b), using simple extenders, found better motility at dilution rates of 1:5 and 1:10 than at 1:20, 1:50 or 1:250. Later, Yubi (1984), using Tris-fructose extender, also found that motility was better at dilution rates of 1:5 and 1:10 than at 1:1 or 1:20. England (1992), using Tes/Tris buffer, found that 1:4 dilution rate was optimal. Somewhat different results were obtained by Foote (1964c) who, using citrate-glucose-glycerine-yolk extender, found considerably greater motility at a dilution rate of 1:30 than of 1:3. Perhaps these differences reflect differences in the ability of various diluents to stabilise spermatozoa during cooling and
storage. In this context, it is noticeable that, in species, such as the boar (having spermatozoa with poor cold-shock resistance), high dilution rates are frequently associated with acrosomal damage and cell death (Pursel et al., 1972).

The overall results of these experiments demonstrated that removal of seminal plasma was markedly better than its presence for maintenance of spermatozoal motility at +4°C, although the effect also depended upon glycerolisation temperature and dilution rate. Thus, it was noted that the removal of seminal plasma had a greater effect upon the maintenance of motility when the semen was glycerolated at +4°C than at room temperature. The finding that the effect of removal of seminal plasma was more beneficial at low dilutions (1:1 and 1:2), than at high dilutions (1:4, 1:8 and 1:32) irrespective of any treatment (such as centrifugation and glycerolisation temperature) was, perhaps, predictable since the higher degree of dilution of the seminal plasma would have markedly reduced the final concentration of seminal plasma in the semen suspension.

The question of whether the mechanical process of centrifugation affects semen quality has previously been addressed by several workers. Several of these studies have unequivocally demonstrated that centrifugation does not affect either pre-freeze (Pérez Bodega and Mayenco-Aguirre, 1996) or post-thaw (Platz and Seager, 1977; Olar, 1984) semen quality in dogs. Interestingly, however, the work of Pérez Bodega and Mayenco-Aguirre (1996) demonstrated that semen quality (percentage motility, acrosomal status and viability) was markedly impaired when it was repeatedly centrifuged.

A number of previous studies have also investigated the interaction between dilution rate and the presence of seminal plasma. In one of the earliest of such studies, Martin (1963b) used a range of dilution rates (1:5, 1:10 and 1:20), plus 8% glycerol, but observed no effect of seminal plasma upon post-thaw motility. Platz and Seager (1977), who studied the effects of removal of seminal plasma (before 1:4 dilution in an extender containing 4% glycerol), found that motility was slightly better in the absence of seminal plasma.

Subsequently, Oettle (1982) reported that removal of seminal plasma before dilution and cryopreservation resulted in a significantly longer maintenance of post-thaw motility (during incubation at 32°C) than in controls, which did contain seminal plasma.
Conversely, Olar (1984), using semen at about 1:20 dilution in the extender (containing 4\% glycerol), found that the initial post-thaw motility was slightly higher in centrifuged samples than in neat semen, although the opposite occurred after incubation at 37°C for 1 h.

The foregoing findings are in general agreement with the results of the present investigations. Although the details of Experiment 1a differed from the protocols used in the above trials, the trend in motility responses to the presence or absence of seminal plasma was evident. Such trends were most clearly seen when effects of the presence of seminal plasma were examined over an extended period of incubation. Summarising the present trial, it was noted that; (i) whilst the absence of seminal plasma was beneficial for dilution rates <1:8, for higher dilutions the presence of seminal plasma was beneficial and (ii) there was a substantial reduction in motility values between time zero and 2 h and between 24 and 48 h. Given the interactions between dilution rate and centrifugation, these results suggest that the removal of seminal plasma by centrifugation can be beneficial for maintenance of spermatozoal motility at 4°C for 48 h. Indeed, the suggestion also agrees well with the previous findings of England and Allen (1992b), who demonstrated the adverse effect of seminal plasma on routine semen quality parameters (motility, morphology, live/dead ratio and velocity) following prolonged incubation at 37°C.

There may be a number of other benefits that might accrue from the removal of seminal plasma. These include:

- It ensures that spermatozoa are exposed to similar concentration of the components in the extender, regardless of dilution rate. This factor is of importance to the latter stages of the present thesis, where high levels of extension were needed.
- The total number of spermatozoa in an insemination dose can be regulated more efficiently.

Freiberg (1935) suggested that dilution of 1:8 was optimal for dog semen. It is also interesting to note that with this dilution rate, in the present study, the spermatozoal motility was virtually unaffected either by glycerolisation temperature or by centrifugation. Additionally, Yubi (1984), using Tris-fructose extender, found that the dilution rates of 1:5 and 1:10 caused less deterioration (as compared to 1:1 and 1:20).
England (1992), using Tes/Tris buffer only (no glycerol), found that 1:4 dilution rate was optimal in a range of 1:1 to 1:32. Subsequently, he found that dilution rate of 1:4 was favoured over neat semen or dilutions at 1:16 and 1:32 (England, 1993). However, in another study, Foote (1964c), using citrate-glucose-glycerine-yolk extender, found considerably greater motility at a dilution rate of 1:30 compared to 1:3. In the present study, with centrifuged samples that were glycerolised at +4°C the motility was the highest at a dilution rate of 1:4. It should be remembered that there was marked interrelationship between dilution rate (in Tris-fructose extender) with treatment (centrifugation and glycerolisation) in the present study. Overall, these studies suggest that optimum dilution rate may vary between the extenders used and it is correlated with the seminal treatments (glycerolisation and centrifugation). It should also be noted that high dilution might result in acrosomal damage and increased sensitivity to cold shock, as seen in the boar (Pursel et al., 1972). Therefore, it is logical to include membrane-stabilising agents (e.g. Equex STM Paste) or proteins (e.g. Proline) to protect spermatozoal membranes and promote the motility in the extender when semen is to be diluted especially at higher dilutions. It is also recommended that, following centrifugation or high dilutions, care should be practised during initial cooling to/at +4°C and subsequent freeze-thawing.

2.3.1.2- Glycerolisation regimen

The literature on optimal conditions for glycerolisation is diverse and contradictory. Many different diluents have been used in the various published trials, and perhaps in consequence, the ideal conditions of glycerol concentration and its addition temperature varied greatly between studies. Thus, although there is an exhaustive literature on the subject, it was necessary to repeat this investigation for the particular diluent (Tris) that had been chosen for use in this thesis.

Experiment 1b examined the tolerance of various concentrations of glycerol. The results demonstrated that spermatozoa could tolerate a wide range of concentrations of glycerol without seriously adverse effects. The effect of glycerol concentration was statistically significant (P<0.001), in that lower motilities were recorded at higher glycerol concentrations, yet between 0% and 6% glycerol, a decrease of motility of only 4 percentage points was observed (0% glycerol: 76% ± 1% versus 6% glycerol: 73 ± 1%). Hence the biological effects upon the motility at the range of concentrations of
glycerol used were little. Moreover, there was no significant (P=0.94) interaction between glycerol concentration and dilution rate (over the narrower range of 1:2 to 1:8 used in Experiment 1b); again indicating a wide tolerance of moderate concentrations of glycerol by canine spermatozoa based on motility status.

When one considers the maintenance of sperm motility at +4°C, there are a number of reports using different extenders and various concentrations of glycerol. Foote (1964a) found that the optimum glycerol concentration was 6-8% in citrate buffer, 8-11% in Tris buffer. Using citrate-glycine-glucose-yolk extender, up to 8% glycerol (w/v) could be included with only a slight reduction in motility for short periods of storage (Foote and Leonard, 1964). However, Foote (1964b) observed that 8% glycerol was more detrimental to semen than 4% with the same extender. He also noted that higher levels of glycerol were required as the concentration of buffer increased. Province et al. (1984), using various extenders (i.e. skimmed-milk, Tris-yolk, caprogen and Cornell), observed that whilst 6% glycerol depressed spermatozoal motility during storage at +4°C for 4 days, 3% had no adverse effect. England (1992, 1993) showed that 2% glycerol was superior to 4 and 8% in Tes-Tris extender or saline. Similarly, Bruyas et al. (1996), using Tris, Laiciphos and skimmed-milk extenders, observed that the addition of 3-4.8% glycerol does not improve the preservation of canine semen. Hay et al. (1997b), using Tris-glucose extender containing 4% glycerol, observed that the addition of glycerol made no change in acrosomal status or motility but resulted in a decline in the number of spermatozoa bound per oocyte, indicating subtle changes to the head of spermatozoa.

Neither of the experiments reported in this Chapter investigated the concentration of glycerol needed for cryopreservation. Nevertheless, it is highly pertinent to consider some of the studies of the concentrations of glycerol required for successful cryopreservation, for these investigations, taken together with the results of the present experiments, are of importance in determining the glycerolisation regimens that are to be used in the remaining experiments of this thesis. Thus, Martin (1963a), using Ringer-citrate-egg yolk extender, noted that 4% glycerol was superior to 2 or 8% for spermatozoal velocity in neat semen. However, he also observed that 8% glycerol was apparently superior to 4% for post-thaw motility. Subsequently, he compared 7.5 and 10% glycerol in skimmed-milk diluent and showed that post-thaw motility was slightly
higher with 10% glycerol (Martin, 1963b). In other studies, Christiansen and Schmidt (1980) used 8% glycerol in a Tris-fructose extender and achieved high levels (from 50 to 70%) of post-thaw motility. Later, Smith (1984) suggested that 9% glycerol in Pipes extender was optimum for freezing, but Olar et al. (1989) suggested that semen should contain only 2-4% glycerol for freezing. Fontbonne and Badinand (1993b) suggested that glycerol concentrations should be between 1.6 and 3.2%, but this may vary among different extenders. Mayenco-Aguierre and Gomez-Cuétara Aguilar (1996) observed that post-thaw spermatozoal survival was better in 8% glycerol compared to 4%. Likewise, Rota (1998), using Tris-glucose extender containing 0.5% Equex STM Paste, observed that the reduction in glycerol concentration from 5 to 3% had a negative effect on spermatozoal survival after thawing and during incubation for 5 h. Furthermore, Peña et al. (1998b), using Tris-fructose extender containing 2-8% glycerol, found that post-thaw semen quality (motility, acrosomal integrity) was superior in 8% glycerol while the 2% was the poorest.

Conversely, Günzel-Apel et al. (1993) found little difference in post-thaw motility between 4, 6 and 8% glycerol. Likewise, Kim and Kim (1995) observed that there was no difference in post-thaw semen quality between 5 and 10% glycerol.

It is also interesting to note that Martin (1963b) observed that, after cryopreservation of semen in 7.5 or 10% glycerol, the post-thaw motility was very poor until spermatozoa were resuspended in glycerol-free diluent. Likewise, Ottilé (1982), who froze semen by using 4% glycerol in Tris-fructose extender, resuspended the samples in glycerol-free diluent after thawing and successfully achieved pregnancy in a bitch following intravaginal insemination.

In addition to the effects of glycerol concentration per se, the temperature at which it is added to semen and the presence or absence of seminal plasma also affect the survival of spermatozoa. The results of Experiment 1 demonstrated that glycerolisation at +4°C compared to room temperature was markedly superior for maintenance of spermatozoal motility irrespective of whether the seminal plasma had been removed. However, there were significant interactions between temperature of glycerolisation, presence of seminal plasma and dilution rate. In summary, these were that; (i) at a dilution rate of 1:1, glycerolisation at +4°C, after removal of seminal plasma, was associated with
significantly (P<0.05) higher motility than other treatments, (ii) at dilutions between 1:2 and 1:16, there were little effects of the glycerolisation temperature, providing that the seminal plasma had been removed, but where glycerolisation had been undertaken at room temperature, in the presence of seminal plasma, motility was significantly (P<0.05) lower than for other treatments, and finally (iii) at a dilution of 1:32, glycerolisation at room temperature was associated with significantly (P<0.05) lower motility than when undertaken at +4°C.

In the literature, a number of workers also investigated the effect of glycerolisation temperature upon the semen quality. Martin (1963a,b) found that the addition of glycerol at +5°C was superior to 30°C which was better than 35°C. By contrast, others (Foote, 1964b; Foote and Leonard, 1964; Rohloff et al., 1978) found no difference between glycerolisation of semen at different temperatures. England (1992) compared one- and two-step glycerolisation at 39°C and found that there was no difference between treatments upon the semen quality; thereby concluding that glycerol toxicity was not associated with the sudden change in osmotic pressure caused by a one-step dilution. Additionally, Fontbonne and Badinand (1993b) showed that, as far as post-thaw motility is concerned, glycerol can be added at room temperature and that it is not necessary to add it in several steps. Finally, Peña et al. (1998b) diluted semen at 1:4 dilution rate in an extender which contained 8% glycerol (v/v) and compared three glycerolisation regimens; (i) one-step; at 37°C, (ii) two-step; at +4°C after 1 h cooling, and (iii) two-step; at +4°C after 2 h cooling. Their finding was that there was no difference of the treatments upon the post-thaw semen quality during 4 h storage at 39°C.

Glycerol has both cryoprotectant and toxic effects. It is a highly osmotically active molecule, so high concentrations may cause osmotically-induced membrane damage either during its addition or removal (Watson, 1995). On the other hand, Gao et al. (1995) estimated that, for human spermatozoa, the relatively low percentage of glycerol (i.e. ~0.5 M [3.25%, w/v] or less) used during cryopreservation, osmotic stress may not be a problem. Likewise, Fiser and Fairfull (1986) observed that the method of glycerol addition or removal (by using single or multiple steps) did not affect cryosurvival of ovine spermatozoa. However, they did find that temperature of glycerolisation was important, in that deterioration of spermatozoal motility and acrosomal integrity was
more apparent when samples were glycerolised at 30°C rather than at +4°C. Their conclusion was that osmotic effects of glycerol are less important than its toxicity. However, Gao et al. (1995) showed that significant osmotic injury in human spermatozoa was evident when 1 M (7.5%, w/v) glycerol was added at 22°C in less than four steps and removed in less than eight steps (considering 4-, 6-, 8-steps removal). In this context, Watson (1995) considered that until the 'reflection coefficient' (i.e. an expression of the relative permeabilities of water and glycerol) of spermatozoa is known with certainty for all species, it may be premature to conclude that osmotic stress is of little importance.

2.3.1.3- Conclusions

The conclusions drawn from Experiments 1a and 1b were, therefore, as follows:

- There was little overall effect of dilution rate upon the survival of spermatozoa for 24 h at +4°C. After 48 h storage, motility results were poorer in 1:1 and 1:32 dilutions.
- Glycerolisation at +4°C was superior to room temperature, irrespective of the presence of seminal plasma. At intermediate dilution rates (1:2 to 1:16), the impairment of motility that occurred with glycerolisation at room temperature was worse when seminal plasma was also present.
- At dilution rates of 1:2 to 1:8, there were no significant interactions between glycerol concentration and dilution rate for the maintenance of spermatozoal motility. However, higher concentrations of glycerol (>2%) were associated with a slight but statistically significant reduction in motility.

2.3.2- Experiment 2a

In this part of the study, a method was developed for the induction and assessment of acrosome reactions *in vitro*, by (i) examining the effects of incubating spermatozoa for various times in the presence of different concentrations of calcium ionophore, A23187 and (ii) evaluating the use of naphthol yellow S/aniline blue staining as an alternative to the established method of phase contrast microscopy.

2.3.2.1- Assessment of acrosome reaction

An ideal assay for acrosome reactions should be accurate, consistent, rapid, applicable to small numbers of cells, innocuous to spermatozoal function, usable in all biological
fluids and environments in which the spermatozoa are found and capable of distinguishing false from normal acrosome reactions (Cross and Meizel, 1989). Definitive evaluation of the acrosome reaction can only be performed by electron microscopy (Yanagimachi, 1994), and despite being laborious and time consuming, it is usually the standard against which any new assay is measured (Cross and Meizel, 1989).

More commonly, routine evaluation of acrosome reactions has been undertaken by light microscopy, especially in species having spermatozoa with a large acrosome (e.g. the guinea pig and Chinese hamster), which can be readily examined by PC or DIC microscopy (Yanagimachi, 1994). The acrosome of dog spermatozoa has also been found to be amenable to monitoring the acrosomal changes by PC microscopy (Mahi and Yanagimachi, 1976, 1978). Although fresh spermatozoa can be examined in this way, it is generally easier to observe the acrosome if the cells have been fixed with formaldehyde (Cross and Meizel, 1989). Phase contrast microscopy has the advantages of being both relatively simple and well established as a method of examining the acrosome. The advantages of PC microscopy are:

- Intermediate stages of the acrosome reaction are often discernible.
- Spermatozoa can be monitored both in the laboratory and in the field.
- It is cost-effective, simple and quick.
- Acrosomal scoring is relatively easy, especially with fixed (as compared to non-fixed) samples.

The disadvantages are:

- Acrosomal scoring cannot be delayed, as the wet preparations have only a short life.
- The scoring in the dog is relatively difficult compared with some other species (e.g. bovine).
- The spermatozoa may not always be found in focal plane under the microscope, which can make their assessment difficult.
- Simply scoring the complete loss of the acrosomal material may lead to errors (false negatives) if there are substantial number of spermatozoa in their early stages of the acrosome reaction.

Spermatozoa of many mammals (e.g. bull, ram, dog) have very thin or tightly fitted
acrosomes, and consequently, their acrosome reactions are difficult to detect in the live cells (Yanagimachi, 1994). Nigrosin/eosin stain (developed by Campbell et al., 1956 for bull, ram and boar spermatozoa) is a useful stain for detecting abnormalities and live/dead ratios of dog spermatozoa (Seager, 1986). However, it does not specifically stain the acrosome, as the eosin stain is only taken up by dead spermatozoa (Campbell et al., 1956). Furthermore, Root Kustritz et al. (1998) obtained a significantly lower percentage of normal spermatozoa by using eosin B/eosin Y with nigrosin than by either PC microscopy or by Giemsa stained samples. The more common strategy for staining the acrosome is to use multiple stains: typically, using an acrosomal stain and a nuclear counterstain to provide contrast in the post-acrosomal region (Cross and Meizel, 1989). An example of such a stain is eosin, Congo red and gentian violet (Ivanova-Kicheva et al., 1995) or Bismark brown, rose bengal and trypan blue (Talbot and Chacon, 1981). The latter stain, which is widely used in many species, has been used in dogs for both assessing the acrosome reaction in fresh semen (Kawakami et al., 1993a) and acrosomal damage in frozen samples (Ivanova et al., 1999). A combination of triple fluorescent dyes such as carboxy-SNARF-1 (identifying live cells), propidium iodide (identifying dead cells or cells with damaged membranes) and fluorescent isothiocyanate-conjugated *Pisum sativum* agglutinin (identifying acrosomal content of spermatozoa with damaged plasma and outer acrosomal membrane) has also been used (Peña et al., 1999; also see below). Examples of dual staining include aniline blue/eosin B (Casarett, 1953; Kawakami et al., 1988), trypan blue and Giemsa (Rodríguez-Gil et al., 1994), commercial Spermac stain (developed by Oettlé, 1986a) as used with fresh (Guérin et al., 1999) and frozen (Ström et al., 1997) samples. The other dual stains used in assessing the acrosome reaction include naphthol yellow S in combination with either erythrosine B (Kumi-Diaka and Harris, 1994) or aniline blue (developed by Christensen et al., 1994 for bovine spermatozoa, and used in the present study). Christensen et al. (1994) concluded that naphthol yellow S and aniline blue (NA) is a suitable stain for evaluation of the acrosome reaction, including the intermediate stage (i.e. the partially acrosome reacted).

The present experiments indicated that a high level of correlation exists between acrosome assessments that were made by PC microscopy and those after NA staining. However, although the NA staining technique has many advantages, there are also some
disadvantages. The advantages of the stain are:

- It allows the making of semi-permanent slides.
- The reagents used are accessible and inexpensive.
- It is possible to use a standard, bright-field microscope.
- It allows straightforward determination of the acrosome reaction, including its initial stages.
- The microscope can be linked to a video recorder allowing the large number of spermatozoa to be assessed quickly.

The disadvantages are:

- The preparation of the slides requires more experience and takes longer time than PC (30 min against 2 min, respectively).
- The correlation of the staining with results obtained by TEM is not known.
- Unless the staining solution is carefully handled, background staining and staining artefacts can occur to an unacceptable extent.

As in previous studies (Christensen et al., 1994), the NA staining was found to be more practical than PC. It was simple, quick, repeatable, easy for differentiating the acrosomal status (i.e. intact, partially or completely reacted), and provided slides which can be stored. The other major advantage was that, even following dilution in Tris-based extender, containing egg yolk and glycerol, and then subsequently freeze-thawing the slides still had very little background staining. This made the NA staining a useful tool for assessing pre- and post-thaw sperm morphology, which might be used as a straightforward alternative to DIC microscopy. However, the staining quality was impaired following freezing and thawing, which might be due to the effects of freeze-thawing damage. In this context, Oettlélé (1986a,b), who used commercial Spermac stain in fresh and frozen dog spermatozoa, observed that as the degree of acrosomal damage increased, the intense staining of normal spermatozoa was lost due to a corresponding loss of the acrosomal contents. Likewise, Guérin et al. (1999), using Spermac and chlortetracycline staining in fresh semen, also observed that there were markedly higher numbers of cells (considered to have acrosome reacted) that were unstained by Spermac especially following prolonged incubation (up to 24 h). Indeed, Watson (1975) considered that the decline of staining (Giemsa) in ram spermatozoa after cooling or freezing may indicate the loss of stainable
material from the membranes or contents of the acrosome, and might therefore constitute further evidence of acrosomal damage. Furthermore, a similar observation was made in boar spermatozoa, whose acrosomes exhibited altered staining characteristics with both nigrosin/eosin (Dott and Foster, 1972), Wells-Awa (Wells and Awa, 1970) and commercial Spermac (Paulenz et al., 1995) stains after freezing and thawing.

The interpretation of the results of any assay of acrosome reactions is greatly simplified if physiological acrosome reactions can be differentiated from the degenerative acrosomal loss that accompanies cell death (Cross and Meizel, 1989). It is possible to differentiate individual spermatozoa that are acrosome reacted and viable (i.e. motile) from those which are non-viable (i.e. immotile) in preparations of live spermatozoa (Mahi and Yanagimachi, 1976). However, it is impractical to assess populations thus due to plane of the focus (Cardullo and Florman, 1993) and the motility of the spermatozoa (Yanagimachi, 1994). Techniques utilising supravital stains (such as the Hoechst stain or propidium iodide; semi-permeable DNA-binding dyes picked up only by the nuclei of dead cells), can also be used in the dog (Kawakami et al., 1993a; Hay et al., 1997a; Hewitt and England, 1997; Peña et al., 1999), but have an inherent problem: The plasma membranes of moribund spermatozoa are often impenetrable to the dyes (especially Hoechst), and thus some moribund spermatozoa may be classified as live and potentially fertile (Yanagimachi, 1994). Indeed, whenever the population of immotile, unstained cells may be large, the use of a supravital stain may lead to an unjustified level of confidence in the results (Cross and Meizel, 1989).

In a similar way, the techniques using fluoresceins (Hay et al., 1997a; Hewitt and England, 1997; Peña et al., 1999) have the common problem that spermatozoa must be killed before examining their acrosomes. Once spermatozoa are killed, it can not be determined with certainty whether the cell under observation was vigorous, weakly motile or totally motionless at the time of fixation unless the same spermatozoon is continuously traced (Yanagimachi, 1994).

The best assessment of viability of acrosome reacted spermatozoa is motility but it is not feasible to assess acrosome reaction with a population of actively moving spermatozoa, nor when there are many immotile spermatozoa (e.g. after cryopreservation, in an ejaculate of poor quality, or after prolonged incubation in vitro). Finally, since spermatozoa
inevitably die following the acrosome reaction (Harrison, 1996), and thus the equatorial segment vesiculates (Oettlé and Soley, 1988; Yanagimachi, 1994) and the post-acrosomal region degenerates (particularly in frozen cells), such changes make the determination of viable, acrosome reacted spermatozoa virtually impossible. It was for these reasons that the present study simply considered the percentage of acrosome reacted spermatozoa. Hence, no further attempt was made in differentiating between viable- and dead-reacted spermatozoa. No technique differentiates between a physiologic or degenerative acrosome reaction. It is also clear that the percentage increase in acrosome reacted spermatozoa (not the absolute percentage) reflects the percentage of viable acrosome reacted sperm.

Further studies in other species have compared staining and direct examination of the acrosomes with similar findings (Steinholt et al., 1991; Christensen et al., 1994).

By contrast, De Leeuw et al. (1991) reported differences in the ability of different populations of spermatozoa to adhere to the surface of the slides, which could explain the differences observed in the present study. Although poly-L-lysine was used for optimising adhesion of the spermatozoa onto the slides (Marzia et al., 1975), it is still possible that some of the spermatozoa were lost during staining. Therefore, care appears to be desirable in preparation of the slides for staining, especially during rinsing steps.

Taken together, these results suggest that, for fresh dog semen, NA staining may provide a useful, possibly less laborious, alternative to PC evaluation. It is less clear that the method can be used for cryopreserved canine spermatozoa unlike the frozen bull semen (Christensen et al., 1994). This limits the usefulness of NA staining for the purposes of this thesis and necessitates the use of direct microscopy of wet preparations where acrosome evaluation is required. In consequence, despite its initially promising results, it was decided that no further use of NA would be made in the experiments described in this thesis.

2.3.2.2- In vitro induction of acrosome reactions

Under in vivo conditions, capacitation of spermatozoa takes place within the female genital tract (Austin, 1951; Chang, 1951). However, it is also possible to induce in vitro capacitation by using appropriate culture media and conditions. Early studies of the induction of capacitation used fairly simple salt solutions supplemented with blood
serum or follicular fluid (Yanagimachi, 1994). Later studies found that the spermatozoa of many mammals can survive and capacitate in artificial media similar to modified Tyrode's and Krebs Ringer's solutions (see Yanagimachi, 1994 for detail). In dogs, many media have been used successfully, including (i) modified Tyrode's medium (TALP) (Hay et al., 1997a; Szász et al., 1997); (ii) modified Krebs Ringer's medium (Mahi and Yanagimachi 1976, 1978), (iii) simple/modified Ham's F10 medium (Mayenco-Aguirre and Pérez-Cortés, 1998; also cited by Mahi-Brown, 1991), and (iv) protein-free tissue culture medium 199 (Hewitt and England, 1997). However, Rota (1998), comparing standard Tris-fructose-citric acid buffer (TFC, without calcium ion) and CCM (both contain BSA), found that the percentage of active and acrosome reacted sperm was markedly lower in TFC buffer. This wide range of media suggests that dog spermatozoa may not have highly specific medium requirements for capacitation and acrosome reaction in vitro, provided that the media contain extracellular calcium. Indeed, the presence of calcium ions in the medium is essential for both viability and the acrosome reaction in dog spermatozoa (Mahi and Yanagimachi, 1978). Furthermore, Szász et al. (1997) showed that the percentage of canine spermatozoa undergoing acrosome reaction varies with increasing concentrations (0.05 to 2 mM/l) of calcium ion, being maximum with the latter. Finally, there appeared to be no detrimental effect of using PVA (rather than BSA) in the TALP, although no direct comparative trials were undertaken to investigate this.

Many studies have examined spontaneous or induced capacitation and acrosome reactions in vitro. The results of these trials are pertinent to the present experiments, so detailed consideration of their results is warranted. The minimum time needed for capacitation in vitro varies considerably from less than 1 h up to several hours, depending on the species. Species differences are likely to be due to the innate differences in the physical and chemical characteristics of the plasma membrane of their spermatozoa (Yanagimachi, 1994). Although there appears no literature upon duration of the capacitation of dog spermatozoa under in vivo conditions, several in vitro studies have reported that the time needed or, more properly, used for capacitation varies from 1 to 24 h (see below for references). In these studies, the criteria considered for the spermatozoa to be judged as 'capacitated' were; (i) the hyperactivated motility and (ii) spontaneous acrosome reaction (Mahi and Yanagimachi, 1976; Kawakami et al., 1991;
Rota, 1998), (iii) ZP penetration/binding capacity (Yamada et al., 1992; Mayenco-Aguirre and Pérez-Cortés, 1998; Ivanova et al., 1999), and (iv) calcium influx (Brewis et al., 1999). Hewitt and England (1997, 1998) further assessed the acrosome intact spermatozoa as non-capacitated or capacitated by fluorescent staining. Mahi and Yanagimachi (1976) observed that the earliest evidence of hyperactivity, acrosome reaction or zona penetration was seen 7 h after insemination and hence concluded that capacitation required 7 h. Similar results were reported by Mahi and Yanagimachi (1978), Wright et al. (1998) and Kawakami et al. (1998a). However, some workers (Szász et al., 1997; Mayenco-Aguirre and Pérez-Cortés, 1998; Ivanova et al., 1999) used a 1 h capacitation time, whilst others used 2 h (Brewis et al., 1999), 4 h (Shimazu et al., 1992; Hewitt and England, 1997; Rota, 1998), 7 h (Kawakami et al., 1993a), 9 h (Kawakami et al., 1991), 10 h (Kumi-Diaka and Harris, 1994), and 24 h (Guérin et al., 1999). All achieved satisfactory capacitation but each used different media and different criteria of “satisfaction”. It is clear that neither minimum nor median times for capacitation are rigidly fixed either in vivo or in vitro, as they depend on both the physiological state of the animal, and the physical and chemical composition of the medium used (Yanagimachi, 1994).

A small number of detailed studies of A23187-induced canine acrosome reactions have been undertaken by Szász et al. (1997), Geussová et al. (1997), Hewitt and England (1998), Brewis et al. (1999) and Guérin et al. (1999) during the time when the experiment described in this thesis was being conducted. Acrosome reaction was also achieved by Metzler (cited by Mahi-Brown, 1991), who used A23187, although no data have been given concerning its concentration. Usually spontaneous acrosome reaction has been studied. A few workers stimulated an acrosome reaction with, for example ZP (Kawakami et al., 1993a), ZP proteins and progesterone (Brewis et al., 1999). Kawakami et al. (1998a) attempted to stimulate the acrosome reaction with oviduct fluid but only achieved capacitation.

The study of Hewitt and England (1998) showed that 10 µM/l A23187 caused capacitation and acrosome reaction. They found that after the start of incubation with A23187 a rapid decline in non-capacitated, acrosome intact cells occurred coincidently with a rapid increase in capacitated, acrosome intact and capacitated, acrosome reacted cells. Control cells exhibited similar changes but at a much slower rate. It was concluded that incubation
for 60 min with ionophore A23187 promoted capacitation and the acrosome reaction in canine spermatozoa. Subsequently, Brewis et al. (1999) found that nearly half of the spermatozoa were acrosome reacted following 30 min of incubation and Guérin et al. (1999) found that in the same time period the majority of the spermatozoa have undergone acrosome reaction. Likewise, in the study of Szász et al. (1997), using various concentrations of A23187 (0.05 to 5 µM/l), the maximal percentage of acrosome reaction was obtained with 5 µM/l A23187 within 30 min with no marked increase by 60 min. The present results cannot be directly compared with the above studies due both to the use of different criteria for assessing acrosome reactions and the use of different culture media. Similar overall trends were observed, as follows:

- There was a significant increase in the percentage reacted cells (from ~54 to 84%) by 15 min in 1 µM/l A23187-treated samples. This indicates that the initiation of the stimulative action of A23187 was very rapid and it agrees well with the observations of Szász et al. (1997), Hewitt and England (1998) and Guérin et al. (1999). Other observations of ZP (Kawakami et al., 1993b), follicular fluid (Kawakami et al., 1998a), lysolecithin and calcium-induced acrosome reactions support the view that the initiation of acrosome reactions occurs within 1-5 min of exposure (see Yanagimachi, 1994 and Brewis et al., 1999 for detail).

- After 15 min, there was no significant difference in percentage of reacted cells between the successive incubation times, as previously noted by Hewitt and England (1998).

- In the present study, there was no significant increase in the percentage of reacted spermatozoa in control samples and in samples incubated in the presence of <1 µM/l during the entire incubation period. This indicated that low concentrations of A23187 were not effective in inducing the acrosome reactions. Additionally, in present trials, the low percentage of reacted spermatozoa (up to 25%) from control samples agrees well with the findings of Szász et al. (1997) and Hewitt and England (1998). Guérin et al. (1999) found that the percentage of spontaneous acrosome reactions was markedly increased following prolonged incubation (3 to 24 h).

Comparison of the present data with those of other species is also of interest. There was no significant difference between the incubation times of 60 and 90 min (16.1 ± 9.0% and 12.1 ± 9.0%, respectively) in control samples of bovine spermatozoa (Christensen et al.,
1994). In 1 µM/l A23187-treated samples, however, there was a significant increase between 60 and 90 min (46.3 ± 16.9% and 87.1 ± 11.4%, respectively), which closely resemble the present observations of the dog.

In summary, it was concluded that, for canine spermatozoa:

- (i) NA staining may be a useful technique for evaluation of the acrosome reaction as an alternative to the established method of PC microscopy, and (ii) both NA staining and PC microscopy are equally sensitive in assessing the acrosome reaction in fresh spermatozoa, although NA is clearly less descriptive in cryopreserved cells.
- Modified TALP medium is sufficient for capacitation and the acrosome reaction.
- The optimum concentration of calcium ionophore (A23187) is 1 µM/l for inducing the acrosome reaction.
- There is a significant increase in the percentage of reacted spermatozoa by 15 min of incubation and further incubation up to 30-45 min may be essential (and enough) to achieve maximum percentage of acrosome reaction.

2.3.2.3- Summary and conclusions

- The Tris-fructose-citric acid diluent containing 20% egg yolk that was used for Experiments 1a and 1b provides a suitable medium for extension and storage of canine semen at +4°C. There is little overall effect of dilution rate upon the survival of spermatozoa for 24 h at +4°C.
- Number of motile spermatozoa is higher after glycerolisation at +4°C than at room temperature.
- The effect of the presence of seminal plasma upon the numbers of motile spermatozoa is marginal, except when semen is glycerolised at room temperature.
- Toxic effects of glycerol are independent of dilution rate, but the numbers of motile spermatozoa are reduced at concentrations >2%.
- NA staining provides an effective means of assessing acrosome reactions in fresh spermatozoa, but is inferior to PC for cryopreserved spermatozoa.
- In the presence of 1 µM/l A23817, incubation of spermatozoa for 15 to 45 min induces acrosome reactions in vitro.
2.3.3- Experiment 2b

2.3.3.1- *In vitro* acrosome reaction in chilled spermatozoa

The presence of different concentrations of A23187 in chilled semen markedly (P<0.001) increased the percentage of acrosome reacted spermatozoa in a time-dependent manner. Furthermore, following chilling, a lower concentration (0.1 µM/l) of A23187 also markedly increased the percentage of reacted spermatozoa. Considering the previous findings with fresh semen, the following comparisons were made:

- In fresh semen, unlike with chilled samples, the interaction between time and A23187 concentration upon the percentage of reacted spermatozoa was not significant.
- At the beginning of incubation (<5 min), there was apparently a higher percentage of reacted spermatozoa in fresh semen with 1 µM/l A23187 compared to the lower concentrations (>50% *versus* <10%, respectively). No such difference was found in chilled semen, in that 40-45% of the cells were reacted for all concentrations (including controls) of A23187.
- As with 1 µM/l A23187, a lower concentration of A23187 (0.1 µM/l) was also effective to induce a marked increase in the percentage of reacted cells in chilled semen during incubation for 30 to 60 min.
- In fresh semen, there was a gradual increase (from 5-10% to ~20-25%) in spontaneous acrosome reactions by 60 min, but no such changes were seen in the percentage of reacted cells in chilled controls (remaining at ~40-45%) over the same period.

Therefore, it is suggested that, even with a 10-fold lower concentration (0.1 *versus* 1 µM/l) of A23187 a higher proportion of acrosome reactions in chilled spermatozoa could be achieved compared to fresh spermatozoa within a relatively shorter period of incubation. It was considered that in chilled semen the two-fold increase (~20-25% *versus* ~40-45%) in the percentage of spontaneous acrosome reaction was due to the subsequent handling processes, i.e. dilution, cooling/equilibration. This is in agreement with previous findings of Oettlé (1986b), who observed a marked acrosomal damage during dilution to equilibration. In contrast, Nizanski *et al.* (1997) showed that there was little deterioration of the acrosome during these pre-freeze handling processes, but they included a detergent (Orvus ES Paste) in the extender to improve the spermatozoal...
membrane quality. It is well known that detergents can increase the proportion of cells having an intact plasma membrane, post-thaw motility and thermostability of dog spermatozoa (Thomas et al., 1992; Rota et al., 1997; Peña et al., 1998c).

Undoubtedly, the foregoing morphological changes of the acrosome result from the ion flux, especially of calcium$^{2+}$, during chilling. There is a species difference in terms of the speed of acrosome reaction (up to 5 min) in fresh spermatozoa (see Yanagimachi, 1994 for detail), which may be even faster during chilling and rewarming (to room temperature and, then to incubation at 39°C). Rota (1998), studying the effect of preservation on capacitation status of dog spermatozoa, found that the time course of capacitation was accelerated (2 h versus 4 h in chilled and fresh samples, respectively). Furthermore, Bailey and Buhr (1995), who studied the regulation of internal calcium$^{2+}$ by chilled spermatozoa in the bull and boar, found that acrosomal morphology of chilled bovine spermatozoa was adversely affected by exogenous calcium$^{2+}$. They also found that the chilling caused an immediate increase in internal calcium$^{2+}$ in boar spermatozoa. Likewise, Zhao and Buhr (1995), studying calcium$^{2+}$ flux of bovine spermatozoa during a temperature challenge (cooling to/at +4°C and return to 25°C), demonstrated that during cooling the rate of calcium$^{2+}$ uptake in all cells increased, and that the rate of intact acrosomes had decreased at the end of temperature challenge. The lowered intact sperm number after temperature change in the present work may therefore have been related to calcium$^{2+}$ flux. It appears that there may be a synergism between the effect of temperature gradient and presence of A23187 upon the speed of acrosome reaction. A23187 causes a rapid influx of calcium ion, and thus promotes acrosomal exocytosis within a relatively short time (Fraser, 1995). Consequently, this could lead to a higher proportion of premature acrosome reactions in chilled semen, as compared to fresh samples. Indeed, Bailey and Buhr (1995) demonstrated that chilling affects the ability of spermatozoa to regulate internal calcium$^{2+}$ stores and to respond to a moderate calcium$^{2+}$ challenge. This effect varies between species. Therefore, the present findings suggest that chilling (cooling to/at +4°C) and return to 39°C (for incubation) might have enhanced the proportion of spermatozoa having undergone the acrosome reaction (even without A23187), through an increased permeability of the plasma membrane, which facilitates the influx of extracellular calcium$^{2+}$ in the medium.
Given the ionic changes, the effects of cooling upon the morphology and enzymes of the acrosome from different species are also of interest. With properly controlled cooling regimens, there was only a slight decrease in the proportion of intact acrosomes of fox (Zalewski and Andersen Berg, 1983), bull (Coulter and Foote, 1974), and ram (Watson and Martin, 1972) spermatozoa as assessed by light microscopy techniques. Chauhan et al. (1994) studied the acrosomal damage and enzyme leakage of goat spermatozoa during dilution and cooling by using enzyme assays and light microscopy. Their finding was that although there was no marked decrease in the proportion of intact acrosomes during both dilution and cooling, phosphatase enzyme was markedly decreased after cooling. They further demonstrated that there was a positively high correlation between enzyme release and acrosomal damage, indicating that the enzymes were of acrosomal origin. In boars, there was a considerable decrease in the percentage of acrosome intact spermatozoa, as centrifuged semen was cooled from 15°C to +4°C (Paulenz et al., 1995).

In a more detailed study, Ortman and Rodriguez-Martinez (1994), using centrifuged boar semen, showed that cooling to +5°C significantly affected the permeability of plasma membrane of spermatozoa, as assessed by supravital fluorescent stain. However, no direct comparison could be made between the present results and those of the other species. This is due to fact that, in the present study, the extender was replaced with TALP, containing calcium$^{2+}$, followed by subjection of semen to acrosome reaction assay. Subtle changes on the acrosome may not be revealed by routine light microscopy techniques, unlike with bioassays such as acrosome reaction. During pre-freeze handling processes, centrifugation followed by resuspension (as used twice in the present study) may lead to further disruption of overlying plasma membrane (Plummer and Watson, 1988). Therefore, the centrifugation itself might increase the acrosomal damage (in dog: Oettlé, 1986b; in boar: Paulenz et al., 1995) through partial loss of semipermeability of the plasma membrane. This might lead to an increased susceptibility of the acrosomes under the pre-freeze handling conditions and acrosome reaction. Hence, the value of centrifugation is still debatable, at least for dog semen.

2.3.3.2- Glycerolisation of chilled semen

The presence of glycerol in the extender or any other interactions between glycerol, incubation times and concentration of A23187 had no significant effect upon the
percentage of reacted spermatozoa. For most species, it is well established that glycerol is the most successful cryoprotectant, and its concentration represents a compromise between protective and toxic effects (Watson, 1979). In the present study, it was used at a final concentration of 2% (v/v). This was previously shown to be optimum for sperm motility in dogs (England, 1992). He demonstrated that during the storage at 39°C, glycerol exerted its marked toxic effect upon the acrosomal morphology only at a concentration of 10%, and that there was no influence of glycerol (including 10%) upon the vital staining. Hay et al. (1997b) studied the effect of glycerol (4%, v/v, final) and found that following equilibration at 0°C, centrifugation and resuspending in TALP, the addition of glycerol had no effect upon the acrosomal status or motility. However, the number of spermatozoa bound per oocyte declined, indicating subtle changes of the plasma membrane of the sperm head. The effect of structural deterioration of spermatozoa leading to the poor oocyte binding might have resulted from using a two-fold higher glycerol concentration than was used in the present study (4% versus 2%). In this context, Murdoch and Jones (1978), studying its effects upon the metabolism and ultrastructure of boar spermatozoa, suggested that glycerol affects fertility by stabilising and then destabilising when it is removed, the spermatozoal membranes to an extent which reduces their capacity to undergo an acrosome reaction or to fuse with vitelline membrane of the ovum. In contrast, Bower et al. (1973), using zero to 8% glycerol (v/v, final) in diluted boar semen, showed that glycerol increases spermatozoal membrane permeability when it was >2%. Slavík (1987), studying the effect of glycerol (zero to 10%) on the penetrating ability of fresh ram spermatozoa with zona-free hamster eggs, demonstrated that the penetrating activity depended on the concentration of glycerol (markedly higher with >5%) and incubation time. The presence of glycerol accelerated induction of the acrosome reaction. The present results revealed that the addition of 2% glycerol had no effect upon the induction of the acrosome reaction, indicating the absence of any substantial damage upon the plasma membrane and acrosome. Previous studies (see Section 2.1.3.3) demonstrated that concentrations of >2% (v/v) glycerol resulted in markedly lower survival than at lower concentrations. Therefore, it appears that the loss of semipermeability of the plasma membrane (accelerating the acrosome reaction) could be less evident with lower concentrations of glycerol (≤2%).
Osmotic gradients across the plasma membrane and resultant cell volume excursions can be more evident with higher concentrations of glycerol which exerts its toxic or protective effects, in a concentration dependent manner (Watson, 1979; England, 1992). The equilibration temperature that was applied in the present study was +4°C compared to 0°C used in Hay’s study (Hay et al., 1997b). There has been no study to determine whether there is an effect on acrosome reaction of cooling to 0°C or holding at +4°C. However, Plummer and Watson (1988) demonstrated that there was a considerable deterioration of the plasma membrane and acrosome of boar spermatozoa (due to cold shock), as temperature was lowered from +4 to 0°C.

In the present study, semen was initially centrifuged. Removal of seminal plasma before cooling and glycerolisation is likely to increase the proportion of spermatozoa having disrupted plasma membranes adding to further damage may be introduced by glycerolisation-equilibration, and further removal of glycerol by second centrifugation and resuspension in the medium.

Compared to the study of Hay et al. (1997b), a higher concentration of extracellular calcium\(^{2+}\) (3.0 versus 1.71 mM/l) in TALP was used in the present trials. This might have led a higher proportion of spermatozoa to undergo the acrosome reaction at the beginning of incubation, and thus possibly masked the effect of glycerol in the present study.

The correlation between the oocyte penetration assay and acrosome reaction and their relevance to in vivo fertility are yet to be firmly established in the dog. Hewitt and England (1997) observed that there was no correlation between the percentages of acrosome reacted spermatozoa and oocytes penetrated. Kawakami et al. (1993b) suggested that both acrosome intact and acrosome reacted dog spermatozoa are capable of binding to the ZP. This does not necessarily mean that spermatozoa will attach to the plasma membrane of the oocyte (following ZP penetration) as they can be lost after binding (Mahi-Brown, 1991; Kawakami et al., 1993b). Mahi-Brown (1991) noted that acrosome intact spermatozoa of dogs adhere loosely to the zona surface, and can be removed by pipetting. These studies thus imply that attachment of dog spermatozoa to homologous ZP (of the bitch) might be independent of the acrosomal status.

The effect of glycerolisation upon the acrosome of spermatozoa from other species is
also of interest. There were no considerable differences in acrosomal status of fresh and glycerolated spermatozoa in the fox (Zalewski and Andersen Berg, 1983), bull (Coulter and Foote, 1974), and ram (Watson and Martin, 1972). However, in a later study, Watson (1975) found a marked difference in acrosomal staining before and after glycerolisation in cooled ram semen, indicating deterioration of the plasma membrane of sperm head and of the acrosome. Similarly, in goat spermatozoa, a marked release of Glutamic oxaloacetic transaminase (GOT) enzyme was observed after equilibration (compared to fresh semen) while there was no obvious change on the acrosomal ultrastructure by scanning electron microscopy (SEM) (Chauhan et al., 1994). Ortman and Rodriguez-Martinez (1994) observed that although there were marked differences in plasma membrane integrity and acrosomal ultrastructure between diluted and equilibrated spermatozoa in boar semen, no differences were found before and after glycerolisation. These results imply that the effect of glycerolisation might not be revealed by light microscopy or even by SEM. Therefore, effects need to be further studied by transmission electron microscopy (TEM) which provides more detail of the structure of spermatozoa than SEM (Yanagimachi, 1994; also to be discussed later).

2.3.3.3- Summary and conclusions

- Unlike with fresh semen, chilling to/at +4°C increases the ability of A23187 to induce the acrosome reaction in vitro, since a lower concentration (0.1 versus 1 µM/l) of A23187 is sufficient for inducing a marked increase in the percentage of reacted spermatozoa.
- In chilled semen, the time of incubation (in TALP medium) is a more important factor, since the A23187-induced acrosome reaction is time-dependent.
- The sequence of the acrosome reaction in chilled semen is unaffected by the presence of 2% glycerol (v/v, final) in Tris based extender.
- As with NA staining and PC microscopy techniques, DIC microscopy is an effective light microscopy technique allowing the assessment of acrosome reaction of dog spermatozoa.
Legends to plates (for NA staining):

**Plate 1a.** Unfrozen sperm with intact (I) acrosome stained as light blue or blue, with a slight but distinct and dark blue acrosomal ridge. Note the equatorial segment (ES) as pale yellow and the post-acrosomal region light blue. Bar=5 µm, Magn. 1,000X.

**Plate 1b.** Frozen sperm with intact (I) acrosome exhibiting light blue or blue colour with a slightly indistinct, dark blue acrosomal ridge. Note the acrosome having indistinctly uneven surface with a slightly lessened contrast.

**Plate 1c.** Unfrozen sperm with the loss of clear outline of the acrosomal ridge as indicative of the partial acrosome reaction (PR). Note swelling of the acrosome in white colour.

**Plate 1d.** Frozen sperm undergoing partial acrosome reaction (PR). Note similar swelling pattern of the acrosome with irregular outlines.

**Plate 1e.** Unfrozen sperm with a complete loss of the overlying blue-stained acrosome revealing a yellow-stained nucleus. Note the presence of slightly dark ES.

**Plate 1f.** Frozen sperm with no acrosome (CR). Note pale yellow stained nucleus and intact ES.
Plate-1. The assessment of naphthol yellow S/ aniline blue- stained pre- and post-freeze spermatozoa undergoing different degrees of acrosome reaction by Bright-Field microscopy.

- **Fresh- intact (I)**
  - (a)
  - 5 µm

- **Frozen- intact (I)**
  - (b)

- **Fresh- partially reacted (PR)**
  - (c)

- **Frozen- partially reacted (PR)**
  - (d)

- **Fresh- reacted (R)**
  - (e)

- **Frozen- reacted (R)**
  - (f)
Legends to plates (for PC microscopy):

Plate 2a. Sperm with intact (I) acrosome (arrow) seen as a dark shadow with a regular, smooth outline. Note clear and dark equatorial segment (ES) and post-acrosomal region (PAR). Bar=5 μm, Magn. 7,000X.

Plates 2b-h. Spermatozoa undergoing various degrees of partial acrosome reaction (PR):

Plate 2b. Sperm with partially reacted acrosome. Note restricted decondensation and swelling (arrows) of the acrosome as starting proximal to the equatorial segment (as recognized to be the 'earliest' sign of acrosome reaction).

Plates 2c-f. Spermatozoa with partially reacted acrosomes. Note the development of decondensation and swelling (arrows) of the acrosome towards the apex of the head of spermatozoa with the ES remaining unchanged.

Plates 2g and h. Spermatozoa with partially reacted acrosomes. Note the completion of decondensation and swelling of the acrosomes (arrows) having irregular outlines, together with intact ES.

Plate 2l. Sperm with completely missing acrosome (CR) as revealing a brighter nucleus (arrows) together with the ES and post acrosomal region (PAR) remaining intact.
Plate-2. The assessment of fresh spermatozoa undergoing different degrees of acrosome reaction by Phase Contrast microscopy.
Legends to plates (for DIC microscopy):

**Plate 3a.** Cooled (unfrozen) sperm with intact (I) acrosome as pale-dark shadow with a slightly distinct, dark acrosomal ridge. Note the equatorial segment and post acrosomal region both as pale-dark or dark shadow. Bar=5 µm, Magn. 1,000X.

**Plate 3b.** Frozen sperm with intact (I) acrosome having an indistinctly less dense contrast with an intact equatorial segment (ES).

**Plate 3c.** Cooled sperm undergoing the partial acrosome reaction (PR). Note swelling and decondensation of the acrosome (seen as a brighter shadow) and the resultant loss of clear outline of acrosomal ridge. The ES remains intact.

**Plate 3d.** Frozen sperm undergoing the partial acrosome reaction (PR) as seen with swelling and decondensation of the acrosome leading to irregular outlines except over the ES.

**Plate 3e.** Cooled sperm with a complete loss of the acrosome (R) revealing the nucleus as a pale shadow. Note the ES and post acrosomal region remaining intact.

**Plate 3f.** Frozen sperm with no acrosome (R). Note the ES and post acrosomal region with no visible changes.
Plate-3. The assessment of pre- and post-freeze spermatozoa undergoing different degrees of acrosome reaction by Differential Interference Contrast microscopy

Fresh- intact (I)  Frozen- intact (I)

Fresh- partially reacted (PR)  Frozen- partially reacted (PR)

Fresh- reacted (R)  Frozen- reacted (R)

ES: Equatorial Segment
CHAPTER 3. OPTIMISATION OF FREEZING REGIMENS FOR DOG SPERMATOZOA

Hypothesis:
Optimisation of freezing regimens, by examining losses of motile spermatozoa through each of the critical stages of the freezing curve in various cooling regimens, will lead to a maximisation of the survival of dog spermatozoa that posses functionally-intact acrosomes, in terms of their ability to undergo the acrosome reaction in vitro.

3.1- Introduction
Having established a number of key methodologies for the extension and evaluation of spermatozoa, the main experiments for optimisation of freezing conditions for canine spermatozoa could be undertaken.

The subject of cryopreservation has been considered exhaustively in the literature, although surprisingly few systematic studies of freezing conditions for canine semen have ever been undertaken. The main direction of the experiments to be described in this Chapter was to examine losses of motile spermatozoa through each of the critical stages of the freezing curve. Then, having established criteria for cooling conditions that appear to give optimal results in terms of motility, it was to validate such motility results in terms of the most critical indicator of spermatozoal functionality, namely the ability to undergo acrosome reaction in vitro.

Firstly, however, it is necessary to consider in some detail the principles and practice of cryopreservation as applied to spermatozoa.

3.1.1- Opportunities and limitations of cryopreservation of spermatozoa
In order to extend the life span of spermatozoa beyond a few days, the temperature must be reduced below 0°C. This exposes the spermatozoa to the additional stresses of freezing and thawing (Watson, 1990). The highest temperature at which physiologic saline solutions and most cryoprotectant solutions used for slow cooling can be made to freeze (see below) is around -3°C (Shaw et al., 1993). However, in the presence of 5-7% glycerol the semen sample can often supercool to -15°C before ice nucleation occurs
(Watson, 1995). It can also be induced at -6°C (so-called ‘seeding’) as is widely used in oocyte and embryo freezing (Shaw et al., 1993). During freezing, cells must survive the critical temperature zone from -10 to -40°C (Watson, 1979), within which substantial cellular damage occurs (especially between -15 and -25°C; Polge, 1957). Indeed, intracellular ice nucleation, which is usually lethal, occurs around these temperature ranges, i.e. -5 to -40°C (Mazur, 1988).

Therefore, it might be expected that an optimum freezing rate through this temperature zone would result in superior post-thaw survival of cells. However, even with an optimum freezing rate (which has not yet been unequivocally determined for spermatozoa of any species) a considerable degree of damage could occur due to other factors such as dilution, rate of cooling above 0°C, and the addition or removal of glycerol (before and after freeze-thawing, respectively). Such damage is attributable to the formation of intra- and intercellular ice as the latent heat of fusion is released, and the osmotic consequences of ice formation, including the occurrence of a substantial osmotic gradient across the plasma membrane which leads to water flow from the cell, increased solute concentrations and changes in pH (Watson, 1995). Furthermore, following freezing to subzero temperatures (below the eutectic point), cells that were suspended in concentrated solutions (due to pulling of pure water for ice formation) will be encased in ice (Mazur, 1984; Grout and Morris, 1987). This might be relatively critical due to the decrease in the size of the unfrozen channels during slow freezing (Mazur, 1984). Inevitably, structural and functional components of the cells will then be changed under such conditions. Examples of possible changes include impaired enzymatic reactions (e.g. decreased Na⁺/K⁺ase activity as likely to result from ATP deficiency) and alterations in the activity of membrane-bound pumps (e.g. depolarisation of voltage-dependent gate leading to Ca²⁺ influx, thereby allowing the phase transition and phase separation of membrane phospholipids resulting in cell damage or death) (Hammerstedt et al., 1990). The effect of such stresses will be a progressive, but usually assumed to be very slow, increase in cell damage as the period of storage in the frozen state is extended.

At temperatures below the glass transition temperature (-139°C for water and higher for aqueous solutions) no recrystallisation of ice will occur and the rates of chemical
reactions and biophysical processes will therefore be too slow to affect cell survival (Grout and Morris, 1987). A frozen-storage temperature of >-139°C (such as at ~-70°C) resulted in poorer survival than at -196°C (Foote, 1964a, in dog; Trummer et al., 1998, in human). Many cells stored above ~-80°C are not stable, probably because traces of unfrozen solution still exist (Mazur, 1970). By contrast, at -196°C, thermodynamic processes are virtually halted, and theoretically the only deterioration of cells arises from the background cosmic radiation, but its accumulation (leading to death of the cells) would take more than one thousand years (Mazur, 1988; Watson, 1990). Below -139 °C, cell viability should be independent of the period of storage (Grout and Morris, 1987), which provides the basis for cryopreservation of biological material in cryogens such as liquid nitrogen (LN2).

3.1.2- A brief review of the mechanisms of cryopreservation

The most significant difficulties in cryopreservation of semen are the processes of cooling and warming rather than the stability of spermatozoa at -196°C. Indeed, cryoinjury to a variety of cell organelles is attributable to the change in temperature, and the formation and dissolution of ice (Watson, 1995). Thus, the process of dehydration of the cells during slow freezing potentially results in cell survival, whereas at faster freezing rates cell death is more likely. Furthermore, the optimal cooling rate depends on cell type (see below). For example, for spermatozoa, the optimal cooling rate between -5 and -45°C lies ~-10 to -80°C/min (Watson, 1995). While maximal cooling rate in pellets (on dry ice) and ampoules (in vaporised LN2) is ~-100°C/min, in straws (in vaporised LN2) it is ~-170°C/min (Graham et al., 1978). Although supercooling does not occur during freezing in pellets and ampoules (resulting in a linear cooling curve), for straws (in vapour freezing) semen samples always show supercooling, thus resulting in a non-linear cooling curve (Graham et al., 1978). Therefore, the difficulty with cooling rates expressed as an average is that the rate may vary considerably from the average at different temperatures. Indeed, the cooling curve is usually sigmoid during the eutectic curve, as the latent heat of fusion is released, or at extremes, e.g. -120°C that was used as an 'end' temperature for controlled-rate freezing in the present study. In this context, a step-wise approach to optimisation of freezing regimens appears desirable. England (1992), who also used straw packaging, obtained a linear cooling curve (avoiding
supercooling and the latent heat plateau phase, as resulting in a superior post-thaw recovery) through a combination of an alcohol bath at -80°C and vapour freezing over the surface of LN₂. For cooling rate, however, there are further interactions with diluent composition, glycerol concentration and thawing rate (Watson, 1979). Thawing is generally achieved at much faster rates of 1000-2000°C/min (Watson, 1995). Rewarming by immersion in a water bath at 37°C (close to 35°C, as used in the present study) is capable of giving a thawing rate of 1000°C/min between -70 and 0°C (Watson, 1990).

The main physical effects occurring in cells during freezing are as follows (adapted from Mazur, 1988):

- **Above -5°C**, the cells and the surrounding medium remain unfrozen both because of supercooling and of depression of the freezing point by protective solutes (such as glycerol) that are frequently present.

- **Between -5 and -15°C**, ice forms in the external medium (either spontaneously or as a result of seeding the solution with an ice crystal), but the cell contents remain unfrozen and supercooled, because the plasma membrane blocks the growth of ice crystals into the cell interior.

- The extracellular ice formation removes water from the extracellular medium, resulting in increasingly high concentrations of solutes in the extracellular medium. In consequence, osmotic pressure of the extracellular medium increases, leading to dehydration of the cell.

Subsequent physical events in the cell depend on the cooling rate (Mazur, 1963; Watson, 1995):

(i) **When cooling rate is sufficiently slow**, the cell is able to loose water fast enough by exosmosis to concentrate the intracellular solutes sufficiently to eliminate supercooling, and maintain the chemical potential of intracellular water in equilibrium with that of extracellular water. This results in cellular dehydration without internal freezing, and thus increases the likelihood of survival. At cooling rates slower than the optimum, however, cells are exposed for too long to 'solution effects': that is, all the changing characteristics of the extracellular solution as ice crystallises out (increased salt concentration, increasing
osmolality, changing pH, altered solution composition as the salts reach their saturation point and crystallise out), and all the cellular consequences of these events.

(ii) At too fast cooling rates, there is insufficient time for water to move out of the cells (especially from the ova and haemopoietic stem cells; see below for detail), leading to supercooling and an increased likelihood of intracellular ice nucleation, which is usually lethal.

There are other theories in which the membrane rupture (leading to cell damage/death) is regarded as the cause (not as a result) of intracellular ice (Litvan, 1972; Muldrew and McGann, 1990). Litvan (1972) suggested that less-than-optimum cooling rates cause excessive dehydration and too rapid rates of cooling result in membrane rupture followed by intracellular ice formation. Muldrew and McGann (1990) suggested that due to critical osmotic gradient across the plasma membrane, the rupture is caused by the 'friction' of water passing through the membrane exceeding its capacity to conduct water.

In addition, during freezing and thawing three biological parameters have a special influence on the survival of individual cells, namely water permeability ($L_p$), its temperature coefficient or activation energy ($E_a$) and the size of the cells, or more properly, its surface-to-volume (of water content) ratio ($A/V$) (Mazur, 1988). Mazur (1988) noted that an increase in $L_p$ produces the same effect as a comparable decrease in cooling rate. Likewise, an increase in diameter of the cell reduces the cooling rate required to produce a given probability of intracellular freezing. He further suggested that all the parameters, needed to compute the curves for kinetics of water loss, can be estimated experimentally without conducting the actual freezing. Once computed the curves could be used to estimate the probability of intracellular ice as a function of cooling rate:

- Cells that have dehydrated close to the equilibrium (between the 'minimum tolerable' amount of intracellular water and extracellular ice) before reaching their ice-nucleation temperature will have a zero probability of undergoing intracellular freezing.
Cells that are still extensively supercooled when cooled to their ice-nucleation
temperature, and hence still hydrated will have a high probability of undergoing
intracellular freezing.

Therefore, it is possible to calculate the maximum cooling rate, which is compatible
with the avoidance of supercooled intracellular water and the likelihood of intracellular
ice. The results of calculated water loss correspond with experimental survival tests for
a number of cell types (other than spermatozoa) with close agreement (Mazur, 1984).
For many years, conventional ways of measuring the variates \( (L_p, E_a, A/V) \) of
spermatozoa necessary for Mazur's theoretical approach to achieve optimum freezing
rate were inadequate because of the irregular shape of sperm cells (see Yanagimachi,
1994). The difficulty was eventually overcome by combining Mazur's approach
(Mazur, 1963) with a newer technology (Watson et al., 1992b). This new technique
relies on the following assumptions:

(i) Cells respond as perfect osmometers (Drevius, 1972).

(ii) The osmotically inactive water is a relatively small proportion of the intracellular
water (Du et al., 1993).

(iii) All cells respond alike (Watson, 1995).

(iv) Permeability is affected by temperature below 0°C in the same way as it is above
0°C (Watson, 1995).

Comparing the \( L_p \) and \( E_a \) values for spermatozoa from different species (fowl, ram,
human, bull, stallion and rabbit) and other cell types (ovum, human haemopoietic stem
cells and erythrocytes) it was found that the estimates of \( L_p \) for spermatozoa are much
higher, and \( E_a \) much lower than those in other cells (Watson, 1995). Therefore, these
values for spermatozoa (except those of rabbit) are more in accord with erythrocytes and
other cells (e.g. kidney tubule cells), which are associated with water transport during
secretion and absorption. This apparently indicates that, in general, the metabolism or,
more properly, survival of spermatozoa \textit{in vivo} and \textit{in vitro} depends on the extracellular
environment (i.e. presence of nutrients, temperature, ionic strength, osmolality, etc.).
The values were also used to estimate the maximum cooling rates for spermatozoa
compatible with maintaining osmotic equilibrium. The results gave cooling rates three
orders of magnitude greater than those demonstrable experimentally (Curry et al., 1994;
also see below). Therefore, Curry et al. (1994) suggested that damage sustained at high cooling rates might be unrelated to intracellular ice. However, further studies would determine the actual value of these estimations (together with the actual effect of intracellular ice) for achieving greater post-thaw survival of spermatozoa.

Undoubtedly, the water itself or its movement inside/outside of the cell play a major role in maintaining osmotic 'equilibrium' within the cell (as dehydration and rehydration) during freezing and thawing. The water content of bull spermatozoa was estimated as 53-81% (Watson, 1979). That is somewhat lower than most mammalian cells (e.g. ovum and erythrocytes; >75%). Spermatozoa behave as perfect osmometers within certain limits, i.e. 100-1600 mOsm/l (Du et al., 1993; Liu and Foote, 1998a,b), and osmotically inactive water is 13% of total (Du et al., 1993). In dog spermatozoa, a value of 150 mOsm/l in the medium (e.g. fructose or sodium citrate) was enough for characteristic hypo-osmotic swelling patterns of the tail (England and Plummer, 1993), indicating that dog spermatozoa are also sensitive to osmotic changes. When spermatozoa are cryopreserved, they are exposed to increasingly higher osmotic conditions (hyperosmolality) as water freezes out. This can increase the osmolality more than 20 times higher than isosmotic levels (i.e. ~300 mOsm/l as physiologic osmolality) (Mazur et al., 1981). As part of the 'solution effect', this high osmolality, together with the likelihood of intracellular ice nucleation due to membrane rupture (Watson, 1995) could partly explain why avoiding (England, 1992) or minimising (Parkinson and Whitfield, 1987) the latent heat plateau phase (during which ice nucleation occurs) is beneficial for survival of spermatozoa after thawing.

Because osmotic change is an important factor it will be discussed in more detail. The cells will be exposed to critically high osmotic gradient across their plasma membrane especially during slow freezing (Mazur et al., 1981; Muldrew and McGann, 1990). Hyperosmotic conditions are detrimental to spermatozoa (Drevius, 1972), but the main cellular membrane damage is observed as the osmolality is decreased (due to water ingress) which occurs during thawing (Gao et al., 1993; Curry and Watson, 1994; Holt and North, 1994). The cells are affected at 2-4 times isosmotic level (Watson, 1995) and much higher osmolality occurs during cryopreservation. Gao et al. (1993) demonstrated that spermatozoa were more resistant to osmotic damage at low
temperatures. Watson (1995) considered that since a proportion of spermatozoa survive slow cooling the membranes must be considerably resistant to hyperosmotic stress at low temperature. Therefore, he eventually hypothesised that for spermatozoa, osmotic stress applied too rapidly, i.e. at supraoptimal freezing (or thawing) rates, causes a sudden movement of water across the cell membrane which requires accommodation of a new volume by displacement of the membrane. This may damage the membrane either by disrupting cytoskeletal elements or bulk transport of the water across the bilayer (Muldrew and McGann, 1990).

Consequently, considering the above and together with the theories of cryoinjury (Mazur, 1965; Meryman, 1970; Mazur and Rigopoulos, 1983; Quinn, 1985) it is concluded that:

- A two-factor understanding of cell damage is clearly appropriate for cryoinjury: while too slow cooling rates result in prolonged exposure to excessive solute concentrations (Mazur's so-called 'solution effect') with cell damage becoming apparent when rehydration occurs (during thawing), too fast freezing rate results in volume changes that are too rapid for the cell membrane to accommodate.

- However, unlike some other cell types (e.g. ovum and haemopoietic stem cells), spermatozoa (including those of dog) are particularly sensitive to osmotic changes, which play a major role in the resultant cryoinjury.

- Bulk water ingress (during and/or after thawing) creates membrane damage (Woolley and Richardson, 1978).

- The lipid reorganisation occasioned by thermal phase events (Hammerstedt et al., 1990; White, 1993) clearly alters membrane responsiveness to the various stresses (e.g. removal of glycerol, further centrifugation, sudden temperature changes, intravaginal environment, etc.).

3.1.3- Freezing rates for cryopreservation of spermatozoa

In the early 1960's, the advent of small-volume plastic straws as the means of storing bovine semen made a great contribution towards the development of relatively simple procedures for semen freezing. The methods of either suspending horizontal racks of well-separated straws (Cassou, 1964) or lowering the containers of vertically located straws (Jondet, 1964) just above the surface of LN₂ are simple and quite effective. These
methods rely upon the vaporisation of LN$_2$ to cause a rapid fall in temperature within the straw. However, both freezing techniques have the disadvantage that the operator has no control over the speed of freezing process beyond setting the initial conditions of vapour temperature and the level of LN$_2$ within the freezing chamber. Although vapour freezing remains one of the standard methods for cryopreservation of semen (Verheyen et al., 1993), programmable, semi-controlled freezers have also been used because of their capability to control cooling rates in a more reliable way. A number of workers have reported better post-thaw survival of bovine (Parkinson and Whitfield 1987), porcine (Hammit and Martin, 1989) and human (McLaughlin et al., 1990; Verheyen et al., 1993) spermatozoa in programmed freezing compared with vapour freezing. Hammit and Martin (1989) observed a smaller variation and slower deterioration of the post-thaw motility and of the acrosomal morphology among straws (from one ejaculate) that were frozen simultaneously with controlled-rate method rather than with vapour freezing. Comparing freezing techniques, survival of spermatozoa is better in vertically positioned straws (McLaughlin et al., 1990) and worse in some individuals (so-called 'bad-freezers'), whose semen do not survive freezing effectively. This could be due either to intrinsic factors (Parkinson and Whitfield, 1987; Verheyen et al., 1993) or genital diseases (Ragni et al., 1990). Moreover, Ragni et al. (1990) considered that slow-staged (programmable) freezing was particularly effective for storage of abnormal human semen. This could be vitally important in cases where semen quality is compromised. On the other hand, Verheyen et al. (1993), using the two techniques given, found only minimal differences in spermatozoal recovery when high quality semen has been examined.

The 'average' value for optimum cooling rates for semen of domestic mammals are in the range of -10 to -100°C/min and -10 to -80°C/min between -5 and -45°C (Watson, 1995). Spermatozoa are less sensitive to the cooling rate because of the more permeable membrane structure than that of most other cell types. The overall survival at optimal cooling rates differs between spermatozoa from different species due to interactions with diluent composition, glycerol concentration, and thawing rate (Watson, 1979; Olar et al., 1989). So far, insufficient attention has been given to the sample dimensions (i.e. L$_p$, E$_a$ and A/V values) in determining both the actual cooling rate affecting spermatozoa
and the removal of heat from the specimen (i.e. the thermal properties of the sample itself and not only the dewar or cooling chamber environment). During the release of heat (as ice nucleation occurs) the temperature of the sample rises rapidly from \(-15^\circ C\) (with 5-7% glycerol) back to the level of freezing point depression \((-0.60^\circ C\) for freezing extenders used in dogs; Smith, 1984). Therefore, the resultant temperature oscillation often yields, with a programmable freezer, a very rapid cooling phase immediately following dissipation of the "latent heat" as the sample is returned to cooling 'ramp' (Watson, 1995). At this point, there is a danger of inducing intracellular ice, although it might be mediated by subsequent very fast cooling. For spermatozoa, the elimination of supercooling by seeding (at \(-6^\circ C\)) to avoid intracellular ice and initiate early dehydration was of no specific benefit in many species (ram: Watson and Martin, 1975; boar: Fiser et al., 1991; dog: Hay et al., 1997b). A very slight improvement in survival rates was obtained in human (Critser et al., 1987), boar (Fiser, 1988) and bull (Chen et al., 1993). An optimal linear freezing rate, avoiding the latent heat plateau, would allow time during cooling for spermatozoa to remain in 'equilibrium' with the environment, i.e. extracellular medium (Watson, 1990), and this would be preferable to equilibration by dehydration of the cell. A linear cooling curve or a sigmoid curve with minimising size of the plateau phase was consistently shown as beneficial in dog (England, 1992) and in bull (Parkinson and Whitfield, 1987) spermatozoa, respectively. Therefore, we hypothesised that the manipulation of cooling rates at a controlled rate (achieved by a semi-programmable biological freezer) might be critical for a superior post-thaw survival of dog spermatozoa.

A considerable number of studies have investigated the effect of different cooling rates upon survival of spermatozoa in many species, including bull (Woelders and Malva, 1998), boar (Bwanga et al., 1991a), stallion (Heitland et al., 1996), ram (Curry et al., 1994), fowl (Seigneurin and Blesbios, 1995), fish (Steyn, 1993; Ohta et al., 1995), and human (Watson et al., 1992a). Likewise, the effects of freezing rate upon dog spermatozoa were also studied (Dobrinski et al., 1993; Hay et al., 1997a; Ström et al., 1997), simply by using unknown cooling rates, or more properly, as mainly adapted from the bull (Foote, 1964a; Seager, 1969; Andersen, 1972a) or commercially-based (Govette et al., 1996).
3.2- Experiment 3. Cooling between +4°C and -35°C

Hypothesis:
Cooling between +4 and -35°C is associated with the highest levels of death of dog spermatozoa.

3.2.1- Rationale
This experiment examined the effects of cooling rates, between +4 and -35°C, upon post-thaw motility of spermatozoa. The initial phase of the freezing between +4 and -35°C is associated with the highest levels of death of spermatozoa (Parkinson and Whitfield, 1987; Liu et al., 1998). Hence, it was decided that since this temperature range was likely to be a critical determinant of the overall success of the freezing process, optimisation of freezing conditions should firstly be achieved over this part of the freezing curve. An initial experiment was undertaken to give approximate indications of the cooling rates that were likely to give reasonable freezing results (Experiment 3a), then a more detailed trial was undertaken to produce more specific results (Experiment 3b).

3.2.2- Materials and Methods

3.2.2.1- Semen collection and evaluation
For Experiment 3a, three ejaculates were collected from each of three dogs (Dogs A, B and C; see Appendix 2.1 for detail) as previously described. For Experiment 3b, single ejaculates were collected from each of the same three individuals.

Initial assessment of semen was previously as described (Section 2.1.2.6). In order to be used, semen had to meet the minimum threshold values of motility and morphology, as described in Section 2.2.2.1 (i.e. >75% progressively motile and >60% morphologically normal).

3.2.2.2- Semen processing
For experiments 3a and 3b semen samples were processed as shown in Figures 3.1 and 3.2.

Firstly, samples of neat semen were centrifuged at 275 g for 5 min to remove the seminal plasma. After aspiration of supernatant, the pellet was resuspended in Tris extender containing 20% egg yolk (Section 2.1.2.5) to its pre-centrifugation volume. It was then further extended (in Tris) to average of 33.44x10^6 sperm/ml (Experiment 3a)
and 21.23 x 10^6 sperm/ml (Experiment 3b) depending on the initial concentration of the sample. Although the results of Experiments 1a and 1b indicated that there was unlikely to be any effect of dilution rate *per se*, dilution was always less than 1:32.

3.2.2.3- Freezing protocols: Experiment 3a

Samples were cryopreserved in a programmable biological freezer (Planer Biomed, R204 Series II, Planer Products Ltd., Sunbury-on-Thames, Middx, England). Three to eight straws were used for each freezing regimen, in which various combinations of cooling conditions were evaluated, as described below. A thermocouple was placed into an additional straw that was filled with extended semen (containing 2% glycerol), in order to determine the changes in temperature that occurred within the straws.

The experiment examined the effects of cooling rates between +4 and -35°C upon post-thaw motility of spermatozoa in a 4 x 4 Latin square experimental design. The semen was cooled from +4°C at either -0.5, -5, -10 or -20°C/min to a series of intermediate temperatures (+2, -1, -5 or -10°C) before being further cooled at various rates (i.e. -10, -20, -30 or -40°C/min) to -35°C. The semen was then maintained at -35°C for a further 2 min to equalise temperatures within the straws. A summary of the experimental procedures is given in Figures 3.1. Thawing and motility evaluations were undertaken as described below.

3.2.2.4- Freezing protocols: Experiment 3b

A Latin square experimental design was again used (see Figure 3.2), in which the semen was cooled at -0.5, -2, -3.5 or -5°C/min, from +4°C to a series of intermediate temperatures (i.e. -3, -5, -7 or -9°C), before being further cooled at various rates (i.e. -30, -40 or -50°C/min) to -35°C. As above, the semen was maintained at -35°C for a further 2 min, and then assessed for post-thaw motility.

3.2.2.5- Assessment of spermatozoal motilities

Straws of semen were thawed in a water bath at 35°C for 1 min.

After thawing, each straw of semen was separately diluted in 2.9% sodium citrate (pH 7.0; 1:2 dilution, semen: citrate). The post-thaw progressive motility was subjectively assessed in duplicate on warmed slides. The slides were assessed (see Section 2.1.2.6) under a routine laboratory microscope at 200x magnification by using dark ground
illumination. The examinations were undertaken in a blind manner (Barth and Oko, 1989) to avoid bias.

3.2.2.6- Analysis of data
Motility data were examined with respect to treatment (initial cooling rate, intermediate temperature, final cooling rate) and individual dogs, by analysis of variance. In Experiment 3a, treatment groups were unbalanced, so, only the main effects could be determined by using generalised linear modelling. In Experiment 3b, motility data were initially subjected to analysis of variance with respect to treatment (i.e. cooling regimen, as cooling rates and intermediate temperatures) in a repeated measure model, in which treatments were nested with individual dogs. Where statistically significant effects were detected, individual means were compared by calculating least significant differences (Snedecor and Cochran, 1967).

In the work of Parkinson and Whitfield (1987), it was found that a highly significant correlation existed between parameters of the freezing curve (notably the eutectic region of the curve) and post-thaw motility of bovine spermatozoa. A number of measurements were therefore made upon the freezing curve (i.e. the temperatures recorded by the thermocouple that had been placed within a diluent-filled straw), as illustrated in Figure 3.3.

Data from the measurements of the freezing curve (i.e. rate1, T1, T2, T1-T2, time, Area and rate2; see Figure 3.3 for detail) and cooling regimen were compared with post-thaw motility by correlation and regression. Pearson correlation and multiple regression analysis (i.e. single/multiple regressions and best subsets) were used in the analysis of these data. In multifactorial regression analysis, different factors were included in the model based, initially, upon their own individual simple correlation/regression with motility. During multiple regression analysis, the effect of each term was examined to determine whether its inclusion had significantly affected the residual variance (residual mean squares). Moreover, since the parameters of the freezing curve were highly correlated, eigenvector (principal component) analysis was used to determine the factors that could, with validity, be included in the model.
SEMEN SAMPLE
(3 individuals x 3 ejaculates)

Initial assessment
(volume, colour, motility, concentration & morphology)

Centrifugation (at 275 g for 5 min as neat semen),
Resuspension (up to pre-centrifugation volume) &
Final extension in Tris [average of 33.44 x10^6 sperm/ml]

Cooling (at +4°C for 1 h) &
Glycerolisation (2%, v/v - final conc.),
Packaging (in 0.25 ml French straws)
& Equilibrating (at +4°C for 1 h)

Freezing:
(from +4°C to -35°C; 3 straws/ treatment)
"in the order of [CR1_TL_CR2] in temperature"

-0.5°C/min _+2°C/-10°C/min
-0.5°C/min _-10°C/-30°C/min
-0.5°C/min _-5°C/-40°C/min
-0.5°C/min _-1°C/-20°C/min

-10°C/min _-5°C/-30°C/min
-10°C/min _-1°C/-10°C/min
-10°C/min _+2°C/-20°C/min
-10°C/min _-10°C/-40°C/min

Holding at -35°C for 2 min
Thawing (at +35°C for 1 min)
Assessing motility
with a routine lab. microscope
(Magn. 200x, dark ground illum.)

Figure 3.1. Experiment 3a: Summary of procedures
**SEmen Sample**

- Initial assessment (volume, colour, motility, concentration & morphology)
- Centrifugation (at 275 g for 5 min as neat semen), Resuspension (to pre-centrifugation volume) & Final extension in Tris [average of 21.23 x 10^6 sperm/ml]
- Cooling (at +4°C for 1 h) & Glycerolisation (2%, v/v - final concentration), Packaging (in 0.25 ml French straws) & Equilibration (at +4°C for 1 h)

**Freezing:**
(from +4°C to -35°C; 3 straws/treatment)
*In the order of [CR1_TL_CR2] in temperature*

---

**Run 1** (3 individuals x 1 ejaculate)

-0.5°C/min -3°C -30°C/min
-0.5°C/min -5°C -40°C/min
-0.5°C/min -7°C -40°C/min
-0.5°C/min -9°C -30°C/min
-2°C/min -3°C -40°C/min
-2°C/min -5°C -30°C/min
-2°C/min -7°C -30°C/min
-2°C/min -9°C -30°C/min
-3.5°C/min -3°C -50°C/min
-3.5°C/min -5°C -50°C/min
-3.5°C/min -7°C -50°C/min
-3.5°C/min -9°C -50°C/min
-5°C/min -3°C -40°C/min
-5°C/min -5°C -40°C/min
-5°C/min -7°C -40°C/min
-5°C/min -9°C -40°C/min

**Run 2** (3 individuals x 1 ejaculate)

-0.5°C/min -3°C -50°C/min
-0.5°C/min -5°C -30°C/min
-0.5°C/min -7°C -30°C/min
-0.5°C/min -9°C -40°C/min
-2°C/min -3°C -30°C/min
-2°C/min -5°C -40°C/min
-2°C/min -7°C -40°C/min
-2°C/min -9°C -30°C/min
-3.5°C/min -3°C -40°C/min
-3.5°C/min -5°C -30°C/min
-3.5°C/min -7°C -30°C/min
-3.5°C/min -9°C -40°C/min
-5°C/min -3°C -40°C/min
-5°C/min -5°C -40°C/min
-5°C/min -7°C -40°C/min
-5°C/min -9°C -30°C/min

**Run 3** (3 individuals x 1 ejaculate)

-0.5°C/min -3°C -40°C/min
-0.5°C/min -5°C -50°C/min
-0.5°C/min -7°C -30°C/min
-0.5°C/min -9°C -50°C/min
-2°C/min -3°C -50°C/min
-2°C/min -5°C -40°C/min
-2°C/min -7°C -40°C/min
-2°C/min -9°C -30°C/min
-3.5°C/min -3°C -30°C/min
-3.5°C/min -5°C -40°C/min
-3.5°C/min -7°C -40°C/min
-3.5°C/min -9°C -40°C/min
-5°C/min -3°C -30°C/min
-5°C/min -5°C -50°C/min
-5°C/min -7°C -30°C/min
-5°C/min -9°C -50°C/min

Holding at -35°C for 2 min, Thawing (at +35°C for 1 min) & Assessing motility with a routine lab. microscope (Magn. 200X; dark ground illum.)

---

Figure 3.2. Experiment 3b: Summary of procedures
Figure 3.3. Measurements made on the eutectic part of the freezing curve by using a thermocouple placed within the straw.

Legends:

rate1- initial cooling (or supercooling) rate (°C/min) with a frequent, sudden fall in temperature just before the amplitude (for ice-nucleation) starts,

T1- lowest temperature point (°C) before the release of the latent heat of fusion causes temperature to rise (i.e. ice-nucleation temperature),

T2- highest temperature point (°C) during the release of latent heat of fusion (i.e. freezing point depression),

T1-T2- difference (°C) of ice-nucleation temperature and freezing point depression,

Amp- amplitude (or magnitude) of the latent heat plateau phase during ice-nucleation; 

time- duration (sec) of the latent heat plateau phase,

Area- total area (°C.sec) of the plateau phase, and

rate2- second cooling rate (°C/min) following freezing point depression.
3.2.3- Results

3.2.3.1- Ejaculates

The characteristics of ejaculates used in the experiment are given in Table 3.1 (Experiment 3a) and Table 3.2 (Experiment 3b):
<table>
<thead>
<tr>
<th>Dog</th>
<th>Ejaculate</th>
<th>No.</th>
<th>(n=3)</th>
<th>concentration (x10^6/ml)</th>
<th>progressive motility (%)</th>
<th>colour</th>
<th>live</th>
<th>normal</th>
<th>abnormal</th>
<th>dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog A:</td>
<td>(Solo)</td>
<td>I</td>
<td>1.45</td>
<td>90</td>
<td>830.0</td>
<td>light milky</td>
<td>81</td>
<td>1</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>1.30</td>
<td>84</td>
<td>187.5</td>
<td>light milky</td>
<td>85</td>
<td>4</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>1.80</td>
<td>90</td>
<td>225.0</td>
<td>light milky</td>
<td>85</td>
<td>4</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Dog B:</td>
<td>(Gillie)</td>
<td>I</td>
<td>0.55</td>
<td>90</td>
<td>200.0</td>
<td>light milky</td>
<td>69</td>
<td>11</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>3.50</td>
<td>85</td>
<td>300.0</td>
<td>light milky</td>
<td>74</td>
<td>4</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>2.30</td>
<td>85</td>
<td>400.0</td>
<td>light milky</td>
<td>79</td>
<td>5</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Dog C:</td>
<td>(Joey)</td>
<td>I</td>
<td>0.54</td>
<td>87</td>
<td>0.27</td>
<td>light milky</td>
<td>0.28</td>
<td>2</td>
<td>0.15</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>0.27</td>
<td>85</td>
<td>0.28</td>
<td>light milky</td>
<td>0.28</td>
<td>2</td>
<td>0.15</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>0.28</td>
<td>90</td>
<td>0.28</td>
<td>light milky</td>
<td>0.28</td>
<td>2</td>
<td>0.15</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Table 3.2. The characteristics of ejaculates used for optimisation of the cooling regimen (between +4 and -35°C) based upon the post-thaw motility of spermatozoa (Experiment 3b)

<table>
<thead>
<tr>
<th>Dog</th>
<th>Ejaculate</th>
<th>colour</th>
<th>volume (ml)</th>
<th>progressive motility (%)</th>
<th>concentration (x10⁶/ml)</th>
<th>morphology (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=3)</td>
<td>No.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>normal</td>
<td>abnormal</td>
</tr>
<tr>
<td>Dog A:</td>
<td>I</td>
<td>light milky</td>
<td>0.90</td>
<td>88</td>
<td>2,000.0</td>
<td>86</td>
<td>6</td>
<td>6 2</td>
</tr>
<tr>
<td>(Solo)</td>
<td>II</td>
<td>light milky</td>
<td>0.75</td>
<td>90</td>
<td>450.0</td>
<td>83</td>
<td>5</td>
<td>7 5</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>light cloudy</td>
<td>1.35</td>
<td>87</td>
<td>100.0</td>
<td>85</td>
<td>1</td>
<td>5 9</td>
</tr>
<tr>
<td>Dog B:</td>
<td>I</td>
<td>light milky</td>
<td>1.55</td>
<td>85</td>
<td>225.0</td>
<td>87</td>
<td>0</td>
<td>11 2</td>
</tr>
<tr>
<td>(Gillie)</td>
<td>II</td>
<td>light milky</td>
<td>1.17</td>
<td>90</td>
<td>200.0</td>
<td>76</td>
<td>4</td>
<td>12 8</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>light milky</td>
<td>0.83</td>
<td>93</td>
<td>950.0</td>
<td>83</td>
<td>2</td>
<td>11 4</td>
</tr>
<tr>
<td>Dog C:</td>
<td>I</td>
<td>light milky</td>
<td>0.20</td>
<td>80</td>
<td>1,750.0</td>
<td>74</td>
<td>2</td>
<td>24 0</td>
</tr>
<tr>
<td>(Joey)</td>
<td>II</td>
<td>light milky</td>
<td>0.65</td>
<td>90</td>
<td>200.0</td>
<td>81</td>
<td>2</td>
<td>14 3</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>light milky</td>
<td>0.50</td>
<td>88</td>
<td>200.0</td>
<td>71</td>
<td>4</td>
<td>18 7</td>
</tr>
</tbody>
</table>
3.2.3.2- Results of Experiment 3a

No significant effects of initial cooling rate, intermediate temperature or second cooling rates were observed, as the design was unbalanced. However, there was a statistically significant (P<0.01) effect of individual dogs upon the post-thaw motility. Post-thaw motility of spermatozoa from Dog C was significantly higher (P<0.05) than Dog B while spermatozoa from Dog A occupied an intermediate position (Table 3.3):
Table 3.3. The effect of individual dogs upon the post-thaw motility of spermatozoa in preliminary investigation of cooling regimen between +4 and -35°C (Experiment 3a)

<table>
<thead>
<tr>
<th>Dog</th>
<th>% motility (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog A</td>
<td>44.16$^{ab}$ ± 0.27</td>
</tr>
<tr>
<td>Dog B</td>
<td>40.23$^a$ ± 0.24</td>
</tr>
<tr>
<td>Dog C</td>
<td>47.55$^b$ ± 0.19</td>
</tr>
</tbody>
</table>

Significance: P<0.01

$^{a,b}$ Means sharing a common superscript are not significantly different from each other (P<0.05). (n=9)
Nevertheless, the trends in the data were sufficiently clear-cut that they could be used to provide a preliminary indication of the cooling rates that could be used in later parts of the trial. Thus, motility appeared to be higher at initial cooling rates of -0.5°C/min (48 ± 2%) than at faster cooling rates (-5, -10 or -20°C/min, overall means: 43 ± 2%), at intermediate temperatures of -5°C and -10°C (46 ± 2% and 45 ± 2%) than of +2°C or -1°C (43 ± 2% for both) and of second cooling rates of -20°C/min (44 ± 2%), -30°C (45 ± 2%) or -40°C (47 ± 2%) than of -10°C (41 ± 2%). These data are illustrated in Figure 3.4(a-c):
Figure 3.4a. The effect of first cooling rate of the freezing regimen between (+4 and -35°C) upon the post-thaw motility of spermatozoa as preliminary results.

\[\text{Cooling rate (°C/min)}\]

\[\text{% motility} \]

-0.5 -5 -10 -20

(n=9, Experiment 3a)
Figure 3.4b. The effect of intermediate temperature of the freezing regimen (between +4 and -35°C) upon the post-thaw motility of spermatozoa as preliminary results

(n=9, Experiment 3a)
Figure 3.4c. The effect of second cooling rate of the freezing regimen (between +4 and -35°C) upon the post-thaw motility of spermatozoa as preliminary results

(n=9, Experiment 3a)
The regression analysis between curve parameters (i.e. rate1, T1, T2, T1-T2, time, area, rate2) and temperature values applied, i.e. the first cooling rate (CR1), intermediate temperature (Ti) and second cooling rate (CR2), against post-thaw motility (see Appendix 3.1) showed that the following parameters measured from the freezing curves and temperature values were highly correlated (when r≥ +/-0.5) (Table 3.4):

| x-variable | y-variable | T1-T2 (r=0.846) | time (r=0.516) | rate1 (r=-0.616), rate2 (r=-0.687), area (r=0.869) | rate2 (r=0.698), area (r=-0.624), CR2 (r=-0.596) | area (r=-0.720), CR2 (r=-0.801) | CR2 (r=0.603) |

However, there was only a poor correlation (P>0.05 for all) for simple regression between post-thaw motility and either the parameter of the freezing curve or the cooling regimen. By contrast, the results from multiple regression analysis showed that there were significant relationships for both:

“CR1 + CR2”
Post-thaw motility (%)= (42.3) + (0.233 CR1) - (0.150 CR2); P≤0.05

and

“rate1 + CR1 + CR2”
Post-thaw motility (%)= (43.2) - (0.153 rate1) + (0.213 CR1) - (0.240 CR2); P<0.05.
3.2.3.3- Results of Experiment 3b: Analysis of variance

A summary of the analysis of variance of the post-thaw motility data is given in Table 3.5:

Table 3.5. Analysis of variance table (Experiment 3b)

<table>
<thead>
<tr>
<th>Term</th>
<th>df</th>
<th>F-ratio</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog (n=3)</td>
<td>2</td>
<td>9.30</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Initial cooling rate</td>
<td>3</td>
<td>7.25</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Intermediate temperature</td>
<td>3</td>
<td>4.71</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>Second cooling rate</td>
<td>2</td>
<td>2.12</td>
<td>NS</td>
</tr>
<tr>
<td>Initial cooling rate x Intermediate temperature</td>
<td>9</td>
<td>1.10</td>
<td>NS</td>
</tr>
<tr>
<td>Intermediate temperature x Second cooling rate</td>
<td>6</td>
<td>1.35</td>
<td>NS</td>
</tr>
<tr>
<td>Initial cooling rate x Second cooling rate</td>
<td>6</td>
<td>12.23</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Initial cooling rate x Intermediate temperature x Second cooling rate</td>
<td>18</td>
<td>5.87</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

There was a significant effect (P<0.001) of individual dogs upon the post-thaw motility. Spermatozoa from Dogs A and C had significantly higher (P<0.05) motility compared to Dog B (Table 3.6):
Table 3.6. The effect of individual dogs upon the post-thaw motility of spermatozoa subjected to different cooling regimens (between +4 and -35°C) (Experiment 3b)

<table>
<thead>
<tr>
<th>Dog</th>
<th>% motility</th>
<th>(mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=3)</td>
<td></td>
</tr>
<tr>
<td>Dog A</td>
<td></td>
<td>21.69&lt;sup&gt;b&lt;/sup&gt; ± 1.50</td>
</tr>
<tr>
<td>Dog B</td>
<td></td>
<td>16.20&lt;sup&gt;a&lt;/sup&gt; ± 0.84</td>
</tr>
<tr>
<td>Dog C</td>
<td></td>
<td>20.44&lt;sup&gt;b&lt;/sup&gt; ± 0.95</td>
</tr>
</tbody>
</table>

Significance P<0.001

<sup>a,b</sup> Means sharing a common superscript are not significantly different from each other (P<0.05). (n=3)
The second order interaction between initial cooling rate, intermediate temperature and second cooling rate was significant (P<0.001) (Figure 3.5). Considering these results in detail, it was noted that:

1. At an initial cooling rate of -0.5°C/min, the effects of intermediate temperature and second cooling rate were:

   For the intermediate temperature of -3°C, motility was significantly (P<0.05) higher at -30°C/min (34 ± 5%) than at either -40°C/min (17 ± 4%) or -50°C/min (14 ± 3%).

   For -5°C, there were no significant differences between motilities at any second cooling rate (-30°C/min: 19 ± 3%; -40°C/min: 31 ± 2%; -50°C/min: 23 ± 4%).

   For -7°C, likewise, there were no significant differences between motilities at any second cooling rate (-30°C/min: 17 ± 4%; -40°C/min: 25 ± 4%; -50°C/min: 15 ± 3%).

   For -9°C, the motility was consistently high, irrespective of the second cooling rate, although there was a non-significant decline in motility as the second cooling rate increased (-30°C/min: 33 ± 6%; -40°C/min: 29 ± 4%; -50°C/min: 27 ± 5%).

2. At the initial cooling rate of -2°C/min;

   For the intermediate temperature of -3°C, motility was significantly (P<0.05) higher at -40°C/min (31 ± 5%) than either at -30 or -50°C/min (12 ± 4% and 15 ± 3%, respectively).

   For -5°C, there were no significant differences between motilities at any second cooling rates (-30°C/min: 27 ± 5%; -40°C/min: 17 ± 4%; -50°C/min: 20 ± 3%).

   For -7°C, likewise, there were no significant differences between motilities at any second cooling rates (-30°C/min: 27 ± 4%; -40°C/min: 8 ± 2%; -50°C/min: 12 ± 3%).

   For -9°C, motility was significantly (P<0.05) higher at -50°C/min (30 ± 6%) than either at -30 or -40°C/min (16 ± 4% and 15 ± 4%, respectively).

3. At the initial cooling rate of -3.5°C/min;

   For the intermediate temperature of -3°C, there were no significant differences between motilities at any second cooling rates (-30°C/min: 19 ± 4%; -40°C/min: 9 ±
3%; -50°C/min: 27 ± 4%).

For -5°C, there was an increase in the motility as the second cooling rate increased. The motility was significantly (P<0.05) higher with the second cooling rate of -50°C/min (26 ± 4%) than at -30°C/min (6 ± 2%) while -40°C/min occupied an intermediate position (12 ± 2%).

For -7°C, likewise, there was an increase in the motility as the second cooling rate increased. Motility was significantly (P<0.05) higher at a second cooling rate of -50°C/min (28 ± 5%) than at either -30 or -40°C/min (8 ± 4% and 12 ± 2%, respectively).

For -9°C, the motility decreased as the second cooling rate increased. Motility was significantly (P<0.05) higher with the lowest cooling rate of -30°C/min (35 ± 5%) than either at -40 or -50°C/min (14 ± 3% and 10 ± 3%, respectively).

4. Finally, at the initial cooling rate of -5°C/min;

For the intermediate temperature of -3°C, the motility was significantly (P<0.05) higher at the second cooling rate of -40°C/min (28 ± 5%) than at -50°C/min (9 ± 3%). Figures for -30°C/min occupied an intermediate position (19 ± 4%).

For -5°C, there were no significant differences between motilities at any second cooling rate (-30°C/min: 9 ± 3%; -40°C/min: 26 ± 5%; -50°C/min: 9 ± 1%).

For -7°C, motility was significantly (P<0.05) higher at second cooling rate of -40°C/min (29 ± 4%) than either at -30 or -50°C/min (11 ± 3% and 10 ± 3%, respectively).

For -9°C, the motility declined as the second cooling rate increased. Motility was significantly (P<0.05) higher with the cooling at -40°C/min (32 ± 5%) than either at -30 or -50°C/min (13 ± 4% and 16 ± 4%, respectively).

Summarising these results, it determined that the lowest initial cooling rate (-0.5°C/min) resulted in significantly (P<0.05) higher motility (overall mean: 24 ± 1%) than at all faster initial cooling rates (overall means for -2°C/min: 19 ± 1%; -3.5°C/min: 17 ± 1%; -5°C/min: 18 ± 1%). These results are summarised in Figure 3.6. The lowest intermediate temperature (-9°C) resulted in significantly (P<0.05) higher motility.
(overall mean: 23 ± 1%) than the intermediate temperatures of -5°C (overall mean: 19 ± 1%) or -7°C (overall mean: 17 ± 1%) (Figure 3.7).

Overall, it appeared that the most favourable cooling regimen was as follows:

- An initial cooling rate of -0.5°C/min (starting from +4°C),
- To an intermediate temperature of -9°C, and
- At a second cooling rate of -40°C/min to -35°C.
Figure 3.5. The effects of initial cooling rate, intermediate temperature and second cooling rate of the cooling regimen (between +4 and -35°C) upon the post-thaw motility of spermatozoa (P<0.001)

a-c Means (± SEM) sharing a common superscript are not significantly different from each other (P<0.05). (n=3, Experiment 3b)
a,b Means (± SEM) sharing a common superscript are not significantly different from each other (P<0.05). (n=3, Experiment 3b)
Figure 3.7. The effect of intermediate temperature of the cooling regimen (between +4 and -35°C) upon the post-thaw motility of spermatozoa (P<0.005)

a,b Means (± SEM) sharing a common superscript are not significantly different from each other (P<0.05). (n=3, Experiment 3b)
3.2.3.4- Results of Experiment 3b: Correlation and regression analysis
Data from the analysis of parameters of the freezing curve and temperature values against post-thaw motility (see Appendix 3.2) showed that the following parameters and temperature values were highly correlated (when $r \geq +/-0.5$) (Table 3.7):

Table 3.7. Correlation matrix for measurements from cooling curve

<table>
<thead>
<tr>
<th>x-variable</th>
<th>v-variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_1$</td>
<td>$T_2\ (r=0.565)$, $T_1-T_2\ (r=0.934)$, rate$_1\ (r=-0.538)$</td>
</tr>
<tr>
<td>time</td>
<td>rate$_1\ (r=-0.579)$, rate$_2\ (r=-0.506)$, area\ ($r=0.869$)</td>
</tr>
<tr>
<td>rate$_1$</td>
<td>rate$_2\ (r=0.523)$, area\ ($r=-0.511$), $T_i\ (r=0.578)$</td>
</tr>
</tbody>
</table>

However, the results from simple regression analysis showed that only the initial cooling rate “CR1” was significantly correlated with post-thaw motility:

Post-thaw motility (%$= (23.1) + (1.33 \text{ CR1}); \ P<0.05.$

Similarly, the results from multiple regression analysis showed that there were a number of significant relationships between parameters of the freezing curve and motility (see Appendix 3.3). Amongst these, it was noticed that two of the curve parameters (time and rate$_1$) were predominant predictors of the motility, which were involved within all regressions at a significant level. The regression between “time, rate$_1$ and $T_i$” against the post-thaw motility appeared to be the highest predictor of motility on the basis of statistical significance:

Post-thaw motility (%$= (1.78) + (0.212 \text{ time}) + (0.264 \text{ rate$_1$}) - (0.887 \text{ Ti}); \ P \leq 0.01.$

3.3- Experiment 4. Interaction of rates of cooling between +4 and -35°C and between -35 and -120°C

Hypothesis:
The cooling rates above -35°C both affect and interact with the cooling rates between -35 and -120°C as assessed by post-thaw motility of spermatozoa.

3.3.1- Rationale
In this experiment, the interaction between cooling rates in the two main segments of the freezing curve was examined. Although both the literature and the results of
Experiments 3a and 3b leave little doubt that it is the cooling rates above -35°C that are the most important in determining overall losses of spermatozoa during cryopreservation. There is also substantial evidence, largely from the early work of Polge and Rowson (1952) and also Parkinson and Whitfield (1987) and Liu et al. (1998) on freezing of bovine semen, that cooling rates above -35°C both affect and interact with the cooling rates between -35 and -120°C. Since the results of Experiments 3a and 3b had provided clear indications of the optimal range of cooling rates for the maximal post-thaw survival of spermatozoa, it was decided to use these cooling rates in combination with a range of cooling regimens between -35 and -120°C.

3.3.2- Materials and Methods

3.3.2.1- Collection, evaluation and processing of semen

Three ejaculates were collected and evaluated from each of the same three dogs (Dogs A, B and C; see Appendix 2.1 for detail), as previously described. Removal of the seminal plasma, extension, glycerolisation, and equilibration were as described in Section 3.2.2.2.

3.3.2.2- Freezing protocols

All samples of semen were cooled at an initial rate of -0.5°C/min from +4 to -9°C. Thereafter, samples were further cooled at -40°C/min to intermediate temperatures of -20, -30, -40 or -50°C. Samples were then cooled at -10, -30, -50 or -100°C/min to -120°C. Straws were maintained at -120°C for 2 min and then plunged directly into LN2 (at -196°C). They remained in LN2 for at least 2 h before thawing and motility assessment (Section 3.2.2.5). A summary of the experimental procedures is given in Figure 3.8.

3.3.2.3- Analysis of data

Motility data were subjected to analysis of variance with respect to treatment (i.e. cooling regimen, as cooling rates and intermediate temperatures) in a repeated measure model, in which treatments were nested with individual dogs. Where statistically significant effects were detected, individual means were compared by calculating least significant differences (Snedecor and Cochran, 1967).
Further measurements were made upon the freezing curve, as described for Experiment 3 (Section 3.2.2.6; Figure 3.3).

Post-thaw motility data were compared for these measurements of the freezing curve and with cooling regimens by correlation and regression. Pearson correlation and multiple regression analysis (i.e. single/multiple regressions and best subsets) were used in the analysis of these data, with significant effects being determined by changes in residual variances.
SEMEN SAMPLE
(3 individuals x 2 ejaculates)

Initial assessment
(volume, colour, motility, concentration & morphology)

Centrifugation (at 275 g for 5 min as neat semen),
Resuspension (to pre-centrifugation volume) &
Final extension in Tris [average of 31.60 x10(6) sperm/ml]

Cooling (at +4°C for 1 h),
Glycerolisation (2%, v/v - final conc.),
Packaging (in 0.25 ml French straws) &
Equilibration (at +4°C for 1 h)

FREEZING
(from +4°C to -120°C in two steps; 3 straws/ treatment)

Step-I Freezing: (from +4°C to -9°C)
-0.5°C/min -9°C
"in the order of [CR1_T11] in temperature"

Step-II Freezing (from -9°C to -120°C)
"in the order of [CR2_T12_CR3] in temperature"

-40°C/min -30°C -100°C/min
-40°C/min -50°C -30°C/min
-40°C/min -40°C -100°C/min
-40°C/min -40°C -50°C/min
-40°C/min -40°C -50°C/min
-40°C/min -30°C -50°C/min

Holding at -120°C for 2 min

Storage in LN2 tank (at -196°C) for 2 h
& Thawing (at 35°C for 1 min)

Assessing motility with a routine lab. microscope
(Magn. 200x, dark ground illum.)

Figure 3.8. Experiment 4: Summary of procedures
3.3.3- Results

3.3.3.1- Ejaculates

The characteristics of ejaculates used for the experiment are given in Table 3.8:
Table 3.8. The characteristics of ejaculates used for optimisation of the cooling regimen (between -35 and -120°C) based upon the post-thaw motility of spermatozoa (Experiment 4)

<table>
<thead>
<tr>
<th>Dog</th>
<th>Ejaculate</th>
<th>colour</th>
<th>volume (ml)</th>
<th>progressive motility(%)</th>
<th>concentration (x10⁶/ml)</th>
<th>morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>normal</td>
</tr>
<tr>
<td>(n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog A:</td>
<td>I</td>
<td>light milky</td>
<td>0.77</td>
<td>90</td>
<td>900.0</td>
<td>89</td>
</tr>
<tr>
<td>(Solo)</td>
<td>II</td>
<td>light milky</td>
<td>1.50</td>
<td>90</td>
<td>550.0</td>
<td>83</td>
</tr>
<tr>
<td>Dog B:</td>
<td>I</td>
<td>light milky</td>
<td>1.30</td>
<td>90</td>
<td>375.0</td>
<td>86</td>
</tr>
<tr>
<td>(Gillie)</td>
<td>II</td>
<td>creamy</td>
<td>1.83</td>
<td>90</td>
<td>300.0</td>
<td>91</td>
</tr>
<tr>
<td>Dog C:</td>
<td>I</td>
<td>light milky</td>
<td>0.50</td>
<td>90</td>
<td>450.0</td>
<td>74</td>
</tr>
<tr>
<td>(Joey)</td>
<td>II</td>
<td>light milky</td>
<td>0.55</td>
<td>85</td>
<td>225.0</td>
<td>73</td>
</tr>
</tbody>
</table>
3.3.3.2- Results of Experiment 4: Analysis of variance

A summary of the analysis of variance of the post-thaw motility data is given in Table 3.9:

Table 3.9. Analysis of variance table (Experiment 4)

<table>
<thead>
<tr>
<th>Term</th>
<th>df</th>
<th>F-ratio</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog (n=3)</td>
<td>2</td>
<td>72.76</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Intermediate temperature</td>
<td>3</td>
<td>0.43</td>
<td>NS</td>
</tr>
<tr>
<td>Cooling rate below intermediate temperature</td>
<td>3</td>
<td>1.64</td>
<td>NS</td>
</tr>
<tr>
<td>Intermediate temperature x Cooling rate below intermediate temperature</td>
<td>9</td>
<td>3.73</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

Analysis of variance with respect to dog, intermediate temperatures and cooling rates showed that there was a statistically significant (P<0.001) effect of individual dogs and a significant (P<0.001) interaction between cooling rate and intermediate temperature upon the post-thaw motility.

Spermatozoa from Dog C had significantly (P<0.05) higher post-thaw motility than from Dog B while both values were also significantly (P<0.05) higher than from Dog A (Table 3.10):
Table 3.10. The effect of individual dogs for optimisation of the cooling regimen (between +4 and -120°C) based upon the post-thaw motility of spermatozoa (Experiment 4)

<table>
<thead>
<tr>
<th>Dog</th>
<th>% motility (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog A</td>
<td>39.08±0.77</td>
</tr>
<tr>
<td>Dog B</td>
<td>47.24±0.93</td>
</tr>
<tr>
<td>Dog C</td>
<td>53.14±0.74</td>
</tr>
</tbody>
</table>

Significance P<0.001

\^c Means having different superscripts are significantly different from each other (P<0.05). (n=6)
The effect of the interactions between cooling rates and intermediate temperatures upon the post-thaw motility were as follows (Figure 3.9):

- At an intermediate temperature of -20°C, there were significant differences (P<0.05) between cooling rates. Cooling at -100°C/min resulted in significantly (P<0.05) higher post-thaw motility (53 ± 3%) than at either -10 or -50°C/min (42 ± 2% and 43 ± 2%, respectively). Values at -30°C/min occupied an intermediate position (51 ± 3%) and were significantly (P<0.05) higher than at -10°C/min.

- At -30°C, there was no significant difference in motility values between different cooling rates (-10°C/min: 46 ± 2%; -30°C/min: 46 ± 2%; -50°C/min: 46 ± 2%; -100°C/min: 45 ± 2%).

- At -40°C, there were no significant differences between motilities at any cooling rates (-10°C/min: 45 ± 2%; -30°C/min: 50 ± 3%; -50°C/min: 48 ± 3%; -100°C/min: 44 ± 3%) nor were there any differences at -50°C (-10°C/min: 48 ± 2%; -30°C/min: 45 ± 3%; -50°C/min: 49 ± 2%; -100°C/min: 44 ± 2%).

Re-examining the data on the basis of cooling rate after the intermediate temperature, the following findings were noted:

- When cooling at -10°C/min, there was a gradual increase in motility as the intermediate temperatures were increased. Motility was significantly (P<0.05) higher at -50°C than at -20°C (48 ± 2% versus 42 ± 2%, respectively), with values at -30°C (46 ± 2%) and -40°C (45 ± 2%) occupying intermediate positions.

- At cooling rates of -30°C/min, there were no significant differences between motilities at any intermediate temperatures (-20°C: 51 ± 3%; -30°C: 46 ± 2%; -40°C: 50 ± 3%, -50°C: 45 ± 3%).

- At -50°C/min there were no significant differences between motilities at any intermediate temperatures (-20°C: 43 ± 2%; -30°C: 46 ± 2%; -40°C: 48 ± 3%; -50°C: 49 ± 2%), although there was an increasing motility trend with decreasing intermediate temperatures.
• At -100°C/min, motility was significantly (P<0.05) higher at an intermediate temperature of -20°C (53 ± 3%) than at lower temperatures (-30°C: 45 ± 2%; -40°C: 44 ± 3%; -50°C: 44 ± 2%).

From these results, it was concluded that the optimum regimen was an intermediate temperature of -20°C followed by cooling at -100°C/min to -120°C.
Figure 3.9. The effect of intermediate temperature and second cooling rate of the cooling regimen (between +4 and -120°C) upon the post-thaw motility of spermatozoa (P<0.001)

a-c Means (± SEM) sharing a common superscript are not significantly different from each other (P<0.05). (n=6, Experiment 4)
3.3.3.3- Results of Experiment 4: Correlation and regression analysis

Analysis of the data for parameters of the freezing curve and temperature values (see Appendix 3.4) against post-thaw motility showed that the following curve parameters were highly correlated ($r \geq +/-0.5$) (Table 3.11):

Table 3.11. Correlation matrix for measurements from cooling curve

<table>
<thead>
<tr>
<th>x-variable</th>
<th>y-variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1:</td>
<td>T1-T2 ($r=0.968$), time ($r=0.775$), rate1 ($r=-0.864$), rate2 ($r=-0.569$), area ($r=0.515$)</td>
</tr>
<tr>
<td>T1-T2:</td>
<td>time ($r=0.779$), rate1 ($r=-0.879$), rate2 ($r=-0.578$)</td>
</tr>
<tr>
<td>time:</td>
<td>rate1 ($r=-0.689$), rate2 ($r=-0.704$), area ($r=0.804$)</td>
</tr>
<tr>
<td>rate1:</td>
<td>rate2 ($r=0.567$), area ($r=-0.577$)</td>
</tr>
<tr>
<td>rate2:</td>
<td>area ($r=-0.640$)</td>
</tr>
</tbody>
</table>

Simple regression analysis demonstrated that there were significant ($P<0.05$) relationships between two of the curve parameters (i.e. T1 and T2) and post-thaw motility:

$$\text{Post-thaw motility (\%) } = (36.50) - (1.06 \text{ T1}); P \leq 0.01$$

and

$$\text{Post-thaw motility (\%) } = (40.40) - (5.08 \text{ T2}); P < 0.005.$$  

Additionally, the results from multiple regression analysis showed that the measurements on the freezing curve parameters alone or in combination with the temperature values applied had a significant predictive power upon the post-thaw motility (see Appendix 3.5). Amongst the regressions, "T1, T2, time, rate1 and rate2" appeared to be the highest predictor of post-thaw motility on the basis of statistical significance:

$$\text{Post-thaw motility (\%) } = (10.70) - (2.73 \text{ T1}) - (3.08 \text{ T2}) + (0.118 \text{ time}) - (0.333 \text{ rate1}) + (0.0591 \text{ rate2}); P \leq 0.001.$$
3.4- Experiment 5. Optimisation of freezing regimen, based upon observations of post-thaw motility

Hypothesis:
The optimum cooling regimen (between +4°C and -120°C) achieved will be repeatable as assessed by post-thaw motility of spermatozoa.

3.4.1- Rationale
The results of Experiments 3a, 3b and 4 showed, in summary, that the components of the freezing regimen between +4°C and -120°C that were associated with highest post-thaw motility rates were:

- Initial cooling rate (from +4°C) -0.5°C/min
- First intermediate temperature -9°C
- Second cooling rate -40°C/min
- Second intermediate temperature -20°C
- Third cooling rate -100°C/min.

Then, after holding the straws that contain the spermatozoa at -120°C for 2 min, they were plunged into LN2 (-196°C).

However, the above freezing regimen is based on an assembly of components which were studied separately. In consequence, it was considered desirable to compare the above freezing regimen with two others that would be expected to give intermediate or poor freezing results, as a final validation step before examining other aspects of spermatozoal functionality through induction of the acrosome reactions in vitro. Hence, a final comparative experiment was undertaken to determine the overall success of the three previous experiments in optimising freezing conditions for dog spermatozoa.

3.4.2- Materials and Methods
3.4.2.1- Collection, evaluation and processing of semen
Single ejaculates were collected from each of three dogs (Dogs A, B and C; see Appendix 2.1 for detail), as described previously (see Section 2.1.2.3). Each ejaculate then underwent routine evaluation for motility (Section 2.1.2.6), concentration and morphology (Section 2.1.2.4), before removal of the seminal plasma, extension, glycerolisation, and equilibration, by the protocol of Section 3.2.2.2.
3.4.2.2- Freezing protocols

The effect of three regimens (identified as giving high, intermediate or low post-thaw motility) were compared. Cooling regimens were as follows:

- **High post-thaw motility:**
  
  +4 to -9°C (first intermediate temperature) \(-0.5°C/min\)
  
  -9 to -20°C (second intermediate temperature) \(-40°C/min\)
  
  -20 to -120°C \(-100°C/min\).

- **Intermediate post-thaw motility:**
  
  +4 to -3°C (first intermediate temperature) \(-10°C/min\)
  
  -3 to -30°C (second intermediate temperature) \(-20°C/min\)
  
  -30 to -120°C \(-50°C/min\).

- **Low post-thaw motility:**
  
  +4 to -5°C (first intermediate temperature) \(-3.5°C/min\)
  
  -5 to -20°C (second intermediate temperature) \(-30°C/min\)
  
  -20 to -120°C \(-10°C/min\).

Straws were maintained at -120°C for 2 min and then plunged directly into LN₂. Following storage in LN₂ for at least 2 h, the straws were thawed and examined for post-thaw motility, as described in Section 3.2.2.5. A summary of experimental procedures is given in Figure 3.10.

3.4.2.3- Analysis of data

As in Experiments 3 and 4, motility data were subjected to analysis of variance with respect to cooling regimen and dogs. Where statistically significant effects were detected, individual means were compared by calculating least significant differences (Snedecor and Cochran, 1967).

Measurements were made upon the freezing curve, as described for Experiment 3 (Section 3.2.2.6).

Post-thaw motility data were compared with these measurements of the freezing curve and with cooling regimens by correlation and regression. Pearson correlation and multiple regression analysis (i.e. single/multiple regressions and best subsets) were used in the analysis of these data, with significant effects being determined by changes in residual variances.
**SEMEN SAMPLE**
(3 individuals x 1 ejaculate)

- Initial assessment
  (volume, colour, motility, concentration & morphology)

- Centrifuging (at 275 g for 5 min as neat semen),
  Resuspension (to pre-centrifugation volume) &
  Final extension in Tris [40 x10(6) sperm/ml]

- Cooling (at +4°C for 1 h),
  Glycerolisation (2%, v/v - final conc.),
  Packaging (in 0.25 ml French straws),
  & Equilibration (at +4°C for 1 h)

- Freezing
  (from +4°C to -120°C)
  "in the order of [CR1_ TH CR2_TII CR3] in temperature"

- "High p/t motility (Aliquot 1)"
  -0.5°C/min-9°C-40°C/min-20°C-100°C/min

- "Intermediate p/t motility (Aliquot 2)"
  -10°C/min-3°C-20°C/min-30°C-50°C/min

- "Low p/t motility (Aliquot 3)"
  -3.5°C/min-5°C-30°C/min-20°C-10°C/min

- Holding at -120°C for 2 min

- Storage in LN2 tank (at -196°C) for 2 h
  & Thawing (at 35°C for 1 min)

- Assessing motility
  with a routine lab. microscope
  (Magn. 200x, dark ground illum.)

Figure 3.10. The summary of experimental steps for comparison of cooling regimens (between +4 and -120°C) as resulted in high, intermediate and low post-thaw motility of spermatozoa (Experiment 5)
3.4.3- Results

3.4.3.1- Ejaculates

The characteristics of ejaculates used for the experiment are given in Table 3.12:
Table 3.12. The characteristics of ejaculates used for comparison of the cooling regimens (between +4 and -120°C) as resulted in high, intermediate and low post-thaw motility of spermatozoa (Experiment 5)

<table>
<thead>
<tr>
<th>Dog</th>
<th>Ejaculate</th>
<th>colour</th>
<th>volume (ml)</th>
<th>progressive motility (%)</th>
<th>concentration (x10⁶/ml)</th>
<th>morphology (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog A:</td>
<td>I</td>
<td>light milky</td>
<td>1.25</td>
<td>85</td>
<td>460.0</td>
<td>85</td>
<td>2</td>
</tr>
<tr>
<td>(Robi)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog B:</td>
<td>I</td>
<td>light milky</td>
<td>1.60</td>
<td>93</td>
<td>287.5</td>
<td>89</td>
<td>3</td>
</tr>
<tr>
<td>(Solo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog C:</td>
<td>I</td>
<td>light milky</td>
<td>1.40</td>
<td>92</td>
<td>150.0</td>
<td>90</td>
<td>2</td>
</tr>
<tr>
<td>(Gillie)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.4.3.2- Results of Experiment 5: Analysis of variance

Results from analysis of variance demonstrated that there were significant effects of individual dogs (P<0.05) and the cooling regimen (P<0.05) upon the post-thaw motility.

While spermatozoa from Dog A had significantly (P<0.05) higher post-thaw motility than Dog C, spermatozoa from Dog B occupied an intermediate position (Table 3.13).

As expected, motility values were significantly (P<0.05) higher when the regimen predicted to result in high post-thaw motility was used compared to the regimen predicted to result in low post-thaw motility (47 ± 3% and 39 ± 2%, respectively) (Figure 3.11).
Table 3.13. The effect of individual dogs for comparison of the cooling regimens (between +4 and -120°C) as resulted in high, intermediate and low post-thaw motility of spermatozoa (Experiment 5)

<table>
<thead>
<tr>
<th>Dog</th>
<th>% motility (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog A</td>
<td>47.70b ± 2.89</td>
</tr>
<tr>
<td>Dog B</td>
<td>41.00ab ± 1.31</td>
</tr>
<tr>
<td>Dog C</td>
<td>37.10a ± 3.64</td>
</tr>
</tbody>
</table>

Significance: P<0.05

a, b Means sharing a common superscript are not significantly different from each other (P<0.05). (n=3)
Figure 3.11. Comparison of cooling regimens expected, on the basis of results from Experiments 3a, 3b and 4 to result in high, intermediate and low post-thaw motility of spermatozoa (P≤0.05)

\[\text{Cooling regimen}\]

<table>
<thead>
<tr>
<th></th>
<th>high</th>
<th>Intermediate</th>
<th>low</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean</td>
<td>49</td>
<td>47</td>
<td>45</td>
</tr>
</tbody>
</table>

\[\text{a,b} \text{ Means (± SEM) sharing a common superscript are not significantly different from each other (P<0.05). (n=3, Experiment 5)}\]
3.4.3.3- Results of Experiment 5: Correlation and regression analysis

Analysis of the data for parameters of the freezing curve and cooling regimens (between +4 and -120°C) against post-thaw motility (see Appendix 3.6) showed that the curve parameters and cooling regimens were highly correlated (when $r \geq +0.5$) (Table 3.14):

<table>
<thead>
<tr>
<th>x-variable</th>
<th>y-variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_1$:</td>
<td>$T_1-T_2$ (r=0.995), time (r=0.527), rate2 (r=-0.585)</td>
</tr>
<tr>
<td>$T_1-T_2$:</td>
<td>time (r=0.558), rate2 (r=-0.584)</td>
</tr>
<tr>
<td>time:</td>
<td>rate1 (r=0.588), rate2 (r=-0.864), area (r=0.808), regimen (r=0.858)</td>
</tr>
<tr>
<td>rate1:</td>
<td>rate2 (r=-0.690), area (r=0.536), regimen (r=0.795)</td>
</tr>
<tr>
<td>rate2:</td>
<td>area (r=-0.557), regimen (r=-0.837)</td>
</tr>
<tr>
<td>area:</td>
<td>regimen (r=0.751)</td>
</tr>
</tbody>
</table>

The correlations for "T2" (r=-0.528) and "Regimen" (r=-0.549) against post-thaw motility were also high. However, data from both single and multiple regression analysis demonstrated that neither the parameters of the freezing curve nor cooling regimens were significantly correlated with post-thaw motility.
3.5- Experiment 6. Evaluation of in vitro acrosome reaction of frozen-thawed dog spermatozoa

Hypothesis:
The optimisation of freezing regimens will lead not only to preservation of high sperm motility but also to retention of functionally intact acrosomes, in terms of their ability to undergo the acrosome reaction in vitro.

3.5.1- Rationale
Experiments 3, 4 and 5 were concerned with the optimisation of the freezing curve in terms of post-freezing motility. However, the main hypothesis underlying this thesis is that optimisation of freezing leads to a maximisation of the retention of spermatozoa that are functionally intact, in terms of their ability to undergo the acrosome reaction, rather than merely upon the preservation of motility. Moreover, the ability to undergo acrosome reaction is a far more objective measure of functionality than is the subjective scoring of motility. Hence, the following experiment is the key investigation to examine the major hypothesis of this thesis, and is the experiment for which all of the preceding work has been preparatory.

3.5.2- Introduction
The process of cryopreservation represents an artificial interruption of the progress of the spermatozoa towards post-ejaculation maturation and fertilisation. Much effort has therefore been expended in attempting to refine the conditions to maximise spermatozoal survival under this non-physiological environment. The assessment of damage upon the cells is not simple, due mainly to technical difficulties in assessing damage to subcellular components (Hammerstedt et al., 1990). Undoubtedly, functional tests of the spermatozoa provide more information on the fertilising potential of spermatozoa than do structural examinations. Hence, the use of the in vitro acrosome reaction (as a physiological function of spermatozoa) could offer a useful tool in assessing the cryogenic damage. It is well known that spermatozoa need to undergo the acrosome reaction to be able to fertilise the ovum both in vivo and in vitro (Yanagimachi, 1994). In this context, Whitfield and Parkinson (1995) have reported a significant relationship between induction of in vitro acrosome reaction in frozen bovine
spermatozoa and the fertility of such spermatozoa in widespread use for artificial insemination.

Within the population of spermatozoa which survive cryopreservation the characteristics of a significant proportion of cells are markedly different from their non-frozen counterparts (Hammerstedt et al., 1990). In dog spermatozoa, considerable acrosomal deterioration (e.g. swelling, decondensation, disruption, complete loss) has been noted after cryopreservation, and whilst some damage occurs during the initial phases of extension, cooling, and equilibration, the majority occurs during freezing and thawing (Oettlé, 1986b; Ström Holst et al., 1998). The acrosomal degeneration that occurs during cryopreservation is accompanied by a decline in motility, but the two events are not closely correlated, since the acrosome appears to suffer more damage than other organelles such as the mitochondria and flagellum (Oettlé, 1986b). Indeed, McLaughlin et al. (1993) concluded that cryoinjury to the acrosome, the plasma membrane and the flagellum of human sperm could occur independently. Similar observations have also been made in other species, e.g. fox (Zalewski and Andersen Berg, 1983), bull (Kjæstad et al., 1993), ram (Watson, 1975) and boar (Paulenz et al., 1995). Electron microscopy studies have shown that frozen-thawed dog spermatozoa present major changes in their morphology, including the loss of acrosomal contents, identified both by swelling and the rarefaction of the acrosome and of the electron dense acrosomal material (Ström Holst et al., 1998). The same occurs in the fox (Hofmo and Andersen Berg, 1989).

There is little evidence to show whether the damage to the acrosome is simply mechanical, or whether the morphological changes that the spermatozoon experiences during cryopreservation affect its physiological capability to undergo the acrosome reaction. Such a question could be resolved by comparing chilled (i.e. extended and glycerolised) and frozen-thawed spermatozoa.

During cryopreservation, the main stresses experienced by spermatozoa are (i) the effects of cooling above the freezing point, (ii) the formation and dissolution of ice (during freezing and thawing, respectively) (Watson, 1995), and (iii) the toxic and osmotic effects of cryopreservatives, e.g. glycerol (England, 1992; Gao et al., 1995). Considering these stressful effects upon spermatozoa, Watson (1995) advanced the hypothesis that cryopreserved mammalian spermatozoa are in a state resembling partial capacitation, which accounts for their relatively reduced viability and readiness to
fertilise the ovum (following the acrosome reaction) \textit{in vitro} without incubation. Likewise, Rota (1998) demonstrated that capacitation time of dog spermatozoa was shortened, i.e. 2 h \textit{versus} 4 h in chilled-rewarmed and frozen-thawed spermatozoa as compared to fresh samples, respectively. Therefore, she concluded that, based on the fluorescent staining and motility patterns, capacitation-like changes seem to be initiated by preservation procedures. Moreover, Drobnis \textit{et al.} (1993) detected that the acrosomal physiology of cryopreserved human spermatozoa was altered after residence in the female reproductive tract (in the cervical mucus), which further shows that at least some of the spermatozoa that remain motile following cryopreservation have sustained sublethal damage that reduces their functional capability \textit{in vivo}.

The functional integrity of the acrosome of cryopreserved bovine spermatozoa was studied by Bailey and co-workers, who conducted a series of studies upon spontaneous and A23187-induced calcium ion fluxes, motility and \textit{in vivo} fertility. Bailey and Buhr (1994) found that cryopreservation alters the calcium ion flux (altering the intracellular pH) thus leading to the acrosome reaction. Furthermore, Bailey \textit{et al.} (1994) compared the relationship between \textit{in vivo} fertility, motility and \textit{in vitro} calcium ion flux, and suggested that the changes in motility and fertility that occur in cryopreserved spermatozoa can both be attributed to the changes in calcium metabolism that occur as a consequence of cryogenic damage. Likewise, Bailey and Buhr (1993) studied calcium ion regulation by cryopreserved spermatozoa in response to ionophore A23187. They observed that cryopreservation affects calcium ion regulation in surviving cells, implying that the response to membrane modulators (e.g. calcium ion, A23187) differs between cryopreserved and fresh spermatozoa. They therefore concluded that calcium ion regulation differs following cryopreservation procedures, possibly causing the reduced fertilisation capacity of commercially cryopreserved semen. McLaughlin \textit{et al.} (1993) also studied the effect of cryopreservation on the acrosomal response of human spermatozoa to A23187. They found no significant differences between fresh and frozen-thawed spermatozoa to A23187 treatment, concluding that acrosome function is maintained after cryopreservation as long as the organelle remains mechanically intact.

In Experiment 2, it was established that acrosome reactions can be induced \textit{in vitro} in canine spermatozoa by the use of calcium ionophore A23187. In Experiments 3, 4 and 5, it was established that freezing regimens can be created that result in the survival of
large numbers of motile spermatozoa. Experiment 6 examines the use of in vitro induction of acrosome reactions with A23187 as a means of testing whether freezing regimens that result in motile spermatozoa also result in cells that are functionally capable of undergoing the acrosome reaction in vitro.

3.5.3- Materials and Methods
The objective of this study was to determine the ability of spermatozoa, subjected to optimised freezing regimens, to undergo the acrosome reaction in vitro.

3.5.3.1- Semen collection and evaluation
One ejaculate from each of three dogs (Dogs A, B and C; see Appendix 2.1 for detail) was collected by manual stimulation (see Section 2.1.2.3).

Semen was held and evaluated as previously described (Sections 2.1.2.4 and 2.1.2.6). This initial evaluation was used to determine that semen attained the minimum threshold values of motility and morphology (see Section 2.2.2.1) prior to further processing.

3.5.3.2- Semen processing
Semen was processed as described in Figure 3.12.

3.5.3.3- Freezing and thawing
The samples were divided into two aliquots and subjected to freezing conditions, as described in Figure 3.12, in a programmable biological freezer (see Section 3.2.2.3). Six to eight straws of semen were subjected to two freezing regimens which previous studies (Experiment 5; Section 3.4.3.2) had established as resulting in a high or relatively low level of post-thaw motility. The cooling regimens were as follows:

- **High post-thaw motility:**
  - +4 to -9°C  
  -9 to -20°C  
  -20 to -120°C  
  -0.5°C/min  
  -40°C/min  
  -100°C/min.

- **Low post-thaw motility:**
  - +4 to -5°C  
  -5 to -20°C  
  -20 to -120°C  
  -3.5°C/min  
  -30°C/min  
  -10°C/min.
All straws were held at -120°C for 2 min, before being plunged into LN₂. Following storage in LN₂ for at least 4 h, the straws were thawed in a water bath at 35°C for 1 min. Post-thaw motility was assessed (Section 2.1.2.6) by using a routine laboratory microscope (Leitz, Wetzlar, Germany) at 200x magnification under dark ground illumination.

3.5.3.4- Induction of acrosome reaction
Frozen-thawed aliquots of semen frozen by both cooling regimens were subjected to a further centrifugation at 275 g for 5 min in 45% Percoll, to remove the freezing diluent. After aspiration of supernatant, the pellet was resuspended in TALP to pre-centrifugation volume. Each sample was then further divided into three aliquots as follows:
- 0 µM/l A23187 (control)
- 0.1 µM/l A23187
- 1 µM/l A23187.

The semen was then incubated at 39°C in 5% CO₂ in the humidified air for 60 min. Samples were withdrawn at 0, 15, 30, 45 and 60 min, fixed in 10% Formaldehyde, and placed on a slide under a coverslip. DPX mounting medium was placed around the coverslip to ensure that the preparations remain wet until they were examined. The assessment of acrosome reactions was undertaken under DIC microscope at 1000x magnification (Section 2.2.2.4).

A summary of experimental procedures is given in Figure 3.12.

3.5.3.5- Statistical analysis
Data were subjected to analysis of variance with respect to freezing regimen, concentration of A23187 and time in a repeated measure model, in which treatments were nested with individual dogs.
Figure 3.12. Experiment 6: Summary of procedures

SEMEN SAMPLE
(3 individuals x 1 ejaculate)

- Initial assessment (volume, colour, motility, concentration & morphology)
- Centrifugation (at 275 g for 5 min as neat semen),
  Resuspension (to pre-centrifugation volume) &
  Final extension in Tris [40 x10(6) sperm/ml]
- Cooling (at +4°C for 1 h) &
  Glycerolisation (2%, v/v - final concentration)
  Packaging (in 0.25 ml French straws)
  & Equilibration (at +4°C for 1 h)

- "High p/t motility"
  - 0.5°C/min from +4°C to -9°C
  - -40°C/min from -9°C to -20°C
  - -100°C/min from -20°C to -120°C
  - Storage in LN2 tank (at -196°C) for 4 h
    & Thawing (at 35°C for 1 min)

  - Centrifugation (at 275 g for 5 min in 45% Percoll) &
    Resuspension in TALP (to pre-centrifugation volume)

    - "Control (Aliquot 1)"
      - Adding TALP only (1:9, v/v - Aliquot:TALP) &
        Incubation (at 35°C in 5% CO2 for 0,15,30,45,60 min)
      - Fixing in 10% Formaldehyde (1:1 dilution)
        DPX-mounting

    - Examining with DIC microscope
      (Magn. 1000x)

    - "A23187-Induced (Aliquot 2)"
      - Adding 0.1 & 1 μM A23187 in TALP (1:9, v/v - Aliquot:A23187 in TALP) &
        Incubation (at 35°C in 5% CO2 for 0,15,30,45,60 min)

- "Low p/t motility"
  - -3.5°C/min from +4°C to -5°C
  - -30°C/min from -5°C to -20°C
  - -10°C/min from -20°C to -120°C
  - Storage in LN2 tank (at -196°C) for 4 h
    & Thawing (at 35°C for 1 min)

  - Centrifugation (at 275 g for 5 min in 45% Percoll) &
    Resuspension in TALP (to pre-centrifugation volume)

    - "Control (Aliquot 3)"
      - Adding TALP only (1:9, v/v - Aliquot:A23187 in TALP) &
        Incubation (at 35°C in 5% CO2 for 0,15,30,45,60 min)

    - Fixing in 10% Formaldehyde (1:1 dilution)
      DPX-mounting

    - Examining with DIC microscope
      (Magn. 1000x)

    - "A23187-Induced (Aliquot 4)"
      - Adding 0.1 & 1 μM A23187 in TALP (1:9, v/v - Aliquot:A23187 in TALP) &
        Incubation (at 35°C in 5% CO2 for 0,15,30,45,60 min)
3.5.4- Results

3.5.4.1- Ejaculates
Characteristics of the ejaculates used in this experiment are given in Table 3.15.

3.5.4.2- Motility
Pre-freeze and post-thaw motilities of spermatozoa are also given in Table 3.15:
Table 3.15. The characteristics of ejaculates used for evaluation of the acrosome reaction of frozen-thawed spermatozoa (Experiment 6)

<table>
<thead>
<tr>
<th>Dog</th>
<th>Ejaculate</th>
<th>colour</th>
<th>volume (ml)</th>
<th>progressive motility (%)</th>
<th>concentration (x10⁶/ml)</th>
<th>morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>normal</td>
</tr>
<tr>
<td>Dog A:</td>
<td>I</td>
<td>light milky</td>
<td>0.98</td>
<td>95</td>
<td>54</td>
<td>47</td>
</tr>
<tr>
<td>(Solo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog B:</td>
<td>I</td>
<td>light milky</td>
<td>1.30</td>
<td>95</td>
<td>64</td>
<td>25</td>
</tr>
<tr>
<td>(Gillie)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog C:</td>
<td>I</td>
<td>light milky</td>
<td>1.40</td>
<td>95</td>
<td>57</td>
<td>49</td>
</tr>
<tr>
<td>(Robi)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.5.4.3- In vitro induction of acrosome reactions

A summary of the analysis of variance of the post-thaw motility data is given in Table 3.16:

Table 3.16. Analysis of variance table (Experiment 6)

<table>
<thead>
<tr>
<th>Term</th>
<th>df</th>
<th>F-ratio</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog (n=3)</td>
<td>2</td>
<td>21.71</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Cooling regimen</td>
<td>1</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>A23187 concentration</td>
<td>2</td>
<td>7.76</td>
<td>P≤0.001</td>
</tr>
<tr>
<td>Time</td>
<td>4</td>
<td>1.25</td>
<td>NS</td>
</tr>
<tr>
<td>Cooling regimen x A23187 concentration</td>
<td>2</td>
<td>2.28</td>
<td>NS</td>
</tr>
<tr>
<td>Time x A23187 concentration</td>
<td>8</td>
<td>0.96</td>
<td>NS</td>
</tr>
<tr>
<td>Cooling regimen x Time</td>
<td>4</td>
<td>1.44</td>
<td>NS</td>
</tr>
<tr>
<td>Cooling regimen x A23187 concentration x Time</td>
<td>8</td>
<td>1.29</td>
<td>NS</td>
</tr>
</tbody>
</table>

The most striking observation from this experiment was that over 90% of the spermatozoa had acrosomal changes following freezing and thawing, irrespective of the freezing regimen applied (Table 3.17). Over 75% of the spermatozoa exhibited various degrees of swelling of the acrosome, leading them to be classified as partially reacted. In consequence, given such very high proportions of spermatozoa that were assessed as being acrosome reacted, there were few statistically significant effects of the experimental regimens that could be discerned.
Table 3.17. Percentage of frozen-thawed spermatozoa exhibiting complete or partial acrosome reaction (Experiment 6)

<table>
<thead>
<tr>
<th>A23187</th>
<th>Time</th>
<th>High motility</th>
<th>Low motility</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM/l</td>
<td>0</td>
<td>94.3 ± 3.7</td>
<td>94.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>96.3 ± 2.7</td>
<td>97.3 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>93.7 ± 7.1</td>
<td>96.0 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>96.7 ± 2.7</td>
<td>94.3 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>90.0 ± 8.6</td>
<td>94.0 ± 4.8</td>
</tr>
<tr>
<td>0.1 µM/l</td>
<td>0</td>
<td>90.7 ± 4.2</td>
<td>97.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>97.0 ± 0.8</td>
<td>87.7 ± 11.0</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>96.0 ± 3.3</td>
<td>88.3 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>94.7 ± 2.9</td>
<td>94.3 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>93.0 ± 4.6</td>
<td>90.3 ± 6.8</td>
</tr>
<tr>
<td>1 µM/l</td>
<td>0</td>
<td>91.3 ± 6.2</td>
<td>95.7 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>97.7 ± 1.8</td>
<td>98.7 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>98.0 ± 2.0</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>98.7 ± 1.8</td>
<td>99.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>96.3 ± 4.2</td>
<td>98.0 ± 2.6</td>
</tr>
</tbody>
</table>

Nevertheless, there was a significant effect (P<0.001) of individual dogs upon the percentage reacted spermatozoa, in that the results from Dogs A and C were significantly (P<0.05) higher than from Dog B (Table 3.18).

There was a significant effect (P≤0.001) of A23187 concentration upon the percentage of reacted spermatozoa (Figure 3.13). Although there was no significant difference in percentage of reacted spermatozoa between zero and 0.1 µM/l A23187 (94.7 ± 1.0% and 93.0 ± 1.2%, respectively), the values were significantly (P<0.05) higher in the presence of 1 µM/l A23187 (97.3 ± 0.7%) compared to other concentrations.

The effects of incubation time, cooling regimen or any interactions between A23187, time and freeze-thawing upon the overall results were not statistically significant.
Table 3.18. The effect of individual dogs upon the acrosome reaction of frozen-thawed spermatozoa (Experiment 6)

<table>
<thead>
<tr>
<th>Dog</th>
<th>% acrosome reaction (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog A</td>
<td>$97.87^b ± 0.32$</td>
</tr>
<tr>
<td>Dog B</td>
<td>$90.87^a ± 1.31$</td>
</tr>
<tr>
<td>Dog C</td>
<td>$96.30^b ± 0.69$</td>
</tr>
</tbody>
</table>

Significance $P<0.001$

$^a,b$ Means sharing a common superscript are not significantly different from each other ($P<0.05$). (n=3)
Figure 3.13. The effect of concentration of A23187 upon the acrosome reaction of frozen-thawed spermatozoa (P<0.001)

Means (± SEM) sharing a common superscript are not significantly different from each other (P<0.05). (n=3, Experiment 6)
3.6- Discussion

3.6.1- Effects of cooling rate upon post-thaw motility of spermatozoa

In this study, a wide range of freezing rates (from -0.5 to -100°C/min) and intermediate temperatures (from +2 to -50°C) were applied as part of "three-step freezing" trials by using a semi-programmable biological freezer. Spermatozoal motility was the criterion of survival as assessed by light microscopy immediately after thawing. The overall results showed that; (i) the optimisation of freezing conditions of dog semen is possible, and (ii) parameters of the freezing curve could be used to estimate post-thaw spermatozoal motility. The details are described below.

3.6.1.1- Initial cooling rate (between +4 and -10°C)

The optimum cooling rate from +4 to -9°C was -0.5°C/min while faster cooling rates (up to -20°C/min) resulted in lower survival. The requirement for such a slow initial cooling rate may indicate that spermatozoal membranes required a considerable period of time (up to 24 min) to tolerate the effects of lowering temperatures below 0°C. In this way, the magnitude of supercooling of the extracellular medium was also smaller, which inevitably lowers the likelihood of excessive supercooling of the water within the cells, thus preventing the formation of intracellular ice, which is usually lethal (Watson 1995). Since the plasma membrane is considered to be a barrier to the extracellular ice (Mazur, 1988), the maintenance of the phospholipid bilayer structure and lipoprotein fraction of the plasma membrane (Singer and Nicolson, 1972) is essential for an optimum cellular dehydration (see below). Superiority of slow initial cooling rate for post-thaw survival might also have been related to the following reasons:

(i) The use of 0.25 ml French straws. The surface area-to-volume (A/V) ratio using a 0.25 ml French straw is maximum (approximately 3292). It is 1.43, 1.48, 2.14, and 2.36 times higher than the A/V ratio of 0.5 ml straws (French and conventional type), 1 ml and 0.5 ml ampoules, respectively (Senger, 1986). It is well known that the A/V ratio of the packaging unit is the 'most important' characteristic determining the method of handling the semen (mainly freezing and thawing). Indeed, the A/V ratio dictates the rate at which the temperature
changes occur within the packaging unit: the higher the A/V ratio the greater the temperature change per unit.

(ii) The use of a high dilution rates (~25 x10^6 sperm/ml at average). This was necessary to obtain a minimum number of straws (3) per treatment (up to 48) per experiment.

(iii) Centrifugation of semen. Although there were no significant differences in maintenance of motility at +4°C for up to 48 h when the samples were diluted at the rates of 1:4 to 1:32, irrespective of centrifugation (see Section 2.1.3.2), the plasma membrane might have been impaired to some extent (e.g. loosing decapacitation factor and/or some of the coating surface proteins, or a slight modification in lipid phase status) during centrifugation.

3.6.1.2- First intermediate temperature (between +4 and -10°C)
The optimum first intermediate temperature was -9°C while higher temperatures (from +2 to -7°C) resulted in poorer survival. It is known that ice nucleation (seeding) can be initiated at ~-6°C (Shaw et al., 1993), but the sample, in the presence of 5-7% glycerol in the extender, can often supercool down to -15°C (Watson, 1995). With 2% glycerol (as used in the present study) the ice nucleation temperature was often ~-10 to -13°C, although it also occurred at ~-6 to -7°C followed by a longer latent heat plateau phase in some cases. The rapid release of latent heat indicates that, to some extent, the avoidance of the plateau phase is achieved, leading to a greater survival (in bulls: Parkinson and Whitfield, 1987; in dogs: England, 1992; also see below). Rota (1998) showed that when the ice nucleation temperature (T1) was slightly lowered, the duration of the plateau phase was shortened 2.5-fold, collectively leading to a markedly (P<0.05) higher motility immediately after thawing. When ice formation is initiated at a high subzero temperature (at -6°C), ice propagates slowly through the extracellular pure water of the solution accompanied by a slow release of the latent heat of fusion (Shaw et al., 1993). Seeding at -6°C (for 10 min) is essential in ovum and embryo freezing, since they have low L_p, high E_a, high water content and require sufficient cellular dehydration. With spermatozoa, the small magnitude of supercooling beyond ~-6°C, or more properly, initiating at ~-9°C is associated with the partial avoidance of subsequent latent heat plateau phase, leading to a greater survival.
3.6.1.3- Second cooling rate (between -9 and -50°C)
The optimum second cooling rate was -40°C/min from -9 to -20°C while other rates (down to -10°C/min or up to -50°C/min) resulted in lower survival. This cooling rate produced a latent heat plateau phase which was generally small in magnitude. Similar results have been found in the bull (Parkinson and Whitfield, 1987). During ice nucleation, achieved by cooling at -40°C/min, there were two types of plateau phase; (i) no supercooling (beyond -7 to -9°C), as is the case in seeding (Shaw et al., 1993), followed by large plateau, or (ii) a small magnitude of supercooling (initiating at ~-7°C and lasting at -13°C as lowest and highest limits, respectively), followed by a smaller plateau phase as occurred in most cases examined. The faster cooling rate probably allowed better cell dehydration and therefore led to better sperm survival. This was found in bull spermatozoa (Liu et al., 1998) and other cell types (Farrant, 1980; Shaw et al., 1993). It is also likely that the 'friction' of the plasma (and/or acrosomal) membrane due to bulk movement of water may be the cause of intracellular ice nucleation (Muldrew and McGann, 1994). The resultant lower survival with a slightly faster cooling rate (-50°C/min) possibly indicates that a small amount of intracellular ice occurred because of faster propagation of extracellular ice. This might have led, to some extent, to bulk movement of intracellular water across the plasma membrane, thus exceeding its capacity to conduct water and damaging the membrane itself and/or the cytoskeleton (Watson, 1995). With slower cooling rates, however, lower survival could either be due to intracellular water that still remains when a faster cooling rate is initiated to complete the nucleation, or prolongation of critical osmotic gradient across the membrane (Muldrew and McGann, 1994). It is known that in most of the plasma membrane, phospholipids are in a lamellar phase. Following the removal of water to below 0.25 g/g lipid (during dehydration), a transition from lamellar to Hexagonal II phase (consisting of tubes of lipid with polar ends oriented into a hydrated core) may occur, contributing to cryoinjury (Grout and Morris, 1987).

3.6.1.4- Second intermediate temperature (between -9 and -50°C)
The optimum second intermediate temperature was -20°C while other temperatures (down to -50°C) resulted in poorer survival. It is known that, during freezing, the critical temperature zone lies between -5 and -45°C (Watson, 1995), within which the
most important part is between -15 and -25°C (Polge, 1957). The present results indicate that the cells had lost enough water within that ‘most’ critical part, by -20°C, so that very fast freezing thereafter was not injurious. This also suggests that the water permeability of the plasma membrane of dog spermatozoa might be quite high, exceeding those of most other species (Watson, 1995) as well as other cell types such as the ovum and embryo (Shaw et al., 1993). Ultrastructural studies of frozen-thawed dog spermatozoa, using the most common cooling and warming regimens, demonstrated that the plasma membrane remained intact in most cases, even though usually exhibiting peculiar plica formation or extensive loosening (Rodriguez-Martinez et al., 1993; Mohanchandran Nair et al., 1998). Therefore, Rodriguez-Martinez et al. (1993) suggested that the plasma membrane of dog spermatozoon responds to cryopreservation in a manner that differs from that of other domestic species. It was noted that the water permeability of the plasma membrane might be linked to reproductive traits of the species concerned, e.g. induced or spontaneous ovulators; duration of fertile life of the ovum, survival of spermatozoa within the glands of the uterus and/or on the epithelium of the oviduct. Apparently, the reason for water permeability is to allow the maintenance of cellular metabolism until the ‘fertilisation task’, i.e. capacitation, hyperactivation, zona binding and its penetration (through acrosome reaction) and eventually fusing with the plasma membrane of the ovum, is completed. Consequently, these results suggest that; (i) the cellular variates (L_p, E_a and A/V ratio) of dog spermatozoa could be more compatible with freezing methods which permit severe osmotic changes to occur during cryopreservation compared to those of other species and (ii) there is a relationship between increasing cooling rates and survival of dog spermatozoa representing an inverted V- (or U) shaped survival curve as with many other species (Farrant, 1980; Parkinson and Whitfield, 1987; Watson, 1995). The cooling rate dependence of other cell types such as erythrocytes, ova and lymphocytes (Mazur, 1988), yeast and alga cells (Grout and Morris, 1987) proves that the speed of cellular dehydration is critical during freezing, and suggests that rehydration during thawing and dilution to isosmotic medium should be adjusted accordingly to minimise possible cryoinjury.
3.6.1.5- Third cooling rate (between -20 and -120°C)

The optimum third cooling rate was -100°C/min while slower rates (from -10 to -50°C/min) resulted in poorer survival. This further confirms that sufficient cellular dehydration had already occurred by -20°C so that subsequent faster cooling was innocuous. At this stage, faster freezing (allowing a rapid propagation of ice nucleation in the extracellular solution) also avoids; (i) excessive dehydration (Litvan, 1972), (ii) the minimum tolerable cell volume (Meryman, 1970), (iii) excessive exposure to the 'solution effects' (Mazur, 1988), and (iv) critical osmotic gradient across the plasma membrane (Muldrew and McGann, 1994) resulting in membrane rupture and intracellular ice that is usually lethal (Watson, 1995). This cooling rate allowed a very rapid change from the latent heat plateau to the cooling 'ramp' leading to a greater sperm survival. This was also found in the bull (Parkinson and Whitfield, 1987).

3.6.1.6- Comparison of cooling regimens

It was further confirmed that the combined cooling regimen chosen resulted in maximum survival. The effect of overall cooling regimens upon spermatozoal motility was not of high significance (P<0.05). This could be due to using only one sample from each individual dog for freezing. Data from the measurements on the freezing curves and the freezing regimens confirmed that the release of the latent heat varied slightly between the freezing regimens in that "T2" and the "regimen" were negatively highly correlated with post-thaw motility (r=-0.528 and -0.549, respectively). This indicates that, to some extent, the regimens that were applied differed from each other, although they had no predictive power upon the motility (P=0.144 and 0.126, respectively). However, it is likely that spermatozoa from different individuals might have tolerated the suboptimal regimens, around optimal, as was previously reported in the bull (Parkinson and Whitfield, 1987) and dog (Dobrinski et al., 1993; Rota, 1998). A higher number of replicates from more than three dogs would provide more reliable results.

It appears from parameters of the cooling curve and cooling regimens that;

- The initial cooling rate (CR1) itself can determine the survival of spermatozoa.
- Since supercooling (rate1), occurring just before the ice nucleation, was a co-existing curve parameter involved in the combinations that were determining the survival, it is suggested that the degree and the magnitude of supercooling of the
semen sample is critical for survival.

- Duration of the latent heat plateau phase was also the second co-existing critical parameter for survival, indicating the importance of exposure time to the "solution effects" as well as the degree of cellular dehydration.

- Both the ice nucleation temperature (T1) and the freezing point depression (T2) can predict survival rate.

- Since the "most critical" predictor of survival was the combined effect of "rate1, T1, T2, rate2 and time" it is suggested that optimisation of the latent heat plateau phase during ice nucleation is required for greater spermatozoal survival, as with other cell types.

3.6.1.7- Comparisons with other cryopreservation studies of spermatozoa in dogs

As discussed earlier (see Section 1.5.3.2), the previous freezing studies in dogs have used either pellet freezing on solid carbon dioxide or, more widely, vapour freezing over the surface of LN2 by using straws. Furthermore, only in a few studies have the freezing rates been controlled in a proper way, i.e. with enforced vapour freezing by using a programmable freezer (Olar et al., 1989; Hay et al., 1997a; Rota, 1998). The step-wise freezing (after initial freezing by vapour, plunging the straws in LN2) was mostly the method of choice, although three-step freezing has sometimes been used (Gill et al., 1970; Rota, 1998). Since glycerol concentrations, extenders and thawing rates have been different amongst the previous freeze-thawing studies, no direct comparisons could be made with the present study. Furthermore, in dogs, no systematic evaluations regarding the effect of freezing rate upon spermatozoal motility have been undertaken (England, 1993).

Since starting the experiments in the current thesis, there have been five new publications on freezing of dog semen. Ström et al. (1997) and Peña et al. (1999) compared one-step (Andersen's method; Andersen, 1975) and three-step (commercial CLONE method; Govette et al., 1996) cooling regimens. The findings of Ström et al. (1997) were; (i) both cryopreservation methods resulted in high initial post-thaw motility and membrane integrity but 'low thermoresistance' (thermolability; as seen especially in CLONE method), with a large proportion of spermatozoa that were undergoing acrosomal degradation, and (ii) as far as post-thaw motility is concerned, the
Andersen's method was markedly better than CLONE method while there were no such differences for plasma membrane integrity or acrosomal morphology. The findings of the latter workers, however, slightly differed from the above study: Although Andersen's method was superior to CLONE method for overall characteristics (i.e. post-thaw motility, integrity of the plasma membrane and of the acrosome) of spermatozoa, marked differences between the methods were observed for post-thaw motility (only after 3 h incubation at 37°C) and acrosomal integrity (only immediately after thawing) in Andersen's method, with no such difference for plasma membrane integrity. There were many differences between the methodologies in the present work and that of Ström et al. (1997) and Peña et al. (1999), e.g. glycerol concentration, straw size and thawing rate.

Hay et al. (1997a) compared five cooling rates using one- or two-step freezing. The findings were; (i) the highest proportion of motility and velocity score resulted from the -12 and -28°C/min freezing rates and the lowest values were obtained by cooling at -214°C/min, (ii) amongst the freezing rates, although there were no apparent differences in the morphology of spermatozoa, the proportions of viable spermatozoa and of undamaged acrosomes were highest for the -12 and -28°C/min. Therefore, they suggested that the optimum cooling rate for freezing canine spermatozoa is ~-30°C/min. Since the extender, concentration of glycerol, straw size and thawing rate were different, no direct comparison could be made with the present study.

Later, Hay et al. (1997b) studied the effects of 'seeding' (achieved at -6°C by touching the straws with forceps cooled in LN2) during freezing with the optimum cooling rate (-30°C/min). They found that whilst freezing caused a further decline in motility, acrosomal status and oocyte penetration, there was no difference between freezing with or without seeding. No such seeding attempt has been undertaken in the present study.

Rota (1998) was the first to report measurements on the cooling curve of dog semen. Using neat semen, Rota (1998) compared fast and slow cooling regimens in three-step freezing (referred to as temperature decrease measured from the freezing chamber): -13 and -5°C/min from +4 to -6°C, the both stopped at -6°C for 1 min, -50 and -10°C/min to -40°C, and both followed by -50°C/min to -140°C for fast and slow cooling, respectively. The findings were; (i) although motility immediately after thawing was
markedly higher with fast freezing rates, the percentage of sperm with intact plasma membrane tended to be higher (P=0.52) with slow freezing, (ii) fast and slow cooling regimens produced similar cooling curves, (iii) the interaction between dog and freezing regimen was significant as assessed by post-thaw motility at 38°C, and (iv) the lack of effect of cooling regimen on sperm motility could have been due to the fact that the two regimens were not significantly different and neither exceeded the optimum cooling rate for mammalian spermatozoa (Watson, 1995). Since the extender, glycerol concentrations, size of straws and thawing rates were different, no direct comparison can be made with the present study.

Therefore, the present results together with previous studies in dog spermatozoa suggest that;

- A "three-step freezing" of dog spermatozoa is appropriate for optimisation of cooling conditions.
- Although dog spermatozoa can tolerate a wide range of cooling regimens, there is an optimum cooling regimen for majority of the individuals.
- The survival curve against increasing cooling rates is an inverted U- or V-shaped, as previously shown (England, 1992; Dobrinski et al., 1993; Hay et al., 1997a).
- There is a high variation in survival of spermatozoa from individual dogs against the freezing rate, as previously shown in dog (Dobrinski et al., 1993; Thomas et al., 1993; Rota, 1998) and in other species (see Section 1.5.3.4). Post-thaw survival can also vary with cooling rate depending on extender type and its constituents, glycerol concentration, thawing rate as well as packaging system.
- Seeding does not improve survival.
- Prior centrifugation and high dilution rate may require a slow initial cooling rate.
- During the critical temperature range (between -5 to -45°C, Watson, 1995), sperm cells can tolerate the cooling rates ranging from -3 to -50°C/min depending on packaging system, thawing rate, glycerol concentration and the extender used.
- As the ice nucleation propagates (allowing sufficient cellular dehydration) subsequent cooling at very fast rates can result in improved survival.
3.6.2- *In vitro* induction of acrosome reactions as a measure of post-cryopreservation functionality of spermatozoa

In this study, we compared different concentrations of A23187 for inducing the acrosome reaction in frozen-thawed spermatozoa in modified TALP medium during incubation at 39°C in 5% CO₂ in humidified air for 60 min.

Following freeze-thawing, it was observed that, irrespective of the freezing regimen applied, over 90% of the spermatozoa had acrosomal changes, of which over 75% were various degrees of swelling. Nevertheless, there was a marked effect of A23187 concentration upon the percentage of reacted spermatozoa. A very small but significant proportion (<5%) of the acrosomes were still retained after thawing. The capability to undergo the acrosome reaction could only be induced when the maximum concentration (1 µM/l) of A23187 was used. Therefore, it is postulated that a large proportion of spermatozoa had undergone some degree of acrosomal damage during the cryogenic processes and that only a relatively small proportion (<5%) retained sufficiently normal acrosomes to undergo classic ionophore-induced reaction *in vitro*. There was no significant interaction between A23187 concentration and freezing regimens used. Although, from Experiment 2a (using fresh semen), 0.1 µM/l of A23187 was ineffective in inducing the acrosome reaction, it was hoped that, if low-grade acrosomal damage had resulted from cryopreservation, a lower concentration of ionophore might be discriminatory between degrees of damage as was the case for chilled samples in Experiment 2b. That expectation was not substantiated by the results of Experiment 6. This might have been due to fact that the proportion of spermatozoa having intact acrosomes was very low (<10%) and that those surviving spermatozoa contained the plasma and/or the acrosomal membranes that were more tolerant of the effects of freeze-thawing and hence presumably more resistant to the stimulative effects of low levels of A23187 than in the chilled spermatozoa. Therefore, it is considered that the post-thaw surviving spermatozoa might have a similar membrane and/or acrosomal structure to fresh samples or different structure as compared to chilled samples so that their capability to undergo the acrosome reaction could be maintained for fertilisation in dogs.

Apart from the marked effect of A23187 upon the acrosome, there were no effects of
incubation time, freezing regimen or any interactions between A23187, time and freezing regimen upon the overall results. The simplest explanation for this could be that following freeze-thawing the percentage of acrosome intact cells was <10%. Within this small, potentially viable population, spermatozoal membranes and the acrosome may exhibit a similar cryoresistance to freeze-thawing, since all of them have already successfully passed the harmful handling processes throughout cryopreservation. Furthermore, it has been demonstrated that Percoll, used in the present study for centrifugation of frozen-thawed spermatozoa just before subjection to acrosome reaction, can reduce the proportion of reacted cells with disrupted plasma membranes (Morris et al., 1999). This might have further improved selection of spermatozoa with stable and intact plasma membranes. This may also partly explain the non-significant differences in percentage of reacted cells between glycerolised and non-glycerolised samples after chilling. Undoubtedly, as frozen-thawed spermatozoa are incubated with 1 µM/l A23187 they can undergo acrosome reaction much faster than that of chilled cells and both are faster than that of fresh sperm due to major structural changes on the plasma membranes and acrosomes that occurred during freeze-thawing (see Sections 4.3.1.2 and 4.3.2.2 for detail). In a high proportion of spermatozoa the speed of acrosome reaction is quite fast (1-5 min) in many species (see Yanagimachi, 1994 for detail), including dogs (Kawakami et al., 1993b). Rota (1998), studying the effect of preservation on capacitation status of dog spermatozoa, demonstrated that capacitation times of spermatozoa were shortened in chilled-rewarmed and frozen-thawed semen compared to fresh semen. Using fluorescent staining, she also showed that, after incubation in CCM containing BSA, there were higher proportions of spontaneous acrosome reaction, in fresh (42%), chilled-rewarmed (44%) and frozen-thawed (50%) samples. Since different assessment criteria, freezing protocols and incubation medium were used in the present trials no direct comparison can be made with the above study. However, these results clearly indicate that capacitation-like changes are also initiated by the preservation procedures. Therefore, it is suggested that, during cryopreservation, each of the handling processes, i.e. chilling and freeze-thawing, increase the proportion of spermatozoa with more labile acrosomes, thereby allowing an increased percentage of acrosome reaction to occur after preservation.
Apart from dogs, spermatozoa from many other species also suffer from major disruptions of their membranes and acrosomal structure during freeze-thawing. With the exception of human spermatozoa, which have an acrosome that appears to be more cryoresistant than the mitochondria (Woolley and Richardson, 1978), most animal species are known to exhibit a variety of changes on their plasma membrane, acrosome and mitochondria (Healey, 1969). The effect of acrosomal damage upon spermatozoal function, including fertility, is of major importance. In the pioneering trials, a highly significant deterioration of the acrosomes was found in both ram and bull spermatozoa after freeze-thawing (Watson and Martin, 1972), which agrees well with the previous ultrastructural findings by TEM (Healey, 1969). Subsequent investigations upon the cryopreservation also revealed a major disruption of the acrosome of spermatozoa from the silver and blue fox (Zalewski and Andersen Berg, 1983), ram (Watson, 1975), bull (Coulter and Foote, 1974), goat (Chauhan et al., 1994), and boar (Ortman and Rodriguez-Martinez, 1994; Paulenz et al., 1995). These studies demonstrate that the main acrosomal damage occurs after freeze-thawing, as in dog spermatozoa (Oettlé, 1986b).

Given the excessive damage upon the acrosomes of many species, Bailey and Buhr (1993) studied calcium$^{2+}$ regulation by cryopreserved bull spermatozoa in response to A23187. They demonstrated that the ability of spermatozoa to regulate internal calcium$^{2+}$ concentrations and response to A23187 is clearly altered following commercial cryopreservation procedures, as follows:

(i) The spermatozoa (fresh/cryopreserved) incubated with exogenous calcium$^{2+}$ alone did not differ in viability but differed in their ability to regulate internal calcium$^{2+}$ concentrations, which suggests that cryopreservation affects calcium$^{2+}$ regulation in surviving cells.

(ii) The cryopreserved cells took up calcium$^{2+}$ immediately after its addition alone which was several seconds earlier than that of fresh cells. This suggests that cryopreserved spermatozoa are more permeable to extracellular calcium$^{2+}$ than fresh cells.

(iii) The effect of the combined addition of calcium$^{2+}$ and A23187 induced a rapid influx of calcium$^{2+}$ into the cryopreserved spermatozoa, which exceeded those of
fresh ones for a brief period (after the treatment), suggesting that cryopreserved spermatozoa have a slightly greater early response, but that, over time, both fresh and cryopreserved cells have a similar pattern of response to calcium$^{2+}$ and A23187.

(iv) In the absence of external calcium$^{2+}$ there was a detectable increase of internal calcium$^{2+}$ in fresh but not in cryopreserved cells following 120 min of incubation, which suggests that cryopreserved bull spermatozoa are unable to mobilise internal calcium$^{2+}$, possibly due to mitochondrial membrane damage.

Their results conclusively demonstrated that cryopreservation affects the ability of spermatozoa to regulate internal calcium$^{2+}$ even in the presence of calcium ionophore A23187. Conversely, McLaughlin et al. (1993), investigating the effects of cryopreservation on the acrosome of human spermatozoa and its response to A23187, found no such relationship between cryopreservation and A23187 on the basis of induction of the acrosome reaction. Although frozen-thawed cells exhibited fewer spontaneous acrosome reactions than those in fresh samples did, they responded to A23187 in a similar way. Therefore, they considered it likely that frozen-thawed spermatozoa succumbed to the effects of A23187 after acrosome reacting rather than dying and then undergoing an acrosome reaction. As in the present study, there was no significant interaction between A23187 concentration and freezing. These studies suggest that the sequence of acrosome reaction in fresh and in cryopreserved spermatozoa from various species might be different from each other.

3.6.3- Conclusions

Overall, the results of the present studies suggest for dog spermatozoa that;

- A programmable, semi-controlled biological freezer is the method of choice to optimise cooling regimens, based upon the retention of post-thaw motility.
- The optimum cooling regimen is -0.5°C/min from +4 to -9°C, -40°C/min to -20°C, -100°C/min to -120°C, followed by direct immersion of the straws in LN$_2$.
- Parameters of the freezing curve can be used to estimate post-thaw spermatozoal motility.
- Most spermatozoa demonstrate some signs of acrosomal damage (or reaction) after cryopreservation, in that induction of the acrosome reaction in vitro by calcium
ionophore (A23187) was unable to discriminate between more- and less-effective freezing regimens.

- Apart from the stimulative effects of A23187 and extracellular calcium$^{2+}$ upon the acrosomal changes, deterioration of the organelle during freeze-thawing can also alter the sequence (acceleration) of acrosome reaction.
- Following freeze-thawing, a very small proportion (<5%) of spermatozoa may retain the capability to undergo the acrosome reaction, which may not be affected by different freezing regimens.
- Although there may be a high proportion of spontaneous acrosome reactions at the beginning of incubation in chilled and frozen-thawed spermatozoa, it may remain virtually unchanged by 60 min.

However, whilst it was of significance to determine that freezing conditions could be optimised, in terms of the proportion of spermatozoa that exhibited motility after cryopreservation, it was disappointing to find that the major hypothesis of the thesis had not been substantiated by the results of Experiment 6. Indeed, it was remarkable to find that, contrary to expectations, the great majority of spermatozoa exhibited acrosomal damage or partial acrosomal reaction after cryopreservation. As the proportion of these spermatozoa was so high, there were few intact cells left to undergo A23187-induced acrosome reaction. Other authors have reported various degrees of cryogenic damage to spermatozoa (Olar, 1986b; Hay et al., 1997a; Rota, 1998). This is the first attempt to evaluate cryopreserved dog spermatozoa through induction of acrosome reactions (with a chemical inducer, A23187).

In consequence, it was deemed imperative to examine the acrosome of cryopreserved spermatozoa in some considerable detail, in order to provide a morphological/ultrastructural basis for the failure to induce acrosome reactions in vitro in cryopreserved cells.
CHAPTER 4. ACROSOMAL ULTRASTRUCTURE OF CHILLED AND FROZEN-THAWED DOG SPERMATOZOA SUBJECTED TO A23187-INDUCED ACROSOME REACTION AS ASSESSED BY SCANNING AND TRANSMISSION ELECTRON MICROSCOPY

Hypothesis:
It is possible to provide ultrastructural basis for the failure of the optimisation of cooling regimens based on the induction of the acrosome reaction in vitro in cryopreserved spermatozoa using Scanning and/or Transmission Electron Microscopy.

4. Experiment 7
4.1- Introduction
The previous experiments demonstrate that the survival of canine spermatozoa after cryopreservation depends upon the (i) composition of extender, (ii) concentration of cryoprotectant within the extender, (iii) temperature at which glycerolisation takes place, (iv) treatment by centrifugation, and most importantly (v) cooling rates that are applied to spermatozoa during freezing. Nevertheless, the present results, in common with many earlier studies of cryopreservation of canine spermatozoa, indicate that survival rates are relatively poor, with only a moderate number of cells remaining sufficiently undamaged to be able subsequently to undergo the acrosome reaction in vitro. Most importantly, the results of Experiment 6 did not substantiate the hypothesis that differences in post-thawing functionality of spermatozoa could be evaluated by in vitro induction of the acrosome reaction with A23187 because a high proportion of cells exhibited spontaneous changes to the acrosome before the start of incubation.

Whilst the damage sustained through cryopreservation causes little effect upon fertility in some species (e.g. cattle, human, rabbit), the results obtained from the use of cryopreserved semen in many other species (e.g. sheep, goat, fox, buffalo, horse) are significantly worse than those of fresh semen (Watson, 1990). The dog, pig and cat have been particularly intractable examples of this problem (Watson, 1990). However, recent progresses in canine AI technology (i.e. precise timing, correct placement of the semen and the use of sophisticated cryopreservation techniques) have made a great contribution
to dog breeding. Hence, fertility rates of frozen-thawed semen were very high, either close (Rota, 1998) or equal (Nöthling and Volkmann, 1993; Govette et al., 1996) to those obtained by natural mating.

Considerable acrosomal deterioration (e.g. swelling, decondensation, disruption, complete loss) has been noted during the cryopreservation of canine spermatozoa which occurs mainly during freezing and thawing but also to a limited extent during extension, cooling and equilibration (Oettlé, 1986b; Hay et al., 1997a). Scanning and/or transmission electron microscopy (SEM and TEM, respectively) of dog spermatozoa have been used to study morphological abnormalities (Oettlé, 1993; Dahlbom et al., 1997), spontaneous (Oettlé and Soley, 1988) or A23187-induced (Geussová et al., 1997; Guérin et al., 1999) acrosome reaction and damage on the acrosome during cryopreservation (Yubi, 1984; Ström Holst et al., 1998; Mohanchandran Nair et al., 1998). Comparable electron microscopic observations on acrosomal damage in frozen-thawed spermatozoa have also been made in the fox (Hofmo and Andersen Berg, 1989), bull (Krogences et al., 1994), ram (Holt and North, 1994), boar (Bwanga et al., 1991b; Kovachev et al., 1994), stallion (Grøndahl et al., 1992; Christensen, 1995), goat (Chauhan et al., 1994), buffalow (Kakar and Anand, 1984a,b), fowl (Harris et al., 1973), chinchilla (Healey, 1969), and human (Woolley and Richardson, 1978; Mahadevan and Trounson, 1984). Of particular interest was the study of Krogences et al. (1994) who examined the ultrastructural alterations on the head membranes during heparin-induced capacitation of frozen-thawed bull spermatozoa. Much less information is available for the effects of cryogenic damage upon the ultrastructure of the midpiece of fowl (Harris et al., 1973), pig (Courtens and Paquignon, 1985), human (Woolley and Richardson, 1978), bull (Kovachev et al., 1994), and horse (Christensen, 1995) sperm. This reflects the relative importance of the acrosome in determining post-cryopreservation fertility of spermatozoa.

The acrosome contains a large number of hydrolytic enzymes that are released simultaneously in the acrosome reaction to facilitate penetration of the ZP (Yanagimachi, 1994; Tulsiani et al., 1998). During the acrosome reaction, stimulatory messengers such as ZP proteins (McLeskey et al., 1997) lead to increased intracellular concentrations of calcium and hydrogen ions (Fraser, 1998), which cause fusion of the outer acrosomal and plasma membranes, thus resulting in vesiculation of the two membranes and release of the
acrosomal content (Yanagimachi, 1994; Tulsiani et al., 1998). Given the crucial role of the acrosome in fertilisation, it could be argued that the most important aspect of acrosomal damage that occurs during cryopreservation is the degree of impairment of the spermatozoal ability to undergo a spontaneous acrosome reaction. Indeed, the ultrastructural changes to the canine acrosome that lead to cryoinjury, as assessed by electron microscopy (Ström Holst et al., 1998), are remarkably similar to those occurring during the spontaneous acrosome reaction or secondary acrosomal changes, e.g. ageing, damage or cell death (Oettlé and Soley, 1988).

There have been a number of studies on the physiology of the acrosome reaction in canine spermatozoa (Mahi and Yanagimachi, 1978; Kawakami et al., 1998b; Hewitt and England, 1998). In vitro capacitation was induced by oviductal fluid (Kawakami et al., 1998a) while the acrosome reaction was achieved either by canine ZP or ZP proteins (Kawakami et al., 1993b; Brewis et al., 1999), progesterone (Brewis et al., 1999) or calcium ionophore A23187 (Szász et al., 1997; Hewitt and England, 1998). However, there is little information on the sequence of morphological or ultrastructural changes of the acrosome during in vitro induction of the acrosome reaction in either cooled or frozen-thawed dog spermatozoa.

Therefore, the objectives of this study were to:
- investigate the effect of 1 µM/l A23187 upon the induction of the acrosome reaction of chilled and cryopreserved dog spermatozoa, and
- illustrate the value of electron microscopy techniques (scanning and transmission) to assess the ultrastructural changes of the spermatozoa during the acrosome reaction.

4.2- Materials and Methods

4.2.1- Semen collection and initial evaluation

Single ejaculates were collected from each of three dogs (Dogs A, B and C; see Appendix 2.1 for detail) by manual stimulation (see Section 2.1.2.3).

The semen was examined as previously described (see Sections 2.1.2.4, 2.1.2.6 and 2.2.2.1).

4.2.2- Semen processing

The semen was processed as shown in Figure 4.1.
4.2.3- Freezing and thawing

The aliquot of semen used for cryopreservation was frozen in a programmable biological freezer (Section 3.2.2.3). Six to eight straws of semen were subjected to a freezing regimen which previous studies (Section 3.4.2.2) had established as resulting in a high level of post-thaw motility. Cooling regimen was as follows:

- +4 to -9°C -0.5°C/min
- -9 to -20°C -40°C/min
- -20 to -120°C -100°C/min.

Straws were then held at -120°C for 2 min before being plunged into LN₂ (-196°C). Following storage in LN₂ for at least 2 h, straws were thawed in a water bath at 35°C for 1 min.

Post-thaw progressive motility was assessed as previously described (Section 2.1.2.6).

4.2.4- Induction of acrosome reaction

The protocol for induction of the acrosome reaction was previously described (Section 2.2.2.3). Samples of chilled and cryopreserved semen were layered onto an equal volume of Percoll solution (Section 2.2.2.2) and centrifuged. After discarding the supernatant and resuspension of the pellet in TALP medium (see Appendix 2.3a,b) to pre-centrifugation volume, each sample was divided into two aliquots:

- Chilled, control (Aliquot 1)
- Chilled, A23187-induced (Aliquot 2)
- Cryopreserved, control (Aliquot 3)
- Cryopreserved, A23187-induced (Aliquot 4).

Aliquots 1 and 3 contained no ionophore, while 1 µM/1 A23187 (Appendix 2.4) was added to Aliquots 2 and 4. Following incubation of each aliquot in capped tubes at 39°C in 5%CO₂ in humidified air for 60 min, 50 µl of suspensions were withdrawn and fixed with 2.5% Glutaraldehyde in TALP (1:10, semen: fixative) for further use in electron microscopy.

A summary of experimental steps before electron microscopy processing is given in Figure 4.1:
Figure 4.1. Summary of procedures prior to electron microscopy
4.2.5- Electron Microscopy

4.2.5.1- Scanning Electron Microscopy (SEM)

Semen samples were processed for SEM by modification of the method of Cooley et al. (1994) as follows:

- Following fixation in 2.5% Glutaraldehyde in TALP for at least 4 h at +4°C, 50 µl of specimens were placed onto circular glass coverslips (13 mm in diameter) that had been pre-washed in 100% alcohol and coated with poly-l-lysine (Sigma).
- Fixed materials on coverslips were then allowed to settle in a humid environment for at least overnight.
- Afterwards, the samples were post-fixed in 1% Osmium tetroxide (Sigma) in 1 M sodium cacodylate buffer (Sigma) for 1 h, washed in distilled water and dehydrated in a series of ethanol (70% ethanol for 30 min, 90% ethanol for 30 min, 100% ethanol for 1 h and a further 2 h).
- Next, the samples were placed in a 1:1 mixture of ethanol and HMDS (1, 1, 1, 3, 3, 3-Hexamethyldisilizane; Sigma) for 1 h and 100% HMDS overnight.
- The HMDS was finally sublimed under nitrogen gas in a freeze dryer.
- Dry samples were mounted onto scanning EM stubs with double sided tape, sputter coated with gold for 2¼ min and examined in the scanning microscope, Stereo Scan 200, at 15 kV.
- Micrographs were recorded (on 120 Ilford FP4 Plus film, black & white) at an average magnification of 7,800x.
4.2.5.2- Transmission Electron Microscopy (TEM)

- Samples for transmission EM were fixed in 2.5% Glutaraldehyde in sodium cacodylate buffer (495 mOsm/l, pH 7.2-7.4) and then were cut into approximately 1 mm cubes.

- Afterwards, specimens were washed twice in a weak (0.1 M) sodium cacodylate buffer for 1 h and rinsed twice for 5 min in the same, but more concentrated (0.2 M) buffer.

- They were then dehydrated in a series of alcohol (70% ethanol for 2 x20 min, 90% ethanol for 2 x10 min, 100% ethanol for 2 x20 min) and embedded in acrylic resin (LR White; London Resin Co., Reading, UK) overnight, that was continued further for 2 x1 h.

- Following the last embedding by using fresh LR White in gelatine capsules (labelled, capped), the samples were allowed to polymerise at 60°C for 24 h.

- Finally, specimens were counterstained with uranyl acetate (saturated in distilled water) for 5 min, and followed by staining with lead citrate (see Appendix 4.1) for 5 min.

- Sections were examined in a transmission EM, Philips EM 201, and photographed by using Kodak 4489 cut film (black & white) at a magnification of 18,900x.
4.3- Results

The characteristics of the ejaculates are given in Table 4.1. All of the samples (one collection from each animal) were used for SEM. The sample from Dog A was also split for SEM and TEM.
Table 4.1. The characteristics of ejaculates used for assessment of the acrosome reaction of spermatozoa subjected to chilling and freeze-thawing by SEM and TEM (Experiment 7)

<table>
<thead>
<tr>
<th>Dog (n=3)</th>
<th>Ejaculate No.</th>
<th>colour</th>
<th>volume (ml)</th>
<th>progressive motility (%)</th>
<th>concentration (x10^6/ml)</th>
<th>morphology (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>normal</td>
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<tr>
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<td>live</td>
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<td></td>
<td>abnormal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>live</td>
</tr>
<tr>
<td><strong>Dog A:</strong> (Gillie)</td>
<td>1</td>
<td>milky</td>
<td>0.77</td>
<td>93</td>
<td>75</td>
<td>250.0</td>
</tr>
<tr>
<td><strong>Dog B:</strong> (Robi)</td>
<td>1</td>
<td>light milky</td>
<td>1.00</td>
<td>90</td>
<td>65</td>
<td>837.5</td>
</tr>
<tr>
<td><strong>Dog C:</strong> (Solo)</td>
<td>1</td>
<td>light milky</td>
<td>1.25</td>
<td>92</td>
<td>58</td>
<td>912.5</td>
</tr>
</tbody>
</table>
4.3.1- Spermatozoal head and acrosome
4.3.1.1- Assessment by SEM

Intact spermatozoa
The chilled intact spermatozoa had a densely packed and clearly shaped head, with a distinct apical ridge. The entire head had a uniform surface with a slightly indistinct equatorial segment and post-acrosomal region (Plates -4a, -5a, -6a). The membranes overlying these regions were intact and distinct, with a clear outline. Frozen-thawed spermatozoa usually exhibited minor degenerative changes. Their surface was uneven, with a non-uniform, slightly reduced contrast, resulting from rarefaction of the acrosomal content (Plates -4b, -6b). Some were not obviously different from chilled sperm (Plate- 5b). The membranes overlying the head were not as smooth as in chilled spermatozoa and, although they remained mainly intact, they typically had some minor breaks (Plate -6b). The acrosomal ridge was not as obvious as in chilled spermatozoa (Plates -4b, -5b, -6b).

Partially reacted spermatozoa
The partially reacted chilled spermatozoa were characterised by marked changes over the acrosome, leading to an irregular ultrastructure. The acrosomal content was rarefied and unevenly distributed. The head exhibited an irregular, unclear outline (Plates -4c, -5c, -6c), swelling (Plates -4c, -5c), small cracks (Plate -6c), or discontinuities (Plates -5c, -6c) of the membranes overlying the acrosome. Particles of varying size were attached to the head (Plates -4c, -6c). The equatorial segment and the post-acrosomal region remained almost normal (Plate -5c) in some spermatozoa, while others had minor changes. These changes included an uneven surface with a lessened contrast (Plates -4c, -6c), or localised areas of minor damage on the equatorial segment (Plate -6c). However, the overlying membranes were mainly intact, with a clear outline (Plates -4c, -6c). No obvious differences were observed between the chilled and frozen-thawed partially reacted spermatozoa on the basis of acrosomal changes (i.e. swollen acrosomes, irregular outlines, uneven surfaces with lessened contrast, attached particles) (Plates -4d, -5d, -6d).
Reacted spermatozoa

The chilled reacted spermatozoa had no distinct overlying membranes on the apical part of the head, revealing a narrow-shaped head with a prominent nucleus, which exhibited darkened contrast (Plates -4e, -5e, -6e). The equatorial segment and the post-acrosomal region usually remained intact, having a uniform contrast with clear, overlying membranes (Plates -5e, -6e). However, in some cases, there were also minor changes such as an uneven surface with a reduced contrast and disintegration of the equatorial segment although their outline membranes remained almost clear (Plate -4e). In frozen-thawed spermatozoa, the lack of the acrosome was as obvious as in chilled spermatozoa, but, in addition, changes also appeared on their equatorial segment and post-acrosomal region (Plates -4f, -5f, -6f).

4.3.1.2. Assessment by TEM

Transmission EM permitted detailed examination of the ultrastructure of all parts of the sperm head (i.e. the plasma membrane, outer acrosomal membrane, acrosomal content, perforatorium, inner acrosomal membrane, nuclear membrane, nucleus, equatorial segment and post-acrosomal region), midpiece and tail (Plates -7a, b, -8a, b).

Intact spermatozoa

In chilled intact spermatozoa, the head appeared as densely packed in shape with a clear, smooth outline (i.e. the plasma membrane), allowing all the underlying compartments to be seen clearly (Plate -7b). Although the nucleus appeared dark, surrounded by a distinct nuclear membrane, all the remaining head organelles (particularly the acrosomal content) had brighter contrast, which facilitated the differentiation of the presence or absence of the acrosome (consisting of the outer acrosomal membrane, the acrosomal content and the inner acrosomal membrane). The acrosomal content appeared uniform and evenly distributed, surrounded by intact, closely attached inner and outer acrosomal membranes. The equatorial segment appeared as a narrowing part at the middle of the head, while the post-acrosomal region, which includes the distinct, underlying post acrosomal dense lamina, was also intact and distinguishable. The perforatorium, a small organelle that forms the apical ridge over the top of the nucleus, was also intact, with a uniform contrast.
The freeze-thawing process led to a number of ultrastructural alterations (Plate -8b). All compartments of the spermatozoal head, except the nucleus, had slightly non-uniform and lessened contrast leading to a poor distinction of each. The plasma membrane appeared mainly intact although the outline was less clear, and there were localised areas of damage. The acrosomal content appeared to be relatively evenly distributed but with a slight rarefaction. The inner and outer acrosomal membranes were less distinct due to slight rarefaction of the acrosomal content and slight irregularity of the plasma and nuclear membranes. The nuclear membrane was also less distinct and broken at a few points forming multiple perinuclear spaces. Finally, the equatorial segment the post-acrosomal region were less distinct but intact and surrounded by a less clear plasma membrane.

**Partially reacted spermatozoa**

In the chilled partially reacted spermatozoa (Plates -7c,d), the changes mainly appeared on the acrosome, which included an irregular, unclear plasma membrane, rarefied and/or swollen acrosomal content, and vesicles originating from the fusion between the plasma and the outer acrosomal membranes. This vesiculation had therefore resulted in disintegration of the acrosomal membranes. The inner acrosomal membrane usually remained intact. In contrast to these changes on the acrosome, the nuclear membrane, the equatorial segment and the post-acrosomal region apparently remained intact. In frozen-thawed spermatozoa, the changes included the formation of a small number of vacuoles and vesicles, which resulted from the fusion of the plasma and outer acrosomal membrane and contained some acrosomal content (Plate -8g), swollen plasma membranes, acrosomal contents and broken nuclear membranes. There were also a partial detachment of the equatorial segment from the nucleus (Plate -8f) and, occasionally, severe damage (Plate -8g) or discontinuation (Plates -8g,h) of both the equatorial segment and post-acrosomal region.

There were also some changes in the frozen-thawed spermatozoa, even when they had not been treated with A23187 (i.e. control samples). The plasma membranes either remained intact (Plates -8c,d) or became disintegrated due to multiple fusions with the outer acrosomal membrane, leading to the vesicle formation (Plates -8d,e), together with a non-clear, irregular outlines (Plate -8e). The acrosomal contents were swollen (Plate -
8e) or rarefied (i.e. enlargement with a reduced contrast) (Plates -8c,d,e). The nuclear membrane was also broken at different levels leading to enlarged perinuclear spaces (Plate -8c).

*Reacted spermatozoa*

In the chilled reacted spermatozoa, with the exception of the equatorial segment which was well preserved and intact, the acrosome was absent (Plate -7e). Likewise, the perforatorium, the nuclear membrane and the post-acrosomal region also remained intact. There was no apparent change in the nucleus, which was intact, uniform and darkly contrasted. In the frozen-thawed reacted spermatozoa (Plates -8i,j), the acrosome was also clearly lacking, whilst there was also discontinuation (Plate -8i) and/or disintegration (Plate -8j) of the equatorial segment, the post-acrosomal region and their overlying plasma membranes. Finally, the perforatorium and the nuclear membrane appeared disintegrated and/or discontinuous or partly damaged.

4.3.2- Midpiece

Apart from the changes on the sperm head due to the acrosome reaction and/or freeze-thawing, there were also some changes in the ultrastructure of the midpiece.

4.3.2.1- Assessment by SEM

The midpiece of chilled spermatozoa appeared as a densely packed structure with a closely attached plasma membrane, which was distinct, intact and had a smooth surface. While many of the frozen-thawed spermatozoa examined appeared very similar, a few others (Plates -4d, -6b) exhibited some localised discontinuity of the plasma membrane on the neck.

4.3.2.2- Assessment by TEM

Only one spermatozoon was found in each of the chilled and frozen-thawed samples which was longitudinally sectioned. These two spermatozoa were compared. In the chilled spermatozoon (Plate -7a), the proximal ring (i.e. the connection point between the head and neck), plasma membrane, fibrous sheath, mitochondria (rectangular, cubic or triangular in shape), outer dense fibre, and axoneme appeared intact and firmly attached to each other. In frozen-thawed spermatozoon (Plate -8a), the proximal ring was damaged and the plasma membrane and fibrous sheath were missing over the neck.
While some of the outer dense fibres remained almost intact, they seemed enlarged together with a loose connection to the neck. The content of the axoneme at this region appeared almost empty. In the lower part, the plasma membrane and the underlying fibrous sheath were almost entirely missing. The majority of the mitochondria at this region had less contrast and appeared varying in shape (i.e. rectangular, rounded, swollen with almost empty contents or disrupted), together with less clear membranes and loose attachments between them.
4.4- Discussion

In the present study, the ultrastructure of dog spermatozoa could be satisfactorily assessed by both the electron microscopy techniques (SEM and TEM). Although SEM allowed a broad assessment to be made of the ultrastructure of intact acrosomes, its resolution was often not adequate to see the minor changes that occurred in either chilled or frozen-thawed spermatozoa due to the acrosome reaction and/or cryogenic damage. Such an opinion was shared by Yanagimachi (1994), who suggested that although SEM gives spectacular images of spermatozoa, its resolution is not enough to examine the details of the spermatozoal membranes. By contrast, with TEM, it was possible to examine the ultrastructure of individual subcellular compartments in detail, particularly the initial changes to the membrane that occurred during the acrosome reaction or following freeze-thawing. Hence, although it is laborious and time consuming, TEM is the only technique that allows detailed examination of the morphological status of the acrosome including the acrosome reaction (Yanagimachi, 1994). Furthermore, it has been suggested that TEM can also provide information on the normality of the acrosomal changes (Cross and Meizel, 1989), for the patterns of membrane changes in normal acrosome reaction are characteristic. One example of such normal changes are those of the membranes at the anterior margin of the equatorial segment, which, in normal acrosome reactions, characteristically exhibit no discontinuities (Bedford, 1970). However, many of the acrosome reacted spermatozoa will not be suitably oriented for this judgement to be made (Cross and Meizel, 1989). The present study did not attempt to differentiate between the causes of acrosome reaction, i.e. normal (true) or abnormal (false) reaction, as the latter may occur due to ageing, cell death or cell damage (Bedford, 1970; Oettlé and Soley, 1988) after poor fixation, cold shock or freeze-thawing.

There have been a number of studies of cryogenic damage to the acrosome using electron microscopy in the dog (Yubi, 1984; Ström Holst et al., 1998; Mohanchandran Nair et al., 1998) and fox (Hofmo and Andersen Berg, 1989). No reports have been published on the ultrastructure of the acrosomal changes in frozen-thawed spermatozoa subjected to A23187-induced acrosome reactions. With fresh samples, Geussovä et al. (1997) studied A23187-induced acrosome reaction of dog spermatozoa using monoclonal antibodies and TEM, but no micrographs were taken from A23187-treated cells. Guérin et al. (1999),
using A23187, simply illustrated the spermatozoa before and after acrosome reaction (as acrosome-intact and -reacted, respectively), but gave no detail on the intermediate stages (partial acrosome reaction). In a pioneering study, Yubi (1984) used SEM to investigate the ultrastructure of spermatozoa in some detail in order to compare various cryopreservation techniques. Subsequently, Mohanchandran Nair et al. (1998) used TEM to study the cryogenic damage upon the acrosome, as did Rodriguez-Martinez et al. (1993), using both SEM and TEM. Ström Holst et al. (1998) also used TEM to investigate the ultrastructural damage following various cryopreservation techniques, and X-ray microanalysis, as previously used by Rodriguez-Martinez et al. (1993), to examine the elemental composition of the head of spermatozoa. Oettlé and Soley (1988) used TEM to study the ultrastructural basis of spermatozoal abnormalities and the acrosomal changes which occur during spontaneous acrosome reaction and cell damage (i.e. ageing, cell death, etc.). Likewise, Oettlé (1993) used TEM for a simple discrimination of spermatozoal abnormalities. This was also used by Koehler et al. (1998) to determine abnormal wolf spermatozoa. However, in none of these studies were the ultrastructural changes of the midpiece assessed.

The results obtained from the present SEM and TEM studies of the acrosome during spontaneous acrosome reaction and cryogenic damage are in general agreement with the above studies. The earlier observations are extended by the present examination of spermatozoa with A23187-induced acrosome reactions. For the unfrozen (herein referred to as 'chilled') intact spermatozoa, the findings are similar to those of Yubi (1984) and Hofmo and Andersen Berg (1989) who, using SEM, showed that the surface of the entire heads of the spermatozoa (including the acrosome) was even having a uniform contrast, together with a distinct and clear outline. When fresh (Oettlé and Soley, 1988; Guérin et al., 1999) or chilled (Hofmo and Andersen Berg, 1989, in fox) intact spermatozoa were examined with TEM, all compartments of the head could be seen clearly, and there were no apparent changes on the acrosome. Subsequently, workers from the same laboratory reported that the apical plasma membrane presented a peculiar plication, which was particularly evident over the acrosomal region, and which extended towards the equatorial segment and (after thawing) over the midpiece (Rodriguez-Martinez et al., 1993; Ström Holst et al., 1998). These workers used 'etching' (i.e. trimming of the cell membranes as
part of a routine EM processing and applied for tissue and small cell cultures) in their EM studies. However, it is not normally used for preparation of semen samples for EM (D.M. Woolley, personal communication). Likewise, the step of etching during EM processes might have provoked the plasma membrane to dry out further (Cooley et al., 1994). Therefore, it is possible that in the studies of Rodriguez-Martinez et al. (1993) and Ström Holst et al. (1998), the plica formation could have been an artefact due to excessive dehydration caused by etching. Indeed, Mohanchandran Nair et al. (1998), who used a different EM technique (without etching), observed that although the plasma membrane of fresh spermatozoa was usually slightly loosened, there was no such plica formation in frozen-thawed samples. This was also the case in the present trials.

In the present study, it was of interest to note, immediately after thawing, that up to 100% of the acrosomes examined were swollen and/or rarely detached. Such a high degree of acrosomal changes, resulting solely from cryopreservation, agrees well with previous findings by light microscopy (Oettlé, 1986b; Hay et al., 1997a,b). There was a similar finding when TEM was used (Ström Holst et al., 1998). Woolley and Richardson (1978) and Courtens and Paquignon (1985) have suggested that the high level of acrosomal damage is due to thawing and/or subsequent handling procedures. These results may further indicate why the post-thaw viability of dog spermatozoa is limited up to 8 h at body temperature (England and Ponzio, 1996) and why such a high number of spermatozoa is generally required for each insemination (see Linde-Forsberg et al., 1999 for detail). However, Jones and Stewart (1979) speculated that the acrosomal swelling should not effect the fertilising capacity of spermatozoa, because swelling is a preliminary stage of acrosome reaction. Indeed, swelling of the plasma membrane and the acrosomal membrane of human spermatozoa is compatible with fertility (Mahadevan and Trounson, 1984). In dogs, a similar observation was made by Ström Holst et al. (1998), using spermatozoa that had been cryopreserved by a method that was known to maintain high fertility (Govette et al., 1996). On the other hand, it could also be considered that if the swelling of the plasma and the acrosomal membranes can be minimised, the maintenance of structural integrity of the entire head is likely to be prolonged (Jones and Stewart, 1979) until the fertilisation is completed. During the SEM assessment of the spermatozoa, which had undergone partial or complete acrosome reaction in vitro, there was no obvious
difference between the acrosomal changes before or after freeze-thawing. However, in almost all the chilled spermatozoa that were examined, the distal part of the head (i.e. the equatorial segment and the post-acrosomal region) remained intact.

Following freezing and thawing, the post-acrosomal region of the acrosome reacted spermatozoa underwent significant changes (rarefaction, non-uniform contrast, uneven surface and attached particles), although the plasma membrane remained intact. Krogenæs et al. (1994) reported similar findings in the bull. With TEM, the acrosomal changes appeared as single/multiple membrane fusions between the plasma membrane and the outer acrosomal membrane, formation of vesicles, swelling, rarefaction and loss of dense acrosomal contents. Similar observations have also been made in the dog (Oettlé and Soley, 1988) and in the bull (Krogenæs et al., 1994) during spontaneous or heparin-induced acrosome reactions, respectively. The acrosomal swelling did not include the equatorial segment, which normally remains intact during, but not after, the acrosome reaction in vitro and in vivo (Yanagimachi, 1994). The equatorial segment may eventually vesiculate and be lost as time passes after the acrosome reaction (Oettlé and Soley, 1988; Yanagimachi, 1994), leading to cell death. Such changes to the equatorial segment were usually seen in frozen-thawed spermatozoa in the present study. As with previous studies, the equatorial segment, together with the post-acrosomal region, revealed various changes from a minor disintegration (Ström Holst et al., 1998; Mohanchandran Nair et al., 1998) to virtually a complete loss.

In frozen-thawed fox spermatozoa, the majority of spermatozoa had extensive vesiculation of the outer acrosomal membrane and detachment of the apical plasma membrane towards the equatorial segment (Hofmo and Andersen Berg, 1989). By contrast, in frozen-thawed dog spermatozoa, it has been reported that the plasma membrane is maintained, although swollen, even in cases where the acrosome was strongly modified (Rodriguez-Martinez et al., 1993; Mohanchandran Nair et al., 1998). Therefore, Rodriguez-Martinez et al. (1993) concluded that the plasma membrane of dog spermatozoa responds to cryopreservation in a manner that differs from other domestic species. Comparison of these findings with the present study is not straightforward as the induction of acrosome reaction in the frozen-thawed spermatozoa largely precluded further observations of the acrosome. It is possible that the plasma and outer acrosomal membrane of spermatozoa are much more vulnerable
to chemical treatment (fixation) than those of uncapacitated spermatozoa (Yanagimachi, 1994). Furthermore, following the induction of acrosome reaction, the plasma membrane fuses with underlying outer acrosomal membrane and forms multiple hybrid vesicles, which lead the membranes to be discontinued and then entirely lost (Yudin et al., 1988). As the acrosome reaction necessitates ultrastructural alterations, leading to the entire loss of the organelle as well as the plasma membrane (Yanagimachi, 1994), no direct comparison could be made.

Following freezing and thawing, many of the acrosome intact (Plate -8b) and partially reacted (Plate -8c) spermatozoa in control samples (i.e. without A23187) had broken nuclear membranes, forming enlarged perinuclear spaces (loosely connected to the underlying nucleus). Such changes were frequently seen in the region proximal to the equatorial segment (Section 2.2.3.1). This is not surprising, since, in many species (i.e. bull, rabbit, hamster, rat and guinea pig), the plasma and outer acrosomal membranes of spermatozoa appear to be least stable in this region (Gaddum-Rosse and Blandau, 1972). Furthermore, according to Watson and Plummer (1986), the outer acrosomal membrane of this region is very rich in calcium-binding sites, indicating the point at which the early stages of acrosomal changes start. This may further facilitate the occurrence of nuclear membrane changes.

It is therefore concluded that the acrosomal changes of spermatozoa represent the effects of chilling (from dilution to equilibration) and, in particular, freezing and thawing that were independent of induction of acrosome reactions with A23187 and calcium. Indeed, almost all of the frozen-thawed spermatozoa examined exhibited various degrees of acrosomal changes in the presence of extracellular calcium, even without A23187 (Plates -8c,d,e). Hence, it is suggested that the processes of freezing and thawing further provoke the acrosome reaction of spermatozoa in calcium-containing medium, most probably through an increased permeability of the plasma membrane (facilitating calcium uptake).

In a similar manner, in frozen-thawed spermatozoa that had undergone A23187-induced acrosome reaction, it was observed that both partially (Plates -8f,g,h) and completely (Plates -8i,j) reacted spermatozoa had nuclear membranes which were discontinuous and/or disintegrated. Hence, it is clear that the nuclear membrane becomes more
vulnerable to the cryogenic damage during or, especially, after the acrosome reaction (eventually resulting in cell death). Some nuclear elements could be lost where the nuclear membrane is damaged, which may lead to interference with spermatozoal function (e.g. acrosome reaction, nuclear decondensation during fertilisation, etc.). In this context, Küpker et al. (1998) noted that altered patterns of chromatin structure and signs of chromosomal decondensation are frequently associated with impaired spermiogenesis and deterioration of the nuclear membranes, as seen after freezing and thawing (Plates -8b,c). Indeed, concentrations of sulphur and phosphorus in dog spermatozoa have been shown to decrease following freeze-thawing, suggesting that chromatin changes are induced (Ström Holst et al., 1998), perhaps by DNA damage. In human spermatozoa, it has been shown that freeze-thawing alters the stability of chromatin structure, resulting in a drastic decrease in fertilising ability (Royere et al., 1991).

An assessment of the midpiece (including mitochondria) of cryopreserved spermatozoa was also undertaken. Swelling of the plasma membrane of the midpiece was previously described as occurring in a few fresh canine spermatozoa during cryopreservation (Mohanchandran Nair et al., 1998) or hypoosmotic swelling (England and Plummer, 1993), although there are no previous reports of such examinations of frozen-thawed semen. There are, however, some similarities with the midpiece changes reported for other species. In the domestic chicken, some spermatozoa showed severe changes such as destruction of the plasma membrane overlying the mitochondria, leading to detachment and swelling of the mitochondria (Harris et al., 1973). In the pig, Larsson et al. (1976) observed that some spermatozoa had rounded and swollen mitochondria with lessened content and broken plasma membranes. Additionally, in freeze-substitution studies, Courtens and Paquignon (1985) found ice in the neck and the flagella of frozen spermatozoa with an unclear, slightly loosened plasma membrane. Following thawing, they also observed unclear microtubules of the axoneme. By contrast, Kovachev et al. (1994), using a new cryopreservation technique, achieved a well preserved midpiece, which had only slightly rounded mitochondria with almost normal contents and an intact plasma membrane. Likewise, in human spermatozoa, Woolley and Richardson (1978) observed that the mitochondria appeared rounded and well preserved. In the horse, Christensen (1995) noted that the outline of the axoneme and the outer dense fibres were
indistinct, although there were no obvious changes either to the plasma membrane or the mitochondria. Hence, the findings of the present study agree well with the observation of Larsson et al. (1976) in the pig, as it was clear that the mitochondria experienced major ultrastructural changes following freezing and thawing. Spermatozoa that have been subjected to the acrosome reaction may experience further ultrastructural changes (likely to extend towards the midpiece) due to cell damage, as they die following the reaction (Harrison, 1996). Since there is no unique cryopreservation technique which can be used for all species, the degree of cryogenic damage upon the entire spermatozoa of different species may also differ (Watson, 1979).

Death of the cell results in ultrastructural alteration of the cell (Oettlé and Soley, 1988). This might explain why the equatorial segment and the post-acrosomal region exhibited ultrastructural changes (Plates -8g,h,i,j) in virtually all of frozen-thawed, A23187-induced spermatozoa. It is therefore considered that extensive damage to these organelles of the spermatozoa might be one of the main reasons of lower fertility following AI using frozen semen, since especially the equatorial segment (the site where spermatozoa fuse with the plasma membrane of the ovum; Bedford et al., 1979) might not be maintained following acrosome reaction. Hence, although marked ultrastructural changes of the entire head of sperm cell usually occur following freezing and thawing, it may not always be possible to differentiate the origin of the acrosomal changes.

As discussed earlier (Section 2.2.1.1), the acrosome reaction is a prerequisite during fertilisation in vivo and in vitro (Yanagimachi, 1994) and A23187-induced acrosome reaction can provide information for prediction of in vitro fertilisation rates in subfertile men (Liu and Baker, 1998). Whitfield and Parkinson (1995), using frozen-thawed bull spermatozoa, have shown that A23187-induced acrosome reaction can predict in vivo fertility. Taken together, these data imply that in vitro induction of the acrosome reaction provides a sufficiently accurate model of the events during fertilisation in vivo, to allow estimates of fertility of spermatozoa to be made, a situation which might also be the case in the dog. Therefore, the present findings may provide basic information upon the sequence of ultrastructural changes during in vivo acrosome reaction, which are necessary to determine the validity of such a bioassay for use with canine spermatozoa.
In conclusion, it is suggested that the processes of freezing and thawing provoked *in vitro* acrosome reaction of dog spermatozoa, resulting in damage to the equatorial segment as well as the post-acrosomal region. Therefore, future studies should be directed towards improving freezing and thawing techniques which maintain the integrity of the entire head of the sperm cell. In addition, it is recommended that TEM should be the method of choice during ultrastructural studies upon dog spermatozoa.
Legends to plates for SEM (in Dog A):

Plate -4a. Chilled intact (I) sperm having a densely packed and clearly shaped head, with a distinct apical ridge and uniform surface over the entire head. Note slightly indistinct equatorial segment (ES) and post-acrosomal region (PAR) and their overlying membranes as both being intact and distinct, with a clear outline. Bar=5 μm, Magn. 7,800X.

Plate -4b. Frozen (I) sperm with minor degenerative changes: uneven surface (especially over the acrosome) with a non-uniform slightly reduced contrast over the acrosome, ES and PAR. Note indistinct acrosomal ridge and intact membranes overlying the ES and PAR. Magn. 7,930X.

Plate -4c. Chilled partially reacted (PR) sperm having marked changes especially over the acrosome: rarefied and unevenly distributed acrosomal content with irregular outlines due to swelling of the overlying membranes and attached particles in varying size. Note the ES and PAR as both having slightly uneven surface with a lessened contrast together with mainly intact overlying membranes. Magn. 7,850X.

Plate -4d. Frozen (PR) sperm exhibiting major acrosomal changes: rarefied and unevenly distributed acrosomal content with irregular, unclear outlines due to swelling and attached particles. Note the ES and PAR as both having less dense, unevenly distributed contrast (especially on the ES), with mainly intact, clear outlines. There is a minor membrane damage (discontinuity) on the neck. Magn. 7,790X.

Plate -4e. Chilled reacted (R) sperm having no distinct overlying membranes on the apical part of the head, revealing a narrow-shaped head with a prominent nucleus exhibiting darkened contrast. Note the ES and PAR with uneven surface having a lessened contrast, together with outlining membranes remaining virtually clear. Magn. 7,730X.

Plate -4f. Frozen (R) sperm having no acrosome with no distinct overlying membranes and exhibiting a narrower shadow, the nucleus with a darkened contrast. Note the ES and PAR as both having slightly uneven surface with a lessened contrast, together with mainly intact, overlying membranes. Magn. 7,790X.
Plate-4. The assessment of pre- and post-freeze spermatozoa undergoing different degrees of acrosome reaction by Scanning Electron Microscopy
Legends to plates for SEM (in Dog B):

**Plate -5a.** Chilled intact (I) sperm having a densely packed and clearly shaped head, with a distinct apical ridge and uniform surface over the entire head. Note slightly indistinct equatorial segment (ES) and post-acrosomal region (PAR) and their overlying membranes as being intact and distinct, with a clear outline. Bar=5 μm, Magn. 7,790X.

**Plate -5b.** Frozen (I) sperm having virtually unchanged surface characteristics over the acrosome, ES and PAR. Note indistinct apical ridge and slightly reduced contrast over the entire head. Magn. 7,680X.

**Plate -5c.** Chilled partially reacted (PR) sperm having marked changes especially over the acrosome: rarefied, unevenly distributed acrosomal content with irregular, unclear outlines due to swelling or discontinuities of the overlying membranes. Note the ES and PAR remaining virtually intact. Magn. 7,400X.

**Plate -5d.** Frozen (PR) sperm having degenerative changes especially over the acrosome: irregular outlines and uneven surface due to attached particles and swelling of the acrosomal content. Note mainly intact ES and PAR together with uneven surface and slightly lessened contrast especially over the ES. Magn. 7,840X.

**Plate -5e.** Chilled reacted (R) sperm having no distinct overlying membranes on the apical part of the head, revealing a narrow-shaped head with a prominent nucleus, exhibiting darkened contrast. Note intact ES and PAR as both having a uniform contrast, with a clear, overlying membranes. Magn. 7,930X.

**Plate -5f.** Frozen (R) sperm lacking acrosome with no distinct overlying membranes revealing a narrower shadow, the nucleus with a darkened contrast. Note the ES and PAR as both having slightly uneven surface with a lessened contrast (especially over the ES), together with mainly intact, overlying membranes. Magn. 7,840X.
Plate-5. The assessment of pre- and post- freeze spermatozoa undergoing different degrees of acrosome reaction by Scanning Electron Microscopy
Legends to plates for SEM (in Dog C):

Plate -6a. Chilled intact (I) sperm having a densely packed and clearly shaped head, with a distinct apical ridge and uniform surface over the entire head, as well as slightly indistinct equatorial segment (ES) and post-acrosomal region (PAR). Note the membranes overlying the ES and PAR as both being intact and distinct, with a clear outline. Bar=5 μm, Magn. 7,890X.

Plate -6b. Frozen (I) sperm with minor degenerative changes over the entire head: slightly uneven surface with a non-uniform slightly reduced contrast over the acrosome, the ES and PAR. Note distinct acrosomal ridge, the ES and PAR as all having few minor breaks. There is a minor membrane damage (discontinuity) on the neck. Magn. 7,400X.

Plate -6c. Chilled partially reacted (PR) sperm exhibiting marked changes especially over the acrosome: rarefied and unevenly distributed acrosomal content with irregular, unclear outlines due to small cracks or discontinuities of the membranes overlying the acrosome and attached particles in varying size. Note the ES and PAR as both having uneven surface, with a lessened contrast and minor damage, while the overlying membranes remaining mainly intact, with a clear outline. Magn. 7,960X.

Plate -6d. Frozen (PR) sperm exhibiting changes over the entire head: irregular outlines and uneven surface of the acrosome due to discontinuity of the acrosomal ridge, attached particles of varying size and less dense acrosomal content. Note mainly intact ES and PAR, together with uneven surface and slightly lessened contrast especially over the ES. Magn. 7,550X.

Plate -6e. Chilled reacted (R) sperm with no distinct overlying membranes on the apical part of the head, revealing a narrow-shaped head with a prominent nucleus, exhibiting slightly darkened contrast. Note the ES and PAR as both remaining intact, with a uniform contrast and a clear, overlying membranes. Magn. 7,730X.

Plate -6f. Frozen (R) sperm having no acrosome with no distinct overlying membranes and exhibiting a narrower shadow, the nucleus with a darkened contrast. Note the ES and PAR as both having slightly uneven surface with a lessened contrast (especially over the ES), together with mainly intact, overlying membranes. Magn. 7,550X.
Plate-6. The assessment of pre- and post- freeze spermatozoa undergoing different degrees of acrosome reaction by Scanning Electron Microscopy.
Legends to plates for TEM in chilled spermatozoa

Plate -7a. Longitudinal section of chilled sperm (in controls) showing damage (discontinuities and swelling) on the plasma membrane (PM), but with remaining other intact organelles [i.e. the outer acrosomal membrane (OAM), inner acrosomal membrane (IAM), nucleus (N)] of the head and mid-piece [i.e. the proximal ring, perinuclear space, basal plate, capitulum, proximal centriol, segmented columns, mitochondria (rectangular, cubic or triangular in shape), PM, fibrous sheath, axoneme, outer and inner dense fibres, as firmly attached to each other]. Bar=1 µm, Magn. 18,900X.

Plate -7b. Detail from chilled intact (I) sperm head having densely packed shape with a clear, intact PM and dark N surrounded by a distinct nuclear membrane (NM). Note the remaining head organelles: The acrosomal content (AC) having uniform, evenly distributed, brighter contrast and surrounded by intact, closely attached OAM and IAM. The ES is appearing as a narrowing width at the middle part of the head, while the PAR is remaining intact and distinguishable with a distinct, underlying post-acrosomal dense lamina. The perforatorium, forming the apical ridge over the nucleus, is also intact, with a uniform contrast.

Plates -7c,d. Chilled partially reacted (PR) spermatozoa exhibiting major acrosomal changes: swelling and decondensation (Plate -7c) of the AC and fusion of the PM and OAM (i.e. vesiculation) resulting in the disintegration of both. Note the perforatorium, IAM, NM, ES together with intact OAM and IAM at this region, and the PAR as all remaining intact.

Plate -7e. Chilled reacted (R) sperm having no acrosome and slightly damaged (unclear) inner acrosomal membrane (IAM). Note the perforatorium, ES (with the OAM and IAM at this region), NM and PAR as all remaining well preserved and intact. There is no apparent change in the nucleus as being intact, uniform and having dark contrast.
The sperm with changes on PM in Control group

Intact (I) Partially Reacted (PR)

Perforatorium
Decondensation

AC

IAM

Swelling

OAM

Vesicle

PM

ES

PAR

N

PM

Intact ES

Intact PAR

(a)

(b)

(c)

(d)

(e)

Proximal ring
Basal plate
Perinuclear space
Segmented columns

Capitulum
Proximal centriol

Mitochondria
Fibrous sheath

PM

Axoneme

Outer dense fibre

Inner dense fibres

Tail

1µm

Swelling

Acrosome lacking

OAM

IAM

Intact ES

Intact PAR

Partially Reacted (PR)

Partially Reacted (PR)

Reacted (R)

Plate 7. The assessment of chilled spermatozoa undergoing different degrees of acrosome reaction by Transmission Electron Microscopy
Legends to plates for TEM in frozen-thawed spermatozoa

Plate -8a. Longitudinal section of frozen-thawed sperm exhibiting major changes on the head [i.e. irregularity and discontinuity of the PM, IAM, OAM, and NM; decondensation and swelling of the AC; damaged ES and PAR with swelling and irregular outlines]. The midpiece has also apparent changes (i.e. damaged proximal ring, missing PM and fibrous sheath over the neck, while some of the outer dense fibres remaining almost intact, but slightly enlarged, together with a loose connection to the neck). Note the axoneme at this region as with almost empty content. In the lower part, the PM and fibrous sheath are almost entirely missing. The majority of the mitochondria at this region have less contrast and are varying in shape (i.e. rectangular and rounded, disrupted or swollen with almost empty contents), together with less clear membranes and loose attachments between them. Bar=1 µm, Magn. 18,900X.

Plate -8b. Detail from frozen-thawed intact (I) sperm with slight changes; all compartments of the head, except the N having a dark and uniform contrast, together with slightly non-uniform and lessened contrast leading to poor distinction of each: The PM appears mainly intact but with a less clear outline and localised areas of damage. The AC shows relatively even distribution but with a slight rarefaction. The IAM and OAM are less distinct due to slight rarefaction of the AC and slight irregularity of the PM and NM. The NM is also less distinct and broken at some points forming multiple perinuclear spaces. Note intact but less distinct ES and PAR as both surrounded by a less clear PM.

Plates -8c,d,e. Sections from frozen-thawed partially reacted (PR) spermatozoa (in controls) exhibiting slight changes: The PM either remained intact (Plates -8c,d) or became disintegrated due to multiple fusions with the OAM, leading to the vesicle formation (Plates -8d,e), together with a non-clear, irregular outlines (Plate -8e). The AC’s were swollen (Plate -8e) or rarefied (i.e. enlargement with a reduced contrast) (Plates -8c,d,e). Note the NM as broken at different levels leading to enlarged perinuclear spaces (Plate -8c).

Plates -8f,g,h. Frozen-thawed (PR) spermatozoa (A23187-treated) with major changes on the head: formation of small number of vacuoles and vesicles of the PM and OAM (Plate -8g), swollen PM and AC and broken NM. Note partial detachment of the ES from the N (Plate -8f) and severe damage (Plate -8g) or discontinuation (Plates -8g,h) of the ES and PAR.

Plates -8i,j. Sections from frozen-thawed reacted (R) spermatozoa with clearly lacking acrosome together with discontinuation (Plate -8i) and/or disintegration (Plate -8j) of the ES, PAR and their overlying PM. Note the perforatorium and NM as both disintegrated and/or discontinuous or partly damaged.
Plate 8. The assessment of frozen-thawed spermatozoa undergoing different degrees of acrosome reaction by Transmission Electron Microscopy

The sperm with major changes on the head and tail in Control

Perforatorium

Intact (I)

Partially Reacted (PR) in Control

Shipping changes on Acrosome

N

Nuclear vacuoles

PM damaged

PM decondensed

N broken

PM

ES

Vesicle

I AM

I AM

O AM

O AM

Nuclear vacuoles

Vesicle

PM

PAR

PAR

Swollen PM

Swollen

Vacuole

AC

AC

PAR

Paracentric sheath

Mitochondria

1μm

(a)

(b)

(c)

(d)

(e)

(f)

(g)

(h)

(i)

(j)
GENERAL DISCUSSION

Background
Research on semen preservation has a long history over two centuries, but most of the work has been carried out in the last four decades (Watson, 1990), especially since the discovery of the use of glycerol in 1949 by Polge and his co-workers (Polge et al., 1949). Although a great deal of success has been achieved in both cryopreservation and AI with chilled/frozen semen, there is still a problem that even with the best preservation techniques to date, the post-thaw survival is restricted to about 50% of spermatozoal population (Watson, 1995; Rodriguez-Martinez et al., 1997). Moreover, most surviving spermatozoa have characteristics different from their unfrozen counterparts. Indeed, it is generally accepted that cryopreserved mammalian spermatozoa are in a state resembling partial capacitation (Watson, 1995; Rota, 1998), which accounts for their relatively reduced survival (especially in dogs: Concannon and Battista, 1989; Peña et al., 1999), and readiness to undergo acrosome reaction or egg penetration without incubation (Watson, 1995). Consequently, AI with frozen semen usually results in poorer fertility than those of fresh semen in most species (Watson, 1990; Linde-Forsberg et al., 1999), which can partially be overcome by inseminating, often on more than one occasion, greater numbers of live spermatozoa (up to a threshold) and/or precise timing of insemination.

Objectives
Therefore, the objectives of this study were to establish for the dog; (i) improved methods of pre-freeze handling of semen, (ii) an in vitro acrosome reaction assay using fresh and chilled semen, (iii) to utilise these data for optimisation of freezing regimens on the basis of post-thaw motility of spermatozoa, and (iv) a method of differentiation of the damage to the acrosome due to a) the acrosome reaction, b) chilling and c) freeze-thawing by means of various assessment techniques (including Bright-Field, Phase Contrast, Differential Interference Contrast, Scanning and Transmission Electron Microscopy).

Criteria for assessment of spermatozoa
In order to obtain data on the effects of treatment upon the spermatozoa, the changes in the acrosomal integrity (for acrosome reaction only) and the motility were assessed. Wide changes in osmolality of treatment solutions are needed before there is damage to the
plasma membrane overlying the acrosome (Gao et al., 1995; Rota, 1998). However, sperm motility will be affected by even small variations in osmolality (Gao et al., 1995). This is why sperm motility has usually been used to assess freezing regimens (Rota, 1998).

It is known that acrosomal changes are characteristic in dogs (Oettlé and Soley, 1988). While substantial acrosomal damage can occur during dilution, cooling and equilibration, the major deterioration of the organelle occurs after freeze-thawing (Oettlé, 1986b; Hay et al., 1997a). However, even in spermatozoa which have no apparent acrosomal damage nor reduction in motility, subtle changes may have occurred. This was concluded by Hay et al. (1997b) who found a decline in the number of spermatozoa binding to oocytes after cooling or freeze-thawing in the dog.

**The effects of seminal plasma, dilution rate and glycerolisation regimen upon the survival of spermatozoa during cold-storage (at +4°C)**

In order to freeze semen and thus prolong the fertile life of spermatozoa, semen needs to be diluted and/or cooled at +4°C for a period of time. The choice of Tris (Rota et al., 1995) as the extender in the present study was based on the fact that it is most widely used in practice and that fertility results after AI are well known (Linde-Forsberg, 1995).

Previous studies have shown that either the removal of the first and the third fractions (England and Allen, 1992a) or the removal of the entire seminal plasma (Martin, 1963a) was beneficial for survival and morphological characteristics of dog spermatozoa, with no adverse effect on fertility (Platz and Seager, 1977). Controversial reports exist of the effect of dilution rates (Foote, 1964c; England, 1992), the concentration (Province et al., 1984; England, 1992; Günzel-Apel et al., 1993) and addition temperature (Martin, 1964a; Fontbonne and Badinand, 1993b) of glycerol upon semen quality. The relationship between the presence of seminal plasma, dilution rate and glycerolisation regimen is also unknown. Since pre-freeze handling steps will affect the success of freeze-thawing (Oettlé, 1986b; Hay et al., 1997a), it was desirable to investigate the effect of each step upon the survival of spermatozoa.

The results of the present study agree well with the previous findings. Tris-fructose-citric acid extender containing 20% (v/v) egg yolk extended survival of spermatozoa at +4°C for up to 48 h depending on dilution rates. Centrifugation was beneficial to spermatozoal
motility. However, there were marked interactions with dilution rate and glycerolisation temperature. Furthermore, spermatozoal survival was markedly higher after glycerolisation at +4°C than at room temperature. Although the beneficial effect of centrifugation upon maintenance of spermatozoal motility was apparent at dilution rates up to 1:8, possible ultrastructural changes upon the plasma membrane and the acrosome should also be considered before final recommendations can be made. However, since dog semen is generally diluted at 1:1 to 1:5 (or up to 1:8) in an extender depending on spermatozoal concentration (Andersen, 1980; Christiansen, 1984), centrifugation appears to be applicable. Concentrations of glycerol that were >2% (v/v, final) resulted in markedly lower survival than at lower concentrations, although up to 14% glycerol (at a dilution rate of 1:20) has been reported in the literature (Foote, 1964a). Therefore, on the basis of spermatozoal motility, tolerance of glycerol up to 2% in Tris-fructose based extender suggests that glycerol can still be used until a less toxic, thus more effective cryoprotectant is to be found.

With Tris extender, the additives such as Orvus EM Paste (Nizanski et al., 1997), Equex STM Paste (Rota, 1998), sodium dedocyl sulphate (Peña et al., 1998c) and Proline (Peña et al., 1998a) have been shown to be beneficial for post-thaw motility, viability and acrosomal integrity. However, there was no significant increase in in vivo fertility after AI with frozen semen containing Equex STM Paste (Rota, 1998). Nevertheless, incorporation of one of these agents and one antioxidant (presumably butylated hydroxytoluene (BHT); see Watson, 1995 for detail) in the extenders may be recommended for a better maintenance of membrane integrity of spermatozoa especially in centrifuged samples. In this way, partial capacitation of spermatozoa leading to premature acrosome reaction may be minimised. This may be particularly critical since the acrosomal changes must result in precisely 'timed' acrosome reaction to fulfil fertilisation task (Tesarik, 1989).

The effects of calcium ionophore (A23187) upon acrosome reaction of spermatozoa as assessed by Bright-Field and phase contrast microscopy

In the literature, different types of chemical (e.g. calcium ionophore, A23187) and/or biological (e.g. progesterone and ZP) components have been used to stimulate the acrosome reaction in dog spermatozoa. It is known that extracellular calcium is essential for motility and acrosome reaction of dog spermatozoa, which can be induced in the
absence of eggs or cumulus cells (Mahi and Yanagimachi, 1978), as with many other species (Yanagimachi, 1994). Recently, Brewis et al. (1999) found that both heat solubilised bitch ZP and progesterone can cause a marked influx of calcium ions, thus, leading to acrosome reaction. In the present study, using modified TALP medium (containing 3 mM/l Ca\(^{2+}\)), optimum concentration of A23187 was found to be 1 µM/l. However, the concentration was higher (i.e. 2-10 µM/l A23187) in previous studies (Geussová et al., 1997; Szász et al., 1997; Hewitt and England, 1998), using lower concentrations (≤1.71 mM/l) of calcium. These studies indicate that there is a relationship between constituents (especially calcium ion) of the medium and concentration of the stimulant to induce the acrosome reaction in vitro. Since A23187 was a suitable agent for induction of the acrosome reaction in dogs, as with other species (e.g. bull: Christensen et al., 1994; human: Liu and Baker, 1998), it is recommended for future use. It might be possible that A23187-induced acrosome reaction could be used to predict in vivo fertility in the dog, as is the case in the bull (Whitfield and Parkinson, 1995). However, the effects of biological (i.e. ZP, hemi-zona binding and progesterone) and non-biological (i.e. A23187) stimulants to induce the acrosome reaction should be compared so that a biological basis of chemically induced acrosome reaction can be established for dog spermatozoa.

Although numerous assessment techniques (with or without staining) have been used for monitoring the acrosome reaction of dog spermatozoa, a novel stain, i.e. naphthol yellow S/ aniline blue was found to be a useful tool in the present study. Compatibility of the stain with fresh (or even diluted) samples and distinguishable characteristics of stained spermatozoa may provide further means for monitoring the acrosomal changes in the dog. However, poorer staining quality of frozen-thawed spermatozoa (in dogs: Oettlé, 1986b; in rams: Watson, 1975; in boars: Paulenz et al., 1995) compared to fresh semen might limit the distinction of the degree of cryoinjury, as with other stains such as Spermac (Oettlé, 1986b; Paulenz et al., 1995) and Giemsa (Watson, 1975).

**The effects of glycerol upon the acrosome reaction of chilled spermatozoa as assessed by Differential Interference Contrast microscopy**

The present results demonstrated that the acrosomal integrity is impaired during chilling (cooling to/at +4°C). Almost half of the cells had already undergone spontaneous acrosome reaction in the incubation medium (modified TALP). This indicates marked
acrosomal damage during pre-freeze handling steps, confirming the previous findings with light microscopy (Oettelé, 1986b). Indeed, Rota (1998) showed two-fold shortening of capacitation time in chilled-rewarmed spermatozoa compared to fresh dog semen. This indicates that pre-freeze cooling is critical for functional integrity of spermatozoa. Therefore, a suitable diluent (e.g. Tris-based; Rota, 1998), incorporation of membrane stabilisers (i.e. detergents: Nizanski et al., 1997; Rota, 1998) and proper cooling (England, 1992; Hay et al., 1997b) may be practised for a superior maintenance of spermatozoal integrity following chilling in future studies. By doing so, the proportion of functionally intact spermatozoa entering to harmful freeze-thawing processes might be increased, leading to a superior success in cryopreservation.

In the literature, glycerol concentrations varied widely (from 0.5 to 14%, v/v; England, 1992 and Foote, 1964a, respectively) and inseminations, resulting in high pregnancy rates, used glycerol in the range of 2-8%. The present results showed that in the presence of glycerol at a final concentration of 2% (v/v) in the extender, there was no significant change in the percentage of acrosome reacted spermatozoa in chilled samples. Since using glycerol up to 2% also resulted in a non-significant decrease in sperm motility at +4°C (for 48 h), the findings suggest that glycerol can be used as cryoprotectant of choice for further handling (freeze-thawing) until it may be substituted by a less- or non-toxic agent (e.g. proteins). In other species, glycerol-free freezing diluents were used in few previous studies (Gibson and Graham, 1969; Abdelhakeam et al., 1991a,b). Using unfrozen turkey semen, Donoghue and Walker-Simmons (1999) demonstrated that heat soluble, dehydration-induced proteins (isolated from dry wheat seed embryos) are capable of protecting spermatozoa during in vitro storage, increasing fertility and hatchability of eggs. These might also potentially improve long term storage of spermatozoa from other species.

The present results imply that acrosome reaction assay may be a helpful tool especially for investigation of the effects of pre-freeze handling steps such as chilling and glycerolisation to semen quality. However, future studies of the effect of glycerol upon the ultrastructure of dog spermatozoa or in vivo fertility are required.

The effects of freezing regimens upon the post-thaw motility of spermatozoa

The effects of freezing rate upon the spermatozoa of virtually all species (including dog) have not yet been investigated systematically with the exception of bovine (Parkinson and
Whitfield, 1987, using a semi-programmable freezer) even though modern cryobiology has started half a century ago, through the introduction of glycerol to semen before freezing (Polge et al., 1949). It is clear that there is no control over the freezing rate above solid carbon dioxide (for pelleted semen) and that although the advent of small-volume straws in 1964 (Cassou, 1964) allowed adaptation of more sophisticated freezing in vaporised liquid nitrogen, freezing rate across the straw may not be uniform (especially in vertical position) unless a controlled-rate, programmable freezer is used (McLaughlin et al., 1990). In dogs, the freezing methodologies were mainly adapted from bovine (Foote, 1964a; Seager, 1969; Andersen, 1975) because of high food/economical demand of this species. Therefore, there have been very limited systematic studies investigating the actual effect of freezing rates upon dog spermatozoa (England, 1993).

In the present study, it was found that; (i) three-step freezing is clearly appropriate, (ii) the optimum freezing regimen (from +4 to -120°C) is -0.5°C/min from +4 to -9°C, -40°C/min to -20°C, -100°C/min to -120°C, followed by direct immersion of the straws in liquid nitrogen, and (iii) parameters of the freezing curve can be used for estimation of post-thaw motility of spermatozoa. These results confirm that the optimisation of freezing conditions of dog spermatozoa is possible. As with the bovine (Parkinson and Whitfield, 1987), minimising the latent heat plateau phase (through its quicker release by faster cooling) was beneficial for survival of spermatozoa, although the initial cooling rate was also one of the main determinants of post-thaw motility, due either to centrifugation, using smallest size of French straws (0.25 ml French type, with high surface/volume ratio) or high dilution rate used in the present studies. Although England (1992) demonstrated that the avoidance of plateau phase during freezing was beneficial, Rota (1998) comparing fast and slow freezing (yielding different magnitudes of plateau phases) found no clear advantage of one freezing rate over the other. This was attributed to freezing rates used that were both in the range between -10 to -80°C/min, considered to be suitable for freezing of spermatozoa (Watson, 1990). Therefore, it would be interesting to compare different magnitudes of plateau phase against its earlier induction by seeding (Hay et al., 1997b) or its avoidance by pellet freezing (England, 1992). Additionally, Olar (1984), comparing different freezing rates, demonstrated that lectin-induced agglutination of dog spermatozoa was markedly altered after freezing. Rota (1998) found a higher incidence of head-to-head agglutination, that tended to be higher when fast freezing rate was used. These freezing-
induced changes might indicate alteration in the acrosome and/or plasma membrane leading to partial capacitation (Watson, 1995, Rota, 1998). Hence, the effect of sperm agglutination during cryopreservation should also be investigated in detail for a better understanding of cryoinjury.

It is known that glycerol decreases salt concentration (through its water binding properties), and thus increases the unfrozen fraction of water during freezing (Watson, 1995). Therefore, it minimises cryoinjury (mainly during slow cooling) and as its concentration is decreased freezing rate must be increased (Farrant, 1980). This can normally be achieved in pellet freezing or by using programmable freezers (McLaughlin et al., 1993; Rota, 1998). Although the incorporation of glycerol in freezing extender could have been avoided completely (Gibson and Graham, 1969; Abdelhakeam et al., 1991a, b), the variation in post-thaw motility of spermatozoa between individual sires then became substantially greater (Gibson and Graham, 1969). However, since dog spermatozoa may survive freezing (i.e. up to 21% post-thaw motility) in the absence of glycerol or any cryoprotectant other than egg yolk (Olar et al., 1989), the optimisation of freezing regimens in a glycerol-free extender might also yield promising results. The development of a newer extender (with no glycerol) coupled with pellet freezing (providing fast cooling rates; Graham et al., 1978) might be needed, as with ram spermatozoa (Abdelhakeam et al., 1991a, b; who achieved >60% post-thaw motility and >50% lambing rate after cervical insemination in natural oestrus). If the results of Abdelhakeam et al. (1991a, b) are confirmed with further studies, it is possible that glycerol might not be essential (Watson, 1995). Therefore, the methodology of England (1992), using a combination of alcohol bath and LN2 coupled with straw packaging (0.25 ml, French type), may be considered for future studies of cryopreservation of semen in the dog.

In cryobiology, prior attention has usually been given to the effects of freezing, but the role of thawing, which also contributes to cryoinjury has generally been ignored. Apart from the cryoinjury during freezing, substantial damage to the cells occurs during or after thawing (Woolley and Richardson, 1978). The effects of warming rate depend on prior cooling rate and cell type (Watson et al., 1992a). However, Rota (1998) found a highly significant interaction between glycerol concentration and thawing rate but not between freezing rate and thawing rate for dog spermatozoa. For cooling rate, the major concern is
whether it was high enough to induce intracellular freezing (requiring fast thawing) or low enough to produce cell dehydration (requiring slow thawing) in cell survival (Mazur, 1988). Woolley and Richardson (1978), using human spermatozoa, found that ultrastructural changes of the plasma membrane, acrosome and mitochondria occur either during or after thawing (to be discussed later). Osmotic studies upon the ram and human spermatozoa showed that the cells are damaged by water ingress (during thawing) rather than by its egress (during freezing) (Gao et al., 1992; Curry et al., 1994; Holt and North, 1994). Similarly, Holt et al. (1992), studying the membrane permeability of ram spermatozoa, found that the membrane injury, as a result of freeze-thawing, was observed only during warming in the range of 2-30°C (well after dissolution of ice and re-dilution of the solute that was concentrated during freezing). Hence, they considered that membrane lipid changes might be involved (as also suggested by Quinn, 1985), but concluded that the freezing injury is different from cold shock injury (see Watson, 1981 for detail). However, due to the limited number of ultrastructural observations (e.g. Woolley and Richardson, 1978; Courtens and Paquignon, 1985) on fresh, frozen and frozen-thawed spermatozoa, freezing injury has not yet been distinguished clearly from those that occur during and/or after thawing. Therefore, for a greater post-thaw survival of dog spermatozoa, the actual effect of thawing or post-thaw dilution to an isotonic solution have to be investigated systemically rather than simply using single, 'fixed' thawing rate (i.e. at 37°C for 1 min) only or comparing several rates (e.g. at +4°C for several minutes, 37°C for 1 min, and 70 or 75°C for either 6, 8 or 12 sec) (also see Watson, 1990 and below).

The effects of freezing regimens upon the acrosome reaction of spermatozoa as assessed by Differential Interference Contrast microscopy

The results of the present study demonstrated that the acrosomal integrity deteriorates during chilling (cooling to/at +4°C) and especially after freeze-thawing. This confirms the previous findings with light microscopy (Oettlé, 1986b, Rota, 1998). Considering the acrosomal changes, it was noted that there were no differences between the various freezing regimens used in the present study, while the motility differed between them. This indicates that spermatozoal motility is more sensitive to osmotic gradient, as generated by various cooling rates (Gao et al., 1995; Liu and Foote, 1998a,b), than the plasma membrane (with underlying acrosome) of the head (Rota, 1998). This proves the accuracy of motility as a useful, but not a single criterion of cryoinjury during optimisation of
freezing conditions. Lack of correlation between the acrosomal (and overlying plasma membrane) damage and post-thaw motility (dog: Oettle, 1986b; Rota, 1998; boar: Pursel et al., 1972; human: McLaughlin et al., 1993; Gao et al., 1995) requires further consideration of both attributes of spermatozoa for a superior cryosurvival.

The previous studies using light microscopy techniques demonstrated that acrosomal changes (i.e. 77 to >80%) are common following freeze-thawing by various regimens (Oettle, 1986b; Hay et al., 1997b). Regardless of freezing regimen applied in the present study, the incidence of acrosomal deterioration was too high (>90%), possibly reducing potentially viable spermatozoa available for acrosome reaction. Considering other difficulties in performing the insemination itself and precise timing of insemination (see Linde-Forsberg, 1995; England, 1998 for detail), future studies are needed to reduce the acrosomal damage to increase the viable population of spermatozoa capable of undergoing 'true' (physiological) acrosome reaction. However, it is possible that thawing itself, as it can exert effects comparable to those of cooling, or post-thaw handling processes (e.g. further centrifugation, re-suspension in an isosmotic medium, TALP containing extracellular calcium, etc.) contributed to substantial changes on the acrosome.

The effects of chilling and freeze-thawing upon the acrosome reaction of spermatozoa as assessed by Scanning and Transmission Electron Microscopy

SEM
SEM provided spectacular images of the entire integrity and surface characteristics of dog spermatozoa, although visualisation of the sub-compartments (e.g. the outer and inner acrosomal membranes and the mitochondria) was usually impossible. Therefore, in future studies it may not be recommended for monitoring the sequence of acrosome reaction especially for the intermediate stages (i.e. the early stages of partial reaction). However, it may be used for determining the presence of the acrosome before or after freeze-thawing as well as assessment of gross morphological abnormalities of spermatozoa.

TEM
Although it was laborious and time demanding, virtually every single compartment of spermatozoa could be visualised by TEM, which confirms its value for assessment of ultrastructure of dog spermatozoa as a standard test (Oettlé and Soley, 1988). Using this technique, the initial stages of acrosome reaction could be illustrated rather easily.
Therefore, for future studies upon cryopreservation of dog spermatozoa, as with other species (Yanagimachi, 1994), priority should be given to TEM to illustrate the actual ultrastructural changes on the acrosome due either to chilling, freeze-thawing or acrosome reaction.

Comparing the chilled and the frozen-thawed cells, there were obvious differences between the integrity of the entire head and mid-piece. On the head, both the equatorial membrane and plasma membrane were generally intact in chilled cells, however various degrees of acrosomal changes usually proceeded towards these regions after freeze-thawing. This accelerates the acrosome reaction in frozen thawed cells, eventually leading to cell death, which may therefore impair the ability of spermatozoa to fuse with the plasma membrane of the ovum at the end of the acrosome reaction (Bedford et al., 1979). In the mid-piece, the plasma membrane and mitochondrial structures were affected by freeze-thawing, although they were well preserved in chilled spermatozoa. Despite the fact that the cooling regimen (given above) was optimal for post-thaw motility, this might not be the case for the plasma membrane or the acrosomal integrity of the head of spermatozoa, as previously shown (Oetttlé, 1986b; Rota, 1998). Therefore, during freeze-thawing, it is relevant to maintain the entire integrity of spermatozoon to achieve superior post-thaw survival.

An optimal cooling regimen should be followed by an optimal warming regimen as the latter is as important as the former in contributing to the eventual cryoinjury (Mazur, 1988). Thawing at around body temperature, i.e. 35-37°C (as widely used in many species; Watson, 1990), might not simply ‘fit’ for every single cooling regimen, even it is an ‘optimal’ one. Therefore, systematic comparisons, or using at least three different rates of thawing (i.e. slow, intermediate and fast rates, as given above) are required. In this way, it is possible to overcome excessive cryoinjury to spermatozoa during or after thawing (Woolley and Richardson, 1978; Courtenas and Paquignon, 1985). It is known that the process of dehydration of cells accompanying slow freezing (which might be an ‘optimal’ one) is potentially associated with cell survival, whereas at more rapid rates cell death is more likely due to likelihood of intracellular ice, which is usually lethal (Watson, 1995). Unless thawing is ‘optimised’, swelling of the entire head of spermatozoa could become inevitable even it was combined with an ‘optimum’ freezing regimen. Indeed, this was the
case in the present trials and many other studies in mammals during thawing (involving rehydration) of spermatozoa (Watson, 1995). Therefore, using a small amount of glycerol (such as 2%, v/v, final concentration) in the extender (Tris) may become relevant, so that the cell volume excursions could be minimised during or after cryopreservation.

Obviously, during post-thaw handling processes (i.e. either re-dilution of semen into an 'isosmotic' medium, acrosome reaction in vitro or direct introduction of semen into the genital tract of female during AI) care should be practised. Post-thaw re-dilution of semen in homologous prostatic fluid, that may result in maximum pregnancy rate (100%) after intravaginal insemination (Nöthling and Volkmann, 1993) should also be considered. However, the actual effect of prostatic fluid upon the frozen-thawed cells is not known with certainty (Nöthling and Volkmann, 1993). Nevertheless, the addition of prostatic fluid does increase the volume of the inseminate. This may overcome the difficulties in AI allowing a substantially larger proportion of spermatozoa to enter into the uterus through the rather tortuous cervix of the bitch. Using this technique, when coupled with a properly timed insemination schedules (that includes monitoring the plasma LH and/or progesterone), the number of inseminations might be decreased to only one during oestrus (Nöthling et al., 1997).

Finally, therefore, appropriate precautions are needed during both pre-freeze and post-thaw handling (e.g. centrifugation, dilution into an extender, cooling and glycerolisation at +4°C, freezing and thawing regimen, re-dilution) and insemination procedures (e.g. timing and the number of inseminations, deposition site of the inseminate) when the preserved (especially frozen-thawed) semen is to be used. In this way, the thermo resistance of dog spermatozoa at body temperature might be improved substantially which allows using a more flexible insemination schedule, yet results in high in vivo fertility.
For the *in vitro* induction of the acrosome reaction, cold storage and freeze-thawing of dog spermatozoa, the present study indicated that:

1. The removal of seminal plasma was beneficial for the survival of spermatozoa in Tris-fructose-citric acid extender containing 20% egg yolk and 8% glycerol (v/v, final concentration) at dilution rates <1:8 (semen: extender). At higher dilutions (>1:8) the presence of seminal plasma was beneficial.

2. A dilution rate of 1:8 was optimal regardless of glycerol concentration in the extender.

3. Glycerolisation at +4°C was beneficial. The maximum tolerable amount of glycerol in the extender was 2% (v/v, final concentration) on the basis of motility.

4. The optimal concentration of A23187 was 1 µM/l for the induction of acrosome reaction in modified TALP medium (containing 3 mM/l calcium) at 39°C, 5% CO2 in humidified air.

5. A 30-45 min incubation was required to induce the acrosome reaction (especially for fresh semen).

6. Naphthol yellow S/aniline blue staining was a useful tool for monitoring the acrosomal changes due not only to the acrosome reaction in fresh semen, but also after freeze-thawing. Moreover, the staining results compared well with that of phase contrast microscopy.

7. Chilling caused deterioration of the acrosomal morphology and thus accelerated the A23187-induced acrosome reaction so that 0.1 µM/l A23187 was enough stimulant for the reaction after 60 min of incubation.

8. The addition of 2% glycerol did not affect the rate of acrosome reaction.

9. DIC microscopy was found to be a useful tool in monitoring the acrosomal changes.

10. The optimal freezing regimen from +4 to -120°C was -0.5°C/min to -9°C, -40°C/min to -20°C, -100°C/min to -120°C, followed by direct immersion of 0.25 ml French straws in the liquid nitrogen.

11. The prediction of post-thaw motility was possible on the basis of the data from parameters of the freezing curve.

12. Freeze-thawing resulted in major acrosomal deterioration. The induction of acrosome reaction, as achieved by 1 µM/l A23187, was similar amongst different freezing regimens.

13. Ultrastructural studies, by SEM and TEM, demonstrated that freeze-thawing further provoked the A23187-induced acrosome reaction, as compared to chilling. In completely reacted cells, the equatorial segment and post acrosomal membrane were usually intact in chilled spermatozoa. However, in partially and in completely reacted cells the entire head integrity was usually devagued: (i) the acrosomal changes (i.e. decondensation and swelling leading to entire loss of the acrosomal content), except vesiculation of the acrosomal membranes, extended to the equatorial segment, and (ii) a further damage also occurred to the post-acrosomal region after freeze-thawing.
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APPENDIX

2. Appendix for Chapter 2 (Preliminary studies)

2.1- Individual dogs used

In the present studies, six clinically healthy dogs were used. While half of the animals (Solo, Gillie and Joey) were of Department of Clinical Veterinary Science and sheltered on the site, other animals (Hands, Callum and Robi) were privately owned. Since there were only three individuals used for each experiment, all the dogs were always referred to as “Dogs A, B and C”, as follows (Appendix 2.1):
### Appendix 2.1. Individual details of dogs used for semen collection

<table>
<thead>
<tr>
<th>Name</th>
<th>Breed</th>
<th>Age</th>
<th>Chapter 2</th>
<th>Chapter 3</th>
<th>Chapter 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Exp. 1a</td>
<td>Exp. 1b</td>
<td>Exp. 2a</td>
</tr>
<tr>
<td>Solo</td>
<td>Greyhound</td>
<td>2 yr</td>
<td>Dog A</td>
<td>Dog A</td>
<td>Dog A</td>
</tr>
<tr>
<td>Gillie</td>
<td>Greyhound</td>
<td>5 yr</td>
<td>Dog B</td>
<td>Dog B</td>
<td>Dog B</td>
</tr>
<tr>
<td>Hands</td>
<td>Cross-Breed</td>
<td>8 yr</td>
<td>Dog C</td>
<td>Dog C</td>
<td>-</td>
</tr>
<tr>
<td>Callum</td>
<td>Labrador</td>
<td>1 yr</td>
<td>-</td>
<td>-</td>
<td>Dog C</td>
</tr>
<tr>
<td>Joey</td>
<td>Greyhound</td>
<td>8 yr</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Robi</td>
<td>Golden Retriever</td>
<td>2 yr</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
2.2- Tris extender

The following formula, modified from Rota et al. (1995), was used for Tris-fructose-citric acid extender containing 20% egg yolk and up to 8% (v/v) glycerol at final concentration, as follows (Appendix 2.2):

Appendix 2.2. Constituents of Tris extender (100 ml)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris (hydroxymethyl)methylamine</td>
<td>3.025 g</td>
</tr>
<tr>
<td>Citric acid</td>
<td>1.700 g</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>1.250 g</td>
</tr>
<tr>
<td>Crystapen</td>
<td>1 mg/ml</td>
</tr>
<tr>
<td>Streptomycin sulphate</td>
<td>1 mg/ml</td>
</tr>
<tr>
<td>Egg yolk (v/v)</td>
<td>20%</td>
</tr>
<tr>
<td>DD water (up to)</td>
<td>100 ml</td>
</tr>
<tr>
<td>Glycerol* (v/v, final concentration)</td>
<td>8%</td>
</tr>
</tbody>
</table>

* Final concentrations varied from zero to 8.0% depending on the experiment conducted.

The pH of suspension was adjusted to 6.70 (Rota et al., 1995) with NaOH and the osmolality was found to be 330 ± 5 mOsm. The antibiotics were added during preparation of the suspension (no egg yolk). The batches of suspension were then kept in a deep freezer at -20°C until it was needed. The frozen batches were thawed in a water bath at 37°C and stored at +4°C up to three months. Following the addition of 20% egg yolk at room temperature, the final mixture (referred to as Tris extender) was stored at +4°C up to five days. However, it was kept at 35°C during the course of experiment.
2.3. TALP medium

The incubation medium (TALP) was based on a modified Tyrode’s medium, previously described for incubation of spermatozoa in vitro (Bavister and Yanagimachi, 1977), as follows (Appendix 2.3a,b):

Appendix 2.3a. Individual stock solutions (100 ml each) as constituents of basic Tyrode’s medium

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (g/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ (1.8 M)*</td>
<td>19.980</td>
</tr>
<tr>
<td>KCl (2.6 M)*</td>
<td>19.370</td>
</tr>
<tr>
<td>MgCl₂ (0.23 M)*</td>
<td>3.860</td>
</tr>
<tr>
<td>NaCl (3 M)*</td>
<td>17.550</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O (0.63 M)*</td>
<td>4.968</td>
</tr>
</tbody>
</table>

*A certain amount from each of these solutions was included in TALP medium (see below).

It was further modified in the following way as described by Christensen et al. (1994):

- Lactate and pyruvate were added to the basic Tyrode’s medium according to the method of Bavister and Yanagimachi (1977).
- Bovine serum albumin (BSA) was replaced by 1 mg/ml polyvinyl alcohol, (PVA) (Parrish et al., 1989), additional D-glucose (5.6 mM/l; Varner et al., 1987) and 3 mM/l CaCl₂ (Shams-Borhan and Harrison, 1981) was also included.
- The HEPES concentration was altered to 40 mM/l, the HCO₃⁻ concentration to 10 mM/l and finally, the pH was adjusted to 7.40 (Parrish et al., 1986) with hydrochloric acid (HCl, BDH) or sodium hydroxide (NaOH, BDH).
# Appendix 2.3b. Constituents of TALP medium (100 ml)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$</td>
<td>100 µl</td>
</tr>
<tr>
<td>KCl</td>
<td>100 µl</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>100 µl</td>
</tr>
<tr>
<td>NaCl</td>
<td>100 µl</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$·H$_2$O</td>
<td>4.500 ml</td>
</tr>
<tr>
<td>HEPES</td>
<td>0.260 g</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>0.016 g</td>
</tr>
<tr>
<td>Lactate (DL Lactic acid, 60% w/w, Na salt; add warm)</td>
<td>0.378 ml</td>
</tr>
<tr>
<td>Pyruvate (Pyruvic acid, Na salt)</td>
<td>11 mg</td>
</tr>
<tr>
<td>Dextrose (D(+)-Glucose)</td>
<td>0.111 g</td>
</tr>
<tr>
<td>PVA (cold water soluble; add at last)</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Crystapen</td>
<td>1 mg/ml</td>
</tr>
<tr>
<td>Streptomycin sulphate</td>
<td>1 mg/ml</td>
</tr>
<tr>
<td>DD Water (up to)</td>
<td>100 ml</td>
</tr>
</tbody>
</table>
2.4- Calcium ionophore A23187

Calcium ionophore (A23187) was based on a chemical inducer, previously described for the acrosome reaction of bull spermatozoa *in vitro* (Christensen *et al.*, 1994), as follows (Appendix 2.4):

Appendix 2.4. Stock solution for A23187

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A23187</td>
<td>1 mg</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.236 ml</td>
</tr>
<tr>
<td>TALP</td>
<td>18.862 ml</td>
</tr>
</tbody>
</table>
3. Appendix for Chapter 3 (Freezing trials)

The correlations between parameters of the freezing curve and cooling regimens (from +4°C to -120°C) on the basis of post-thaw motility of spermatozoa were as follows (Appendix 3.1-6):
Appendix 3.1. The correlations between parameters of the freezing curve and temperature values applied against the post-thaw motility in preliminary investigation of cooling regimen between +4 and -35°C (Experiment 3a)

<table>
<thead>
<tr>
<th></th>
<th>T1</th>
<th>T2</th>
<th>T1-T2</th>
<th>time</th>
<th>rate1</th>
<th>rate2</th>
<th>area</th>
<th>CR1</th>
<th>Ti</th>
<th>CR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>0.485</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>0.846*</td>
<td>-0.055</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>time</td>
<td>0.586*</td>
<td>0.254</td>
<td>0.516*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate1</td>
<td>-0.439</td>
<td>-0.067</td>
<td>-0.463</td>
<td>-0.616**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate2</td>
<td>-0.304</td>
<td>0.034</td>
<td>-0.371</td>
<td>-0.687**</td>
<td>0.698*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>area</td>
<td>0.373</td>
<td>0.326</td>
<td>0.232</td>
<td>0.849*</td>
<td>-0.624**</td>
<td>-0.720**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR1</td>
<td>0.147</td>
<td>0.189</td>
<td>0.052</td>
<td>0.159</td>
<td>-0.088</td>
<td>-0.088</td>
<td>0.103</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ti</td>
<td>-0.121</td>
<td>-0.120</td>
<td>-0.063</td>
<td>-0.072</td>
<td>0.181</td>
<td>-0.184</td>
<td>0.082</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR2</td>
<td>0.197</td>
<td>-0.021</td>
<td>0.241</td>
<td>0.416</td>
<td>-0.596**</td>
<td>-0.801**</td>
<td>0.603*</td>
<td>0.000</td>
<td>0.398</td>
<td></td>
</tr>
<tr>
<td>P/t motility</td>
<td>-0.009</td>
<td>-0.036</td>
<td>0.011</td>
<td>-0.041</td>
<td>-0.019</td>
<td>0.078</td>
<td>-0.057</td>
<td>0.145</td>
<td>-0.094</td>
<td>-0.143</td>
</tr>
</tbody>
</table>

Significance: P=0.913  P=0.665  P=0.896  P=0.628  P=0.822  P=0.356  P=0.498  P=0.084  P=0.260  P=0.087

*Positive high correlation; **Negative high correlation (when r ≥ +/- 0.5).
Appendix 3.2. The correlations between parameters of the freezing curve and temperature values applied against the post-thaw motility in order to investigate the cooling regimen between +4 and -35°C (Experiment 3b)

<table>
<thead>
<tr>
<th></th>
<th>T1</th>
<th>T2</th>
<th>T1-T2</th>
<th>time</th>
<th>rate1</th>
<th>rate2</th>
<th>area</th>
<th>CR1</th>
<th>Ti</th>
<th>CR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>0.565*</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>0.934*</td>
<td>0.233</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>time</td>
<td>0.450</td>
<td>0.461</td>
<td>0.331</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate1</td>
<td>-0.538**</td>
<td>-0.409</td>
<td>-0.457</td>
<td>-0.579**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate2</td>
<td>-0.208</td>
<td>-0.065</td>
<td>-0.217</td>
<td>-0.506**</td>
<td>0.523*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>area</td>
<td>0.154</td>
<td>0.468</td>
<td>-0.021</td>
<td>0.869*</td>
<td>-0.511**</td>
<td>-0.472</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR1</td>
<td>0.049</td>
<td>0.249</td>
<td>-0.050</td>
<td>0.160</td>
<td>0.086</td>
<td>-0.120</td>
<td>0.142</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ti</td>
<td>-0.111</td>
<td>-0.150</td>
<td>-0.066</td>
<td>-0.341</td>
<td>0.578*</td>
<td>0.226</td>
<td>-0.366</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR2</td>
<td>-0.012</td>
<td>-0.006</td>
<td>-0.012</td>
<td>0.150</td>
<td>-0.221</td>
<td>-0.441</td>
<td>0.229</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>P/t motility</td>
<td>0.002</td>
<td>-0.032</td>
<td>0.016</td>
<td>0.121</td>
<td>0.081</td>
<td>-0.068</td>
<td>0.040</td>
<td>0.194</td>
<td>-0.070</td>
<td>0.032</td>
</tr>
</tbody>
</table>

Significance: P=0.981 P=0.702 P=0.846 P=0.147 P=0.334 P=0.416 P=0.637 #P=0.020 P=0.407 P=0.706

*Positive high correlation; **Negative high correlation (when r≥ +/- 0.5). #P= Statistically significant (P<0.05).
Appendix 3.3. The results of multiple regression analysis between parameters of the freezing curve and temperature values applied for the cooling regimen (between +4 and -35°C) against the post-thaw motility of spermatozoa (Experiment 3b)

<table>
<thead>
<tr>
<th>Parameters of freezing curve and temperature values applied</th>
<th>Regression Equation (predicting the mean post-thaw motility)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;time + rate1&quot;</td>
<td>9.13 + 0.213 time + 0.184 rate1</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>&quot;time + rate1 + area&quot;</td>
<td>11.60 + 0.403 time + 0.182 rate1 - 0.0475 area</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>&quot;time + rate1 + Ti&quot;*</td>
<td>1.78 + 0.212 time + 0.264 rate1 - 0.887 Ti</td>
<td>*P≤0.01</td>
</tr>
<tr>
<td>&quot;T1-T2 + time + rate1 + area&quot;</td>
<td>6.94 - 0.842 ‘T1-T2’ + 0.521 time + 0.142 rate1 - 0.0745 area</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>&quot;T1 + T2 + + time + rate1 + rate2 + area + CR1 + Ti + CR2&quot;**</td>
<td>12.30 - 0.290 T1 - 0.04 T2 + 0.464 time + 0.265 rate1 - 0.0743 area + 0.904 CR1 - 1.09 Ti + 0.123 CR2</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

* Predictor at the highest statistical significance level; ** ‘T1-T2’ is highly correlated with other X variables (predictors) and has been removed from the equation.
Appendix 3.4. The correlations between parameters of the freezing curve and temperature values applied against the post-thaw motility of spermatozoa in order to investigate the cooling regimen between +4 and -120°C (Experiment 4)

<table>
<thead>
<tr>
<th></th>
<th>T1</th>
<th>T2</th>
<th>T1-T2</th>
<th>time</th>
<th>rate1</th>
<th>rate2</th>
<th>area</th>
<th>Ti2</th>
<th>CR3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>0.236</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>0.968*</td>
<td>-0.115</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>time</td>
<td>0.775*</td>
<td>0.074</td>
<td>0.779*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate1</td>
<td>-0.864**</td>
<td>-0.040</td>
<td>-0.879**</td>
<td>-0.689**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate2</td>
<td>-0.569**</td>
<td>-0.025</td>
<td>-0.578**</td>
<td>-0.704**</td>
<td>0.567*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>area</td>
<td>0.515*</td>
<td>0.154</td>
<td>-0.491</td>
<td>0.804*</td>
<td>-0.577**</td>
<td>-0.640**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ti2</td>
<td>0.012</td>
<td>0.070</td>
<td>-0.006</td>
<td>0.021</td>
<td>0.038</td>
<td>-0.033</td>
<td>-0.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR3</td>
<td>0.026</td>
<td>-0.015</td>
<td>0.031</td>
<td>0.048</td>
<td>-0.026</td>
<td>-0.342</td>
<td>0.059</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>P/t motility</td>
<td>-0.252</td>
<td>-0.301</td>
<td>-0.182</td>
<td>-0.099</td>
<td>0.108</td>
<td>0.187</td>
<td>-0.083</td>
<td>0.030</td>
<td>-0.030</td>
</tr>
</tbody>
</table>

Significance: *P=0.013  **P=0.003  P=0.077  P=0.335  P=0.295  P=0.069  P=0.423  P=0.770  P=0.770

* Positive high correlation; ** Negative high correlation (when r≥ +/- 0.5). *P= Statistically highly significant (P≤0.01); **P= Statistically highly significant (P<0.005).
Appendix 3.5. The results of multiple regression analysis between parameters of the freezing curve (between +4 and -120°C) and temperature values applied against the post-thaw motility of spermatozoa (Experiment 4)

<table>
<thead>
<tr>
<th>Parameters of freezing curve and temperature values</th>
<th>Regression Equation (predicting the mean post-thaw motility)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;T1 + T2&quot;</td>
<td>33.70 - 0.809 T1 - 3.31 T2</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>&quot;T1 + 'T1-T2'&quot;</td>
<td>33.70 - 5.12 T2 + 4.31 'T1-T2'</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>&quot;T2 + 'T1-T2'&quot;</td>
<td>33.70 - 5.12 T2 - 0.809 'T1-T2'</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>&quot;T1 + time&quot;</td>
<td>26.00 - 1.85 T1 + 0.0809 time</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>&quot;T1 + rate1&quot;</td>
<td>27.10 - 2.64 T1 - 0.391 rate1</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>&quot;T1 + rate2&quot;</td>
<td>36.30 - 0.911 T1 + 0.0139 rate2</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>&quot;T1 + area&quot;</td>
<td>33.70 - 1.20 T1 + 0.0105 area</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>&quot;T2 + area&quot;</td>
<td>41.30 - 4.98 T2 - 0.0061 area</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>&quot;T1 + T12&quot;</td>
<td>37.40 - 1.07 T1 + 0.0266 T12</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>&quot;T2 + T12&quot;</td>
<td>41.70 - 5.14 T2 + 0.0412 T12</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>&quot;T1 + T2 + area&quot;</td>
<td>30.40 - 0.971 T1 - 4.36 T2 + 0.0124 area</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>&quot;T1 + T2 + rate1&quot;</td>
<td>27.70 - 1.95 T1 - 3.44 T2 + 0.272 rate1</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>&quot;T1 + time + area&quot;</td>
<td>25.90 - 2.03 T1 - 0.134 time - 0.0253 area</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>&quot;T2 + time + area&quot;</td>
<td>40.40 - 5.10 T2 - 0.0457 time + 0.0120 area</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>&quot;T1 + T2 + rate2&quot;</td>
<td>33.30 - 0.540 T1 - 4.52 T2 + 0.0233 rate2</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>&quot;T2 + T12 + CR3&quot;</td>
<td>41.30 - 5.15 T2 + 0.0413 T12 - 0.0092 CR3</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>&quot;T1 + time + rate1 + rate2&quot;</td>
<td>8.22 - 3.47 T1 + 0.134 time - 0.437 rate1 + 0.0593 rate2</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>&quot;T1 + T2 + time + rate1&quot;</td>
<td>20.30 - 2.55 T1 - 3.09 T2 + 0.0590 time - 0.272 rate1</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>&quot;T1 + T2 + time + rate2&quot;</td>
<td>19.20 - 1.33 T1 - 4.13 T2 + 0.109 time + 0.0500 rate2</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>&quot;T1 + T2 + time + area&quot;</td>
<td>26.20 - 1.48 T1 - 3.83 T2 + 0.0783 time - 0.0088 area</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>&quot;T1 + 'T1-T2' + time + area&quot;</td>
<td>26.20 - 5.31 T1 + 3.83 'T1-T2' + 0.0088 area</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>&quot;T2 + 'T1-T2' + time + area&quot;</td>
<td>26.20 - 5.31 T2 - 1.48 'T1-T2' + 0.0783 time - 0.0088 area</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>&quot;T1 + T2 + time + rate1 + rate2**</td>
<td>1.070 - 2.73 T1 - 3.08 T2 + 0.118 time - 0.333 rate1 + 0.0591 rate2</td>
<td>*P&lt;0.001</td>
</tr>
<tr>
<td>&quot;T1 + T2 + 'T1-T2' + Ti2 + CR3***</td>
<td>34.70 - 0.804 T1 - 4.39 T2 + 0.0405 Ti2 - 0.0077 CR3</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>&quot;T1 + T2 + 'T1-T2' + time + rate1 + rate 2 + area***</td>
<td>8.79 - 3.47 T1 - 2.19 T2 + 0.185 time - 0.439 rate1 + 0.0544 rate2 - 0.0330 area</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>&quot;T1 + T2 + 'T1-T2' + time + rate1 + rate 2 + area + Ti2***</td>
<td>10.30 - 3.46 T1 - 2.28 T2 + 0.182 time - 0.444 rate1 + 0.0555 rate2 - 0.0314 area + 0.0485 Ti2</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>&quot;T1 + T2 + 'T1-T2' + time + rate1 + rate 2 + area + CR3***</td>
<td>8.30 - 3.48 T1 - 2.19 T2 + 0.191 time - 0.446 rate1 + 0.0629 rate2 - 0.0325 area + 0.0161 CR3</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>&quot;T1+ T2+ 'T1-T2'+ time + rate1+ rate2+ area + Ti2 + CR3***</td>
<td>9.90 - 3.47 T1 - 2.28 T2 + 0.188 time - 0.451 rate1 + 0.0642 rate2 - 0.0308 area + 0.0495 Ti2 + 0.0165 CR3</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

* Predictor at the highest statistical significance level; ** 'T1-T2' is highly correlated with other X variables (predictors) and has been removed from the equation.
Appendix 3.6. The correlations between parameters of the freezing curve and cooling regimens between +4 and -120°C (as resulted in high, intermediate and low post-thaw motility) against the post thaw motility of spermatozoa (Experiment 5)

<table>
<thead>
<tr>
<th></th>
<th>T1</th>
<th>T2</th>
<th>T1-T2</th>
<th>time</th>
<th>rate1</th>
<th>rate2</th>
<th>area</th>
<th>regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>0.497</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>0.995*</td>
<td>0.404</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>time</td>
<td>0.527*</td>
<td>-0.023</td>
<td>0.558*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate1</td>
<td>0.050</td>
<td>0.044</td>
<td>0.048</td>
<td>0.588*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate2</td>
<td>-0.585**</td>
<td>-0.270</td>
<td>-0.584**</td>
<td>-0.864**</td>
<td>-0.690**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>area</td>
<td>-0.031</td>
<td>-0.320</td>
<td>0.005</td>
<td>0.808*</td>
<td>0.536*</td>
<td>-0.557**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>regimen</td>
<td>0.246</td>
<td>0.000</td>
<td>0.259</td>
<td>0.858*</td>
<td>0.795*</td>
<td>-0.837**</td>
<td>0.751*</td>
<td></td>
</tr>
<tr>
<td>P/t motility</td>
<td>-0.254</td>
<td>-0.528**</td>
<td>-0.205</td>
<td>-0.342</td>
<td>-0.462</td>
<td>0.433</td>
<td>-0.197</td>
<td>-0.549**</td>
</tr>
</tbody>
</table>

Significance: P=0.509 P=0.144 P=0.597 P=0.368 P=0.210 P=0.244 P=0.612 P=0.126

Positive high correlation; ** Negative high correlation (when r≥ +/- 0.5).
4. Appendix for Chapter 4 (Electron Microscopy)

To stain the spermatozoa for transmission electron microscopy (TEM), Reynold's lead citrate stain was used, as follows (Appendix 4.1):

Appendix 4.1. Reynold's lead citrate stain

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lead nitrate</td>
<td>1.33 g</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>1.76 g</td>
</tr>
<tr>
<td>Distilled water (up to)</td>
<td>30 ml</td>
</tr>
</tbody>
</table>