STUDIES ON CAPACITATION AND THE EFFECTS OF COOLING AND LOW TEMPERATURE STORAGE ON STALLION SPERM FUNCTION

by

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LIST OF CHAPTERS

ABSTRACT

This study was undertaken to assess stallion sperm function under two sets of conditions 1) liquid storage at 5°C and 2) incubation under conditions which support capacitation. Two aspects of sperm function were studied; motility, using a computerised motility analyser capable of objective and detailed analysis of movement patterns, and the ability to regulate intracellular Ca^{2+}, and assessment of the functional competence of the sperm plasma membrane, using the fluorescent dye Fura-2AM.

It was determined that after cooling and storage at 5°C there was a decline in the percentage of live spermatozoa and a decline in the velocity parameters of the live spermatozoa. A change in the pattern of motion to non-progressive motility was also detected. Concurrently, an increase in intracellular Ca^{2+} was detected; this was believed to indicate a reduction in the functional competence of the sperm plasma membrane. This increase was most marked after cooling and further experiments indicated that intracellular Ca^{2+} increased during the cooling process, most markedly below about 15°C. Examination of the Ca^{2+} regulating ability of individual cells confirmed that mean intracellular Ca^{2+} for the whole population increased but, more importantly, that there was a discrete subpopulation of sperm cells still able to regulate calcium at pre-cooled levels, despite cooling and storage.

Incubation conditions capable of supporting capacitation were established for stallion spermatozoa, as assessed using the dual fluorescent stain chlortetracycline and Hoechst 33258. This involved incubation for 300 minutes in a TALP-milk medium. Expression of hyperactivated motility was examined to evaluate the usefulness of this motility pattern as a biomarker of capacitation. The drug pentoxifylline appeared to accelerate the capacitation process, judged by dual staining, but had no detectable stimulatory effect on motility. Instead, pentoxifylline promoted head-to-head agglutination of the sperm cells in a dose dependent manner.

Further experiments showed that an increase in intracellular Ca^{2+} occurred during prolonged incubation in a TALP-milk medium with or without pentoxifylline. This may be a necessary component of capacitation. Binding studies indicated the presence of progesterone receptors on the plasma membrane of both human and

stallion spermatozoa. A progesterone-mediated calcium influx was stimulated by adding progesterone to washed human spermatozoa. The same effect was only generated with stallion spermatozoa after pre-incubation for 300 minutes under capacitating conditions, in the presence of pentoxifylline. The latter observation would indicate that the progesterone receptor on stallion spermatozoa is only active after pre-incubation under capacitating conditions.

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DEDICATION

I dedicate this work to my father Mr Goriman Khan bin Kalandar Khan and my mother Puan Tamnah Khatoon bt Abdul Kadir

DECLARATION

I certify that this thesis does not contain any material previously published or written by any other person, except when referred to in the text. I certify that the work of which this thesis is a record, is my work, and has not previously been submitted in any form to any institution for an award of a degree.

Mohd Azam Khan bin Goriman Khan

CHAPTER 1

LITERATURE REVIEW

1.1 The Preservation of Equine Spermatozoa for Artificial Insemination - An Historical Perspective

Reference has been made to equine artificial insemination as early as 1322, when it was reported that an owner served his mare with semen stolen from a stallion owned by a hostile neighbour (Watson, 1990). However, the first documented scientific research was not published until 1780, when an Italian clergyman called Spallanzani reported the artificial insemination of a bitch (Watson, 1990) and later evaluated the procedure in horses (Brinsko and Varner, 1992). Spallanzani is also credited with the discovery of cryopreservation through his experiments on cooling stallion, human and frog semen with ice (Watson, 1990). In the late 1800's the French veterinarian, Repiquet, advised the use of artificial insemination for the treatment of infertility in mares (Brinsko and Varner, 1992). By the early part of the present century the use of artificial insemination was expanding through the work of Professor Iwanoff, Director of the State Institute of Veterinary Experimental Medicine in Moscow. Due to his influence, by 1928, one hundred and twenty thousand mares had been artificially inseminated in Russia (Iwanoff, 1930). The widespread use of frozen semen was predicted by Mantegazza in 1866, who saw the potential for transporting frozen semen rather than live animals (Watson, 1990). However, the first foal was not born from cryopreserved semen until 1957 (Barker and Gandier, 1957).

While breeding horses by artificial insemination has become popular in the United States, China and Japan, use of the technique is still limited overall, and the use of cryopreserved semen is very limited. The exception appears to be China which has developed an extensive artificial insemination programme, with six hundred thousand mares inseminated in 1959 alone (Brinsko and Varner, 1992) and eleven hundred thousand mares inseminated with cryopreserved semen in between 1980 and 1985 (Amann and Picket, 1987).

Methods for semen collection evolved over time. Semen was recovered from the vagina of the mare either directly or using an intravaginal sponge, or collected from the stallion using "Breeder's Bags" or rubber condoms. In the early 1930's, an artificial vagina was constructed by Milovanov to facilitate semen collection, but it

was reported to be cumbersome and difficult to handle (Frank, 1950). In 1938, Berliner produced a more pliable version which could be pressed close to the mare during collection (Berliner, 1940). A modified version of this artificial vagina is the Missouri model which is in common use today (Love, 1992).

Despite the early interest in artificial insemination, use of the technique has never been as widespread as in cattle. In the 1950's, successful cryopreservation methods were developed for bovine semen which facilitated the rapid expansion of cattle artificial insemination (Polge and Rowson, 1952). Comparable conception rates using cryopreserved stallion semen have still not been achieved. The long period of oestrus and the difficulty of predicting ovulation in the mare makes the correct timing of insemination difficult. However, the principal reason for slow technological progress is the attitude of most horse Breed Societies, which has been to either restrict or prohibit the use of artificial insemination.

1.2 Pregnancy Rates Using Preserved Semen

There are relatively few publications reporting pregnancy rates from artificial insemination of the mare. Pregnancy data from artificial insemination using chilled liquid semen is summarised in Table 1.1 and using cryopreserved semen in Table 1.2. Trials using chilled liquid semen reported pregnancy rates of between 50% and 91%. Using cryopreserved semen pregnancy rates ranged between 23% and 67%.

This huge variation in pregnancy rate data can be attributed to a number of factors including selection of stallion, selection of mares and evaluation of only a small number of animals. Differences in insemination dose and in timing of insemination relative to ovulation will affect the outcome, as will the choice of extending medium and semen storage method. The method of data presentation, which may be as a *per cycle pregnancy rate* or as an *end of season pregnancy rate*, often makes direct comparison of data impossible. Methods of assessing pregnancy also differ. In some trials pregnancy may be established by manual examination and in others, by foaling. Moreover, many of these studies were conducted before ultrasonography was commonly used to determine the time of ovulation.

If attention is focused on the more recent pregnancy rate data which is reported as single cycle pregnancy rates, rates for chilled semen vary from 59-87% (Douglas-Hamilton *et al.*, 1987; Heiskanen *et al.*, 1994). Results for cryopreserved semen vary from 29-56% (Cristanelli *et al.*, 1984; Amann and Pickett, 1987). It appears that results for liquid chilled semen compare favourably with natural service but there is still room for improvement in the results obtained using cryopreserved semen.

Table 1.1: Summarised pregnancy rate data from 7 trials using liquid, chilled semen.

Author		Pregnancy rate
Douglas-Hamilton, 1984	3 cycle pregnancy rate	91%
Province *et al.*, 1984		52%
van der Holst, 1984	foals registered	70-80%
de Vries, 1987	season	76%
Douglas-Hamilton *et al*, 1987	first cycle pregnancy rate	59%
Heiskanen *et al.*, 1994	one cycle pregnancy rate	65-87%
Bedford *et al.*, 1995	7 day embryo recovery rate	50%

Table 1.2: Summarised pregnancy rate data from 13 trials using cryopreserved semen.

Author		Pregnancy rate
Nishikawa and Shinomiya, 1976		47-67%
Ellery *et al.*, 1971		41%
Klug *et al.*, 1975		23%
Martin *et al.*, 1979		63%
Tischner, 1979	7 day embryo recovery rate	32%
Fomina, 1980		61%
Muller, 1982		37%
Loomis *et al.*, 1983	season	45%
Cristanelli *et al.*, 1984	one cycle pregnancy rate	56%
Amann *et al.*, 1987	one cycle pregnancy rate	29-56%
Muller, 1987		49-63%
Evain, 1989; (cited in Pickett and Amann, 1993).		38-40%
Arns *et al.*, 1987		32%

1.3 Factors Associated with the Use of Artificial Insemination

The principle advantage of artificial insemination is the increased utilisation of a selected stallion. A single ejaculate can be divided and used to inseminate a number of mares. This allows more mares to be booked to an individual stallion without overuse occurring. It is possible for breeding to continue while a stallion is competing at shows or performance events or while recovering from injuries or illness. Semen from prized stallions will still be available in the event of death of the animal. Other advantages include early detection of stallion infertility through routine semen analysis and control of the transmission of bacterial diseases where the male is the carrier, by addition of antibiotics to seminal extenders. With subfertile stallions, the supportive and protective nature of seminal extenders may improve pregnancy rates. In cases where the stallion is not able to achieve full tumescence or penetration because of injury, artificial insemination may be used to reinforce natural service. Risk of injury to stallion and the mare is decreased in artificial insemination and the selection of available semen is widened giving mare owners access to genetically superior stallions.

There are also disadvantages associated with artificial insemination. Successful management of an AI programme requires a higher degree of knowledge and skill than the management of a natural service programme. If not applied properly, techniques for processing, cooling, freezing and packaging can reduce semen quality. Poor insemination technique will result in low pregnancy rates. Within the breeding industry as a whole, some stud owners would face loss of income from stud fees and mare boarding fees. Most importantly, from the viewpoint of certain breed societies, increased production of offspring from popular stallions would reduce the monetary value of the individual animals.

1.4 Reproductive Anatomy and Physiology of the Stallion

The male reproductive system consists of two testis, each suspended by a spermatic cord and external cremaster muscle, two epididymides, two deferent ducts each with an ampulla, paired vesicular glands, a prostate gland, paired bulbourethral glands, the penis and the associated urethralis, ischiocavernosus, bulbospongiosus and retractor penis muscles. The reproductive system is supported within the pelvic cavity by the hammock-like genital fold and externally by the scrotum and prepuce (Amann, 1981).

The scrotum comprised two lateral pouches, which serve to cover, protect and thermoregulate the testis by vascular transfer of heat and changes in position of the testis relative to the abdominal wall. This function is dependent upon the combined action of the scrotum, pampiniform plexus, tunica dartos muscle and external cremaster muscles (Amann, 1981).

The testis are slightly laterally compressed ovoid structures, each situated in a separate pouch within the scrotum. Their primary functions are the production of spermatozoa (spermatogenesis) and the production of steroid hormones (steroidogenesis). In most animals the testis descend from their point of origin, near the kidney, into the scrotum before birth. The testicular parenchyma consists of seminiferous tubules surrounded by interstitial tissue. These seminiferous tubules are lined with Sertoli cells and developing germ cells. The developing germ cells are spermatogonia, which undergo mitotic division and the primary spermatocytes, secondary spermatocytes and spermatids, cell which divide meiotically to produce the haploid spermatid. Spermatids do not divide further but undergo morphological changes to tranform from round cells to the more familiar elongated shape of the spermatozoa. The spermatid acquires an acrosomal cap, the nucleus condenses, a flagellum forms and mitochondria spiral round the cranial portion of the flagellum, to form the midpiece. The different stages of spermatogenesis are enclosed in invaginations of the Sertoli cell. Finally, the spermatozoon is released into the lumen of the seminiferous tubule (Amann, 1981). Further maturation occurs during travel through the epididymis. The interstitial tissues contains steroidogenic Leydig cells,

fibroblasts, lymphocytes, blood vessels and lymphatics.

Rapid growth of the testis occurs at about 18 months of age when the production of androgen by the Leydig cells and spermatogenesis gradually begin. This development culminates in puberty, after which both the quantity and quality of the spermatozoa produced increase over time. Some studies suggest that the testis continues to grow throughout life (Thompson *et al.*, 1979; Johnson and Neaves, 1981; El Wishy *et al.*, 1982) while others suggest that it attains maximum size by 5 to 7 years of age (Amann *et al.*, 1979; Johnson and Thompson, 1983).

The epididymis is a highly convoluted duct, which is divided into the head or caput, body or corpus and the tail or cauda. It originates at the convergence of the efferent ductules leaving the testis and terminates at the beginning of the deferent duct. It is characteristically highly convoluted, and very long (Amann, 1981). Epididymal functions include concentration, maturation, storage and transport of spermatozoa. Spermatozoa are conducted to the pelvic urethra via the deferent ducts.

The penis of the stallion is of the musculo-cavernous type and consists of three parts: the root, the body and the glans. It is anchored at the roots by paired crura and the ischiocavernosus muscles and suspended from the ventral surface of the pelvis by two short, strong suspensory ligaments (Amann, 1981), which provide the lateral stability necessary for normal intromission of the erect penis. The penile musculature on the caudal or ventral surface of the penile urethra comprises paired bulbospongiosus muscles. These muscles evacuate the penile urethral contents during urination or ejaculation. Rhythmic pulsation of the bulbospongiosus muscle indicate ejaculation during semen collection or natural service. The pulsations are palpable as a thrill of turbulent fluid coursing along the ventrum of the penis and are usually accompanied by simultaneous "flagging" of the tail. The retractor penis muscles also lie on the ventral surface of the penis for most of its length. These muscles are responsible for returning and maintaining the detumescent penis within the prepuce. The glans of the stallion penis consists of the mushroom shaped *corona glandis*. The neck of the glans is termed the *collum glandis*. The urethral process extends beyond the surface of the glans and is surrounded by the *fossa glandis*.

The prepuce serves to protect the non-erect penis and consists of an internal and an external preputial fold. The external fold forms the preputial orifice, whilst the

internal fold forms the preputial ring. When the penis is erect the preputial orifice is situated at the base of the penis and the preputial ring is represented by a mid-shaft fold of skin.

The seminal vesicles, ampullae of the deferent ducts, prostate glands and the bulbourethral glands make up the accessory sex glands (Setchell, 1977). Secretions from these glands, the seminal plasma, constitute approximately 95% of the ejaculate volume (Pickett *et al.*, 1983) and are known to contain factors which may inhibit (Bass *et al.*, 1983; Padilla and Foote, 1991) or stimulate (Muller and Kirchner, 1978; Overstreet *et al.*, 1980) spermatozoal function, and are thought to be beneficial, though not essential, to fertility (Amann, 1981). The seminal plasma functions as a vehicle of sperm transport (Rodger, 1975; Varner *et al.*, 1987a; Yanagimachi, 1988) and may also serve antioxidant, bactericidal and immunoprotective functions (Mann, 1975; Rodger, 1975). However, prolong exposure is detrimental to sperm survival. Semen collected using the artificial vagina should thus be mixed with an extender containing nutrients to prolong survival during *in vitro* storage.

Several studies concerning semen storage at 5°C show a negative impact of seminal plasma on the viability of spermatozoa (Pickett *et al.*, 1987; Jasko *et al.*, 1992a), demonstrated by a decline in sperm motility in whole ejaculates compared to samples in which all or a high fraction of seminal plasma is removed by centrifugation. Other studies in which contact of spermatozoa with seminal fluids was prevented by vesiculectomy (Webb *et al.*, 1990) or by collection of only the sperm rich fraction of the ejaculate (Varner *et al.*, 1987a) showed improved motility in frozen-thawed and cooled samples and the phenomenon of sperm agglutination was reduced.

The natural act of mating or copulation in the stallion normally occurs with two physiologically distinct events, termed emission and ejaculation. Emission is the release of spermatozoa and accessory gland fluids into the pelvic urethra. Ejaculation is the forceful expulsion of the combined fluids from the pelvic and penile urethra. The reflex emission follows a series of seven to nine intravaginal thrusts of the erect penis. Smooth muscle of the cauda epididymides, deferent ducts, ampullae, vesicular glands, prostate and possibly bulbourethral glands contract with resultant release of spermatozoa into the urethra. At the same time the bladder neck contracts, keeping

the urethral orifice of the bladder tightly closed. These actions are primarily alpha-adrenergically mediated (McDonnell, 1992).

Ejaculation of semen from the urethra is mediated via the pudendal and sacral segment at the spinal cord. It results in rhythmic contraction of the ischiocavernosus, bulbospongiosus, urethralis and other striated pelvic musculature. Simultaneously, the anal sphincter contracts rhythmically and ejaculation in stallion is a series of between five to ten jets of semen, each jet expelled with a subsequent decrease in pressure, volume and sperm concentration (Kosiniak, 1975; McDonnell, 1992). The ejaculate is composed of three fractions. The first, or pre-sperm fraction, is watery in appearance and contains no spermatozoa. This is followed by several jets of milky white the 'spermatozoa rich' fraction. The third fraction contains large quantities of gel and citric acid but few spermatozoa.

1.5 The Stallion Spermatozoon

1.5.1 Basic structure of the spermatozoon

Spermatozoa exhibit diversity of form, size and structure. A typical eutharian spermatozoa can easily be distinguished into three regions, the head, neck and tail, with the latter further subdivided into the middle piece, the principle piece and the end piece (Bedford and Hoskins, 1990)

The head of the spermatozoon consists of a nucleus with its nuclear envelope, the acrosome and post-acrosomal region and a plasma membrane. The nucleus contains highly condensed chromatin, DNA complexed with protamine, and is enclosed by a double-layered nuclear envelope containing nuclear pores. The rostral portion of the nucleus is overlain by the acrosome, a membrane limited vesicle which houses several hydrolytic enzymes. The acrosome, in turn, is overlain by the plasma membrane. Although the plasma membrane is continuous over the surface of the spermatozoon except after the acrosome reaction, or with senescence and death, the nature and function of the plasma membrane differs regionally (Bedford and Hoskins, 1990).

The neck connects the head and the middle piece. It comprises a connecting piece, the proximal centriole, several small mitochondria and a redundant nuclear envelope (Bedford and Hoskins, 1990). The middle piece is characterised by the presence of numerous mitochondria arranged end to end in a continuous double spiral, and extends from the caudal end of the neck distally. Central to the mitochondria are nine dense fibres, extending from their origin in the neck of the spermatozoon throughout the length of the middle piece and most of the principle piece. These fibres gradually taper away in the caudal principle piece and are not present in the end piece.

Central to the dense fibres is the axoneme. The axoneme is the propulsive element of the spermatozoon. It consists of a central pair of microtubules surrounded by a ring of 9 doublets. The role of the central pair is unclear. Each doublet is composed of two subfibres A and B. The principle structural protein of the

microtubules is tubulin. Tubulin molecules are arranged in rows to form protofilaments. Subfibre A contains 13 such protofilaments and subfibre B contains 9 or 10 such protofilaments. Subfibre A also contains two longitudinal rows of arms pointing to the next doublet. These arms contain dynein, which is rich in ATPase and transduces chemical energy to mechanical motion. The entire doublet is about 70% tubulin and 15% dynein. The doublets are interconnected by nexin links and a series of 9 radial spokes extend from the central pair to the doublets. The nexin links maintain symmetry during motion and the spokes probably provide structural support. The dense fibres and axoneme of the middle piece continue through the principle piece, although the dense fibres becomes narrower and terminate in the caudal principal piece. The doublets and central pair of the axoneme continue intact about halfway through the end piece and then taper out over a short distance of 1 to 2 μm. The 9 + 2 pattern is lost in the caudal end of the end piece (Bedford and Hoskins, 1990).

The stallion spermatozoon has a similar basic structure to spermatozoa of other mammals. However, some features, the asymmetric head, small volume of the acrosome and the abaxial position of the tail (Bielanski and Kaczmarski, 1979) distinguishes it from spermatozoa of bull (Jones, 1975), ram and boar (Bielanski and Kaczmarski, 1979).

Measurements of stallion spermatozoa gave the following average dimensions (± s.d); ***head:*** length - 5.75 ± 0.47 μm; maximum width - 2.93 ± 0.34 μm; length of post-nuclear cap - 1.65 ± 0.39 μm; width at the base - 1.45 ± 0.22 μm; surface area - 12.86 ± 2.17 μm^2; ***midpiece:*** length including the neck - 10.50 ± 1.27 μm; diameter - 0.60 ± 0.11 μm; ***main piece:*** length - 67.16 ± 3.99 μm; terminal piece: length 2.79 ± 0.90 μm (Bielanski and Kaczmarski, 1979).

When a spermatozoon leaves the testis the neck is surrounded by remnants of spermatid cytoplasm, the cytoplasmic droplet. Depending on the position of the cytoplasmic droplet on the tail in relation to the neck, it can be classified as a proximal or distal cytoplasmic droplet. Normally when spermatozoa pass through the epididymis, the cytoplasmic droplet will move down the mid-piece and is completely lost by the time of ejaculation. This movement and complete stripping of the cytoplasmic droplet is part of the maturation process and the presence of large

numbers of spermatozoa with protoplasmic droplets in an ejaculate is an indication of a failure of the maturation process (Dott, 1975).

The sperm plasma membrane is similar to other cell membranes in terms of the lipid bilayer arrangement of hydrophilic head groups and hydrophobic tail groups, but varies in its composition of lipids (mainly phospholipids and cholesterol) and proteins (peripheral and integral). Proteins make up more then 50% of the total weight of the spermatozoon. Most are histone protein involved in DNA packaging. Others are in the form of enzymes in the acrosome and receptors on the plasma membrane or participate in ion and carbohydrate transport during normal membrane function. Other proteins make up the cytoskeletal structure which gives shape to the head and contributes to the filaments of the tail (Mann, 1964)

About 14% of the total dry weight of the stallion spermatozoon is lipid. Even though this value is not different from the bull or boar, the composition is characteristically different, with cholesterol contributing 23% to sperm lipid in the stallion compared with 14% and 13% in the bull and boar, respectively (Mann, 1975). In stallion spermatozoa the cholesterol to phospholipid ratio of the plasma membrane is 0.36. The composition, distribution and nature of the fatty acyl side chain of the phospholipids also differ within certain regions of the plasma membrane and this determines the fluidity of the membrane. Under certain conditions, such as cooling, the lipids may change into crystalline arrays and proteins become aggregated, making membranes unstable and possibly irreversibly damaged (Hammerstedt *et al.*, 1990). This change in physical structure is termed 'lipid phase transition'.

The biochemical characteristics of stallion semen are similar to that of other domestic species. The pH of normal stallion semen ranges between 6.2 and 7.8, with osmolality between 300 and 334 mOsm/kg. Stallion semen differs from bull or ram in that it contains considerably higher concentrations of glucose (Polakoski and Kopta, 1982), sorbitol and lactic acid (Mann, 1975). Semen contains glyceryl phosphoryl choline (GPC), ergothioneine and citric acid. Glycerol phosphoryl choline is secreted mainly by the epididymis and may play a role in the sperm maturation process (Mann, 1975). Citric acid is mainly secreted by the seminal vesicles and the concentration found in semen is influenced by seasonal fluctuations. It is thought that the function of citric acid may be to bind with calcium and contribute

to the buffering capacity of semen. Ergothionein is secreted by the ampullary glands, and may play a role in protecting spermatozoa from the immobilising action of oxidising or peroxidising agents (Mann, 1964; Amann and Graham, 1993).

1.5.2 Sperm metabolism

The two main metabolic processes within semen are glycolysis and respiration; the rate of these processes being determined by the number of spermatozoa and the degree of sperm motility (Mann, 1964). Under conditions of semen storage for artificial insemination, that is in the absence of oxygen, spermatozoa rely primarily on exogenous substances; glucose, fructose, lactic acid, glycerol, fatty acids and amino acids to meet their energy requirements (Mann, 1964). There are some species specific differences in the ability of spermatozoa to utilise the metabolic pathways. Bull and ram spermatozoa are able to make use of anaerobic respiration at a higher rate than stallion spermatozoa. Stallion spermatozoa readily metabolise glucose but are limited in their capacity to use fructose. They are equally incapable of utilising sorbitol which is a major component of equine seminal plasma. Glucose breakdown provides the major source of adenosine tri-phosphate (ATP) in the stallion spermatozoon (Amann and Graham, 1993). The utilisation of ATP has been studied in bull spermatozoa and about 60% of ATP is used to maintain motility (Hammerstedt, 1983). The aerobic pathway generates 36 ATP per glucose molecule (Amann and Graham, 1993), but the oxygen dissolved in the extender or seminal plasma is soon exhausted, forcing the spermatozoa to rely on anaerobic metabolism. This occurs, for example when semen is cooled from body temperature to 5°C.

Whilst oxygen is essential, 'excess' oxygen causes peroxidative damage. Extensive lipid peroxidation destroys the structure of the lipid matrix resulting in membrane instability (Holland and Storey, 1981; Alvarez and Storey, 1984; Aitken and Clarkson, 1988). After oxygen is depleted, only the glycolytic pathway is used, and the end product of anaerobic metabolism, lactic acid accumulates and reduces the pH of the medium (Mann, 1964). This may cause a decrease in metabolism and ATP production which eventually results in loss of motility. Maintaining the appropriate

intracellular pH may seem like the most likely means of sustaining motility, but this is complicated by the fact that the pH of the extender is affected by the temperature, buffers in the extender, buffer capacity, changes in ionic strength of the medium and cryoprotectants present.

1.5.3 Sperm transport in the female reproductive tract

The site of deposition of the ejaculate varies between species; it may be deposited in the vagina, cervix or uterus. In the stallion the ejaculate is deposited directly into the uterus. Whatever the site of deposition, the ability of spermatozoa to migrate through the female reproductive tract to the site of fertilisation and eventual ability to fertilise the egg is controlled by (i) the properties of the female reproductive tract and egg investments and (ii) sperm kinematics and surface properties (Katz *et al.*, 1989).

Sperm movement through the female reproductive tract involves both passive and active transport mechanisms. Generally, sperm must pass through the cervix, uterus, uterotubal junction and oviductal isthmus before reaching the oocyte in the oviductal ampulla. Different regions of the female tract have physiologically different environments which influence capacitation and may affect fertilisation by influencing sperm movement (Suarez *et al.*, 1983; Katz *et al.*, 1989).

In those species where the ejaculate is deposited intravaginally the cervix provides the first barrier to sperm transport. Cervical mucus serves to exclude seminal plasma and is said to selectively exclude sperm cells showing morphological and functional abnormalities (Hanson and Overstreet, 1981). The cervix also provides a sperm reservoir (Katz *et al.*, 1989).

In the mare, semen is deposited directly into the uterus at copulation. The only other large domestic animal in which this occur is the pig. The result is that in this species, spermatozoa and male accessory sex gland secretion are rapidly distributed in the uterine horns. Therefore, unlike many other domestic animals in which capacitation and selection of spermatozoa takes place during the transport from cervix through the uterus, it is believed that this function must occur at the level of the uterotubal junction in the mare and sow (Boyle *et al.*, 1987).

The uterotubal junction has a narrow diameter and the walls are highly folded (Katz *et al.*, 1989; Suarez *et al.*, 1990). On reaching the junction spermatozoa must swim actively through. Those which succeed become inactive at the lower isthmus of the oviduct (Overstreet and Cooper, 1975; Hunter *et al.*, 1982; Smith *et al.*, 1987), and it is at this site that spermatozoa reside, attached to the wall of the oviduct, forming another reservoir (Smith and Yanagimachi, 1990; Pollard *et al.*, 1991). The duration over which spermatozoa remain inactive and attached to the oviductal wall depends on the timing of mating or insemination in relation to ovulation (Smith *et al.*, 1987). At about the time of ovulation, spermatozoa detach from the walls of the oviduct to start swimming actively towards the ampulla. Of the millions of spermatozoa in each ejaculate, thousands will reach the isthmus, but only tens may reach the ampulla (Suarez *et al.*, 1990).

In most domestic species secondary oocytes are released at ovulation. However in the mare, and the bitch, immature primary oocytes are released. These oocytes undergo further division after ovulation, before fertilisation. Oocytes are fertile for about 24 hours and spermatozoa survive within the reproductive tract for between 6 to 48 hours in most species and between 130 and 144 hours in dogs and horses (Hunter, 1988). For successful fertilisation to occur, spermatozoa must first become capacitated, and in most species this process can occur between 1 to 6 hours in the reproductive tract (Hunter, 1988).

1.6 Sperm Physiology

1.6.1 Capacitation

The original independent observations of Austin and Chang in 1951 and subsequent studies confirmed that the environment of the female reproductive tract changes the spermatozoon, conferring on it the ability to penetrate the vestments of egg, and eventually fertilise it (Austin, 1951; Chang, 1951; Bedford, 1983; Florman and Babcock, 1991). These changes, now collectively termed capacitation, comprise events which involve both the surface and intracellular components of the spermatozoon. These changes are not restricted to the sperm head only, but also involve the sperm tail (Vernon *et al.*, 1985). It is believed that the changes involve dissociation or neutralisation of negative regulators on the sperm surface membrane, and gradual removal, modification and redistribution of surface membrane proteins and lipids (Johnson and Hunter, 1972; Snider and Clegg, 1975; O'Rand, 1979; Llanos and Meizel, 1983; Myles *et al.*, 1984; Langlais and Roberts, 1985; Go and Wolf, 1985; Wolf *et al.*, 1986) as well as redistribution of lectin binding sites (Koehler, 1981 and Ahuja, 1985). The term "capacitation" is now generally accepted as comprising all prefertilisation changes occurring in the spermatozoon prior to the acrosome reaction.

David and Aitken, (1989) showed capacitation to be associated with a progressive rise in cAMP concentrations which precedes the onset of hyperactivation, and that the rise in cAMP concentrations is severely attenuated in the absence of exogenous calcium. These changes in intracellular Ca^{2+} are important for the transition from the uncapacitated to the capacitated state, and for triggering acrosomal exocytosis. Low traces of Ca^{2+}, at micromolar concentrations, are sufficient to promote capacitation of guinea pig sperm (Fraser, 1995), slightly higher, but still micromolar concentrations, will support the capacitation of mouse spermatozoa (Fraser, 1987) while millimolar concentrations are needed to capacitate human spermatozoa (Stock and Fraser, 1989; Das Gupta *et al.*, 1993). In contrast, all the mammalian species investigated required millimolar concentrations of Ca^{2+} for

maximal acrosomal exocytosis (*guinea pig* - Yanagimachi and Usui, 1974; *ram* - Shams-Borhan and Harrison, 1981; *hamster* - Yanagimachi, 1982; *mouse* - Fraser, 1987; *human* - Thomas and Meizel, 1988).

1.6.1.1 *In vivo* studies

Capacitation is a post-ejaculatory modification of sperm function which occurs in the female genital tract. The site at which it occurs varies depending on where semen is deposited at coitus. In species in which semen is deposited in the vagina at coitus (rabbit, human) capacitation may begin as spermatozoa passes through the cervix or cervical mucus. When it is deposited in the uterus (rodents, pig, horses) the principal site of capacitation may be the oviduct (Yanagimachi, 1994). More recent research has provided evidence that the isthmic region of the oviduct may be the major site for capacitation. These studies, using hamsters, indicated that only uncapacitated spermatozoa attached to oviduct mucosa, and that once capacitated they lost this affinity for the mucosa and were released, implying that some component of oviduct cell secretion is responsible for capacitation (Smith and Yanagimachi, 1991).

1.6.1.2 *In vitro* studies

In 1963, Yanagimachi and Chang reported that hamster epididymal spermatozoa, which had never been exposed to the female tract could fertilise eggs *in vitro*, indicating the possibility of *in vitro* capacitation (Yanagimachi and Chang, 1963). Early studies used oviduct and follicular fluids to induce *in vitro* capacitation until the development of synthetic media (Yanagimachi, 1994). Media commonly used today for *in vitro* capacitation are modified Tyrodes or Krebs-Ringer solution supplemented with energy sources (glucose, pyruvate and lactate) and albumin (Chang, 1984). Commercially available tissue culture media (e.g. TC 199 and Hams F-10) supplemented with serum are also commonly used for human and pig. There is, however, no known medium capable for supporting capacitation for all species. For instance, it has been reported that Krebs-Ringer solution supplemented with glucose,

pyruvate and bovine serum albumin supports mouse sperm capacitation and fertilisation but hamster spermatozoa incubated in the same medium die within a few hours. For the capacitation of hamster spermatozoa the medium requires supplementation with taurine and hypotaurine (Cornen and Meizel, 1978). Other agents such as heparin (*bull*; Parrish *et al.*, 1988) and caffeine are also known to facilitate capacitation (Mrsny *et al.*, 1979).

The time required for completion of capacitation *in vitro* varies depending on the species. Notable differences have been reported between individual human sperm donors (Perreault and Rogers, 1982). However, other workers reported that the usual time required for capacitation of human spermatozoa is between 2.5 and 5 hours (McMaster *et al.*, 1978). Minimum capacitation times for rabbit, golden hamster and mouse spermatozoa were estimated to be about 5 to 6 hours, 2 hours and 1 hour respectively (cited in Yanagimachi, 1988).

Another factor which appears to influence *in vitro* capacitation is the concentration of spermatozoa incubated in chemically defined media. A concentration of 5 million/ml has been recommended for human spermatozoa (McMaster *et al.*, 1978).

1.6.1.3 Measurement of capacitation

It has proven difficult to measure capacitation because there are no obvious morphological alterations to the spermatozoa, and because capacitation is defined as events occurring before, but not including the acrosome reaction. Bioassays have been developed to determine the ability of the spermatozoa to penetrate the zona pellucida of non-living oocytes or zona-free hamster eggs (Yanagimachi *et al.*, 1976; Overstreet *et al.*, 1980) or to fertilise cumulus-free oocytes (Wang *et al.*, 1995). Both of these assays are difficult to perform and correlate weakly with fertilising ability. In many other studies the ability of the spermatozoa to respond to inducers of the acrosome reaction have been used to quantify capacitation. Spermatozoa do not naturally undergo the acrosome reaction unless capacitated, hence the acrosome reaction can be used as a reliable indicator of successful capacitation. However,

capacitation can be bypassed by the use of inducers like Ionophore A23187 or, in some situations, capacitated spermatozoa may be prevented from undergoing the acrosome reaction (Yanagimachi, 1988).

A more recent development is the use of chlortetracycline fluorescence patterns to assess the capacitation state (Ward and Storey, 1984; Fraser and McDermott, 1992; Das Gupta *et al.*, 1993; Fraser, 1995; Wang *et al.*, 1995). This fluorescent staining technique appears to produce three distinct staining patterns characteristic of uncapacitated acrosome intact cells, capacitated acrosome intact cells and acrosome reacted cells. This technique is discussed in greater detail later (Chapters 2, 3 and 5).

1.6.2 The acrosome reaction

At the time of fertilisation the mammalian spermatozoa must undergo an acrosome reaction. This involves fusion of the plasma membrane overlying the acrosome with the outer acrosomal membrane. Vesiculation occurs and there is a release of acrosomal enzymes. The inner acrosomal membrane is exposed, and with this, new receptor sites (Yanagimachi, 1988; Yudin *et al.*, 1988). This occurs, physiologically, on the zona pellucida of the egg and is triggered, it is believed, by zona receptors (Bleil and Wassarman, 1983; Cross *et al.*, 1988; Florman and First, 1988). It has been shown clearly that the acrosome reaction is dependent on an influx of calcium ions (Yanagimachi and Usui, 1974).

The central role of calcium in the events of membrane fusion was first reported in 1974 (Yanagimachi and Usui, 1974). The mechanism by which the plasma membrane and outer acrosomal membrane fuse has been studied intensively. Several calcium dependent consequences leading to a modification of the phospholipid composition of membranes occur. These include activation of phospholipase C (Roldan and Harrison, 1989), activation of phospholipase A_2 (Roldan and Fragio, 1993), activation of protein kinase C (Breitbart *et al.*, 1992), and activation of enzymes involved in cAMP metabolism (De Jonge *et al.*, 1991). Since calcium is known to bind to the outer acrosomal membrane (Chandler and Battersby,

1976; Berrutti *et al.*, 1986; Watson and Plummer, 1986) it has been proposed that calcium may interact with the phospholipid polar head groups and allow the close approximation of two membranes or it may cause condensation of polar phospholipid head groups, thus increasing the hydrophobic attraction forces between the membranes (Niles and Cohen, 1991).

Although acrosome morphology varies from species to species, the basic structure and function are similar. It is evident in all species studied so far that the successful completion of the acrosome reaction is an absolute prerequisite to fertilisation. Individuals whose sperm display mutations of the acrosome are infertile (Jeyendran *et al.*, 1985; Sotomayor and Handel, 1986).

1.6.2.1 *In vivo* studies of the acrosome reaction

In studies where spermatozoa were recovered from naturally mated or artificially inseminated females, the majority of free swimming spermatozoa recovered from the ampullary region of the oviduct were still acrosome intact (Overstreet and Cooper, 1979; Cummins and Yanagimachi, 1982; Suarez *et al.*, 1983). Spermatozoa already associated with the cumulus complex were either unreacted or undergoing the acrosome reaction (Yanagimachi, 1970; Bedford, 1972). Spermatozoa associated with the zona pellucida were undergoing acrosomal change or had completed the acrosome reaction (Cummins and Yanagimachi, 1982).

Premature occurrence of the acrosome reaction will result in failure of the individual spermatozoon to fertilise. Studies have found spermatozoa undergoing acrosomal exocytosis in different regions of the female reproductive tract. This premature occurrence, while having no significance in the penetration of the zona pellucida, is thought to be an important selection process ensuring that only the most viable, acrosome intact spermatozoa, with optimal fertilising potential, arrive at the zona pellucida (Suarez *et al.*, 1983, 1984; Storey *et al.*, 1984; Cherr *et al.*, 1986; Cummins and Yanagimachi, 1986; Corselli and Talbot, 1986, 1987).

1.6.2.2 *In vitro* studies of the acrosome reaction

There has been a great deal of interest in assessing the ability of substances to induce acrosomal exocytosis, in order to study the cohort of capacitated sperm. This has led researchers to examine the induction of acrosomal loss by physiologically available substances such as follicular fluids (Tesarik, 1985), cumulus oophorus cells (Tesarik, 1985; Siiteri *et al.*, 1988), mural granulosa cells (Siiteri *et al.*, 1988), progesterone and 17a-hydroxyprogesterone (Osman *et al.*, 1989; Thomas and Meizel, 1989). Other approaches have utilised the calcium ionophore A23187 (Talbot *et al.*, 1976; Ward and Storey, 1984; Wolf *et al.*, 1985; Aitken *et al.*, 1994) and the fusogenic agent lysophosphatidylcholine (Green, 1978; Fleming and Yanagimachi, 1981; Byrd and Wolf, 1986; Hochi *et al.*, 1996).

It has been suggested that progesterone, found in high concentrations in the cumulus matrix, follicular fluids and in the oviducts around the time of ovulation, may act as a physiological stimulus for the acrosome reaction in human (Meizel *et al.*, 1990) and other spermatozoa (Shi and Roldan, 1995; Libersky and Boatman, 1995). Progesterone has been shown to enhance acrosome loss (Osman *et al.*, 1989) and is said to affect sperm motility (Mbizvo *et al.*, 1990). Recent work has shown the presence of progesterone receptors on the sperm plasma membrane; these receptors mediate a progesterone induced increase in intracellular calcium in human and other spermatozoa (Blackmore *et al.*, 1990; Meizel and Turner, 1991; Tesarik *et al.*, 1992). Whilst some reports indicate that progesterone may influence motility as shown by a rise in hyperactivation (Uhler *et al.*, 1992; Mbizvo *et al.*, 1990), other workers have reported an increased proportion of acrosomal loss without a corresponding rise in hyperactivation (Kay *et al.*, 1994).

1.6.2.3 Measurement of the acrosome reaction

Because the acrosome reaction is essential for fertilisation, information concerning the acrosome status is important in semen quality control for artificial insemination and fertilisation studies. Several techniques have been described for the

assessment of the acrosomal status. Most involve visualisation of the acrosome under the microscope. The acrosome of hamster and guinea pigs can be assessed using phase contrast microscopy (Yanagimachi, 1981), but the acrosome of most mammalian species is too small to be examined directly by phase contrast microscopy. Consequently, techniques for staining the acrosome have been developed.

Many stains have been used for acrosomal visualisation, such as Giemsa (Saacke and Marshall, 1968; Watson, 1975; Didion *et al.*, 1989), naphtol-yellow S/erythromycin B (Bryan and Akruk, 1977) and rose bengal/bismarck brown (Talbot and Chacon, 1981). Techniques using fluoresceinated lectins (Talbot and Chacon, 1980), monoclonal antibodies and polyclonal antisera have also been developed (reviewed in Cross and Meizel, 1989). The fix vital stain technique (de Leeuw *et al.*, 1991) allowed differentiation between physiological and degenerative acrosome loss, by the addition of the supravital stain H33258 which only stains the nuclei of non-viable spermatozoa. Chlortetracycline (CTC) fluorescence assays have also been described (Ward and Storey, 1984; Das Gupta *et al.*, 1993; Fraser *et al.*, 1995). A recent development is the use of flow cytometry, but though this has been described as an accurate and efficient way to differentiate acrosome intact spermatozoa from acrosome free (Miyazaki *et al.*, 1990), visual methods are simpler and more widely practiced in the clinical andrology laboratory.

1.6.3 Capacitation related motility changes - hyperactivation

In the terminal stages of the capacitated state, spermatozoa of many different species express an altered pattern of motility termed "hyperactivation" (reviewed Yanagimachi, 1981, 1988). In 1970, Yanagimachi first described a change in the motility of hamster spermatozoa capacitated *in vitro,* which he termed 'activation'. He later changed the terminology to 'hyperactivation' to differentiate from the initiation of motility at ejaculation (Yanagimachi, 1981). Since then, hyperactivated spermatozoa have been observed directly in the oviductal ampulla of hamsters (Katz and Yanagimachi, 1980) and rats (Saling and Philips, 1988). This pattern has also

been observed in spermatozoa recovered from the oviducts of rabbits (Cooper *et al.*, 1979a; Suarez *et al.*, 1983), hamsters (Cummins and Yanagimachi, 1982), mice (Olds-Clarke, 1986; Suarez, 1987; Suarez and Osman, 1987) and sheep (Cummins, 1982). Hyperactivation has also been observed in human (Burkman, 1984), dog (Mahi and Yanagimachi, 1976), bull (Singh *et al.*, 1983), boar (Nagai *et al.*, 1984) and stallion spermatozoa (Ellington *et al.*, 1993a, 1993b) during culture *in vitro*.

In this change to an activated state, the spermatozoa adopted a highly conspicuous and vigorous motility pattern, with increased flexion of the flagellum, increased amplitude of flagellar waves, and reduced frequency of beats. Episodes of progressive motility may alternate with episodes of non-progressive motility, and the pattern of motility has been described as "boobing" (Gwatkin and Anderson, 1969), "high amplitude" (Yanagimachi, 1970), "serpentine" (Yanagimachi, 1972), "whiplash" (Cooper *et al.*, 1979b), "figure-of-eight" (Fraser, 1977), and "darting" (Corselli and Talbot, 1986). This pattern of motility may generate forces which facilitate sperm detachment from oviduct cells and migration within the oviductal lumen (Katz *et al.*, 1989; DeMott and Suarez, 1992) and may increase contact and penetration of the cumulus or zona pellucida (Suarez *et al.*, 1991; Suarez and Dai, 1992). It has been suggested that hyperactivated motility is typical of capacitated spermatozoa and that it shortly precedes the acrosome reaction (Robertson *et al.*, 1988).

1.6.3.1 *In vivo* studies of hyperactivation

As stated above, hyperactivated spermatozoa have been observed in the oviductal ampulla of hamsters (Katz and Yanagimachi, 1980) and rats (Saling and Philips, 1988). This has been possible due to the transparent nature of the oviducts of these species, allowing direct visualisation after transillumination. *In vivo* studies of mouse spermatozoa in the female reproductive tract showed that spermatozoa in the uterus, uterotubal junction and isthmus appeared to adhere to the epithelium most of the time, only breaking away occasionally. When not attached to the epithelium, they often appeared to be hyperactivated, because their motility was vigorous and not

linear. Sperm samples from the same male, after *in vivo* and *in vitro* incubation, displayed the same acute flagella bends after a period of incubation (Suarez and Osman, 1987).

Reported evidence suggests that hyperactivation offers an advantage to spermatozoa in terms of detaching from the oviductal mucosa (Suarez *et al.*, 1983; Suarez, 1987) and for penetrating viscoelastic substances occupying the oviductal lumen and the hyaluronate matrix of the cumulus oophorus (Suarez *et al.*, 1993). It has been calculated that hyperactivation also increases the amount of thrust that the spermatozoon can develop against the zona pellucida, hence conferring an advantage during penetration of the zona (Drobnis *et al.*, 1988).

The mechanisms regulating hyperactivation, which entails an increase in flagellar-bend amplitude (Suarez *et al.*, 1991) and flagellar beat asymmetry (Aoki *et al.*, 1994) remains obscure but are believed be linked to calcium (Yanagimachi and Usui, 1974; Fraser, 1977; Neill and Olds-Clarke, 1987) and cAMP (Morton and Albagli, 1973; Fraser, 1981; Mrsny and Meizel, 1990).

1.6.3.2 *In vitro* studies of hyperactivation

Hyperactivation *in vitro* is dependent on glycolytic metabolism (Fraser and Quinn, 1981) and on the presence of calcium ions (Yanagimachi and Usui, 1974). Hamster and mouse spermatozoa can be hyperactivated in defined solutions lacking protein or cAMP, but they do not acrosome react or fertilise under such conditions (Bavister, 1981; Fraser, 1981). Conversely, acrosome reactions were induced using calcium ionophore (Green, 1978), but hyperactivation did not normally occur unless serum albumin was added to remove excess ionophore (Shams-Borhan and Harrison, 1981). Mohri and Yanagimachi (1980) were able to induce hyperactivated movements in uncapacitated, demembranated guinea pig, hamster and human spermatozoa simply by exposing them to suitable concentrations of ATP and cAMP, thus implying that hyperactivation occurs as a result of changes in plasma membrane consequent upon capacitation.

Specific incubation conditions which support hyperactivation *in vitro* have

been developed for different species. Factors of importance in the initiation of hyperactivation are calcium (Fraser, 1977), potassium and sodium (Fraser, 1983), bicarbonate (Neill and Olds-Clarke, 1987) and albumin (Yanagimachi, 1970; Mack *et al.*, 1989). A range of commercially available media including TCM 199 (Blottner *et al*, 1989), modified BWW medium (Grunert *et al.*, 1990), Hams F-10 (Robertson *et al*, 1988) and other media used for *in vitro* fertilisation support hyperactivation. These media are usually supplemented with an albumin source such as fetal calf, bovine or human serum albumin. Supplementing the medium with other substances is also common; addition of calcium ionophore (Grunert *et al.*, 1990;) caffeine, taurine and prolactin (Burkman, 1984), progesterone (Mbizvo *et al.*, 1990) and pentoxifylline (Kay *et al.*, 1993) have all been shown to increase hyperactivation in human spermatozoa. Short incubation periods of 4 hours (hamsters), or 3 hours (mice) at 37°C in medium capable of supporting capacitation, are sufficient to induce hyperactivation (Neill and Olds-Clarke, 1987), although the optimal incubation period is not known for other species.

Under *in vitro* conditions hyperactivation has been associated with the penetration of zona-free eggs and fertilisation of zona-intact eggs (Fraser, 1981, Lambert, 1981; Fleming and Yanagimachi, 1981; Cummins, 1982).

1.6.3.3 Measurement of hyperactivation

Hyperactivated motility is considered an integral part of the process of capacitation and thus, a prerequisite for fertilisation. It has been shown to correlate with fertilising ability (Coddington *et al*, 1991), suggesting that hyperactivation may be a useful biomarker of functional ability. This is particularly relevant to the diagnosis of infertility in the medical field, and to semen preservation studies in medical and veterinary fields. To this end techniques were developed for the objective measurement of hyperactivated spermatozoa, utilising computerised motility analysis (Robertson *et. al.*, 1988; Burkman, 1991).

Early studies of representative motility patterns were made using high speed microcinematography (Cummins, 1982), videomicrography (Johnson *et al*, 1981),

stroboscopic illumination (Cooper and Woolley, 1982) and videomicrography with computer assistance (Neill and Olds-Clarke, 1987). These techniques were time consuming, assessed relatively small numbers of cells and were not readily applicable to routine clinical evaluation. The advent of computerised motility has allowed the rapid measurement of a range of motility parameters in a large number of individual sperm. It has also facilitated the detection of individual hyperactivated spermatozoa, recognised by a combination of certain motility parameters. The development of algoriths for the detection of hyperactivation and their incorporation into motility analyser software allows the rapid detection and measurement of hyperactivated subpopulations of cells. This is discussed further in section 1.8.5, in computerised motility analysis.

1.6.4 Calcium and sperm function

Although many cations (Ca^{2+}, Na^{+}, K^{+} and H^{+}) have been implicated in the modulation of sperm function (Fraser, 1995), current evidence suggests that Ca^{2+} plays a critical role. Calcium ions are involved in promoting sperm motility (Yanagimachi, 1982; Garbers, 1989; Lindemann and Kanous, 1989), capacitation (White and Aitken, 1989; Zhou *et al.*, 1990; Das Gupta *et al.*, 1993), hyperactivation (Yanagimachi, 1982; White and Aitken, 1989; Suarez *et al.*, 1993), the acrosome reaction (Yanagimachi, 1982; Thomas and Meizel, 1988), sperm-egg fusion and egg activation and also plays a central role in the activation of phospholipases and in membrane fusion. Ca^{2+} ions act as a trigger and second messenger for co-ordinating the above events. These effects, in turn, are regulated by Ca^{2+} ions crossing the sperm plasma membrane.

1.6.4.1 Calcium and motility

The precise mechanisms underlying the modulation of sperm motility are still obscure, but is known that calcium in the external medium plays a regulatory role (Singh *et al.*, 1978; Triana *et al.*, 1980; Singh *et al.*, 1980). Optimal external concentrations vary from species to species. In ram, mouse, rabbit and guinea pig the absence or presence of calcium at less than 5 mM had little effect on motility (Morita and Chang, 1970; Quinn *et al.*, 1970; Bredderman and Foote, 1971; Hyne and Garbers, 1981). With chimpanzee and bull spermatozoa (McGrady *et al.*, 1974) motility was inhibited in a dose dependent manner at calcium concentrations above 1 mM. At a concentration of 1 mM hamster sperm motility was stimulated (Morita and Chang, 1970; Morton *et al.*, 1974). At 1.7 mM the motility of rat sperm was optimal; both higher and lower concentrations were inhibitory (Davis, 1978). In the 1-10 mM range which prevails both in sea water and in mammalian tissue fluid a steep inward calcium concentration gradient exists. Since the intracellular free calcium of the spermatozoon is about 0.1 to 1 μM (Breitbart and Rubinstein, 1983) the sperm plasma membrane is responsible for maintaining low intracellular levels by on-going active extrusion of Ca^{2+}.

1.6.4.2 Calcium and capacitation

The development of culture systems which support *in vitro* fertilisation, hence capacitation *in vitro*, has enabled studies of the specific ionic requirements for the stages of sperm function to be carried out. Most attention has focused on cations, particularly Ca^{2+}, Na^{+}, K^{+}, and H^{+} ions. The requirement for Ca^{2+} has been discussed earlier. In studies of sperm function using CTC, it has been shown that in Ca^{2+}-deficient medium mouse sperm fail to complete capacitation and are non-fertilising. Chlortetracycline patterns indicated that the majority of spermatozoa remained in the uncapacitated state (Fraser, 1982; Fraser and McDermott, 1992). Preincubation of mouse sperm suspensions in medium with higher than normal concentration of Ca^{2+} - (3.6 mM vs. 1.8 mM) stimulated acrosomal exocytosis, hyperactivated motility and

fertilising ability *in vitro* (Fraser, 1987; Fraser and McDermott, 1992; Das Gupta *et al.*, 1993). Bull spermatozoa showed similar staining patterns in response to ionophore (Fraser *et al.*, 1995). Other studies (not using CTC) have also shown the involvement of increased concentrations of intracellular calcium (bovine: Parrish *et al.*, 1989). Newer types of calcium indictors allow the determination of Ca^{2+} concentration in small cells (Tsien, 1988; Grynkiewicz *et al.*, 1985). These indicators have been used to measure calcium influx during capacitation (White and Aitken, 1989; Zhou *et al.*, 1990) or in the assessment of calcium influx after manipulation by compounds which affect its movement (Simpson and White, 1988).

1.6.4.3 Calcium and the acrosome reaction

In early studies the acquisition of direct evidence of an increased intracellular concentrations of Ca^{2+} was impeded by the lack of detector systems for cells such as spermatozoa. Experiments demonstrating that the calcium ionophore A23187, in the presence of calcium, would induce the acrosome reaction (Summers *et al.*, 1976; Reyers *et al.*, 1977; Green, 1978) led to the assumption that the immediate cause of acrosome reaction was an increase in the cytoplasmic free calcium concentration (Green, 1978). Specific extracellular concentrations required to support capacitation and the acrosome reaction have been listed earlier. Some of this external extracellular Ca^{2+} must be internalised, and this has been demonstrated by using A23187. Induction of acrosomal exocytosis using A23187 has been described in a number of studies (Talbot *et al.*, 1976; Jamil *et al.*, 1982; Varner *et al.*, 1987b). When millimolar concentrations of extracellular calcium are present, ionophore is said to promote both capacitation and exocytosis. In the presence of micromolar concentrations, however, it is said that capacitation proceeds but neither the acrosome reaction nor *in vitro* fertilisation is supported, judged by CTC staining patterns (*mouse*.- Fraser, 1987; Fraser and McDermott, 1992). Although it has been suggested by some researchers that treatment with ionophore bypasses capacitation, CTC analysis reveals that the fluorescence patterns change from the 'uncapacitated' to 'capacitated-acrosome intact' to 'acrosome reacted' state and not 'uncapacitated' to the 'acrosome reacted' state directly. Thus, the initial rise in Ca^{2+} following

ionophore introduction leads to an increase in the number of 'capacitated-acrosome intact' cells and as intracellular Ca^{2+} continues to increase it passes the critical threshold to trigger acrosomal exocytosis (*mouse* - Fraser and McDermott, 1992; *human* - Das Gupta *et al.*, 1993; *bull* - Fraser *et al.*, 1995; *ram* - Perez *et al.*, 1996).

1.6.4.4 Methods for measuring calcium

Methods which are used to measure calcium in a wide range of biological systems can be broadly categorised into indirect and direct. Examples of indirect methods are: (i) use of radioisotopes such as $^{45}Ca^{2+}$ (Robertson and Watson, 1987) (ii) estimates based on physiological responses (Portzhel *et al.*, 1964) (iii) using electrical methods (Adams and Gage, 1979) (iv) by biochemical means (Means and Dedman, 1980) (v) pharmacological methods (Kauffman *et al.*, 1980) and (vi) ultrastructural methods (Carafoli and Crompton, 1976).

Direct method studies entail inserting some form of a Ca^{2+} sensor into a cell. The first of these used photoproteins, for example, aequorin (Ridgway and Ashley, 1967), to measure intracellular calcium. Photoproteins which were obtained from the luminescent organs of coelenterates emitted light in a reaction which was catalysed by Ca^{2+}. This method was first used to measure intracellular calcium by Ridgway and Ashley (1967). Subsequently, two other major methods have developed; Ca^{2+} sensitive microelectrodes (reviewed in Thomas, 1982) and the Ca^{2+} sensitive dyes or metallochromic indicators.

Metallochromic indicators were first used to measure free Ca^{2+} in 1963, when Ohnishi and Ebashi utilised murexide (a first generation calcium indicator) to monitor the uptake of Ca^{2+} by isolated sarcoplasmic reticulum (Ohnishi and Ebashi, 1963) but its use was never particularly widespread. Several different groups of workers subsequently worked with arsenazo III and antipyrylazo III (second generation indicators) and established their effectiveness as intracellular calcium indicators (Brown *et al.*, 1975; Thomas and Gorman, 1977). It was, however, difficult to monitor dynamic changes in $[Ca^{2+}]_i$ in individual cells accurately until the synthesis of Quin-2, Fura-2AM and Indo-1 by Tsien and colleagues (Tsien, 1980, Tsien *et al.*, 1982, Grynkiewicz *et al.*, 1985)

Quin-2 was the first of the third generation of calcium indicators, developed by Tsien in 1980. Quin-2 has been used for the direct measurement of intracellular calcium in a number of cell types (Grynkiewicz *et al.*, 1985). Although much important biological information was revealed using Quin-2, severe limitations were acknowledged. The short excitation wavelength of 339 nm excited significant autofluorescence from cells, and penetrated microscope optics poorly. It required high loading concentrations, which in some cell types dampened the response to changes in intracellular calcium. Quin-2 signals calcium shift by an increase in fluorescence intensity without much shift in either excitation or emission wavelengths. It was realised that a better indicator would be one which responded to calcium by shifting wavelengths while maintaining strong fluorescence intensity. Fura-2AM and Indo-1 were considered to be an improvement over Quin-2 for this reason (Grynkiewicz *et al.*, 1985).

Since their development Indo-1 and Fura-2AM have been used widely to estimate intracellular cytoplasmic free calcium [$(Ca^{2+})_i$] in various cell types (Blatter and Wier, 1990). Fura-2AM has become the most popular fluorescent probe for monitoring dynamic changes in cytosolic free calcium in intact living cells (Roe *et al.*, 1990). The fluorescent dyes (Fura-2AM or Indo-1) are combined with acetoxymethyl ester groups to form a lipophilic derivative which can cross cell membranes. Inside the cells Fura-2AM is hydrolysed by cytosolic esterases to form the fluorescent dye. The assessment of fluorescence signals at specific wavelengths, which depended on the choice of fluorescent dye used, can be used to interpret calcium distribution within the cytosol of sperm cells. For Fura-2AM, the peak excitation wavelength changes when Fura-2 binds Ca^{2+}. As a consequence, measurement of fluorescence at the two excitation wavelengths (340 and 380 nm) can be used to obtained a measure of $[Ca^{2+}]_i$ which is independent of dye concentration, cell thickness, excitation light intensity and camera sensitivity. The ratio of the fluorescence intensity of the two excitation wavelengths may be used to calculate $[Ca^{2+}]_i$ according to the equation originally derived by Grynkiewicz *et al.*, (1985) (explained further in Chapter 2).

There are, however, recognised limitations to the use of Fura-2AM and many of these problems are cell-type specific. Among these problems are (a) an incomplete hydrolysis of Fura-2 AM by cytosolic esterases, and the presence of

unidentified contaminants in some commercial preparations, (b) sequestration of Fura-2AM in non-cytoplasmic compartments, (c) active or passive dye loss from loaded cells, (d) quenching of Fura-2 fluorescence by heavy metals, (e) photobleaching and photochemical formation of fluorescent non-calcium sensitive Fura-2 species, (f) shifts in the absorption and emission spectra as a function of either polarity, viscosity, ionic strength or temperature of the probe environment and (g) accurate calibration of Fura-2 signal within the cell.

The fluorescent indicators, Quin-2, Indo-1 and Fura-2AM have been used to measure intracellular free calcium in various mammalian sperm species (Irvine and Aitken, 1986; Mahanes *et al.*, 1986; Rigoni and Deana, 1986; Babcock and Pfeiffer, 1987; Simpson and White, 1988; Blackmore *et* al., 1990; Meizel and Turner, 1991; Ashizawa *et al.*, 1992; Bailey and Buhr, 1993; Mendoza and Tesarik, 1993; Shimuzu *et al.*, 1993; Bailey *et al.*, 1993; Weyand *et al.*, 1994; Bonaccorsi *et al.*, 1995; Foresta *et al.*, 1995; Mendoza *et al.*, 1995; Zhao and Buhr, 1995). These studies measured intracellular calcium using dual emission spectrofluorimeters, and hence were useful for mapping changes which occurred in the population of sperm cells within a cuvette. Individual cell responses can also be measured using a detection system built around an inverted microscope with an epifluorescence port for the incoming excitation light and a side port for collecting the emitted fluorescence. At this time very few studies have been carried out using single sperm cells (Suarez *et al.*, 1993; Plant *et al.*, 1995).

1.7 Semen Processing

1.7.1 Semen collection

The development of the artificial vagina (AV) for use in semen collection has allowed for the collection of an ejaculate representative of that emitted during natural service. An AV is the most common method of semen collection and most stallions can be trained to ejaculate into an AV. In general, the AV consists of an outer casing and an inner latex liner. Warm water, and in some cases air, is infused between the casing and the liner to provide the proper pressure and temperature for collection. A collection receptacle is placed on the end of the liner to receive the semen during collection. Once assembled, the AV is filled up with warm water and the temperature allowed to equilibrate to between 44 and 48°C. The pressure within the AV is sufficient to allow contact and stimulation of the penis without hindering penetration or expansion of the penis to its fully erect size. Just before collection the mouth of the AV is lubricated with a small amount of non-spermicidal lubricant. Prior to collection, the erect penis should be washed with clean, warm water to reduce the contamination of semen with bacteria and debris from the surface of the penis. The stallion is allowed to mount a hobbled oestrual mare or a phantom. Once the stallion has mounted the mare or the phantom, the penis is deflected to the side and directed into the artificial vagina. During collection, the hand of the collector is placed on the underside of the penis to detect urethral pulsations. When ejaculation begins the AV is tilted to allow semen to flow into the receptacle and prevent exposure of the ejaculate to temperatures above 43°C. Exposure of semen to temperatures above 43°C results in a decline in sperm motility. After collection the water in the AV is released to allow semen trapped between the walls of the liner to drain into the collecting receptacle (Pickett, 1993b).

1.7.2 Semen extenders

Many different extenders have been formulated for the dilution and storage of equine semen. The most commonly used are TRIS extender (Pickett and Voss, 1975), Cream-Gel, Skim Milk and Skim Milk Gel extenders (Voss and Pickett, 1976), Dried Milk Solids-Glucose extender (Kenney *et al.*, 1975), variations of the Eastern Europe extenders (cited in Pickett and Amann, 1993), H-20 extenders used in Japan (cited in Amann and Pickett, 1987) and Lactose-EDTA-egg yolk extenders. All have some common components such as milk, egg yolk, various sugars, electrolytes, antimicrobial agents, osmolarity and pH regulators. Simple sugars, for example glucose, serve as an energy source for spermatozoa. Sodium citrate and TRIS act as buffers to compensate for changes in pH caused by lactic acid formation. Egg yolk minimises damage to sperm membranes during rapid temperature changes. Sugars like trehalose, mannose, raffinose and lactose also serve as non-penetrating cryoprotectants when semen is frozen. Antibiotics may be added to minimise the proliferation of bacteria (Kenney *et al.*, 1975).

Extender composition varies depending on type of storage; liquid versus frozen. Freezing extenders generally have glycerol as a cryoprotecting component, though dimethylsulfoxide (DMSO) and propylene glycol have also been used (Pickett and Amann, 1993).

Milk extenders have been evaluated extensively for liquid storage of stallion semen (Kenney *et al.*, 1975; Province *et al.*, 1984; Douglas-Hamilton, 1984; Province *et al.*, 1985; Heiskanen *et al.*, 1987; Squires *et al.*, 1988; Padilla and Foote, 1991; Wockener *et al.*, 1992; Heiskanen *et al.*, 1994). The protein fraction of milk in milk based diluents acts as a buffer against changes in pH (Jones, 1969; Watson, 1979) as well as a chelating agent for heavy metal ions (Jones, 1969). It also protects spermatozoa during dilution (Blackshaw, 1953) and cooling for storage (Choong and Wales, 1962).

Recently the addition of bovine serum albumin to equine extenders has been shown to improve the maintenance of sperm motility (Goodeaux and Kreider, 1978; Kreider *et al.*, 1985). Other studies using equine relaxin as an additive, showed improved motility of cryopreserved equine semen post-thaw (Casey *et al.*, 1991).

1.7.3 Semen separation techniques

Semen separation procedures are performed in order to remove seminal plasma, to isolate motile spermatozoa from dead sperm and cellular debris and to produce a fraction of sample as homogeneous as possible with respect to both motility and morphology.

Sperm separation methods involve either centrifugation, be it through medium or gradients, or utilisation of motility to separate highly motile from less motile cells. Repeated washing by centrifugation is a time honoured practice (Harrison and White, 1972; Jones and Holt, 1974). Centrifugation through gradients has utilised Ficoll (Harrison, 1976; Bongso *et al.*, 1989) continuous and discontinuous Percoll gradients (Drobnis *et al.*, 1991) and Nycodenz (Gellart-Mortimer *et al.*, 1988; Serafini *et al.*, 1990). Separation by utilising motility encompasses swim-up techniques (Parrish *et al.*, 1988; Bendvold, 1989), glass wool (Paulson *et al.*, 1979), Sephadex columns (Drobnis *et al.*, 1991), migration-sedimentation technique (Lucena *et al.*, 1989; Yerner *et al.*, 1990) and columns of glass beads (Lui *et al.*, 1979; Daya *et al.*, 1987). The 'classical' sperm preparation technique involves multiple cycles of sperm washing (Harrison and White, 1972; Jones and Holt, 1974). This can be followed by a swim-up step to prepare a subpopulation of morphologically homogeneous and highly motile spermatozoa (Aitken and Clarkson, 1988).

Results with the numerous methods employed had been contradictory and confusing. Comparisons between the swim-up technique and a discontinuous Percoll gradient (Guerin *et al.*, 1989) applied to human semen showed no significant differences in the concentration of spermatozoa after treatments, with both treatments showing improved curvilinear (VCL) and straight line (VSL) velocities when compared to fresh extended semen. A similar comparison by McClure *et al.*, (1989) showed a significant improvement in sperm morphology, numbers of motile sperm, and motility with the Percoll method. He noted that Percoll density gradients give good sperm recovery in normal and abnormal samples. In another publication from Drobnis and co-workers (Drobnis *et al.*, 1991) which compared the separation of cryopreserved spermatozoa using Sephadex columns, washing and Percoll gradients, it was noted that recovery of motile spermatozoa was highest with Sephadex and

lowest with washing. Sperm motility however, was highest with Percoll and lowest with washing but Sephadex and washing were able to maintain motility better than Percoll.

Other reports have also indicated successful recovery of enhanced populations of spermatozoa following migration through Percoll gradients (Pousett *et al.*, 1986, Ord *et al.*, 1990). Percoll prepared spermatozoa were more successful in achieving hamster egg penetration (Berger *et al.*, 1985: Ford *et al*, 1992). Other workers have also shown that Percoll appeared to improve the fertilising capacity of normal human spermatozoa (Forster *et al.*, 1983; Guerin *et al*, 1989). In agreement with this, Van Der Zwalmen *et al.*, (1991) demonstrated a higher pregnancy rate with Percoll separated semen compared to swim-up procedures. Some reports suggests that Percoll separation may result in alterations to sperm function, such as motility changes similar to hyperactivation and early fusion with zona-free hamster oocytes. Arcidiacono *et al.*, (1983) reported membrane swelling and enzyme leakage following selection of motile human spermatozoa on continuous Percoll gradients. It has been proposed that these changes may exacerbate damage done by cryopreservation.

Several of the procedures, for example, glass wool and Sephadex filtration (Samper *et al.*, 1991), discontinuous Percoll gradient (Del Campo *et al.*, 1990; Buzby *et al.*, 1993), sodium hyaluronate migration, semen filtration column and swim-up (Buzby *et al.*, 1993) have been use to isolate equine spermatozoa. Based on the results from these studies, it was concluded that the success of these selection procedures was dependent on stallion individuality. Separation using Percoll gradients resulted in the highest proportion of progressively motile spermatozoa harvested post-separation, but the spermatozoa became agglutinated within 30 minutes. More work is required to evaluate these methods of separation for stallion semen before a suitable one can be used routinely in any stallion semen assisted reproductive programme.

1.7.4 Packaging of semen

A number of packaging systems have been used for stallion spermatozoa, among them glass ampoules (Pace and Sullivan, 1975), or vials capable of holding from 1 to 10 ml of extended semen (cited in Pickett and Amann, 1993), plastic bags (Ellery *et al.*, 1971), plastic straws (0.5, 1.0 and 4.0 ml) (cited in Pickett and Amann, 1993), polypropylene straws (Aliev, 1981), vinyl straws (Nishikawa, 1972, 1975; Nishikawa and Shinomiya, 1976; Loomis *et al.*, 1983; Cristanelli *et al.*, 1984) and aluminium packets (Aliev, 1981; Muller, 1982, 1987).

1.7.5 Preservation of stallion semen

Stallion semen can be stored either in the frozen or liquid state. Regardless of the state in which it was stored, successful preservation will depend on; (1) mixing semen with an appropriate extender (2) cooling to around 4°C for liquid storage or to -196°C if frozen, at optimal cooling rates, (3) choice of packaging and (4) rewarming rate. A number of problems are associated with these processes and are elaborated below.

1.7.5.1 Centrifugation of semen

For all types of packaging, centrifugation of semen is a routine part of the processing procedure. Centrifugation of spermatozoa will (1) eliminate most or virtually all of the seminal plasma present in the ejaculate and (2) provide a suspension of high concentration which will allow packaging such that the volume required in one insemination dose is minimised. The process of centrifugation, however, is deleterious (Pickett *et al.*, 1975; Baumgartl *et al.*, 1980) but damage may be minimised by using low centrifugal forces (Nishikawa and Shinomiya, 1972,1976) or by dilution of semen before centrifugation (Buell, 1963; Rajamanan *et al.*, 1968; Nishikawa and Shinomiya, 1972, 1976; Martin *et al.*, 1979; Cochran *et al.*, 1984). It has been reported that spermatozoa from certain individual stallions may be

excessively damaged by centrifugation (Cochran *et al.*, 1983). More recent studies (Aitken and Clarkson, 1988) indicated that techniques which involved the repeated centrifugation of unselected sperm populations generated cell suspensions with significantly reduced motility and an impaired capacity for fertilisation. Shannon and Curson (1972) stated that dead bovine spermatozoa were toxic to living cells because of an amino acid oxidase which became active only after the death of the cell. Jones and Mann (1973) reported their findings of a peroxidase that was aerobically produced during centrifugation. The centrifugation step may be omitted from processing, if only the sperm rich fraction of the ejaculate is collected (Tischner, 1979; Varner *et al.*, 1987a).

1.7.5.2 Dilution of semen

No specific guidelines are available regarding the extent to which stallion semen can be extended. The effect of extensive dilution was a subject of great interest in the early years of sperm research (Blackshaw, 1953; Tampion and Gibbons, 1963) and it was shown that high dilution ratios produced damaging effects (Bredderman and Foote, 1971; Harrison *et al.*, 1982). Mammalian spermatozoa respond to high dilution rates by an initial increase in activity, followed by a rapid loss of motility (Mann, 1964). This loss of motility is an effect of cell injury attributed to leaching of intracellular components, as a consequence of the dilution of a "protective agent" in seminal fluid (Watson, 1979) or the absence of proteinaceous motility stimulants from the seminal plasma (Harrison *et al.*, 1982). The data of Ashworth *et al.*, (1994), from experiments performed on ram spermatozoa, suggested that the effect of dilution was dependent on the type of dilution medium used. Those workers found that when ram spermatozoa were diluted in a sucrose based medium or in saline medium supplemented with 10% seminal plasma, survival of spermatozoa was greatly improved. The protective effect of seminal plasma, it was suggested, resided in a 5-10 kDa fraction. They concluded that spermatozoa tended to die following washing and high rates of dilution in simple isotonic saline-based medium.

Whilst excessive dilution has been shown to decrease sperm viability, low

rates of dilution have been shown to improve sperm motility. One trial indicated that progressive dilution of semen increased sperm motility, and dilution to a concentration of 25 million per millilitre prolonged motility (Varner *et al.*, 1987a). Impairment of sperm velocity generally did not occur until the concentration fell below 20 million per millilitre.

1.7.5.3 Cooling rates

Liquid storage of spermatozoa involves cooling extended ejaculates from body temperature to a storage temperature of 5°C and it has become increasingly obvious that the rate of cooling affects the subsequent fertility of the samples. Previous studies have shown that 'cold shock' (see below) damages plasma membranes (Plummer and Watson, 1985; Watson and Plummer, 1985; Watson *et al.*, 1987) while studies focusing on motility showed that there is a significant decline in stallion sperm motion after rapid cooling (Moran *et al.*, 1992). Data comparing the effect of cooling rates of 0.5°C, 0.3°C, 0.05°C and 0.012°C per minute on stallion sperm motility showed that cooling rates of 0.05°C and 0.012°C per minute affected motility to a similar extent and that those cooling rates were superior to cooling at 0.5°C and 0.3°C per minute (Douglas-Hamilton *et al.*, 1987; Kayser *et al.*, 1992; Pickett, 1993a). Kayser (1990) has shown that no immediate or latent damage occurs to the motility of equine spermatozoa cooled rapidly from 37°C to 20°C (cited in Pickett, 1993a). Bedford *et al.*, (1995) were not able to demonstrate a difference in motion characteristics of spermatozoa cooled in milk extender whether cooled slowly or rapidly.

1.7.5.4 The effect of rapid cooling - 'cold shock'

Spermatozoa are susceptible to damage if rapidly cooled to low temperatures. This phenomenon was termed 'temperature shock' by Milovanov in 1934 (cited by Watson, 1990) and is now commonly called 'cold shock'. The severity of damage caused by rapid cooling varies between species. Bull, ram, boar and stallion

spermatozoa are highly susceptible to cold shock; other species, such as human, less so (reviewed Watson, 1981). The plasma membrane is considered to be the site at which injury due to cooling is initiated (Morris, 1987).

Maintenance of plasma membrane integrity is crucial to the spermatozoon, as a number of independent aspects of sperm function involve the internal organelles. The motility apparatus of the tail relies on controlled membrane permeability, and the process of capacitation appears to involve extensive modification of both integral and surface components of the plasma membrane. Ultimately the membrane fusion events of the acrosome reaction require the participation of the plasma membrane. Therefore the preservation of spermatozoa by cooling should ideally cause minimal damage to the plasma membrane, allowing it to resume normal function when required.

Early studies showed that cooling led to an increased incidence of plasma membrane swelling, acrosomal swelling and mitochondrial disintegration (Jones and Martin, 1973). Other effects included loss of membrane-selective permeability and membrane fluidity (Canvin and Buhr, 1989; Buhr *et al.*, 1989), leakage of cellular components such as phospholipids and proteins, disruption of metabolism, irreversible loss of motility (Watson, 1981), increased uptake of $[Ca^{2+}]$ (Karagiannidis, 1976; Simpson *et al.*, 1986, 1987; Robertson *et al.*, 1988,1990) and decreased fertilising ability (Watson, 1979). More recent evidence has shown that the severity of these changes is dependent on the rate of cooling, being generally most severe in the range of 2°C to 12°C (Watson, 1995), and that grossly there was very little cellular change associated with cooling at a slow, controlled rate (Holt and North, 1984).

Early studies had shown many changes associated with cold shock; diminished anaerobic glycolysis (Chang and Walton 1940; Mayer *et al.*, 1951; Blackshaw and Salisbury, 1957; Blackshaw, 1958), diminished levels of ATP (Mann and Lutwak-Mann, 1955), leakage of intracellular enzymes (Murdoch and White, 1968, Pursel *et al.*, 1970; Harrison and White, 1972; Moore *et al.*, 1976) and loss of phospholipids (Blackshaw and Salisbury, 1957; Pickett and Komarek, 1967). Cold shock was also shown to induce cation movements, with Na^+ and Ca^{2+} gained and K^+ and Mg^{2+} lost by spermatozoa (Quinn and White, 1966; Hood *et al.*, 1970; Robertson and Watson, 1987).

Other changes attributed to cooling are the lateral redistribution and regionalisation of lipids and proteins in the post acrosomal region (Holt and North, 1984). Freeze fracture studies have shown that these changes are consistent with a lipid phase transition (Drobnis *et al.*, 1993). Marked effects of cooling were also observed on the flagellum where extensive regions of particle-free plasma membrane were formed in the middle and principle piece. It is said that the rearrangement of membrane components which occur upon cooling below the phase transition temperature would alter the biological and biophysical properties of a membrane. These effects might be explicable in terms of increased membrane permeability (Holt and North, 1984). It appears that these membrane changes are not completely reversible upon restoration of the physiological temperature and might then interfere with sperm motility, or with later events of capacitation and acrosome reaction.

More recent studies using the fluorescent indicators Indo-1 showed that significant rates of calcium uptake occur during cooling and at 5°C, and that Ca^{2+} was extruded during rewarming (Zhoa and Buhr, 1995). This may be due to the calcium pumps being less active at low temperatures, thus leading to an accumulation of intracellular calcium or may also be an effect of protein clustering which may adversely affect the function of these pumps. It has been suggested that upon the rewarming of plasma membranes the lateral phase separations induced by cooling are reversed (de Leeuw *et al.*, 1990, 1991) and the calcium pumps are then able to extrude the accumulated calcium (Zhoa and Buhr, 1995).

1.7.5.5 Problems associated with rewarming - 'warm shock'

Rewarming exerts effects on sperm survival in a manner similar to cooling and this also appears to be rate dependent. Rapid warming of slowly cooled cells is said to induce a form of osmotic shock. Bamba and Cran (1985, 1988) showed that warming caused visible sperm plasma membrane surface changes and acrosomal damage in bull, boar and rabbit spermatozoa. These changes were describe as roughening or wrinkling of sperm head surface, invagination of acrosomal membranes into their matrix, the formation of small punctuate ruptures through which acrosin may be released, or the removal of surface membranes with the loss of contents. The

loss of plasma membrane integrity, a decline in motility and leakage of ATP have also been reported (Holt *et al.*, 1992).

These consequences of rewarming are likely to affect fertility. However, it is difficult to compare the thawing procedures employed in different studies because of variation in sperm concentration, packaging material, package volumes, cooling methods and rates, type of freezing medium used, thawing rates and assessment protocols (Arns *et al.*, 1987; Padilla and Foote, 1991; Casey *et al.*, 1991).

1.8 Evaluation of Stallion Semen

A detailed semen analysis would consist of a record of volume, sperm concentration, a visual estimate of motility and an assessment of morphology. It might also comprise examination of the acrosome, other membrane staining patterns, biochemical tests of sperm function and computerised analysis of motility patterns (Watson, 1990). Fresh stallion semen should appear watery, opaque and near-white in colour. This appearance varies with sperm concentration. The ejaculate volume varies with age and season, but generally falls between 15 and 100 ml. Concentration may vary from 50 to 700 x 10^6 spermatozoa per ml with total number of spermatozoa per ejaculate ranging from 1 to 20 x 10^9. Semen longevity should change little during the first 30 minutes of incubation. Sperm pH immediately after collection should lie between 7.2 and 7.6. Morphological abnormalities not exceeding 10% of total is acceptable (Jasko, 1992). Seminal characteristics reported in several studies are summarised in Table 1.3. Morphological classification systems used for stallion sperm are summarised in Table 1.4 and seminal characteristics of fertile stallions summarised in Table 1.5.

1.8.1 Visual assessment of stallion semen motility

A grading system for visual gross motility assessment has not been defined for stallion spermatozoa. Long *et al.* (1993) scored stallion spermatozoal motility in neat semen as follows; 1 (excellent), 2 (good), 3 (fair) and 4 (poor). What each score describes (whether rapid, sluggish or immotile) was not indicated.

To determine the percentage of progressively motile spermatozoa, neat semen was first diluted in non-fat dried skim milk extender (1:20). Extension of semen prevents clumping of spermatozoa (agglutination) of sperm and reduces the influence of sperm concentration and seminal pH on the subjective appraisal of sperm motility. Suspensions are restricted to a depth of between 10 and 20μl can be prepared by the placement of either a 6 or 10μl drop of semen on a slide and placing either a 18 or 22 mm^2 coverslip, respectively, over the drop (Jasko, 1992).

Table 1.3 Mean (SD or SEM) of stallion seminal characteristics reported in several studies (nr = not reported).

Characteristics	Reference 1 Mean (SD)	2 Mean (SD)	3 Mean (SD)	4 Mean (SD)	5 Mean (SEM)	6 Mean (SEM)
Gel free volume (ml)	45 (30)	45 (31)	58 (24)	44.1 (27.8)	42 (3.4)	60.6 (7.4)
Sperm concentration (10^6/ml)	335 (232)	178 (168)	173 (118)	168.8 (108.6)	264 (38.9)	199 (32.7)
Total sperm/ejaculate (10^9)	11.9 (9.0)	7.2 (6.9)	9.2 (5.9)	5.8 (4.8)	8.3 (0.9)	9.8 (1.1)
Motile sperm (%)	nr	nr	70 (17)	36.7 (13.3)	nr	nr
Progressively motile (%)	53 (15)	72 (16)	53 (24)	53.3 (17.3)	55.9 (3.4)	48.8 (4.0)
Percentage live (%)	nr	nr	nr	70.5 (7.1)	nr	nr
Morphologically normal (%)	51(15)	nr	55 (19)	nr	38.4 (3.4)	48.2 (3.5)
Morphologically abnormal (%)	nr	nr	nr	28.6 (12.8)	-	-

1 to 3 - Adapted from Jasko, 1992

4 - Rousset *et al.*, 1987

5 - Long *et al.*, 1993 (ejaculate, early breeding season)

6 - Long *et al.*, 1993 (ejaculate, late breeding season)

Table 1.4. Morphologic classification systems used for stallion sperm. (Adapted from Jasko, 1992).

Reference		
Kenney et al., 1983	**Dowsett et al., 1984**	**Jasko et al., 1990**
Normal sperm	Abaxial mid-pieces	Normal sperm
Abnormal acrosome	Abnormal heads	Abnormal heads
Abnormal heads	Loose heads	Detached heads
Detached heads	Proximal droplets	Proximal droplets
Proximal droplets	Distal droplets	Distal droplets
Distal droplets	Abnormal mid-pieces	Abnormal mid-pieces
Bent or coiled mid-pieces	Abnormal tails	Abnormal tails
Other mid-piece abnormalities		
Hairpin or bent tails		
Coiled tails		

Table 1.5. Seminal characteristics of fertile stallions reported in several studies (Adapted from Jasko, 1992).

References	Seminal Characteristics
Bielanski, 1956	Minimal requirements: volume, 16 ml; motile, 40% in raw semen; sperm concentration, 50 million/ml
Bielanski, 1975	Morphologic features: 1% abnormalities,<10%; *in vitro* survival time of semen diluted 1:3 in 7% glucose at 4°C for a minimum of 24 hr
Bielanski, 1982	Morphologic features: normal sperm,>80%; droplets, <10%; tail loops, <3%; loose heads, 3%; and other abnormalities, <1%
Dowsett and Pattie, 1982	Minimal requirements: gel-free volume, 25 ml; sperm concentration, 20 million/ml; total sperm per ejaculate, 1.3 billion; total live sperm, 1.1 billion
Kenney, 1975	Minimal requirements for single ejaculate collected between April and July: total sperm, 4 billion; progressive motility, 60%; morphologically normal sperm, 60%; longevity of motility, raw semen maintain-10% progressive motility for 6 hours at 23°C
Kenney *et al.*, 1983	Minimal requirements for the second of two ejaculates collected an hour apart in each month of the year after 1 week of sexual rest: 1 billion morphologically normal, progressive motile sperm.

1.8.2 Microscopic examination of semen

A number of techniques have been developed to estimate sperm viability and membrane integrity. These techniques are used to examine unstained specimens with differential interference (DIC) microscopy or stained specimens using standard light microscopy.

The examination of unstained specimens under DIC optics was developed for assessing acrosomal damage to bull semen (Saacke and Marshall, 1968). The method involved the preparation of sperm suspensions by mixing a few drops of raw semen in buffered formol-saline or gluteraldehyde based fixatives. This type of microscopy reveals the structure of the acrosome and mid-pieces clearly, and has been used successfully for examining stallion spermatozoa. (Colenbrander *et al.*, 1992a). The main advantage of the method is that no artifactual damage is caused to the sperm membrane by the sample preparation method.

The nigrosin-eosin stain is the most common staining technique for distinguishing live and dead spermatozoa in a dried smear (Hancock, 1951; Campbell *et al.*, 1956; Dott and Foster, 1972). Other established staining methods include Blom's or Wells-Awa's procedure (Harasymowycz *et al.*, 1976), eosiniphilic staining (Johnson *et al.*, 1980) and Spermac stain (Oettle, 1986).

Other staining techniques were developed in order to distinguish the presence or absence of an acrosome. Giemsa stain has been used to detect changes in the acrosomes of ram spermatozoa (Watson, 1975). The dye combination 'napthol yellow S and erythrosin B' is reported to stain the acrosome but fails to distinguish live from dead spermatozoa (Bryan and Akruk, 1977). A triple stain technique, using Trypan blue, Bismark brown and Rose Bengal stains, was designed to evaluate the acrosomal status of live spermatozoa while differentiating live from dead spermatozoa (Talbot and Chacon, 1981).

Fluorescent probes have been used to evaluate plasma membrane integrity. One type detects intracellular acrosome-associated material and therefore requires that the cell be permeabilised before labelling. The second type can be use without permeabilising the cells. Lectins are used in the former manner, binding to glycoconjugates of the acrosomal matrix or outer acrosomal membrane (Saacke and

Marshall, 1968; Blanc *et al.*, 1991). Others used in this manner include dyes such as carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) (Garner *et al.*, 1986; Harrison and Vickers, 1990), certain monoclonal antibodies (Wolf *et al.*, 1985; Moore *et al.*, 1987; Blach *et al.*, 1989; Wockener *et al.*, 1992,) and Hoechst 33258 (Cross *et al.*, 1986).

The second group of probes consists of reagents used without permeabilising the cells; certain monoclonal antibodies (Saling and Lakoski, 1985; Saling *et* al., 1985; Blach *et al.*, 1989) and chlortetracycline (CTC) staining (Fraser and McDermott, 1992; Das Gupta *et al.*, 1993).

Transmission and scanning electron microscopy have been used in many studies of sperm morphology (Plummer and Watson, 1985; de Leeuw *et al.*, 1990; Magistrini and Palmer, 1991). While supplying much useful information, electron microscopy is a specialist technique with use confined to the research laboratory.

1.8.3 Biochemical examination

Cells with damaged membranes are not able to maintain the required intracellular concentrations of ions and solutes and lose essential metabolites and enzymes into the extracellular medium. Biochemical examinations entail measurement of the components of the entire intracellular or extracellular compartment. A measure of the distribution of hyaluronidase (Mancini *et al.*, 1964; Gould and Bernstein, 1973), acrosin (Johnson and Pursel, 1974; Church and Graves, 1976), arylsulphatases and aspartate-aminotransferase (Bower *et* al., 1973; Larsson *et* al., 1976; Moore *et* al., 1976; Colenbrander *et al.*, 1992b) and rate of influx of Ca^{2+} ions into spermatozoa can provide information on sperm membrane integrity. The enzyme asparatate aminotransferase, which plays a role in the biochemical processes connected with sperm motility, was evaluated as an indicator of stallion semen freezability (Kosiniak and Bittmar, 1991). They concluded that poor freezability of an ejaculate is related to high enzyme activity. Their study agreed with an earlier observation (Amann *et al.*, 1987) that asparatate aminotransferase concentrations may help determine the freezability of stallion semen.

1.8.4 Functional examination

Functional tests are usually performed to see if an intact plasma membrane is capable of carrying out normal function. Common methods of functional assessment are the hypo-osmotic swelling test (Check and Check, 1991; Colenbrander *et al.*, 1992a), zona pellucida binding (Berger *et al.*, 1989; Arns *et al.*, 1991; Yoshizawa *et al.*, 1994) and the zona-free hamster oocyte penetration test (Yanagimachi, 1984; Blasco, 1984) The latter test is potentially useful as an indicator of sperm fertility (Yanagimachi *et al.*, 1976; Berger and Parker, 1989) and it was found to be highly correlated with the fertility of bull spermatozoa used for artificial insemination (Davis *et al.*, 1987; Graham *et al.*, 1987). This test is said to monitor the ability of spermatozoa to undergo capacitation, the acrosome reaction and penetration of an egg (Yanagimachi, 1984). Brackett and co-workers first reported the penetration of zona free hamster oocytes by *in vitro* capacitated stallion spermatozoa in 1982 (Brackett *et al.*, 1982). More recent work using zona-free hamster oocyte penetration test as a means to assess sperm functional capacity has been reported by several groups (Graham *et al.*, 1987; Okolski *et al.*, 1987; Blue *et al.*, 1989; Sampar *et al.*, 1989; Zhang *et al.*, 1989; Padilla *et al.*, 1991).

1.8.5 Computerised motility analysis

Early studies of pattern and velocity of sperm motion were performed manually (Yanagimachi, 1970; Phillips, 1972). This involved examination of sperm preparations with dark field or dark medium-phase contrast objectives. Observations were recorded cinematographically; the film was then projected frame by frame and consecutive positions of individual spermatozoon drawn on paper. Tracings of the points showed the pattern of movement. This method was very time consuming. Since then, much progress has been made in the automation of these procedures. An immediate improvement over the manual method was the use of semi-automated systems. In essence, the semi-automated analysis was similar to the conventional method, in that sperm tracks were traced manually by the operator, but utilised a

digitising tablet, and usually video-recorded images or polaroid photographs system (Overstreet *et al.,* 1979; Katz and Yanagimachi, 1981). The digitiser relayed the measurements to a computer for data processing, which increased the speed of analysis. With the more recent computerised image analysis systems, image detection and data input are carried out automatically.

Several automatic computer-assisted systems (CASA) are currently available *e.g.* Cellsoft (Cryo Resources Ltd, New York, NY), CellTrak (Motion analysis Corp., Santa Rosa, CA), Hamilton-Thorne (Hamilton-Thorne Research Inc., Beverly, MA, USA), Autosperm (Amsaten, NVSA., Corp., Deprite, Belgium) and Hobson (Sense and Vision, Electronic System Ltd, Sheffield, UK) motility analysers.

Computerised sperm motility analysis (CASA) has been used to determine sperm motion characteristics in semen from human (Mack *et al.,* 1988; Mortimer *et al.,* 1988), bull (Budworth *et al.,* 1988), stallion (Amann, 1987; Jasko *et al.*, 1988; Blach *et al.*, 1989; Varner *et al.*, 1991; Palmer and Magistrini, 1992) and a range of laboratory species. Using CASA, several parameters can be analysed from the individual trajectory. Examples of these parameters are:

(i) curvilinear velocity (VCL, μms^{-1}) which is the total distance between each head point for a given cell during the acquisition period, divided by the time elapsed

(ii) straight line velocity (VSL, μms^{-1}) which is the straight line distance between the first point and the last head point divided by the acquisition time

(iii) average path velocity (VAP, μms^{-1}) a smoothed path constructed by averaging several neighbouring head positions (5 or 9 points) on the track and joining the averaged positions

(iv) amplitude of lateral head displacement (ALH, μm), intended to give a measure of sperm head oscillation and calculated from the maximum sperm head departure from the average path in μm, and since this represents the departure from the path in only one direction this figure is doubled to give the full width amplitude

(v) beat cross frequency (BCF, Hz), determined by measuring the frequency with which the sperm track crosses the average path in either direction

(vi) linearity (LIN, %) which measures departure from linear progression and is defined as (VSL/VCL)*100 with 100% representing an absolute straight track and

(vii) straightness (STR, %) measured using the ratio (VAP/VCL) * 100.

During incubation of human spermatozoa in synthetic media three distinct patterns of motility have been identified; (i) forward progressive or non-hyperactivated motility, essentially a forward straight progressive trajectory with uniform flagellar waves, (ii) hyperactivated or "star-spin" motility, characterised by a non-progressive trajectory with large flagellar waves, causing sperm cells to undergo successive rapid flexions and (iii) 'transitional' motility which is intermediate between the first two described (Burkman, 1984; Mortimer *et al.*, 1984).

Robertson *et al.* (1988) defined hyperactivated motility as sperm cells showing a curvilinear velocity (VCL) of $\geq 80\ \mu ms^{-1}$, a linearity (LIN) of $\leq 19\%$ and a dancemean (mean ALH/LIN x 100%) $\geq 17\ \mu m$. Burkman (1991) defined hyperactivation according to the criteria, VCL $\geq 100\ \mu ms^{-1}$, ALH $\geq 7.5\ \mu m$ and LIN $\leq$ 65%. This definition of Burkman is now widely used to identify hyperactivation, mainly because the Hamilton-Thorne motility analyser on which the definition was derived, is currently the most widely used analyser. This definition can be pre-programmed into an analyser, facilitating automatic identification and quantification of hyperactivated cells.

The use of CASA has overcome some of the long standing limitations of visual semen analysis, namely, observer bias and subjective judgement. CASA has also superseded the time consuming analysis using manual or semi-automated systems, allowing more fields and cells to be analysed per sample. However, it has become apparent that motility parameters vary depending on (i) the set-up parameters used (Knuth *et al.*, 1987) *e.g* the threshold grey level setting or brightness and velocity setting (Mack *et al.*, 1988), length of the trajectory analysed (Aanesen and Bendvold, 1990), sample chamber depth (Mack *et al.*, 1988), temperature and composition of medium (LeLannou *et al.*, 1992;), concentration of sample (Mortimer *et al.*, 1988; Mack *et al.*, 1989), viscosity (Suarez *et al.*, 1991), dilution rate (Farrell *et al.*, 1996) and video frequency used (Mortimer *et al.*, 1988; Mortimer and Swan, 1995; Morris *et al.*, 1996). These limitations can be overcome with careful instrument calibration, quality control and standardisation of protocol *e.g.* by defining a universally accepted value for normal and abnormal sperm motion, and establishing a standard of CASA practice and performance. The use of CASA in semen assessments should also be in corroboration with other techniques, as expression of motility is not

an entirely reliable parameter of sperm integrity.

1.9 Objectives of the Study

Research involving equine spermatozoa has generally been directed towards the practical improvement of techniques for preservation, focusing on areas such as extender formulation and cooling procedures. In comparison to other species, relatively little basic research has been carried out. For example there is no widely used *in vitro* fertilisation system; reports of successful IVF exist but are isolated (Palmer *et al.*, 1991). Consequently, basic functions such as capacitation and fertilisation have not been as intensively studied as they have in human, bovine or laboratory species. Nor has stallion sperm function been examined in detail after preservation in liquid, chilled or cryopreserved form. Given the paucity of basic information this study was undertaken to assess stallion sperm function under two sets of conditions (1) liquid storage at 5°C and (2) incubation under conditions which support capacitation.

Two aspects of sperm function were studied: motility, using a computerised motility analyser capable of objective and detailed analysis of movement patterns, and ability to regulate intracellular Ca^{2+}, an assessment of the functional ability of the sperm plasma membrane.

Further, the studies were designed to test the hypothesis that the ejaculate is composed of a heterogeneous cell population, within which there may be discrete subpopulations of physiological importance.

1.10 Structure of the Thesis

This thesis is divide into two experimental sections with the first, described in Chapter 4, investigating the effects of cooling and storage of stallion spermatozoa at 5°C, upon sperm function. The second experimental section, encompassing Chapters 5 and 6 are studies of aspects of sperm capacitation. Detailed discussion of the individual experiments is presented at the end of the appropriate chapters. An overall discussion is presented in Chapter 7.

CHAPTER 2

MATERIALS AND METHODS

2.1 Experimental Animals

Three pony stallions were used in the experiments presented in this thesis. One stallion, Max, was already trained to use the artificial vagina at the onset of this work. Max was an 18 year old Welsh pony stallion standing at 12.2 hands and a proven sire. Two other stallions were trained especially for the study. A 6 year old Dartmoor pony called Cheeko, standing at 12.2 hands, which had been running with mares prior to this work, as had Teddy, a 6 year old Shetland pony standing at 9.3 hands. The pony mares used for teasing prior to semen collection belonged to the Glasgow Veterinary School. From an original pool of seven, three were selected for repeated use on the basis of their temperament.

The stallions were housed in loose boxes throughout the study period. They were fed *ad lib* hay and water and depending on body condition received a ration of horse and pony cubes. Stallions were exercised in isolated paddocks when the weather permitted. Mares were housed in loose boxes and fed *ad lib* hay and water. The mares also received a ration of horse and pony cubes depending on body condition. During summer, mares were allowed to run on available paddocks.

In order to train the stallions to use the artificial vagina a mare in standing oestrus was used as a 'teaser' and a 'phantom' or dummy mare was used for the collection. The phantom mare comprised a railway sleeper secured onto two 6 foot fence posts with iron brackets. The ends and edges of the sleeper was covered with sections of steel radial tyres and padded with straw. This was then covered with canvas sheeting and secured firmly. The stallion was encouraged to mount the teaser mare, but the mare was positioned such that the body of the stallion was in contact with the phantom. At this point the penis was introduced into the artificial vagina. If the stallion dismounted the procedure was repeated until ejaculation occurred. Semen samples were analysed and new stallions were used for experiments after three successive collections of semen with good motility.

2.2 Semen Collection

Semen used in the experiments described in this thesis was acquired from either human and equine donors.

2.2.1 Human semen samples

Samples were obtained from healthy male volunteers. Fresh samples were collected by masturbation into sterile, wide-mouth plastic containers provided by the laboratory. Samples were allowed to liquefy for approximately 30 minutes at room temperature, and were processed immediately after liquefaction.

2.2.2 Stallion semen samples

Semen was collected using a Cambridge model artificial vagina (AV) (Figure 2.1). This AV comprised an outer and an inner rubber liner supported by a rigid plastic casing (Figure 2.2). The plastic casing and rubber liner together formed a water jacket. Before collection the AV was filled with hot water (>60°C), such that the luminal temperature was in the range 44°C to 48°C. The luminal pressure of the AV was adjusted to provide uniformly good contact around the penis, without interfering with penile penetration. This was achieved by filling the water jacket with hot water then pushing a hand through the AV to expel excess water through the filling cap. The inner liner was lubricated with non-spermicidal KY jelly, (Johnson and Johnson Ltd, Maidenhead, United Kingdom). The collecting receptacle was a clean plastic bag, fitted with a milk filter to strain the ejaculate, separating gel and minimising contamination with cellular and non-cellular debris. This collecting receptacle was kept warm in a cover. After collection the sample was extended in Kenney's medium at a ratio of 1:1.

Figure 2.1 Semen collection.

Figure 2.2 Cambridge model artificial vagina.

2.3 Visual Motility Assessment

2.3.1 Motility

After semen collection all samples were inspected visually for motility. Poorly motile samples were not used for experimental purposes. Where visual inspection was used within an experiment, sperm motility was assessed by microscopic examination of a drop of diluted semen at low power (100x) on an Olympus BH2 light microscope. The drop was examined using pre-warmed glassware, under a coverslip, on a heated microscope stage at 37°C. Vigour of the motility was scored on a scale of 0-4 as described below;

Score	Criteria
4	rapid motility, many forward progressive
3	rapid motility, mostly circling
2	sluggish motility, forward or circling progressive
1	sluggish motility
0	tail beating without motility

and the percentage of motile cells estimated to the nearest 10%. In order to avoid bias, two operators were involved in the motility assessment. The slides were prepared and randomised by the first operator and assessed by the second operator.

2.3.2 Assessment of "head to head" agglutination

'Head to head' agglutination or clumping was observed when stallion spermatozoa were extended and incubated in medium (Bavister TALP-Milk) supplemented with pentoxifylline. The agglutination was scored as described below:

- no clumping.
+ majority - sperm heads attached in pairs.
++ majority - between 2 and 5 sperm heads agglutinated.
+++ majority - more than 5 sperm heads agglutinated.

2.4 Medium

2.4.1 Kenney's medium

Kenney's medium is a non-fat, dried skimmed milk-glucose extender that consists of 2.4 g of non-fat dried skim milk (Sanalac™, Premier Beverages, Knighton Adbaston Stafford, UK), 0.272 M glucose, 2 ml of 7.5% $NaHCO_3$ and 2 ml of gentamycin sulfate (50 mg/ml) in 100 ml of millicure water (Kenney *et al.*, 1975). During preparation the antibiotic was added after mixing the other chemicals to prevent curdling of the milk due to the acidic nature of gentamycin. The final pH of this medium was 6.9.

2.4.2 Graham TALP

This medium was recommended by Dr J. K. Graham (personal communication). The optically clear nature of the medium made it suitable for the measurement of fluorescence intensity after Fura-2 'loading'. Graham's TALP medium comprised 37 mM NaCl, 10.6 mM KCl, 1.2 mM KH_2PO_4, 35.7 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.7 mM $CaCl_2.2H_2O$, 9.2 mM HEPES, 84.4 mM Fructose, 5.5 mM Glucose, 0.18 mM Na pyruvate, 0.37 millilitres Na lactate (60% syrup) and 3 mg/ml bovine serum albumin. The final osmotic pressure was 322 mOsmol/kg and the pH was adjusted to 7.3. Where required Graham's TALP was mixed 1:1 (v/v) with Kenney's medium.

2.4.3 Bavister TALP

The optically clear nature of this medium also made it suitable for the measurement of fluorescence intensity after Fura-2 'loading'. Bavister TALP medium consisted of 114 mM NaCl, 3.16 mM KCl, 2 mM $CaCl_2.2H_2O$, 0.5 mM $MgCl_2.6H_2O$, 10 mM Na lactate syrup (60%), 0.3 mM $NaH_2PO_4.H_2O$, 5 mM Glucose, 25 mM $NaHCO_3$, 10 mM HEPES, 0.25 mM sodium pyruvate and 6mg/ml bovine serum albumin (Bavister, 1989). The final osmotic pressure was 302 mOsm/kg and the pH adjusted to 7.3. Where required Bavister Milk medium was made by mixing Bavister TALP and Kenney's medium, 1:1 (v/v).

2.4.4 TALP-egg yolk medium

TALP-egg yolk medium was made by mixing Bavister TALP and TEST-yolk medium at 1:1 (v/v), as described in Meyers *et al.*, (1995). This medium was reported to support stallion sperm capacitation *in vitro*.

2.4.5 TEST-yolk medium

This medium was prepared as described in Bolanos *et al.*, 1983. It consisted of 188 mM N-Tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES), 824 mM hydroxymethyl aminomethane (Tris), 1.11 mM dextrose, 0.013 g streptomycine sulfate and 0.008 g Penicillin-G, dissolved in 50 ml of water twice distilled in glass. The yolk from a chicken egg was then added to make a 20% egg yolk solution. This medium was then centrifuged at 840*g* for 10 minutes, after which the pellet formed was discarded. The pH of the supernatent was adjusted to between 7.35 and 7.45 by the addition of Tris. The medium was then modified as in Meyers *et al.*, (1995), by heat treatment (56°C, 30 minutes) to inactivate the proteases, and by centrifugation (840*g*, 20 minutes) and filtration (Millel - HV disposable filter, Millipore (UK) ltd.) to remove the egg yolk granules.

2.4.6 Culture medium for human spermatozoa

Medicult culture medium number 1031 (Medicult Ltd, Copenhagen, Denmark) was used for human sperm incubations. It consisted of Earle's balanced salt solution, a defined synthetic serum replacement, 0.8 mM sodium pyruvate, 2.2 g/l sodium bicarbonate, 50 IU/ml penicillin, 50 μg/ml streptomycin and 1% human serum albumin with an osmolarity of 280 mOsm/kg and pH 7.4. This culture medium was routinely used at the Glasgow Royal Infirmary in the preparation of spermatozoa for IVF and associated techniques.

2.5 Tissue Culture Ware

Unless otherwise stated 14 ml polystyrene Falcon Grade centrifuge tubes (Falcon 2095, Becton Dickinson Labware, New Jersey) were used for all sperm preparation procedures such as centrifugation, swim-up preparation, Fura-2AM loading and incubation throughout the study. Flat bottom, tissue culture treated polystyrene cell wells (Cell Wells, Corning, Corning Glass Works, Corning, New York) were used for incubation of stallion semen in Chapter 5, section 5.2.1.

2.6 Chemicals

2.6.1 **Pentoxifylline** [1-(5-oxyhexyl)-3-7-dimethylxanthine]

A stock solution of pentoxifylline (Sigma Chemical Company, Poole, Dorset, England) was made by dissolving 20 mg of pentoxifylline in 10 ml Bavister TALP to give a concentration of 2 mg/ml. This solution was further diluted in medium to give

the concentrations required in individual experiments. Fresh solutions were made on the day of use.

2.6.2 **Progesterone** (4-Pregnene-3, 20-dione)

A stock solution was made by dissolving 1 mg of progesterone (Sigma Chemical Company, Poole, Dorset, England) in 1 ml of dimethylsulphoxide (DMSO; Sigma Chemical Company, Poole, Dorset, England) to give a concentration of 1 mg/ml and stored at -70°C in eppendorf tubes. The stock solutions were further diluted as described for individual experiments.

2.6.3 **Ionophore A23187**

A stock solution was made by dissolving 10 mg of Ionophore A23187 (Sigma Chemical Company, Poole, Dorset, England) in 5 ml of DMSO. This was stored as 50 µl aliquots in foil-wrapped eppendorf tubes at -70°C. This stock solution was diluted in TALP medium to a working concentration of 0.5 µM immediately before use.

2.6.4 **Fura-2AM** ({1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-2'-amino-5'-methylphenoxyethane-N,N,N'N' tetraaceticacid pentaacetoxymethyl ester})

A stock solution of Fura-2AM was made by sonicating 0.25 g Pluronic F127 (Molecular Probes Inc., Eugene, USA) in 1 ml of DMSO until the detergent dissolves completely. One ml of this solution was then transferred into a light-shielded bottle containing Fura-2AM (1000 µg; Calbiochem-Novabiochem (U.K) Ltd, Beeston, Nottingham), mixed and dispensed into eppendorf tubes in 50 µl aliquots of 1 mM Fura-2AM. These tubes were stored at -70°C, in the dark, and thawed individually for use.

To form the working solution 50 µl aliquots of stock solution were mixed with 5 ml of medium (Graham's TALP or Bavister TALP) to give a working Fura-2AM concentration of 10 µM. This was mixed 1:1 with sperm suspension.

2.7 Cooling of Semen

A programmable freezing unit (Kryo 10 Series, Planer Biomed, Planer Products Ltd, Sunbury-ON-THAMES, England) (Figure 2.3) was used to cool semen samples. Plastic straws of 0.5 ml capacity were filled with sperm suspension, sealed with P.V.P powder (IMV, rue Clemenceau, Casou, France) and maintained in a water bath at 37°C. The straws were then transferred onto prewarmed racks which were placed in the cooling chamber of the programmable freezer. The machine was programmed to lower the temperature from a selected starting temperature to 5°C at a rate of 0.05°C per minute. Straws were then removed from the straw racks, immersed in a waterbath and maintained at 5°C in a refrigerator for up to 72 hours.

2.8 Semen Preparation Methods

2.8.1 Sperm concentration

Sperm concentration was determined using a haemocytometer as described in the World Health Organization Laboratory Manual (WHO, 1987) and adjusted as required for individual experiments by centrifugation (300*g* for 10 minutes) and resuspension in the appropriate medium. For counting, semen samples were diluted 1:10 in a counting solution consisting of 5 g of sodium bicarbonate and 5 ml of formalin in 500 ml of distilled water. The sample was mixed thoroughly and 10 µl aliquots placed in each counting chamber and allowed to settle for 1 minute. The

spermatozoa in 5 large squares were counted in each chamber and, in order to determine the concentration of the original sample in millions/ml the sperm count was divided by two.

2.8.2 Swim-up migration

The swim-up migration was performed by layering 500 μl of liquefied semen (human) or extended semen (stallion) at the bottom of a 14 ml test tube containing 1 ml of culture medium, using a 1cc syringe and 18G needle. After 45 minutes of incubation at 37°C in 5% CO_2 in humidified air, the upper layer was carefully removed without disturbing the interface.

2.8.3 Removal of seminal plasma by centrifugation

Unless otherwise stated, for the majority of stallion sperm experiments, seminal plasma was removed by centrifugation at 300*g* for 5 minutes after 1:1 dilution in Kenney's medium. Seminal plasma was removed and the pellet re-suspended in medium. Removal of seminal plasma by centrifugation through discontinuous Percoll gradients is described in detail in Chapter 3.

Figure 2.3 Programmable freezing unit (Kryo 10 Series).

Figure 2.4 Hamilton-Thorne motility analyser set-up with video and computer.


REPROD.
KRYO 10 SERIES
PLANER
REPROD.
REPROD.
LAB
SERIES

2.9 Viability Staining

Sperm viability was assessed using either an eosin-nigrosin stain (Hancock, 1951) or the dual staining method described below.

2.9.1 Eosin-nigrosin staining

The eosin-nigrosin stain consisted of 0.67 g of eosin Y and 10 g nigrosin dissolved in 100 ml of water. The stain was mixed with an equal volume of sperm suspension for 1 minute. A drop was then transferred to an alcohol-cleaned slide, smeared and allowed to air dry. Slides were randomised and at least 100 spermatozoa were examined on each slide under oil immersion. Unstained spermatozoa were classified as 'live' and those showing any degree of pink, indicating that the stain had permeated the plasma membrane, were classified as 'dead'.

2.9.2 Dual staining with Chlortetracycline and Hoechst 33258

A stock solution of Hoechst 33258 (2'-[4-Hydroxyphenyl]-5-[-4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole); (Sigma Chemical Company, Poole, Dorset, England) was prepared by dissolving 4 mg of Hoechst 33258 in 1 ml of millicure water. Aliquots (10 µl) were then stored in foiled wrapped eppendorfs at -70°C. A working solution was prepared by diluting stock solution in 10 ml of medium (Medicult for human, TALP for stallion) in foiled wrapped tubes. For experiments using human spermatozoa the medium used was Medicult. TALP was used for experiments with stallion spermatozoa.

Phosphate buffered saline (PBS) containing 2% polyvinylpyrrolidone-40 (PVP-40, Sigma Chemical Company, Poole, Dorset, England) was prepared by mixing 2 g PVP-40 in 100 ml PBS and adjusting the pH to 7.4. This solution was stored as 10 ml aliquots in Falcon grade tubes at -70°C and warmed to 37°C prior to use.

Chlortetracycline buffer was prepared by mixing 20 mM Tris

(hydroxymethyl)methylamine[2-amino-2-(hydroxymethyl)propane-1,3-diol]), 130 mM NaCl and 5 mM cysteine at pH 7.8 and stored in 50 ml aliquots at -70°C. Chlortetracycline powder (CTC; $C_{22}H_{23}ClN_2O_8$.HCl; Sigma Chemical Company, Poole, Dorset, England) was weighed into tin foil packets in portions of 19.5 mg and stored at -70°C. The working stain solution was prepared by adding the contents of a foil packet to 50 ml buffer.

Paraformaldehyde fixative was prepared by adding 1.25 g paraformaldehyde (Sigma Chemical Company, Poole, Dorset, England) to 10 ml of 0.5 M Tris-HCL buffer at pH 7.4 to give a 12.5% solution. Aliquots of 1 ml were stored at -70°C. DABCO (1,4-Diazabicyclo[2.2]octane) fluorescence retarder was prepared by adding 0.2469 g of DABCO to 10 ml of glycerol to give a final concentration of 0.22 M and stored as 1 ml aliquots at -70°C.

Staining Method

One aliquot of Hoechst 33258 (H258) stock solution was diluted in Bavister TALP medium to give a working concentration of 4 μg/ml. This working solution was kept foil wrapped and held at the same temperature as the samples. To stain the sperm cells a 50 μl sample of sperm suspension was mixed with an equal volume of H258 solution for 2 minutes. The sperm suspension was than washed free of dye by centrifugation through 1.5 ml of 2% PVP-40 in PBS at 900*g* for 5 minutes. The supernatant was removed and the pellet resuspended in 20 μl of medium. This solution was mixed with 20 μl of CTC working solution for 30 seconds. After 30 seconds, 1.6 μl of 12.5% paraformaldehyde and 5 μl of DABCO were added. A 5 μl aliquot was then placed on a clean warm slide, and a coverslip carefully applied. The coverslip was squashed gently to exclude extra liquid, and the edges sealed with nail varnish. Slides were stored in a light-protected container and evaluated immediately or up to 2 days later if kept at -70°C, protected from light.

2.9.3 Computerised motility analysis

2.9.3.1 Cell chambers

Aliquots of sperm suspension (5 µl) were used to make preparations of 32 µm depth as described by Mack *et al.* (1988). Cell chambers were made by applying dry transfer circles (13 mm diameter, Chartpak # 49, Leeds, MA 01053-9732) to microscope slides, previously cleaned with absolute alcohol and layered with 10% bovine serum albumin (BSA), for the analysis of stallion spermatozoa, or 1% collodion nitrocellulose in amyl acetate for the analysis of human spermatozoa. The latter step was designed to minimise sperm adhesion to glass. To ensure correct chamber depth, 5 µl of the sample was placed in the middle of the circle and a coverslip applied to force the contents to fill the chamber and spill over the sides of the O-ring.

2.9.3.2 Recording procedure

For each experiment, recordings of at least 200 spermatozoa from two or more slides were made on a video recorder (Ferguson Videostar Model FV 32L). Videotapes were stored for subsequent analysis.

2.9.3.3 Computerised analyser

The Hamilton Thorne Motility Analyzer model 2030, version 7.1, (Hamilton-Thorne Research Inc., Beverly, MA, USA) was used to measure motility parameters (Figure 2.4). The analyser comprised an internal microscope and an automated heated microscope stage, which positioned the sample while maintaining a user defined temperature. The optical assembly used phase contrast optics. Images were acquired by a CCD sensor and digitised. The digitised image was then analysed by a microprocessor and displayed on a colour graphics monitor.

2.9.3.4 Parameter settings

The parameter setting used for both human and stallion spermatozoa throughout this study are summarised in Table 2.1. The parameters for human sperm were determined previously (Kay *et al.*, 1993). Derivation of parameters for stallion sperm is described in Chapter 3. For each sperm track a set of movement characteristics was determined. These were amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), curvilinear velocity (VCL, μms^{-1}), average path velocity (VAP, μms^{-1}) and straight line velocity (VSL, μms^{-1}). Individual cell data generated by the HTM analyser were captured by a portable personal computer (Toshiba T3100) using a HDATA software (HDATA Communications Package for HTM motility analyser). Descriptions of these movement parameters are given more fully in Chapter 1. Using visual assessment and CASA systems it had been possible to identify three general patterns of stallion sperm motility similar to that reported for human spermatozoa (Robertson *et al.*, 1988):

1) Forward progressive or non-hyperactivated motility involves an essentially straight progressive trajectory with uniform flagellar waves (Figure 2.5)

2) Transitional phase motility also involves an essentially straight progressive trajectory, though flagellar waves are larger and irregular (Figure 2.6)

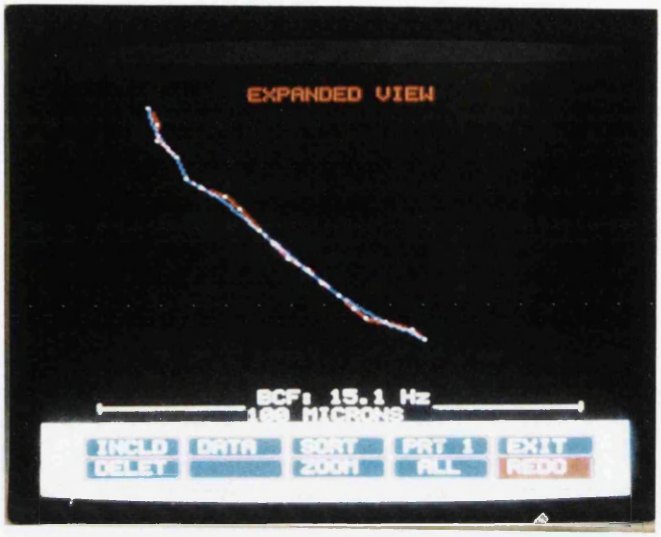
3) Hyperactivated or 'star-spin' motility is characterised by non-progressive trajectory with large flagellar waves, which cause the cells to undergo successive rapid flexions (Figure 2.7)

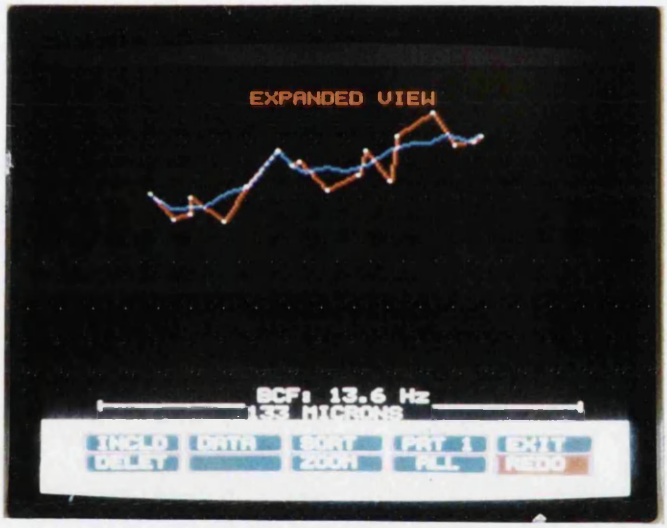
Initial measurements of hyperactivated motility were made using the criteria of Burkman (1984) i.e. ALH > 7.5 μm, VCL > 100 μms^{-1} and LIN < 65%.

Figure 2.5 Spermatozoal motility pattern - forward progressive.

Figure 2.6 Spermatozoal motility pattern - transitional progressive.

Figure 2.7 Spermatozoal motility pattern - hyperactivated.


EXPANDED VIEW
BCF: 15.1 Hz
INCLD
DATA
SORT
PRT 1
EXIT
DELET
ZOOM
ALL
REDO


EXPANDED VIEW
BCF: 13.6 Hz
INCLD
DATA
SORT
PRT 1
EXIT
DELET
ZOOM
ALL
REDO

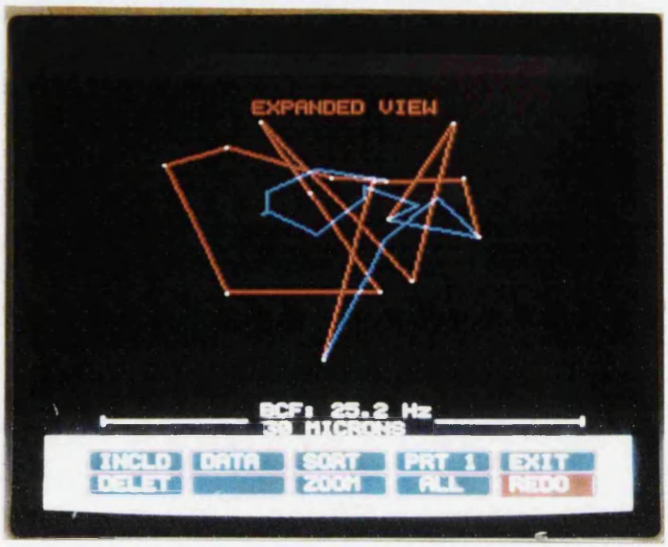

EXPANDED VIEW
BCF: 25.2 Hz
INCLD
DATA
SORT
PRT 1
EXIT
DELET
ZOOM
ALL
REDO

Table 2.1. Machine settings used for motility analyses performed using the Hamilton-Thorne motility analyser. The derivation of parameter settings for stallion spermatozoa is described in Chapter 3.

Parameter	Human	Stallion
Temperature	37°C	37°C
Image type	Phase contrast	Phase contrast
Field Selection	Manual	Manual
Calculate ALH	yes	yes
Frames (min)	13	13
Frames (max)	20	20
Acquisition rate	25 Frames/sec	25 Frames/sec
Minimum contrast	6 pixels	5 pixels
Minimum size	6 pixels	4 pixels
Low size gates	0.4	0.5
High size gates	1.6	1.2
Low intensity gates	0.5 contrast units	0.5 contrast units
High intensity gates	2.0 contrast units	1.2 contrast units
Non motile head size	8 pixels	12 pixels
Non-motile intensity	201	170
Magnification factor	2.17	2.17
Minimum VCL (μms^{-1})	0 μms^{-1}	40 μms^{-1}
Minimum VAP (μms^{-1})	5 μms^{-1}	30 μms^{-1}
Minimum VSL (μms^{-1})	0 μms^{-1}	20 μms^{-1}

2.9.4 Intracellular calcium measurements using Fura-2AM

2.9.4.1 Loading sperm cells with intracellular dye - 'Fura loading'

In order to measure intracellular free calcium the sperm concentration was adjusted to 100 million sperm per ml and aliquots of 2 ml were prepared. To each aliquot was added an equal volume of 0 or 10 μM Fura-2AM in medium. The aliquot containing 0 μM Fura-2AM served as a control sample for sperm autofluorescence. Experimental samples contained a final concentration of 5 μm Fura-2AM in a volume of 4 ml. This suspension was incubated in a foil wrapped test tube at 37°C for 30 minutes to allow Fura-2AM to penetrate into cells. At the end of the incubation period, samples were centrifuged (300*g*, 10 minutes) and the supernatant discarded to remove extracellular dye. The pellet was then re-extended in either Graham TALP or Bavister TALP medium to give a final sperm suspension of 50 million/ml in a 4 ml cuvette.

2.9.4.2 Intracellular calcium measurement - population studies

The fluorescence intensity of sperm samples within a cuvette was measured using a dual-wavelength spectrofluorimeter (diffraction grating monochromator, Perkin Elmer LS-3B, Perkin-Elmer Corporation, Norwalk, USA; Figures 2.8 and 2.9). Cuvettes were placed into a sample chamber which was maintained at 37°C by a circulating water jacket. Sperm suspensions were stirred continuously by a magnetic flea in the cuvette. The excitation wavelengths of the spectrofluorimeter were set to 340 and 380 nm, respectively, and the emitted fluorescence was recorded at 510 nm. The machine was set to make measurements of intensity (f) at a rate of 1 reading every 3.5 seconds alternately for each of the excitation wavelengths (f_{340} and f_{380}). Prior to reading each experimental sample, background intensity was established by reading the autofluorescence control at 3.5 second intervals for approximately 1 minute. The period of reading of experimental samples is detailed in the appropriate Chapter.

The ratio of the fluorescence intensities (R), was obtained after first

subtracting background intensity (sperm autofluorescence) as shown in the following formula;

$$R = \frac{(f_{340(loaded)} - f_{340(unloaded)})}{(f_{380(loaded)} - f_{380(unloaded)})}.$$

Intracellular free Ca^{2+} was calculated by incorporating the ratio of intensity from the experiment into the equation described by Grynkiewicz *et al.*, (1985). The dissociation constant K_d of 225 nmol l^{-1} and $\beta = 7.3$ are standard calibration factors.

$$[Ca^{2+}]_i = \frac{(R - R_{min})}{(R_{max} - R)} * Kd * \beta$$

R_{max} of 16.3 and R_{min} of 0.81 are values related to maximum and minimum intracellular calcium from previous calibration. R_{max} was obtained by adding 8 μM ionomycin, a calcium ionophore, to cell suspension with known concentration of extracellular calcium (1.8 mM), to saturate the dye within the cell; this was followed by adding 40 mM EDTA to sequester all calcium, hence R_{min} is the limiting value that R can have at zero $[Ca^{2+}]$.

For example; if the fluorescence intensity after subtracting background intensity is 579 and 346 at 340 nm and 380 nm respectively, then the ratio of intensities (derived by dividing 579 by 346) is 1.67. This value of 1.67 is then incorporated into the formula, thus;

$$[Ca^{2+}]_i = \frac{(1.67 - 0.81)}{(16.3 - 1.67)} * 225\,nM * 7.3$$

$$= 97\ \text{nM}$$

2.9.4.3 Intracellular calcium measurement - single cell studies

The measurement of the intracellular free calcium of individual sperm cells was performed using an inverted fluorescence microscope (Nikon Diaphot, Tokyo,

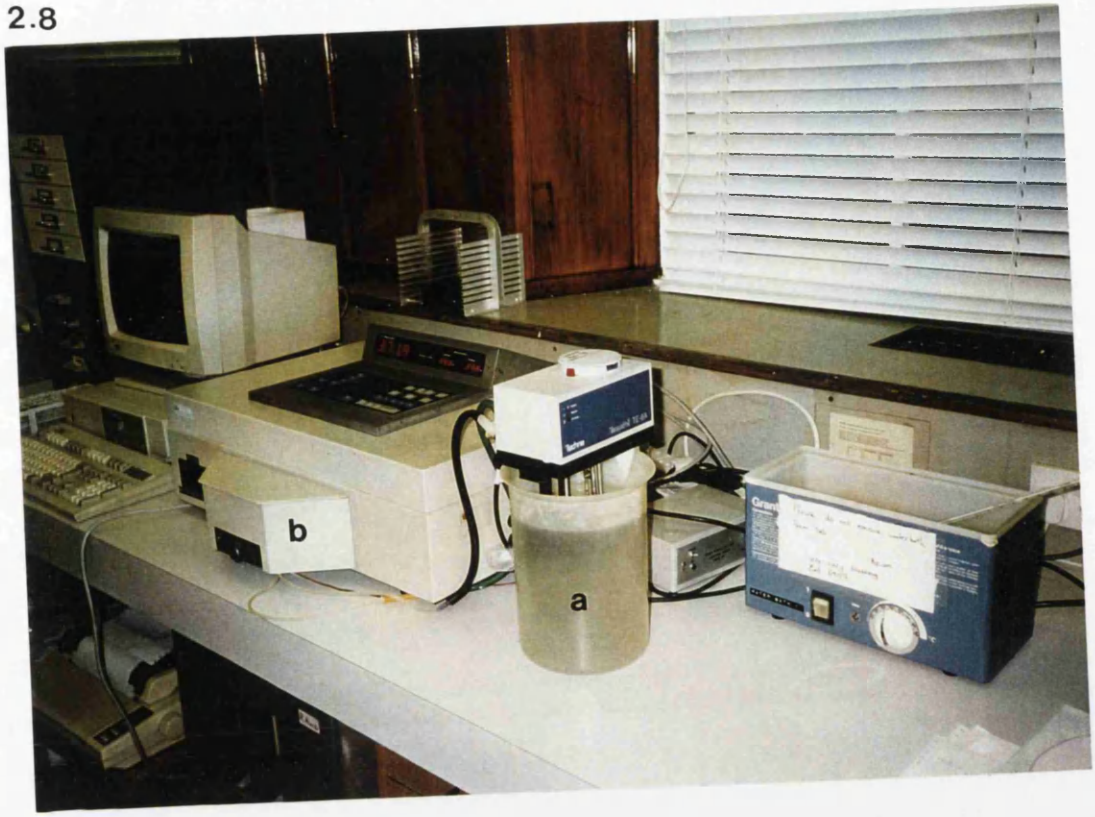
Japan). A schematic diagram is shown in Figure 2.10. An intense broad-spectrum light was supplied by a xenon lamp (a) and a low voltage shutter (b) limited the illumination time by allowing illumination to be turned on and off without the need to reignite the lamp. A spinning wheel (c) (Cairn Research, Sittingbourne, Kent) equipped with six filters, all having half-bandwidths of 10 nm and alternated at 120 Hz, reduced the spectrum to narrow bands and allowed rapid sequential measurement at two (or more) excitation wavelengths. After passing through the filter, the excitation light was reflected by a dichroic mirror (d). This had a half-pass wavelength of 400 nm for Fura-2. The reflected light was focused onto the preparation by the 100x Fluor objective of the inverted microscope (e). The longer wavelength light emitted by the fluorescent indicators was transmitted by dichroic mirror. The light path passed a sliding mirror (f) which directed the light to the eye-pieces of the microscope (g) or allowed it to pass through an adjustable rectangular diaphragm (h) which could be positioned to ensure that light only from the region of interest was measured. The light then passed through a short pass filter (half pass wavelength 700 nm) mounted at 45° which allowed light emitted by the fluorescent indicator to go to the photomultiplier (j) and ensured that the much longer visible wavelength light from the normal microscope illumination did not reach the photomultiplier but was directed to the video camera (k) used for monitoring the sperm and adjusting the diaphragm. The rapidly switched signals from the photomultiplier were integrated using timing pulses derived from the spinning wheel. An output voltage corresponding to intensity ratio was produced with a hardware divider circuit. Both intensity signals and the ratio signal were recorded on paper chart for later analysis.

2.9.4.4 Preparation of sperm suspension and slide chamber for single cell measurements

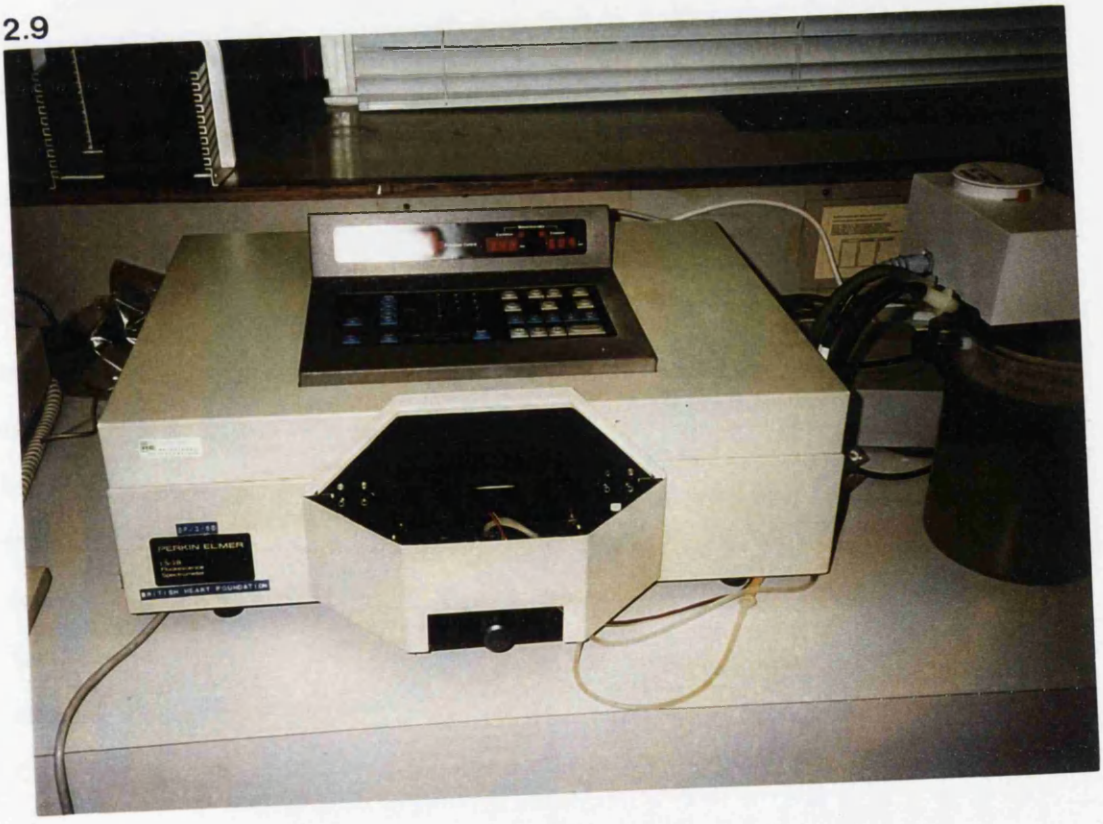
Preliminary experiments were required to establish a means of immobilising spermatozoa and a protocol for the measurement of intracellular calcium. This process is described in Chapter 3.

Figure 2.8 - 2.9 Perkin - Elmer LS-3B dual wavelength spectrofluorimeter with water bath (a) set in order to regulate temperature of cuvette held in the cuvette housing (b) at 37°C.

2.8



2.9



2.10 Radioligand Binding Assay Studies - Principle and Procedure

The principle of this assay is that radioligand will bind to both the receptor and non-receptor sites. Binding to a receptor site occurs in a saturable manner whereas binding to non-receptor sites is in a less saturable manner. Thus, the amount of isotope adhering to tissue in the absence of unlabelled radioligand represents the *total binding* (receptor + non-receptor). When tissues are incubated in a fixed concentration of radiolabelled ligand in the presence of a saturating concentration of unlabelled ligand, the unlabelled ligand will selectively inhibit radiolabelled binding to the ligand but not influence binding to other membrane constituents. This represents the *blank.* The difference between total and blank is taken as a measure of the isotope attached to the receptor (Enna, 1985; Motulsky, 1995).

The radiolabelled ligand used in these studies was [1,2,6,7-^{3}H] progesterone (Amersham, Life Science, Amersham International, Little Chalfont, Bucks, England). The stock radioactive concentration was 1.0 mCi/ml which was further diluted in ethanol to give a concentration (expressed as counts per minute) of 18,250 cpm/µl. This was stored at -79°C in a freezer used for storing radioactive material only.

The binding assay involved layering a 20 µl aliquot of radiolabelled progesterone onto 20 µl of DMSO in 1.5 ml eppendorf tubes, and air drying the mixture for 30 minutes to saturate the DMSO with labelled ligand. This was carried out for both total and blank designated tubes. One hundred microlitres of sperm suspension was then added to the total binding and the non-specific binding tubes. In addition, 10 µl of 1 mg/ml unlabelled progesterone was added to the non-specific binding tubes to give a final concentration 3.18 µM. The tubes were mixed well and incubated at 37°C for 45 minutes. At the end of the incubation period, the tubes were centrifuged (Jouan microcentrifuge, Model A14, Saint-Herblain, France) at 14000 G for 5 minutes. After centrifugation the supernatant was discarded and the pellet re-extended in 100 µl of medium. Centrifugation was repeated and the pellet formed by this second washing step re-extended in 50µl medium, transferred into scintillation vial inserts (Packard Instrument Company, Meriden) and topped up to 4 ml with

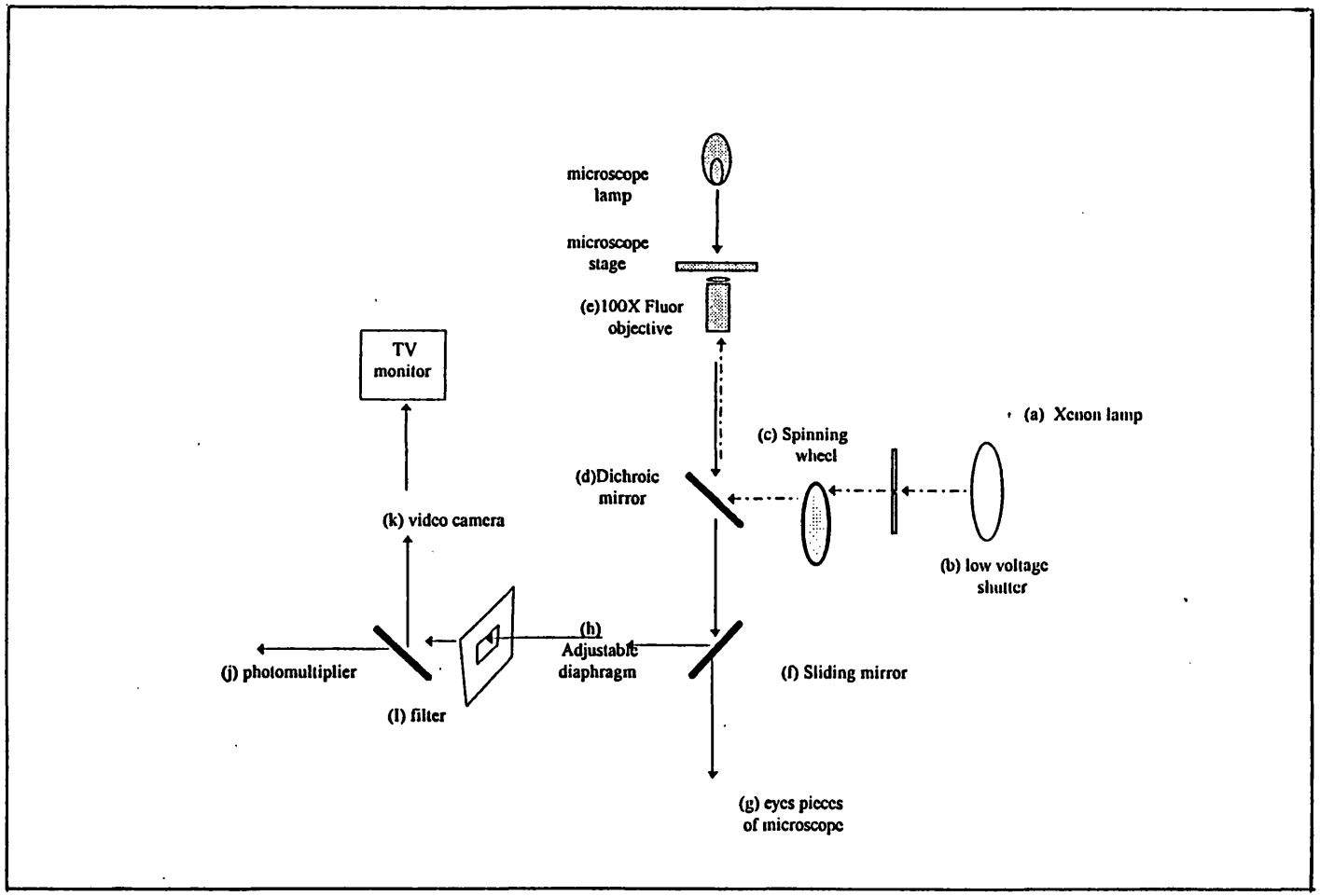
scintillation medium (Ecoscint A, National Diagnostics, Atlanta, Georgia, 30336). The radioactivity in the tubes was read using a Liquid Scintillation Analyser (Model 1600TR, Packard Instrument Company, Meriden). The same procedure was used for human and stallion spermatozoa.

2.11 Statistical Analysis

Statistical analysis was carried out using the statistical package GraphPad InStat. When comparing data from populations with equal standard deviation (SD) an ordinary Analysis of Variance (ANOVA) was performed. When comparing data from population exhibiting different SD, a Kruskal Wallis non-parametric ANOVA was performed. With only two sets of data, a Student's t test was performed. Conversely, an alternate Welch t test was carried out if SD of the two set of data were significantly different. Where appropriate a correlation analysis was performed to examine the nature of the relationship between variables.

Figure 2.10 A schematic diagram of the set-up for measurement of intracellular calcium in single sperm cell.

Figure 2.10: A schematic diagram of the set-up for measurement of intracellular calcium in single sperm cell.



CHAPTER 3

DEVELOPMENT OF METHODS

3.1 Introduction

The experiments reported in this Chapter were performed to validate techniques which had not previously been evaluated for use with stallion spermatozoa or were modified for use in this study.

3.2 Validation of the Hamilton-Thorne Computerised Motility System for the Analysis of Stallion Spermatozoa

Three experiments were conducted to validate the Hamilton-Thorne system for analysis of equine spermatozoa. It was necessary to optimise the detection of motile spermatozoa by the appropriate setting of the "Main Gates" (Experiment 1). Setting the "Main Gates" involved modifying certain parameters in order to optimise the correct identification of motile spermatozoa and minimise misidentification of debris as spermatozoa. The "Main Gate" parameters were the size of the object, in pixels, and contrast of the object, in arbitrary units of brightness above background intensity. It was also necessary to set "Lo/Hi" size gates, which set size limits and "Lo/Hi" intensity gates which set intensity limits for immotile cells. Default values were used to define immotile cells if there were fewer than five motile cells in a field.

Having selected the appropriate "Main Gate " settings, minimum velocity thresholds were imposed to minimise the mis-identification of moving particles as motile cells (Experiment 2). Finally, the repeatability of the analysis was tested (Experiment 3). These experiments are described below.

3.2.1 Experiment 1 - Setting "Main Gates" for equine sperm motility analysis

Semen from a single ejaculate was used in this experiment. Collection of semen was as described in Chapter 2. The concentration was determined using a haemacytometer and semen extended in Kenney's medium to a final concentration of 25 x 10^6 / ml. At this concentration between 5 and 25 spermatozoa were visualised on the Hamilton-Thorne screen per field. Five microlitre samples were loaded into slide chambers as described in Chapter 2 and placed onto the slide stage of the Hamilton-Thorne H2030 unit. The digitised image of sperm motion was observed on the screen of the analyser, and recorded onto a video tape for repeated analysis.

A range of settings were examined and these settings are tabulated in Table 3.1. Approximately 1000 sperm cells were examined for each setting using the video PLAYBACK facility on the Hamilton-Thorne unit. For this experiment velocity parameters were set at minimum values. On digitising the image the centroid of sperm cells is marked with a red point if motile or a blue point if immotile. The number of cells correctly marked, incorrectly marked or omitted from the analysis were recorded.

Table 3.1: Summary of the "Main Gate" settings examined in Experiment 1.

Parameter Set-up	1	2	3	4	5
Motile Cells					
Minimum size	5	5	4	4	6
Minimum contrast	4	5	6	5	5
Immotile Cells					
Minimum (LO) size	0.5	0.5	0.5	0.5	0.4
Maximum (HI) size	1.2	1.2	1.2	1.2	1.8
Minimum (LO) intensity	0.5	0.5	0.5	0.5	0.3
Maximum (HI) intensity	1.2	1.2	1.2	1.2	1.5
Default size	12	12	12	12	11
Default intensity	170	170	170	170	175

3.2.1.1 Results

Numbers of spermatozoa which were correctly marked, mismarked either as immotile or incorrectly identified as motile, motile but omitted and immotile but omitted are summarised in Table 3.2. Overall, Setting 5 generated the lowest number of total errors when motile and immotile cells were considered (6.3%). Setting 4, however, generated the lowest number of errors involving motile spermatozoa, i.e. incorrectly marked immotile and missed motile and omitted (3.2%). This parameter set-up was selected for use in all subsequent stallion semen motility analysis presented in this thesis.

Table 3.2: Summary of the type and percentage of errors observed for each of the settings evaluated.

Set Classification	1	2	3	4	5
correctly identified	1007	1012	1012	884	942
incorrectly marked immotile	102	140	202	14	6
missed motile	3	1	6	14	42
missed immotile	30	6	76	47	11
% error	13.4	14.5	28.1	10.3	6.3
% incorrectly marked immotile and missed motile	10.2	13.9	20.5	3.2	5.1

3.2.2 Experiment 2 - Establishing lower velocity thresholds to minimise the misidentification of immotile cells as motile

It was apparent from Experiment 1 that misidentification of immotile cells as motile or classification of debris as motile cells was a considerable problem. One hundred of these incorrectly identified cells were counted using the video PLAYBACK facility and EDIT TRACK module which displayed motility measurements for each individual cell. Data were recorded onto a personal computer

linked to the HTMA and the velocity distribution of the curvilinear, average path and straight-line velocities (VCL, VAP, VSL; μms^{-1}) of these cells were plotted.

3.2.2.1 Results

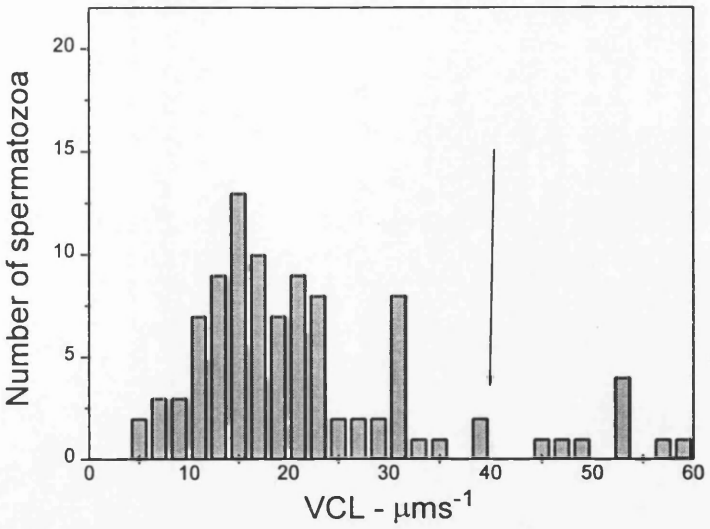
Velocity distribution plots are shown in Figures 3.1a-c. It was observed that 91% of moving debris and cells showed a VCL of $< 40\ \mu ms^{-1}$, 95% a VAP $< 30\ \mu ms^{-1}$ and 96% a VSL $< 20\ \mu ms^{-1}$.

Figure 3.1a: Distribution plot of curvilinear velocity (VCL) of immotile cells or debris misidentified as motile cells. The 91% of cells had aVCL of less than 40 μm/sec.

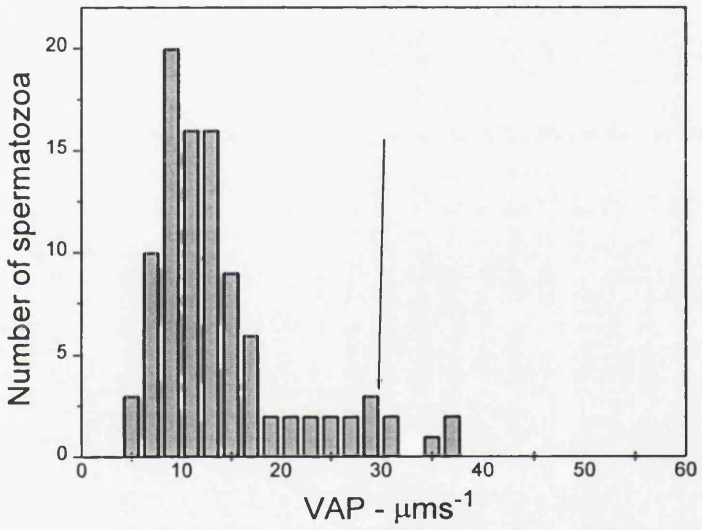
Figure 3.1b: Distribution plot of average path velocity (VAP) of immotile cells or debris misidentified as motile cells. The 95% of cells had a VAP of less than 30 μm/sec.

Figure 3.1c: Distribution plot of straight line velocity (VSL) of immotile cells or debris misidentified as motile cells. The 96% of cells had a VSL of less than 20 μm/sec

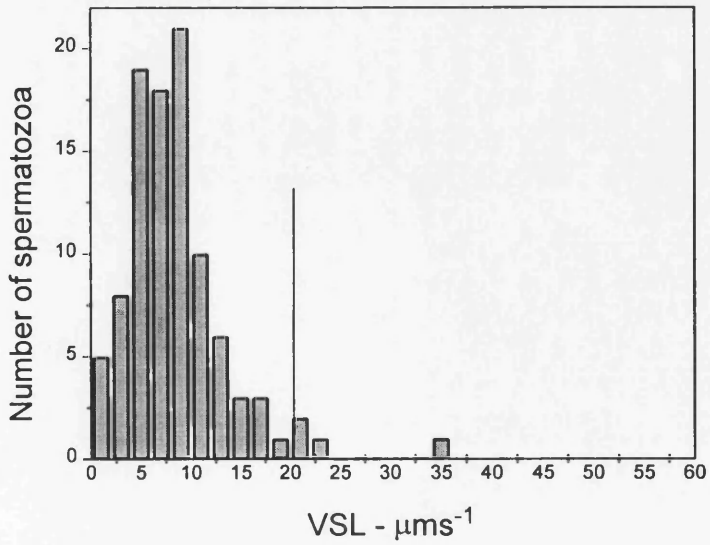
3.1a


Number of spermatozoa
0
5
10
15
20
0
10
20
30
40
50
60
VCL - μms-1

3.1b


Number of spermatozoa
0
5
10
15
20
0
10
20
30
40
50
60
VAP - μms-1

3.1c


Number of spermatozoa
0
5
10
15
20
0
5
10
15
20
25
30
35
40
45
50
55
60
VSL - μms-1

3.2.3 Experiment 3 - Testing the effect of imposing low velocity thresholds

Using the low velocity thresholds derived in section 3.2.2, sections of videotape were analysed with or without the implementation of the thresholds. Approximately 200 cells were analysed for each parameter set-up.

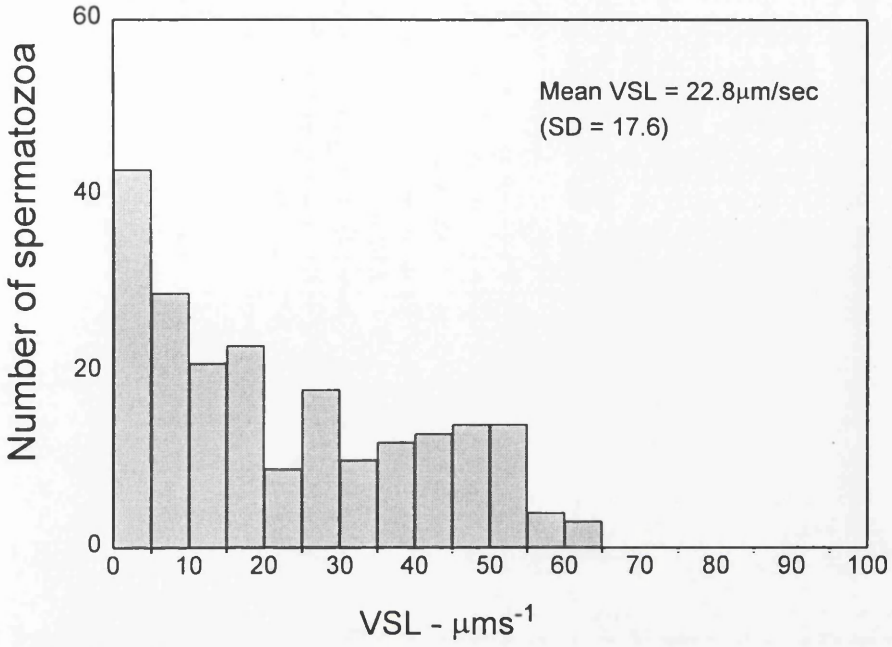
3.2.3.1 Results

Distribution plots were constructed for VCL, VAP and VSL for the first 200 cells analysed on a section of videotape. When low velocity thresholds were not omitted 55, 51 and 54% of cells fell into the low velocity categories for VCL, VAP and VSL, respectively. Visual inspection confirmed that this population of cells consisted of 'moving' immotile cells and debris or cells stuck to glass and showing very poor motility. These cells were eliminated from analysis by the application of the low velocity thresholds. Mean values of VCL, VAP and VSL increased because immotile cells were eliminated from the analysis. Velocity distribution plots for VSL with and without thresholds are shown in Figure 3.2a-b.

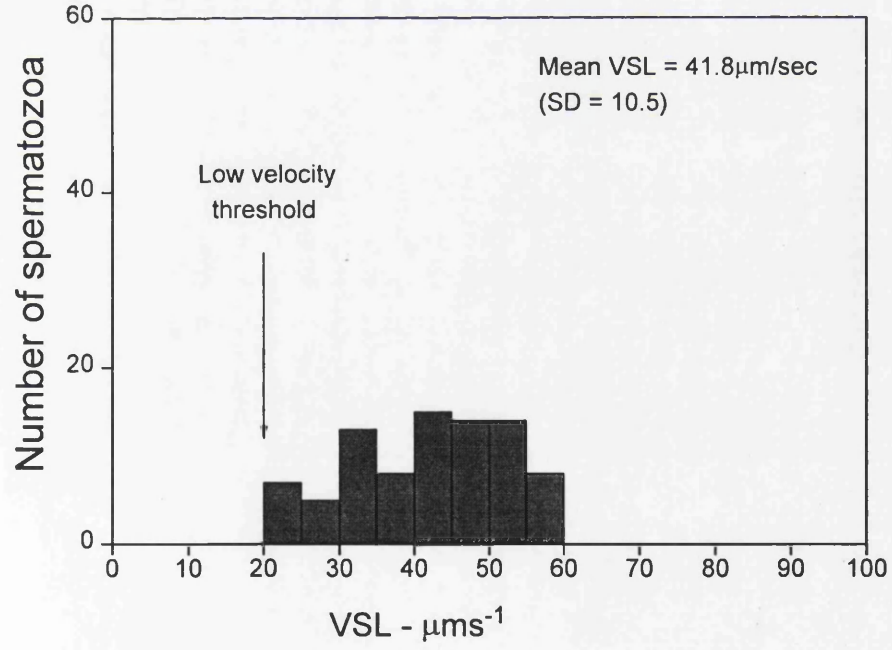
Figure 3.2a: Distribution plot of straight-line velocity (VSL) for 200 cell analysed before the application of low velocity thresholds. Note the high proportion of cells (54%) with velocities of < 20 μm/sec. Visual inspection confirmed that these cells were immotile, stuck to the slide or were moving debris.

Figure 3.2b: Distribution plot of straight-line velocity (VSL) for 200 cells analysed from the same videotaped sample after the application of low velocity thresholds. The mean VSL increased after motile artefacts were deleted from the analysis.

3.2a


Mean VSL = 22.8μm/sec
(SD = 17.6)
Number of spermatozoa
0
20
40
60
0
10
20
30
40
50
60
70
80
90
100
VSL - μms-1

3.2b


Mean VSL = 41.8μm/sec
(SD = 10.5)
Low velocity
threshold
Number of spermatozoa
0
20
40
60
0
10
20
30
40
50
60
70
80
90
100
VSL - μms-1

3.2.4 Experiment 4 - Repeated analysis of the same videotape

This experiment was performed to determine the accuracy of repeated analysis using the settings derived in Experiments 3.2.1 and 3.2.2. A total of approximately 200 cells, with a minimum of 13 trackpoints, from the same section of videotape were analysed in triplicate and the coefficient of variation (CV, %) of the three analyses was calculated.

3.2.4.1 Results

Table 3.3: Measurement of ALH, VAP, VCL and VSL in triplicate for 200 cells from the same section of videotape.

	Tracks recorded	ALH	VAP	VCL	VSL
1st analysis	206	7.65	115.5	131.7	93.6
2nd analysis	203	7.48	114.9	132.0	90.7
3rd analysis	202	7.92	116.1	134.6	91.2
Mean		7.69	115.5	132.8	91.8
Std Deviation		0.23	0.60	1.61	1.56
CV (%)		2.99	0.52	1.21	1.70

3.3 Separation of Spermatozoa from Seminal Plasma

Because seminal plasma is believed to affect capacitation and calcium regulatory mechanisms preliminary experiments were carried out to test three different methods of separating spermatozoa from seminal plasma. One experiment was carried out to assess three different separation methods, centrifugation, swim-up from seminal plasma and the use of a discontinuous Percoll gradient for separation of human and equine spermatozoa from seminal plasma. Efficacy of separation was judged by the percentage of the initial sample which was viable after separation and the quality of the motility. An additional experiment was carried out to assess three different discontinuous Percoll gradients with ejaculates from one individual stallion because of failure to produce a motile sample from this animal after separation through a 50/100% gradient.

3.3.1 Preparation of Percoll gradients

A stock solution of Percoll was purchased (Pharmacia, Uppsala, Sweden) and an isotonic solution, termed 100% Percoll, was prepared by mixing 45 ml of Percoll with 5 ml of 10x Earles balanced salt solution (Sigma Chemical Company, Poole, Dorset, England), 0.75 ml of 4.5% BSA, 0.185 ml Na lactate (60% syrup) and 1.5 mg Na pyruvate. Further Percoll dilutions were made by diluting 100% Percoll with medium as shown in Table 3.4, to give dilutions ranging from 100% to 40% Percoll. Medicult culture medium was used for all human sperm experiments and TALP was used for all equine experiments.

Table 3.4: Percoll dilutions were made by mixing 100% Percoll with the appropriate volume of medium as shown below.

	100%	90%	80%	70%	50%	40%
100% Percoll	10 ml	9 ml	8 ml	7 ml	5 ml	4 ml
Medium	0 ml	1 ml	2 ml	3 ml	5 ml	6 ml

3.3.2 Experiment 1 - Separation of human and equine spermatozoa from seminal plasma by three different methods

One ejaculate was collected from each of 3 human donors and the initial concentrations were determined using a haemocytometer as described in Chapter 2. Three aliquots from each ejaculate were treated as follows. A swim-up was prepared as described in Chapter 2 by layering 500 µl of liquefied semen beneath 1 ml of Medicult and incubating for 45 minutes. The supernatant was then recovered. A 500 µl aliquot of semen was diluted by the addition of 1.5 ml Medicult, centrifuged at 300*g* for 5 minutes, the supernatant discarded and the pellet reconstituted to 500 µl. A 2-step discontinuous Percoll gradient was formed by carefully layering 3 ml of 50% Percoll over 3 ml of 100% Percoll. A measured aliquot of 500-1000 µl of semen was layered above the Percoll and the sample centrifuged at 500*g* for 20 minutes. The supernatant was then discarded and the pellet formed below the 100% Percoll was washed by centrifugation at 200*g* for 6 minutes. Again the supernatant was discarded and the pellet reconstituted to the original volume. Duplicate sperm counts were performed on aliquots of the separated samples, duplicate eosin-nigrosin smears were made and samples were videorecorded for motility analysis.

This experiment was then repeated for one ejaculate from 3 different stallions. For equine spermatozoa Bavister-TALP was used as the diluting medium.

3.3.2.1 Results

The sperm count (millions/ml), percentages live and percentage recovered after separation of seminal plasma by one of three different methods are summarised in Tables 3.5 and 3.6. For human samples there was no difference in the percentage recovered after centrifugation or Percoll separation. Recovery after swim-up was significantly ($p<0.05$) lower than either, at 21% compared with 68 and 62%, respectively. The percentage live was unchanged by centrifugation or Percoll separation but was numerically higher by swim-up preparation.

For equine samples the highest recovery was seen after centrifugation and recovery after swim-up was significantly ($p<0.05$) lower at 22% than after separation by the other two methods. No enhancement of the percentage live was observed for any of separation methods.

Movement analysis of human samples generally showed an increase in ALH and velocity after separation from seminal plasma regardless of method (Table 3.7). This increase was most marked using 'swim-up'. There was no clear increase in movement parameters of equine spermatozoa after separation. No motility was recorded for one stallion (Table 3.8, stallion 2) after separation through Percoll due to sperm agglutination.

Table 3.5. Sperm count (millions/ml), percentage live and percentage recovered from three human donors after separation of human sperm from seminal plasma by one of three different methods.

	Donor	Count	% live	% recover
Fresh	1	125	53	-
	2	122	80	-
	3	162	78.5	-
	Mean (SEM)	**136.3 (12.8)**	**70.5 (8.8)** [a]	-
Centrifuge	1	100	62	80%
	2	83	77	68%
	3	91.5	74	56%
	Mean (SEM)	**91.5 (4.9)**	**71 (4.6)** [a]	**68% (6.9)** [a]
Swim-up	1	24	89	19%
	2	20.5	99	17%
	3	45.5	99.5	28%
	Mean (SEM)	**30(7.8)**	**95.8 (3.4)** [a]	**21% (3.4)** [b]
Percoll	1	67	62	53%
	2	65	91	53%
	3	130	85	80%
	Mean (SEM)	**87.3 (21.3)**	**79.3 (8.8)** [a]	**62% (9.0)** [a]

different letters ***a, b*** between rows shows significant difference ($p < 0.05$)

Table 3.6. Sperm count (millions/ml), percentage live and percentage recovered after separation of equine sperm from seminal plasma by one of three different methods. Mean and (SEM) are also shown.

	Stallion	Count	% live	% recover
Fresh	1	325	52	-
	2	95	73	-
	3	175	73	-
	Mean (SEM)	**198.3 (67.4)**	**66 (7.0)** [a]	-
Centrifuge	1	255	59	78%
	2	75	66	78%
	3	132	70	75%
	Mean (SEM)	**154 (53.1)**	**65 (8.4)** [a]	**77 (1.0)** [a]
Swim-up	1	68	62	21%
	2	7.5	65	8%
	3	62.5	77	36%
	Mean (SEM)	**46 (19.3)**	**68 (4.6)** [a]	**22 (8.1)** [b]
Percoll	1	218	56	67%
	2	44	69	46%
	3	142	65	81%
	Mean (SEM)	**134.7 (50.4)**	**63.3 (3.8)** [a]	**64.6 (10.2)** [a]

different letters ***a, b*** between rows shows significant difference ($p < 0.05$)

Table 3.7. Mean and SEM for amplitude of lateral head deviation (ALH, μm) and velocities (VCL, VAP and VSL; μm/s) of human spermatozoa before and after separation from seminal plasma by one of three different methods.

	Donor	ALH	VCL	VAP	VSL
Fresh	1	5.1	73.8	59.9	51.1
	2	8.3	116.8	85.1	75.1
	3	5.9	89.7	72.8	64.9
	Mean (SEM)	**6.4 (1.0)**	**93.4 (22.1)**	**72.6 (7.3)**	**63.7 (7.0)**
Centrifuge	1	5.5	82.7	68.7	60.2
	2	8.5	121.6	86.7	75.4
	3	7.1	107.3	85.7	77.6
	Mean (SEM)	**7.0 (0.9)**	**103.9(11.4)**	**80.4 (5.8)**	**71.1 (5.5)**
Swim - up	1	6.9	102.1	83.1	70.4
	2	9.5	137.5	97.2	85.9
	3	7.7	112.0	90.0	79.7
	Mean (SEM)	**8.0 (0.8)**	**117.2(10.5)**	**90.1 (4.1)**	**78.7 (4.5)**
Percoll	1	5.2	76.1	61.3	53.3
	2	9.0	114.3	76.4	63.5
	3	7.8	104.9	80.0	68.1
	Mean (SEM)	**7.3 (1.1)**	**98.4 (11.5)**	**72.5 (5.7)**	**61.6 (4.4)**

Table 3.8. Mean and SEM for amplitude of lateral head deviation (ALH, μm) and velocities (VCL, VAP and VSL; μm/s) of equine spermatozoa before and after separation from seminal plasma by one of three different methods.

	Horse	ALH	VCL	VAP	VSL
Fresh	1	8.0	105.1	86.0	62.6
	2	7.6	81.5	53.3	34.2
	3	4.1	63.7	53.2	46.7
	Mean (SEM)	**6.5 (1.2)**	**83.5 (12)**	**64.1 (10.9)**	**47.8 (8.2)**
Centrifuge	1	6.9	87.6	71.3	49.3
	2	8.3	84.4	57.5	35.0
	3	5.5	70.0	58.3	48.0
	Mean (SEM)	**6.9 (0.8)**	**80.6 (5.4)**	**62.3 (4.5)**	**44.1 (4.6)**
Swim - up	1	7.1	108.3	92.8	65.7
	2	8.3	89.2	72.4	40.0
	3	4.3	67.4	55.4	44.2
	Mean (SEM)	**6.6 (1.2)**	**88.3 (11.8)**	**73.5 (10.8)**	**49.9 (8.0)**
Percoll	1	7.4	110.6	93.0	62.7
	2	-	-	-	-
	3	5.3	80.0	68.2	55.2
	Mean (SEM)	**6.4 (1.1)**	**95.0 (15.1)**	**80.5 (12.4)**	**59.0 (3.8)**

3.3.3 Experiment 2 - Separation of stallion spermatozoa through different Percoll gradients

A second experiment was conducted to test 3 ejaculates from Stallion 2 centrifuged through three different discontinuous Percoll gradients. A 50/100% gradient was prepared as before. A two-step 40/80% gradient was constructed using the dilutions in Table 3.4. A six-step discontinuous gradient was formed by layering successive 1 ml aliquots of 100%, 90%, 80%, 70%, 50% and finally 40% in a Falcon tube. Two ml of semen was layered over this gradient and samples centrifuged and washed as before. Duplicate eosin-nigrosin smears were made and live/dead counts are shown in Table 3.9.

3.3.3.1 Results

The percentage live was significantly ($p<0.001$) reduced when compared to fresh ejaculates after centrifugation through any of the Percoll gradients. No motility recordings were made after centrifugation because all spermatozoa were agglutinated and essentially immotile.

Table 3.9: Summary of the percentage of live spermatozoa in fresh ejaculates (n = 3) and after separation through three discontinuous Percoll gradients.

	Eosin-nigrosin			
Ejaculate	Fresh	50/100% Percoll	40/80% Percoll	6-step Percoll
1	81.5	46.2	49	56.7
2	79.5	48.5	53.5	52
3	72	41.5	44.5	45
Mean + SEM	**77.7 (2.9)**	**45.4 (2.1) ***	**49 (2.6) ***	**51.2 (3.4) ***

* - significantly different from fresh ($p<0.001$)

3.4 Intracellular Calcium Measurements Using the Fluorescent Calcium Indicator Fura 2-AM

A series of experiments were carried out to validate the techniques used for intracellular loading of Fura-2AM. Specifically, the ability of dead sperm to load Fura-2AM was examined. Further, the effect upon fluorescence intensities of sperm concentration, method of sperm separation from seminal plasma and the suspension medium was assessed.

3.4.1 Experiment 1- Fura 'loading' of dead spermatozoa

A preliminary series of experiments was performed to test whether the fluorescent Calcium indicator Fura 2-AM 'loaded' into dead spermatozoa. Semen was collected from one stallion on three different days, prepared by centrifugation and re-extended in Kenney's medium to give a final concentration of 100 million spermatozoa per millilitre and split into two 8 ml aliquots. One aliquant was repeatedly immersed in liquid nitrogen. Eosin-nigrosin staining indicated that all spermatozoa from this aliquant stained pink, and hence was classified as dead. The second fraction was maintained in a water bath at 37°C and was classified as live.

Six treatments were evaluated. Samples 1 and 4 were not loaded with Fura-2AM and represented sperm autofluorescence or background intensity. Treatments 2 and 3 were live sperm samples, 'loaded' and maintained at 37°C or 5°C, respectively. Treatments 5 and 6 were dead sperm samples, treated as for samples 2 and 3. Fura-2AM 'loading' was carried out as described in Chapter 2.

The values of emitted intensities read at 340 nm and 380 nm were recorded as described in Chapter 2. The ratio of the intensities for the different treatments is shown in Table 3.10.

3.4.1.1 Results

The mean values for R, ratio of fluorescence intensity, for treatments 5 and 6, killed spermatozoa, were not significantly different from Treatment 1 (control, sperm autofluorescence) control indicating that dead spermatozoa did not load Fura-2AM. Treatments 2 and 3 showed significantly ($p<0.001$) higher ratios of 1.17 and 1.39 respectively, indicating Fura-2AM 'loading'. The higher ratio of intensity seen with treatment 3 than treatment 2 suggested that intracellular calcium was higher when the spermatozoa were held at 5°C than at 37°C.

Table 3.10: Ratio of intensity (f_{340}/f_{380}) of Fura-2 (mean ± SEM, n = 3), determined for live stallion spermatozoa incubated at 37°C (Treatment 2), 5°C (Treatment 3) or killed using liquid nitrogen and incubated at 37°C (Treatment 5) and 5°C (Treatment 6). Treatments 1 and 4 represent autofluorescence controls.

Treatment	Ratio of Intensities (R)
1 autofluorescence	0.953 ± .01 [a]
2 live (37°C)	1.170 ± .03 [b]
3 live (5°C)	1.390 ± .04 [c]
4 autofluorescence	0.995 ± .01 [a]
5 killed (37°C)	0.998 ± .01 [a]
6 killed (5°C)	1.000 ± .03 [a]

abc Different letters within column means value differ ($p<0.001$)

3.4.2 Experiment 2 - Effect of changes in sperm concentration on Fura-2AM loading

This experiment was performed to test if the ability of sperm cells to regulate $[Ca^{2+}]_i$ was affected by changes in sperm concentration. Semen was collected from one stallion on three different days, prepared by centrifugation and re-extended in Kenney's medium to give a final concentration of 500 million spermatozoa per millilitre. Fura-2AM 'loading' and measurement of fluorescence intensities was conducted as described in Chapter 2. A serial dilution was carried out to give aliquots containing 500, 250, 125, 63, 31 and 16 million spermatozoa per ml. These aliquots were diluted to 4 ml in cuvettes with Grahams TALP, giving final sperm concentrations of 125, 63, 31, 16, 8, and 4 million/ml. The $[Ca^{2+}]_i$ was measured as described in Section 2.9.4.2.

3.4.2.1 Results

Results are summarised in Table 3.11. The fluorescence intensities declined with the number of spermatozoa in the cuvette declined. The ratio of intensities was stable 125 and 16 million/ml after which a small decline was evident. When transformed to nM $[Ca^{2+}]_i$ there was no significant difference across the range of concentrations.

Table 3.11: The effect of varying the sperm concentration within the cuvette upon the intensities, the ratio of intensities and the average intracellular calcium (mean ± SD for 3 experiments) is shown below. f_{340} and f_{380} are the fluorescence intensities at 340 and 380 nm respectively, R is the ratio of these intensities.

Concentration million/ml	f_{340}	f_{380}	R (f_{340}/f_{380})	Intracellular calcium (nM)
125	579	346	1.67	97 ± 8 [a]
63	307	188	1.63	92 ± 7 [a]
31	173	104	1.66	95 ± 10 [a]
16	100	59	1.69	99 ± 10 [a]
8	67	53	1.55	82 ± 7 [a]
4	63	43	1.47	73 ± 10 [a]

a Letter within column means values do not significantly differ

3.4.3 Experiment 3 - Effect of semen separation method upon Fura-2AM loading

Duplicate aliquots from one ejaculate from three stallions were treated by swim-up migration and centrifugation as described previously, to separate the sperm fraction from seminal plasma. Fura-2AM loading was then carried out as described in section 2.9.4.

3.4.3.1 Results

The results of the experiment are summarised in Table 3.12. The fluorescence intensities of loaded samples prepared by swim-up migration did not exceed background intensity in any of the samples. In contrast, the fluorescence intensities at both wavelengths, of all samples separated by centrifugation were 2-3 fold higher than background autofluorescence intensities.

Table 3.12: Fluorescence intensities of loaded and unloaded sperm prepared by swim-up migration or centrifugation.

		(a)	(b)	(c)	(d)	(c)-(a)	(d)-(b)	$\frac{[(c)-(a)]}{[(d)-(b)]}$
Separation Method	Ejaculate	Unloaded f_{340}	Unloaded f_{380}	Loaded f_{340}	Loaded f_{380}	(Loaded f_{340})-(b/ground f_{340})	(Loaded f_{380})-(b/ground f_{380})	Ratio
	1	29	32	28	24	*	*	
Swim-up	2	27	27	27	27	*	*	
	3	23	24	25	25	*	*	
	1	62	64	231	181	169	117	1.44
Centrifuge	2	49	49	194	154	145	105	1.38
	3	69	70	328	191	259	171	2.14

* - the fluorescence intensities of loaded samples were close to that of sperm autofluorescence

3.4.4 Experiment 4 - Effect of medium composition upon Fura-2AM loading

This experiment was performed to evaluate Fura-2AM "loading" of spermatozoa suspended in three different medium. Triplicate aliquots from one ejaculate from each of three stallions were suspended to 100 million per ml in either TALP-egg, TALP-milk or Kenney's medium. Fura-2AM loading and measurement of intracellular calcium was carried out as described in Sections 2.9.4 and 2.9.4.2 respectively.

3.4.4.1 Results

The fluorescence intensities measured at 340 nm and 380 nm were consistently higher for spermatozoa suspended in TALP-milk and Kenney's medium when compared to intensities measured in TALP-egg yolk medium. This difference was significant for TALP-milk ($p<0.05$). The ratio of intensity, however, was not significantly different between the three medium. These results are summarised in Table 3.13.

Table 3.13: Florescence intensities of loaded and unloaded sperm extended in Kenney's, TALP-Milk and TALP-egg yolk media.

		(a)	(b)	(c)	(d)	(c)-(a)	(d)-(b)	$\frac{[(c)-(a)]}{[(d)-(b)]}$
Medium	Ejaculate	Unloaded f_{340}	Unloaded f_{380}	Loaded f_{340}	Loaded f_{380}	(Loaded f_{340})-(b/ground f_{340})	(Loaded f_{380})-(b/ground f_{380})	Ratio
Bavister TALP-egg yolk	1	60	61	157	131	97	70	1.38
	2	68	68	161	134	93	66	1.40
	3	78	78	183	146	105	68	1.54
Bavister TALP-milk	1	59	58	235	186	176	128	1.37
	2	48	48	224	158	176	110	1.60
	3	69	70	314	215	245	145	1.69
Kenney's medium	1	63	64	206	159	143	95	1.50
	2	57	57	208	152	151	95	1.59
	3	71	69	305	210	234	141	1.66

3.4.5 Immobilisation of spermatozoa for single cell studies

It was necessary to immobilise spermatozoa for intracellular calcium measurements in the single cell studies to allow stable ratio measurements to be made. The approach taken by other workers (Plant et al., 1995) was to immobilise human spermatozoa in 10% gelatine. However, it was evident that gelatine had a greater intrinsic fluorescence than TALP. It was therefore necessary to use the lowest concentration of gelatine which could immobilise equine spermatozoa while maintaining low background fluorescence. Empirical experimentation indicated that spermatozoa were immobilised at a concentration of 10% gelatine provided the gelatine was cooled to room temperature. For all single cell studies the following protocol was used. An equal volume of sperm suspension in TALP and 20% gelatine were mixed to give a final sperm concentration of 25 million/ml and a final gelatine concentration of 10%. Five microlitre aliquots were transferred onto alcohol cleaned microscope slides to which dry transfer circles had been applied as previously

described. A coverslip was carefully applied to avoid formation of air pockets and excess fluid removed. Intracellular calcium measurements were made at room temperature (23-25°C); in this temperature range the gelatine solidifies and immobilises the spermatozoa. To assist in location of Fura-2AM loaded spermatozoa, the raw intensity signals were amplified and played through a loudspeaker. By moving the microscope stage, a loaded cell was moved into the field of measurement and initially identified by an increase in sound volume, hence, facilitating rapid measurements of cells. A ploughwise transversal of the slide ensured that cells were measured only once. Care was taken to measure only the head region of the cell by adjusting a diaphragm to exclude background. Clumps of cells were avoided.

3.5 Comparison of Eosin-Nigrosin Viability Staining With Chlortetracycline/Hoechst 33258 Dual Staining

A preliminary experiment was carried out to compare sperm viability estimation using the established Eosin-nigrosin staining method with a Chlortetracycline/Hoechst 33258 dual staining method.

3.5.1 Experiment 1

Aliquots from one or two ejaculates from three stallions were extended in Kenny's medium then plunged into liquid nitrogen to supply samples of dead spermatozoa. A second aliquot from each ejaculate was maintained at 37°C to supply samples of live spermatozoa. Live and dead spermatozoa were then mixed in various proportions to approximate 0, 25, 50, 75 and (nominally) 100 percent live spermatozoa. Duplicates subsamples were then stained with eosin-nigrosin or the combined CTC/H258 fluorescent stain as described in Chapter 2. Slides were randomly coded and for each slide 100 spermatozoa were evaluated. For eosin-nigrosin staining spermatozoa were classified as "dead" if stained pink. For dual staining spermatozoa were classified as "dead" if the nucleus stained bright blue.

3.5.1.1 Results

The results are summarised in Table 3.14. The dual staining method correlated strongly with eosin-nigrosin staining ($r = 0.92$).

Table 3.14: Comparisons of percentage live (%; Mean ± SEM; n = 5 ejaculates) stained by eosin/nigrosin or the CTC/H258 dual staining method.

% Live	Eosin/Nigrosin	Dual Stain
0	3.2 ± 1.6	3.4 ± 2.0
25	22.3 ± 2.6	24.1 ± 1.7
50	41.2 ± 2.9	34.2 ± 2.9
75	47.1 ± 2.9	54.9 ± 2.9
100	61.9 ± 2.8	68.7 ± 4.1

3.6 Discussion

The advent of computerised sperm motility analysis has provided a new opportunity to study sperm movement using a rapid, objective method of semen analysis. While this represents a major advance in the objective assessment of semen quality, accuracy of data collection is dependent upon setting the machine parameters appropriately. Other workers have highlighted differences between machines from different manufactures (Mahony *et al.*, 1988). More importantly it has become apparent that different results are obtained according to the setting of sperm detection parameters (Aanesen and Benevold, 1990) or according to the videoframing rate in use by the machine (Morris *et al.*, 1996). The same type of machine used under controlled conditions in multicentre trials still produced variable results. Satisfactory calibration was only achieved when a single videotape was used as a calibration control by the different centres (Davis *et al.*, 1992). Machine setting parameters for use with stallion spermatozoa on a HTM have been suggested by others (Jasko *et al.*, 1988; Varner *et al.*, 1991; Palmer and Magistrini 1992) including the manufacturer (Hamilton Thorne Research, Beverly, MA, USA). However, these settings were not always appropriate for an individual machine with specific software or hardware upgrades. For this reason a series of experiments were carried out to determine optimal parameters for the detection of motile stallion spermatozoa using this individual machine.

The first step in setting parameters was to set Main Gates to optimise sperm detection. The machine software is designed to track any moving object of a pre-set minimum size and brightness. If minimum size or intensity gates were set at too high a level, objects not meeting minimum values for these parameters would not be considered for analysis. Conversely, if gates were set too low, a single cell could be included twice, detected once for the head signal and a second time for the blur caused by movement. The parameters selected in Experiment 1 optimised the detection of motile spermatozoa at the expense of immotile sperm for two reasons. Firstly, immotile sperm were irrelevant to movement analysis and secondly, it is accepted that this type of machine is poor at detecting immotile cells. Recent versions from the

same manufacturer include fluorescent optics and advocate fluorescent staining of cells to facilitate total sperm counts, acknowledging inadequacies in the ability to detect immotile sperm (Hamilton Thorne IVOS version 10, Hamilton Thorne Research, Beverly, MA, USA).

Visual inspection of the selected motile cells revealed a high proportion of slow moving debris and immotile cells. This artefact was usually caused by collisions with motile cells and was particularly obvious in milk diluent. This problem has been identified with other species (*bovine* - Robertson and Middleton, 1992) and the approach taken was to impose low velocity thresholds designed to eliminate slow moving debris. In Experiment 2, a database of moving debris and immotile cells was collected and velocity distributions plotted. Thresholds which eliminated approximately 95% of debris were selected. These thresholds were tested on other samples and markedly reduced skewing of the data towards the low velocity end of the measurement scale.

Finally, after optimising the "Gate" settings and applying low velocity thresholds, a triplicate analysis of the same section of the videotape was carried out. The analysis generated low coefficient of variation values, reflecting a low variation between the repeated measurement of the same videotapes. These settings were used for all further movement analysis.

Because later Chapters involved incubation of spermatozoa under conditions which would support capacitation it was necessary to select a method for removal of spermatozoa from seminal plasma. Three methods were evaluated, centrifugation, swim-up migration from seminal plasma and centrifugation through a discontinuous Percoll gradient. The principal of swim-up migration is that of isolating a self-selecting population of highly motile spermatozoa. Percoll is a medium composed of colloidal silica particles coated with polyvinylpyrrolidone. The principle of using Percoll gradients is to isolate motile spermatozoa in an area of a specific density while trapping debris and dead cells in gradient interfaces of a different densities. Efficacy of separation was judged by the proportion of the original sample recovered, by the percentage viable and by movement analysis of the motile fraction. The separation techniques were used firstly on human ejaculates because these techniques were well documented for spermatozoa of this species and because the techniques were already

in use in this laboratory.

Results for human ejaculates indicated that recovery after centrifugation and Percoll separation was comparable (68 vs. 62%) and that there was a slight enhancement of the percentage viable after Percoll separation. However, as might have been expected the greatest enhancement of percentage live spermatozoa was for the swim-up treatment where samples were up to 99% live. For these samples, however, the percentage of the original sample recovered was very low, with a mean of only 21%. Movement analysis showed a trend towards an increase in mean velocities and ALH after separation from seminal plasma. This was most marked for the swim-up but not consistent for samples centrifuged through Percoll.

For equine spermatozoa centrifugation yielded the highest percentage recovery and swim-up yielded the lowest at 22%. There was a wide variability in recovery after Percoll separation ranging from 46-81%. No enhancement of the percentage live was observed for any separation method. Nor was there a consistent increase in the velocities or the ALH after separation from seminal plasma. One observation was that the sperm sample of one stallion was agglutinated after separation through Percoll. Further investigation using three different discontinuous Percoll gradients confirmed that for this individual animal the percentage of live spermatozoa was actually reduced by centrifugation through the Percoll gradients and that the recovered samples live were consistently immotile. For all further experiments, unless stated otherwise, sperm samples were separated from seminal plasma by centrifugation since this method gave the most consistent results for equine spermatozoa.

Fluorescent calcium indicators have been used in a number of sperm studies, as described in Chapter 1. However, this technique has not been applied to equine sperm studies nor has it been used extensively for examining spermatozoa preserved at low temperatures, so a number of problems had to be addressed prior to the experimental work. Because the experiments in Chapter 4 involved, prolonged low temperature storage, a decline was predicted in the percentage of live spermatozoa present during the experiment. Therefore, it was necessary to establish that Fura-2AM did not load into dead spermatozoa. Secondly, it was necessary to establish that fluorescence intensities were still detectable when live sperm

concentrations were low, such as after 72 hours of incubation. Data from the experiment described in section 3.4.1, indicated that dead spermatozoa did not load Fura-2AM since there was no difference in fluorescence intensities between the killed sperm samples and the autofluorescence control samples, which were not exposed to Fura-2AM. Results from the experiment described in section 3.4.2 indicated that $[Ca^{2+}]_i$ measurements were unchanged over a concentration range from 125 to 16 million sperm/ml and stable down to 4 million/ml.

Since experiments in Chapter 5 involved sperm capacitation it was also necessary to derive a method for removing sperm from seminal plasma without affecting the subsequent measurement of fluorescence intensities. A comparison was made between sperm separation using swim-up migration and centrifugation. It was apparent that the fluorescence intensities of samples loaded after swim-up were not different from sperm autofluorescence. This was in contrast to centrifugation, where the fluorescence intensity at both wavelengths was consistently several fold higher than background. Given the previous experiments which indicated that only 36% or less of the original sperm numbers were recovered after swim-up this finding was attributed to low sperm numbers in the sample cuvette, producing fluorescence intensities at levels too low to be consistently detected by the spectrofluorometer. For all subsequent experiments spermatozoa were removed from seminal plasma by centrifugation, a method which consistently supplied sperm samples with fluorescence intensities within the detection limits of the equipment.

Finally an experiment was conducted to determine the effect of different types of media, Kenney's, TALP-Milk and TALP-egg yolk on Fura-2AM 'loading'. This experiment showed a consistently lower measure of fluorescence intensity in sperm extended in TALP-egg yolk medium in comparison to TALP-milk or Kenney's medium. A significantly ($p<0.05$) higher fluorescence intensity was measured in sperm extended in TALP-milk medium in comparison to TALP-egg yolk medium. This would tend to suggest that some component of the egg yolk based medium dampened the fluorescent signal emitted by the indicator. The ratio of intensity (R), which is a function of intracellular calcium measurement however was not significantly different between the three media, suggesting that the signal was equally affected at both wavelengths. However, the decision was taken not to use egg yolk-

based cryopreservative diluents in any further experiments.

For the single cell calcium measurement studies it was necessary to immobilise the spermatozoa prior to making intensity measurements. The approach taken was that of Plant et al., (1995) who used 10% gelatine to render human sperm immotile. The TALP solutions used for population or cuvette studies had a low intrinsic fluorescence. Unfortunately, gelatine was found to have a significant intrinsic fluorescence. To minimise background fluorescence, it was therefore desirable to use the lowest gelatine concentration required to immobilise the spermatozoa. At 37°C gelatine remained fluid at unworkably high concentrations, so it was also necessary to reduce the temperature to accelerate cross-linking within the gelatine matrix. The gelatine/sperm suspension was therefore allowed to cool to room temperature (23-25°C). At this temperature 10% gelatine was found to be sufficient to halt sperm forward movement, though limited rotational movement was still apparent in some spermatozoa. With this degree of immobilisation individual sperm heads were kept within a small rectangular aperture for long enough to allow a level reading to be taken.

Hoechst 33258 (H258), a fluorescent DNA binding dye with limited membrane permeability, was used in combination with chlortetracycline (CTC) staining to assess changes associated with capacitation and the acrosome reaction in live spermatozoa. The idea of dual staining with H258 and a second stain was developed by others (Wolf *et al.*, 1985; Cross *et al.*, 1986) for the purpose of detecting dead spermatozoa, excluding them from consideration, and thus differentiating between 'physiological' and 'degenerative' acrosome reactions in human sperm populations. This dual H258/CTC protocol was developed in this laboratory for use with human spermatozoa (Kay, *et al.*, 1994); this specific protocol has not been applied to stallion spermatozoa before. For this reason the experiment detailed in section 3.5.1 was performed to compare sperm viability counts using H258/CTC dual staining with eosin-nigrosin staining, a recognised technique used to identify the proportion of live and dead spermatozoa (Hancock, 1951; World Health Organisation, 1987). The study indicated that results from these two methods were strongly correlated ($r=0.92$) and that H258/CTC dual staining was able identify non-viable cells, allowing their exclusion from the assay.

CHAPTER 4

CHANGES IN STALLION SPERM FUNCTION DURING SLOW COOLING AND LOW TEMPERATURE STORAGE

4.1 Introduction

Fertility results using cryopreserved stallion semen are poor (see Table 1.1). Accordingly, artificial insemination has mainly utilised semen stored in the liquid form. Liquid semen is commonly diluted in a preservative extender and cooled to 5°C for storage and transport. Treated in this manner, stallion semen retains fertilising ability for up to 96 hours and reasonable pregnancy rates have been reported (see Table 1.2). Despite these promising results there is limited information available concerning stallion sperm function during cooling and low temperature storage. The following experiments were undertaken to gain more detailed information on the effects of cooling and low temperature storage on two different aspect of sperm function; motility and the ability to regulate intracellular free calcium $[Ca^{2+}]_i$. In Experiment 1, the effect of cooling to 5°C and storage for up to 72 hours on motility was examined. Two cooling protocols were assessed. In Experiment 2, the ability of the live population of cells to regulate intracellular calcium after cooling and storage was studied. Experiment 3 was designed to monitor intracellular calcium accumulation during the cooling process. Recognising the heterogeneous nature of the live sperm population, Experiment 4 was undertaken to examine the ability of single cells to regulate $[Ca^{2+}]_i$ under the test conditions.

4.2 Materials and Methods

Except for Experiment 3, semen used in other Experiments in this Chapter was collected from a single stallion. Methods for stallion semen collection and preparation, media preparation, viability staining, computerised motility analysis, Fura-2AM loading and intracellular calcium measurement are described in Chapter 2. Plastic straws of 0.5 ml capacity were filled with spermatozoa suspended in Kenney's medium to a concentration of 50 million/ml.

4.3 Experiments

4.3.1 Experiment 1 - Stallion sperm motility parameters after cooling and storage at 5°C for up to 72 hours

For Cooling Protocol A, filled straws were cooled from 37°C to 22°C at approximately 0.5°C/minute, then cooled from 22°C to 5°C at 0.05°C/minute using the controlled rate freezer. For Cooling Protocol B straws were cooled from 37°C to 5°C at 0.05°C/minute using the controlled rate freezer. Straws were then maintained at 5°C in a water-bath in a refrigerator for up to 72 hours. Samples were analysed prior to cooling, immediately after cooling and at the end of 24, 48 and 72 hours of storage. For motility analysis, two straws were removed from the refrigerator (5°C) and placed in a water bath (37°C) for 1 minute. The content of the straws were pooled in a pre-warmed test-tube and maintained at 37°C for a further 15 minutes prior to motility analysis. Motility parameters were measured for approximately 200 spermatozoa from each of 5 ejaculates, for both Cooling Protocol A and B.

4.3.1.1 Results

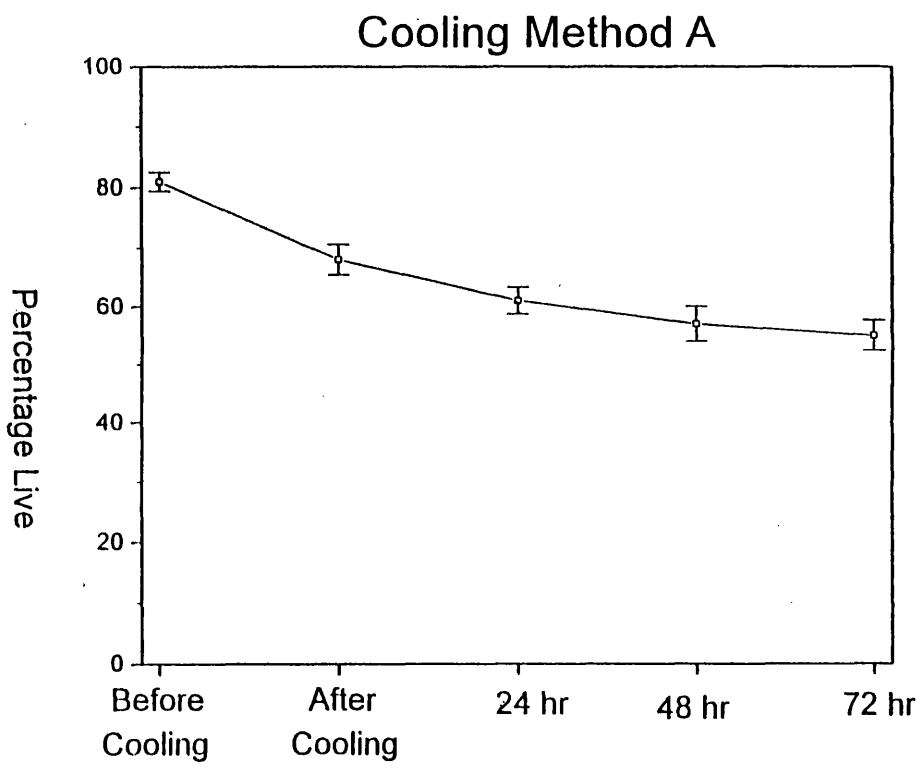
Cooling Protocol A: The percentage of live spermatozoa (Figure 4.1a) declined significantly from 81% before cooling, to 68% immediately after cooling and continued to decline to 61%, 57% and 55% after 24, 48 and 72 hours respectively ($p<0.01$). Analysis of the movement parameters of the living cells show a significant decline in the mean velocity of the parameters VCL, VAP and VSL after cooling and with subsequent low temperature storage (Figure 4.2a). The greatest decline was in VSL, which had declined by 14% after cooling ($p<0.01$). The other velocity parameters showed a similar though smaller decline (VCL 11%, VAP 11%; $p<0.01$). Further decline in mean velocity parameters were observed during subsequent storage (range 0-5%). Mean ALH also decreased after cooling and during subsequent storage (Figure 4.3a). Linearity was essentially unaffected by cooling or storage (Figure 4.4a).

Cooling Protocol B: The percentage of live spermatozoa (Figure 4.1b) declined significantly from 75% before cooling, to 64% immediately after cooling and continued to decline to 55% ,42% and 35% after 24, 48 and 72 hours respectively ($p<0.01$). Analysis of the movement parameters of the living cells show a significant decline in the mean velocity of the parameters VCL, VAP and VSL after cooling and with subsequent low temperature storage (Figure 4.2b). The greatest decline was in VSL, which had declined by 25% after cooling ($p<0.01$). The other velocity parameters showed a similar though smaller decline (VCL 6%, VAP 11%; $p<0.01$). Further decline in mean velocity parameters were observed during subsequent storage (range 0-9%). Mean ALH increased slightly after cooling (+ 1%) but showed a marked decline of 21% during the first 24 hours of storage ($p<0.01$) with no change thereafter (Figure 4.3b). Trajectories were also less linear immediately after cooling (61% compared with 71%) (Figure 4.4b).

Figure 4.1a Percentage live of stallion spermatozoa (%; Mean ± SEM; n = 5) cooled from 37°C to 5°C using Cooling Protocol A and analysed at 0, 24, 48 and 72 hours after the cooling process.

Figure 4.1b Percentage live of stallion spermatozoa (%, Mean ± SEM; n = 5) cooled from 37°C to 5°C using Cooling Protocol B and analysed at 0, 24, 48 and 72 hours after the cooling process.

4.1a


Cooling Method A
Percentage Live
100
80
60
40
20
0
Before Cooling
After Cooling
24 hr
48 hr
72 hr

4.1b

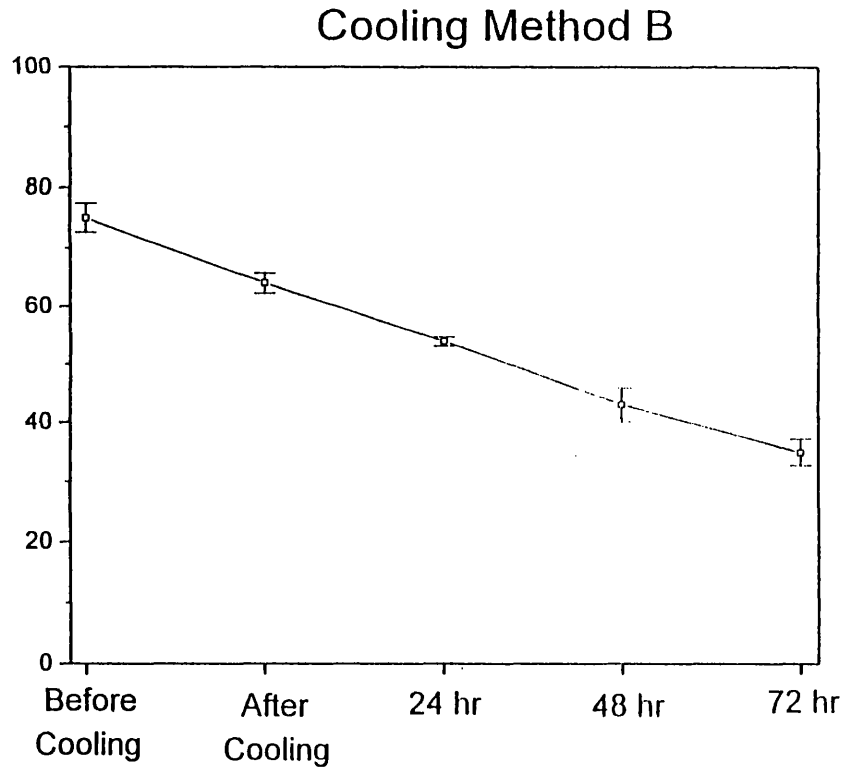
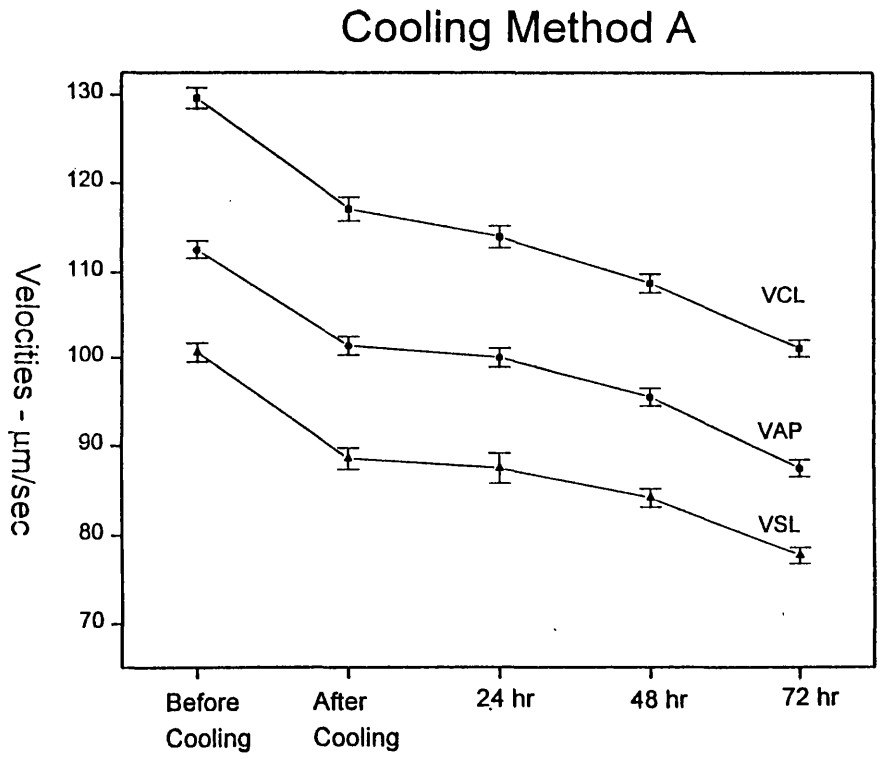

Cooling Method B
100
80
60
40
20
0
Before Cooling
After Cooling
24 hr
48 hr
72 hr

Figure 4.2a Velocities of stallion spermatozoa (μms^{-1}; Mean ± SEM; n=5) cooled from 37°C to 5°C using Cooling Protocol A and analysed at 0, 24, 48 and 72 hours after the cooling process.

Figure 4.2b Velocities of stallion spermatozoa (μms^{-1}; Mean ± SEM; n = 5) cooled from 37°C to 5°C using Cooling Protocol B and analysed at 0, 24, 48 and 72 hours after the cooling process.

4.2a


Cooling Method A
Velocities - µm/sec
130
120
110
100
90
80
70
VCL
VAP
VSL
Before Cooling
After Cooling
24 hr
48 hr
72 hr

4.2b

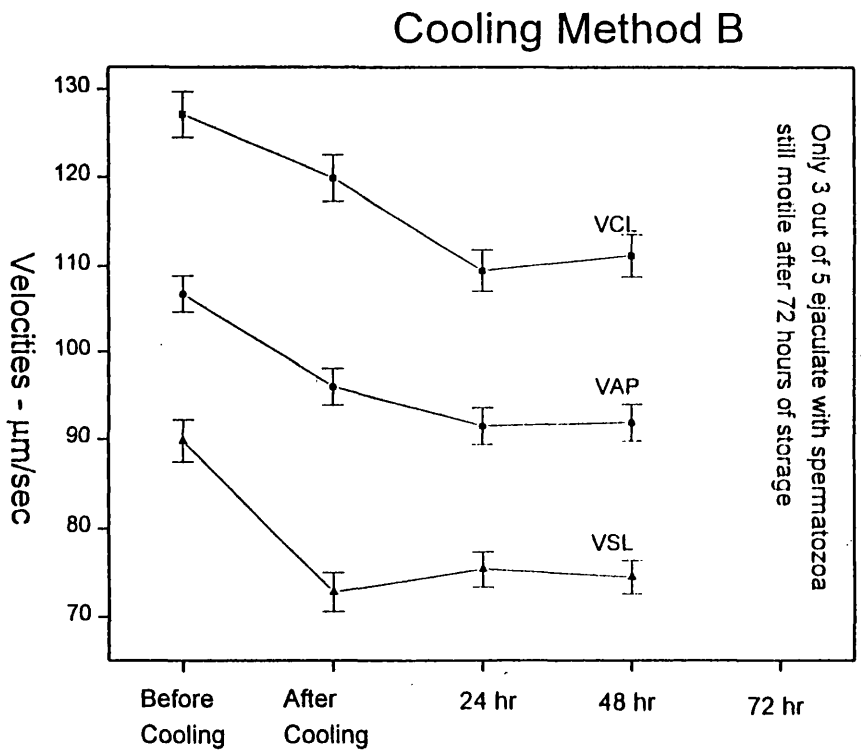
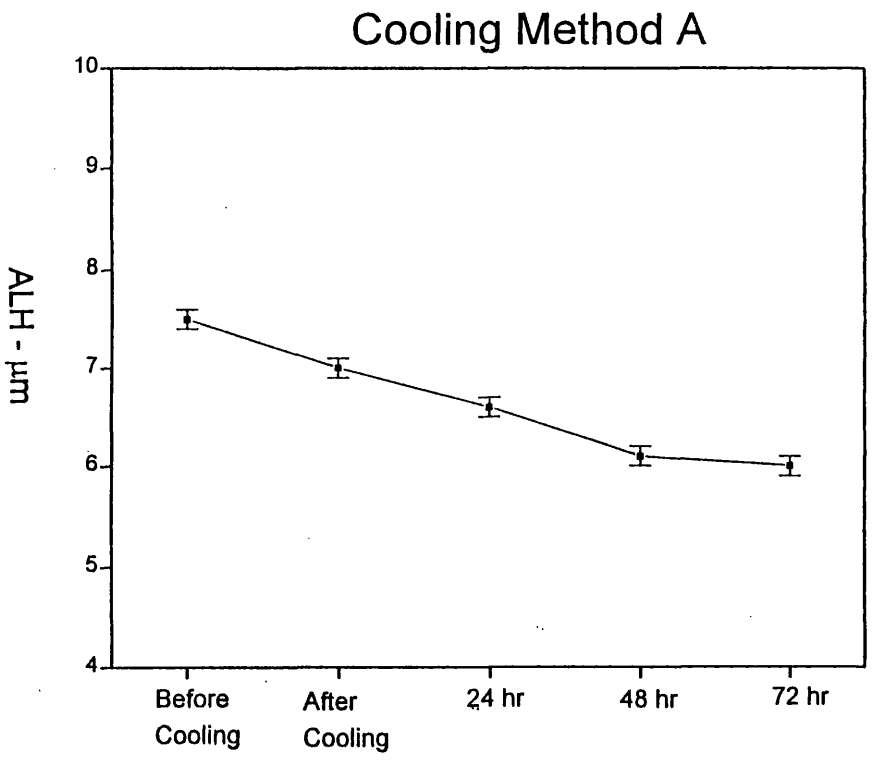

Cooling Method B
Velocities - µm/sec
130
120
110
100
90
80
70
VCL
VAP
VSL
Only 3 out of 5 ejaculate with spermatozoa still motile after 72 hours of storage
Before Cooling
After Cooling
24 hr
48 hr
72 hr

Figure 4.3a ALH of stallion spermatozoa (μm; Mean ± SEM; n = 5) cooled from 37°C to 5°C using Cooling Protocol A and analysed at 0, 24, 48 and 72 hours after the cooling process.

Figure 4.3b ALH of stallion spermatozoa (μm; Mean ± SEM; n = 5) cooled from 37°C to 5°C using Cooling Protocol A and analysed at 0, 24, 48 and 72 hours after the cooling process.

4.3a


Cooling Method A
ALH - µm
10
9
8
7
6
5
4
Before Cooling
After Cooling
24 hr
48 hr
72 hr

4.3b

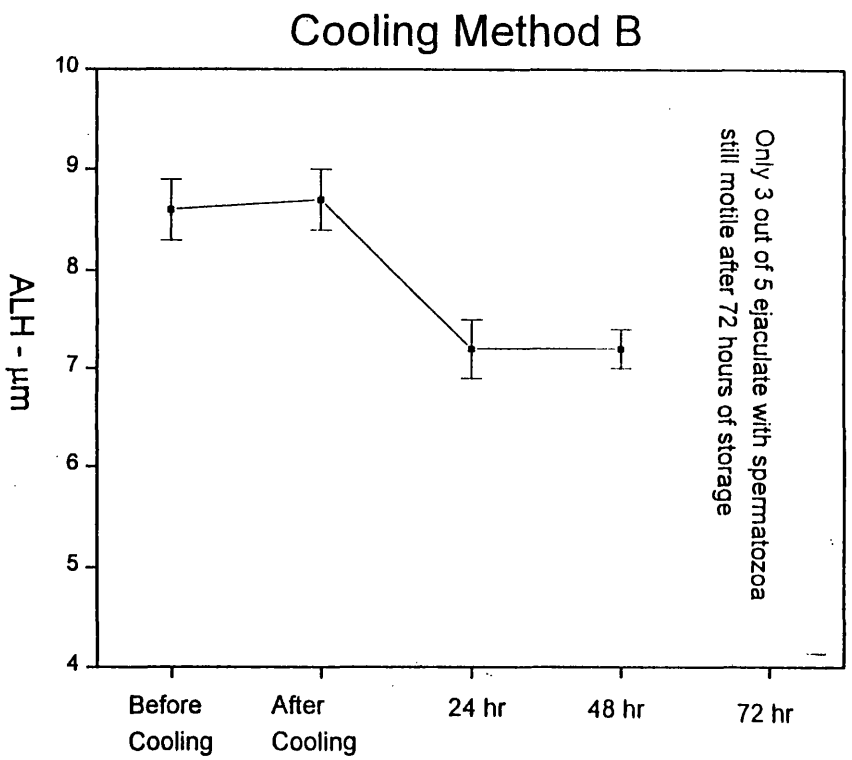
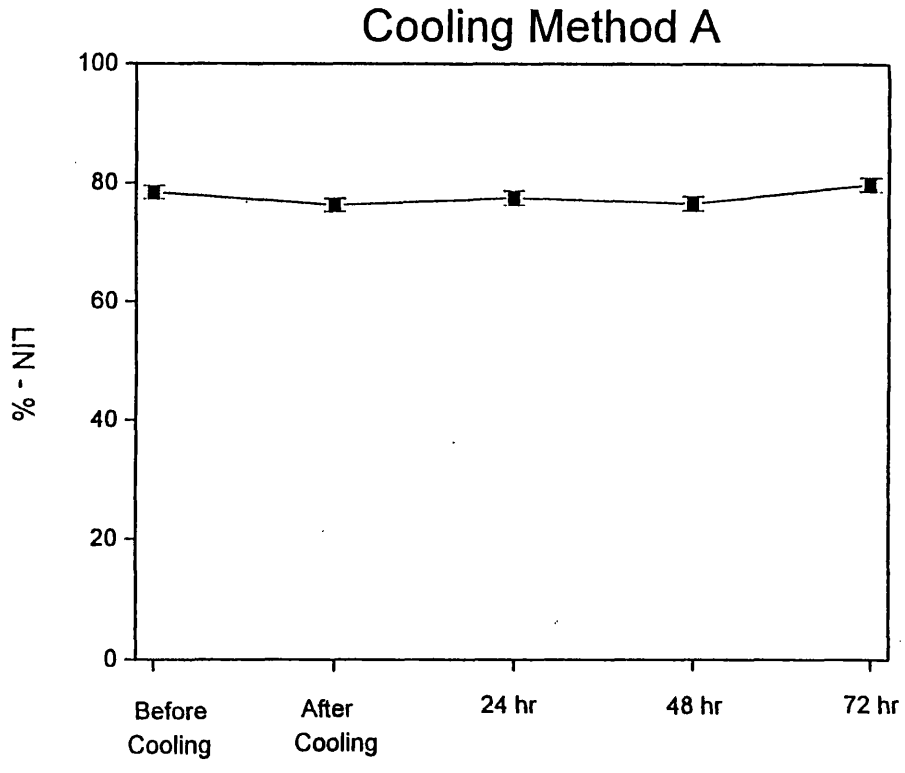

Cooling Method B
ALH - µm
10
9
8
7
6
5
4
Only 3 out of 5 ejaculate with spermatozoa still motile after 72 hours of storage
Before Cooling
After Cooling
24 hr
48 hr
72 hr

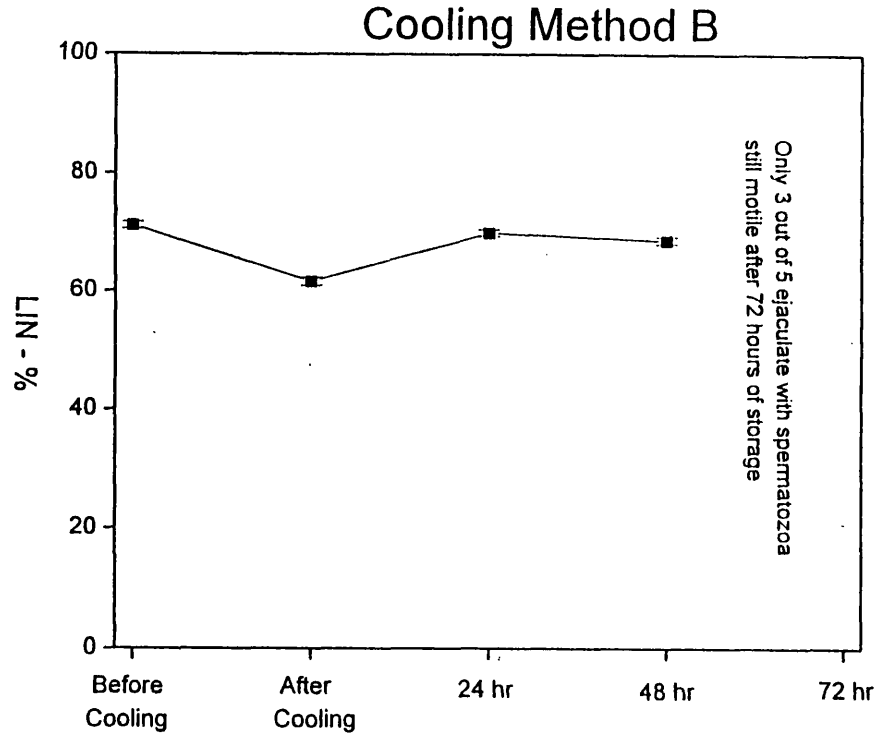
Figure 4.4a LIN of stallion spermatozoa (%;Mean ± SEM; n = 5) cooled from 37°C to 5°C using Cooling Protocol A and analysed at 0, 24,48 and 72 hours after the cooling process.

Figure 4.4b LIN of stallion spermatozoa (%;Mean ± SEM; n = 5) cooled from 37°C to 5°C using Cooling Protocol A and analysed at 0, 24,48 and 72 hours after the cooling process.

4.4a


Cooling Method A
LIN - %
100
80
60
40
20
0
Before Cooling
After Cooling
24 hr
48 hr
72 hr

4.4b


Cooling Method B
LIN - %
100
80
60
40
20
0
Only 3 out of 5 ejaculate with spermatozoa still motile after 72 hours of storage
Before Cooling
After Cooling
24 hr
48 hr
72 hr

4.3.2 Experiment 2 - Measurement of $[Ca^{2+}]_i$ in the sperm suspension after cooling and storage at 5°C for 48 hours

Plastic straws of 0.5 ml capacity and filled with sperm suspension as described previously were cooled from 37°C to 5°C at 0.05°C/minute using either Cooling Protocol A or B. Calcium measurements were made prior to cooling, after cooling to 5°C and after 24 and 48 hours of storage. At the sampling times 8-10 straws were re-warmed to 37°C, and their contents transferred to a test-tube. This suspension was mixed well and further divided into two, 2 ml aliquots (final concentration 50 million/ml) for Fura-2AM loading and intracellular calcium measurement. Measurements were made for suspensions from 3 ejaculates cooled by each of Cooling Protocol A and B.

4.3.2.1 Results

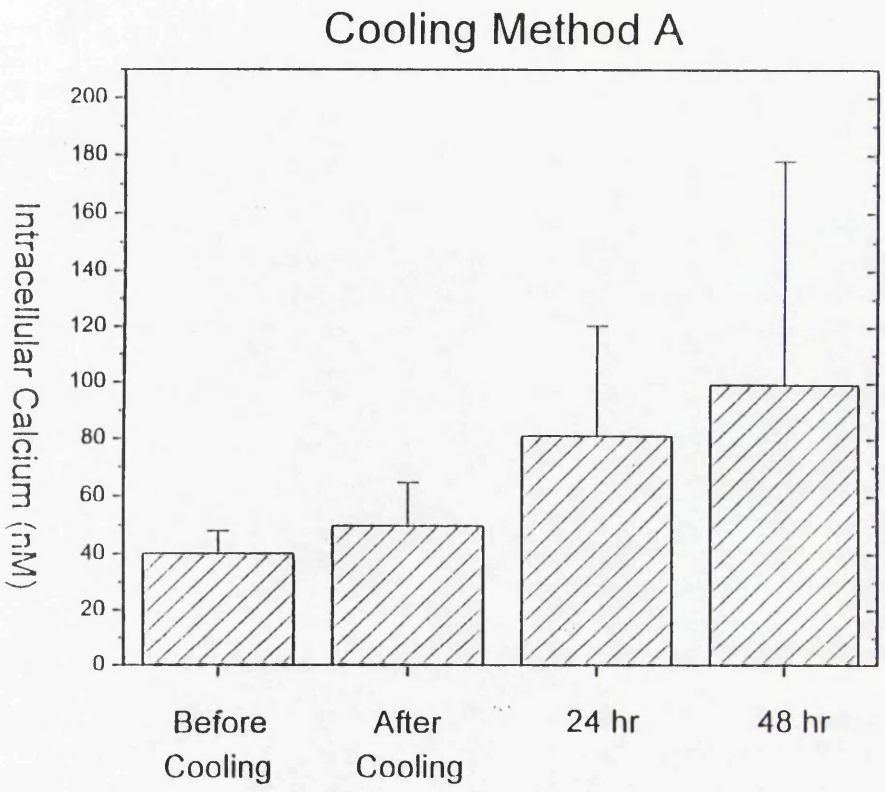
Cooling Protocol A: Measurement of $[Ca^{2+}]_i$ in cell suspensions indicated that $[Ca^{2+}]_i$ increased by 25% over the cooling period. The greatest increase in $[Ca^{2+}]_i$ was seen after 24 hours of storage at 5°C. Intracellular calcium concentrations continued to increase during storage up to 48 hours (Figure 4.5a).

Cooling Protocol B: Measurement of $[Ca^{2+}]_i$ in cell suspensions indicated that $[Ca^{2+}]_i$ increased by 152% over the cooling period. The increase in $[Ca^{2+}]_i$ continued during the first 24 hours of storage, reaching a plateau with little further increase during the second 24 hours of storage at 5°C (Figure 4.5b).

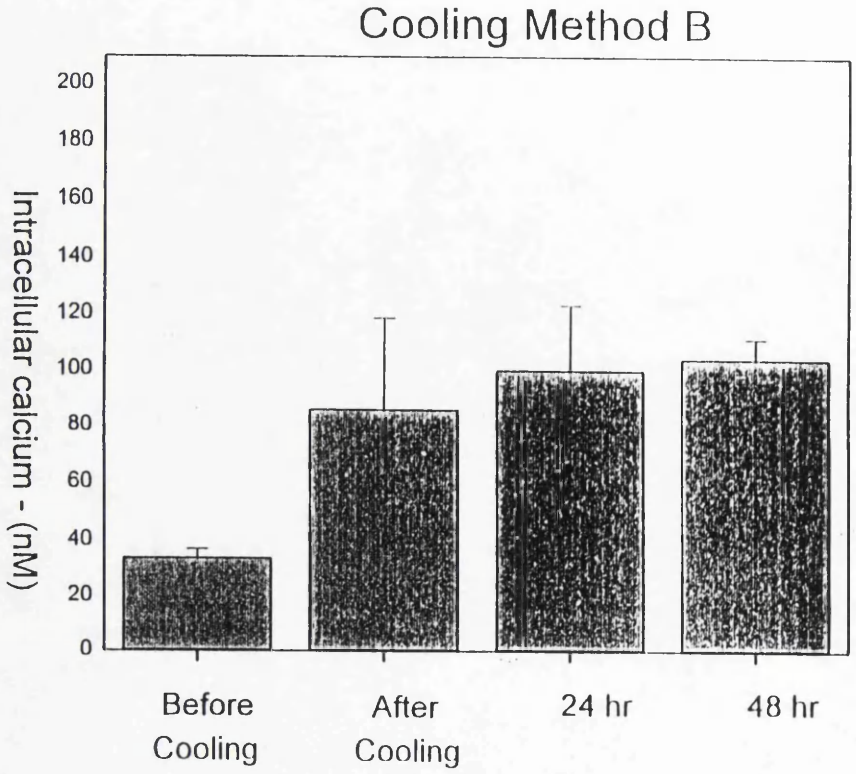
Figure 4.5a Intracellular calcium (nM; Mean ± SEM; n = 3) of stallion spermatozoa cooled from 37°C to 5°C using Cooling Protocol A and analysed at 0, 24, 48 and 72 hours after cooling.

Figure 4.5b Intracellular calcium (nM; Mean ± SEM; n = 3) of stallion spermatozoa cooled from 37°C to 5°C using Cooling Protocol B and analysed at 0, 24, 48 and 72 hours after cooling.

4.5a


Cooling Method A
Intracellular Calcium (nM)
200
180
160
140
120
100
80
60
40
20
0
Before Cooling
After Cooling
24 hr
48 hr

4.5b


Cooling Method B
Intracellular calcium - (nM)
200
180
160
140
120
100
80
60
40
20
0
Before Cooling
After Cooling
24 hr
48 hr

4.3.3 Experiment 3 - Calcium changes during cooling to 5°C

To test the hypothesis that intracellular calcium increased during the cooling process, rather than during storage at 5°C, an experiment was carried out to directly monitor calcium concentrations during cooling from 37°C to 5°C. Three ejaculates, one from each of three stallions were used in this experiment. Sperm preparation, Fura-2AM loading, and measurement of fluorescence intensities was as described previously. Cooling was achieved by adding ice to the waterbath which regulated the water jacket surrounding the cuvettes within the spectrofluorimeter. In this manner, the temperature of the cuvette was reduced from 37°C to 5°C over a period of 115 minutes. The cooling rate averaged 0.3°C/minute. Temperature within a cuvette was monitored using an electronic thermometer (Radio Spares Components Limited, Glasgow).

4.3.3.1 Results

Figure 4.6 is a graphical representation of the increase in $[Ca^{2+}]_i$ which occurred during cooling. During cooling from 37°C to about 16°C, intracellular calcium concentrations were in the range of 72 nM to 87 nM. Below 16°C a gradual increase in intracellular calcium occurred, which became more rapid when the temperature dropped below 13°C. At 5°C intracellular calcium concentrations were significantly higher than at 37°C (186 nM compared with 80 nM)

4.3.4 Experiment 4 - Measurement of $[Ca^{2+}]_i$ in single cells after cooling and storage at 5°C for 48 hours

Three ejaculates from a single stallion were used in this experiment. Straws were filled as in previous experiments and cooled from 37°C to 5°C at 0.05°C/minute and maintained at 5°C for up to 48 hours. The contents of two straws were pooled and analysed prior to cooling, immediately after cooling and at the end of 24 and 48 hours of storage. At these time rewarmed sperm suspensions were loaded with Fura-2AM as described previously. For single cell studies spermatozoa were immobilised in 20% gelatine for fluorescence microscopy. Measurement of sperm $[Ca^{2+}]_i$ was made for a total of 250 individual cells from 3 ejaculates.

4.3.4.1 Results

The ratio (R) of the intensities (340/380 nm) for any single cell is an indication of the free intracellular calcium concentration for that cell. Results are summarised in Figure 4.7. Before cooling the majority of spermatozoa (91%) were regulating intracellular Ca^{2+} at $R < 0.5$. The population of cells was essentially normally distributed around a mean value of $R = 0.36$. After the cooling, the mean R increased to 0.48, increasing again to 0.54 and 0.58 after 24 and 48 hours of storage, respectively. The percentage of cells regulating $[Ca^{2+}]_i$ at an $R < 0.5$ reduced from 91% to 70% after cooling and 60% during subsequent storage. The proportion of cells regulating $[Ca^{2+}]_i$ at an $R = 0.36$, the mean value prior to cooling, fell from 68% to 42% after cooling and 33% then 18% with 24 and 48 hours storage, respectively.

Figure 4.6 Intracellular calcium (nM; Mean ± SEM; n = 3) of stallion spermatozoa during cooling from 37°C to 5°C.

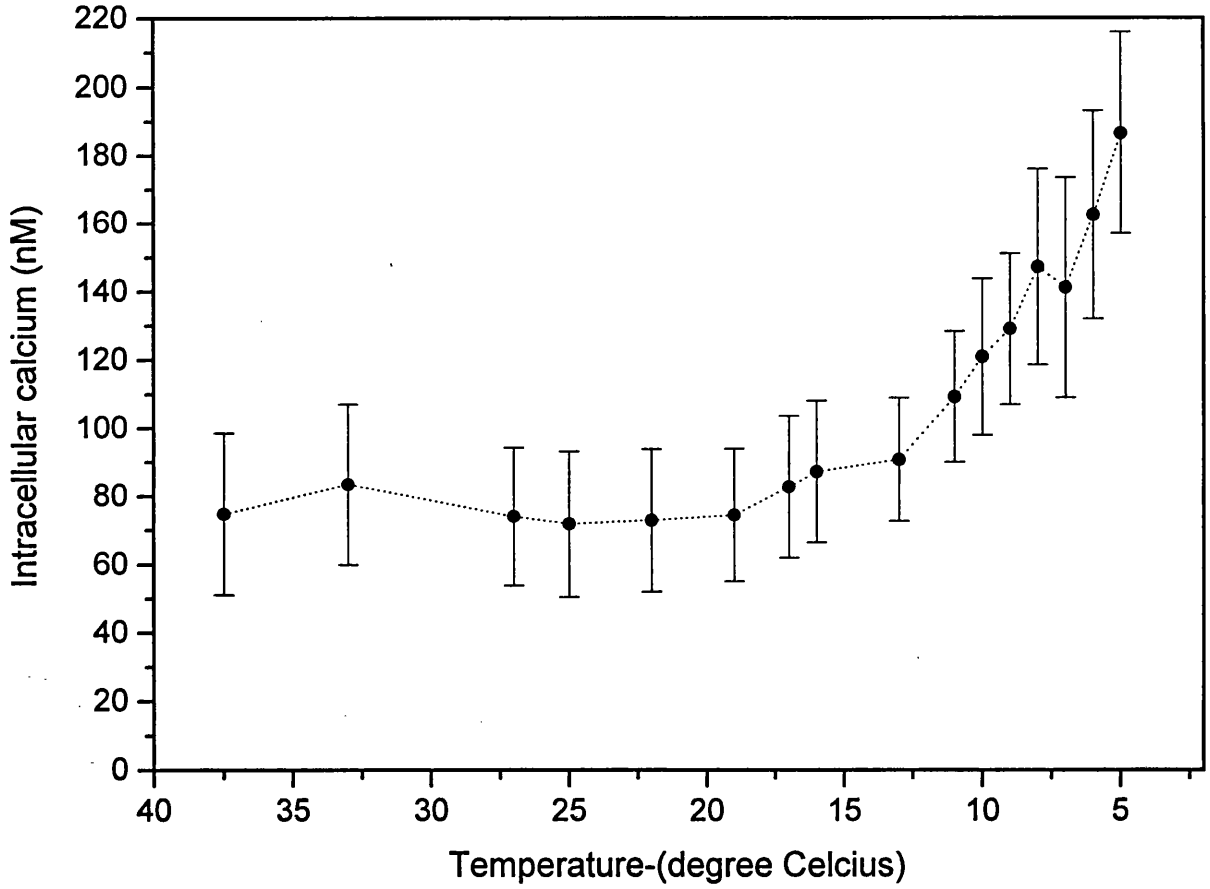
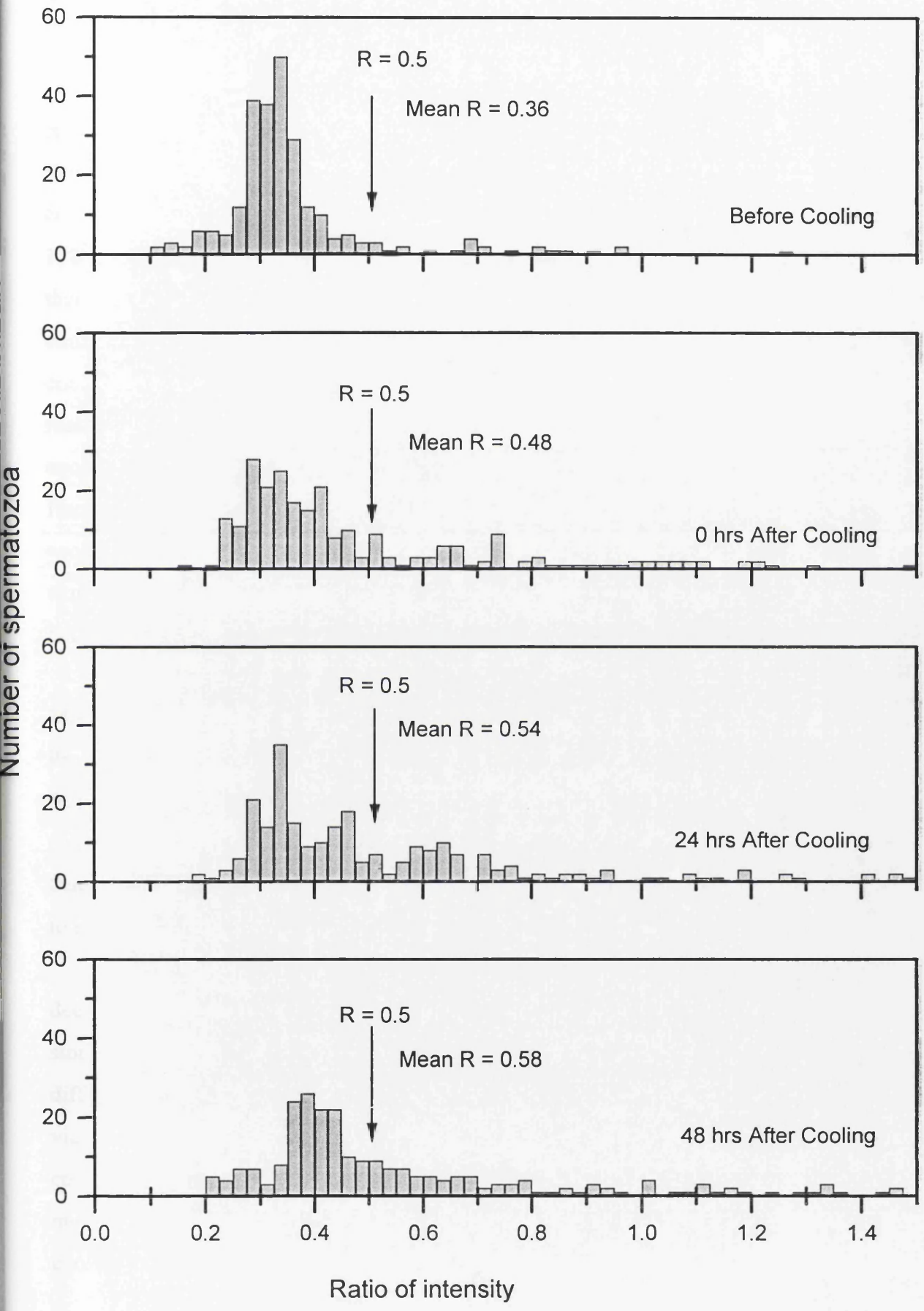

Intracellular calcium (nM)
220
200
180
160
140
120
100
80
60
40
20
0
40
35
30
25
20
15
10
5
Temperature-(degree Celcius)

Figure 4.7 Ratio of intensity (f_{340}/f_{380}) measured from 250 individual stallion spermatozoa cooled from 37°C to 5°C, measured before cooling and at 0, 24 and 48 hours after the cooling process. Mean values for R were 0.36, 0.48, 0.54 and 0.58 respectively.


Number of spermatozoa
60
40
20
0
R = 0.5
Mean R = 0.36
Before Cooling
R = 0.5
Mean R = 0.48
0 hrs After Cooling
R = 0.5
Mean R = 0.54
24 hrs After Cooling
R = 0.5
Mean R = 0.58
48 hrs After Cooling
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Ratio of intensity

4.4 Discussion

Because of the poor fertility which results from using cryopreserved semen, equine artificial insemination most commonly utilises liquid semen chilled to 5°C. Stallion semen is usually diluted in a dried skim milk-glucose extender prior to cooling (Kenney *et al.*, 1975; Douglas-Hamilton, 1984; Douglas-Hamilton *et* al., 1987; Varner *et al.*, 1989; Jasko *et al.*, 1992a; Malmgren *et al.*, 1994). Cooling can then be achieved using a controlled rate freezer or simpler, commercially available, cooling flasks (e.g. Equitainer, Hamilton Thorne Research). It has been reported that cooling at a linear cooling rate of less than 0.05°C/minute between 20°C and 5°C maintained maximal percentage of motile spermatozoa and that stallion spermatozoa cooled thus provided acceptable fertility rates (Douglas-Hamilton, 1984; Douglas-Hamilton *et al.*, 1987). Reports have indicated that careful use of controlled rate cooling between the temperatures of 21-22°C and 5°C is necessary for successful storage (Kayser, 1992) but less emphasis has been placed on controlled rate cooling between 37°C and 22°C.

This study utilised the milk-based extender in most common use, Kenney's Medium, (Kenney *et al.*, 1975) and two cooling protocols. The first, called Cooling Protocol A, used bench top cooling from 37°C to 22°C, with controlled rate cooling from 22°C to 5°C (Moran *et al.*, 1992; Bedford *et al.*, 1995). The second protocol, Cooling Protocol B, used controlled rate cooling from 37°C to 5°C. It is worth noting that Protocol A took approximately five and a half hours and Protocol B eleven hours to reach 5°C.

Viability staining of spermatozoa after cooling and during storage indicated a decline in the number of viable spermatozoa after cooling, which continued during storage. The greatest decline in numbers viable was detected after cooling. No difference was observed between Cooling Protocol A and B. The motility of the viable spermatozoa also changed after cooling and during storage. Mean velocities consistently declined; the greatest decline was immediately after cooling. The numerically greatest decline was a 23% reduction in mean straight line velocity after cooling by Protocol B. The much higher decline in VSL as compared to VAP and

VCL immediately after cooling indicated that the majority of sperm cells were moving in circles. This circular motion may be a manifestation of the cooling injury to stallion sperm during the cooling procedure, although the effect of rewarming must also be considered. It is possible that cooling and rewarming may damage the axonemal elements of the tail, though this has generally been considered resistant to the effects of cooling and rewarming. Holt *et al.*, (1988), in cryomicroscopic studies of cold shock, found that on cooling to 16°C, 50% of ram spermatozoa exhibited a rigid bowing of the midpiece with loss of all progressive motility below this temperature. These workers reported that a number of spermatozoa also acquired a localised swelling of the flagellum, in the region of the midpiece and endpiece. Spermatozoa with swollen endpieces were motile on rewarming, but their flagellar beat was impaired.

To summarise, a decline in sperm viability and mean velocity of the live fraction was observed after cooling and storage at 5°C. Extending the period of cooling at the slower rate of 0.05°C/minute to cover the entire cooling period from 37°C to 5°C (Protocol B) did not result in a marked conservation of viability or velocity, instead, it appeared to increase the proportion of motile cells showing non-progressive motility. One interesting observation was of the maintenance of high mean ALH after cooling by Protocol B. High amplitude lateral head movement is associated with hyperactivated motility, a motility pattern which is regulated by increases in intracellular calcium. It has also been reported that high ALH was correlated with increased intracellular calcium in cryopreserved bull spermatozoa, as a consequence of freezing; in this situation high intracellular calcium, in turn, correlated with reduced fertility (Bailey *et al.*, 1993). The circumstantial evidence, then, associates high ALH with increased intracellular calcium and this is corroborated by the experimental evidence from the fluorescence measurements discussed below.

These experiments applied the fluorescent indicator Fura-2AM to the study equine spermatozoa for the first time, and found that the ability of the stallion spermatozoa to regulate $[Ca^{2+}]_i$ was clearly compromised by cooling and low temperature storage. With Cooling Protocol A, $[Ca^{2+}]_i$ had increased by 25% at the end of cooling and by 64% after the first 24 hours of storage. Using Protocol B, the

internalised calcium concentrations measured immediately after cooling showed a massive increase of 152% compared to precooled samples, with only a small increase during subsequent storage.

Since Fura-2AM does not load into dead spermatozoa (see Chapter 3, Section 3.4.1) the increase in intracellular calcium could not be attributed to a rise in the percentage dead, but instead to a huge increase in the $[Ca^{2+}]_i$ of the live population. In Experiment 4.3.3 it was possible to directly observe $[Ca^{2+}]_i$ increase during the process of cooling. While it was not possible to replicate Cooling Protocol A or B because cooling within the cuvettes could not be controlled to a rate of 0.05°C/minute over 11 hours, it was possible to cool at a rate of 0.3°C/minute. Figure 4.6 shows that $[Ca^{2+}]_i$ was maintained at a fairly steady rate until a temperature of around 15°C was reached. Below this temperature the $[Ca^{2+}]_i$ increased markedly.

Freeze fracture studies have demonstrated that cooling of ram or blackbuck spermatozoa from 30°C to 5°C (Holt and North, 1984) or those of bulls or boars from 38°C to 0°C (de Leeuw *et al.*, 1990) resulted in morphological membrane changes consistent with a lipid phase transition. Fluorescence polarisation studies with sperm plasma membrane vesicles (Holt and North, 1986; Canvin and Buhr, 1989) have demonstrated changes in viscosity of the plasma during cooling. More recent studies using Fourier transform infrared spectroscopy to measure lipid phase transition in shrimp, boar and human spermatozoa during cooling, provided direct evidence that lipid phase transitions occurred during cooling (Drobnis *et al.*, 1993). In boar sperm, major phase shifts were seen between 23°C and 14°C, centred at approximately 18°C. In shrimp sperm (more resistant to cold shock than boar sperm), the lipid phase transition shifts occurred between 13°C and -2°C, centred at 6°C. These lipid phase transition shifts were not detected in human spermatozoa, a species more resistant to cold shock than either boar or shrimp. It is interesting to note that the $[Ca^{2+}]_i$ of stallion spermatozoa increased rapidly below 15°C, perhaps indicating a lipid phase transition around this temperature. Moran *et al.*, (1992) demonstrated that stallion spermatozoa were most sensitive to cold shock between 19°C and 8°C which may be considered as further evidence that stallion spermatozoa undergo a phase transition in this temperature range.

Summary of the data from the single cell studies clearly supports the findings of the whole population studies, of an accumulation of intracellular calcium immediately after cooling, continuing during low temperature storage. Interestingly, however, the data from the single cell studies showed that while the mean R value (ratio of intensity 340 nm: 380 nm) of the 250 cells sampled increased during cooling and with subsequent storage, there were individual cells or a discrete sub-population still capable of regulating calcium across their membranes at a level comparable to precooled levels, while other individual cells showed extremely high levels of $[Ca^{2+}]_i$.

Proper control of $[Ca^{2+}]_i$ flow across the spermatozoa membrane is crucial to successful fertilisation. The ability to regulate $[Ca^{2+}]_i$ may be compromised in two ways during cooling. The accumulation of calcium during the cooling process may be due to the occurrence of lipid phase transitions, as discussed above, which render the plasma membrane 'leaky' and unable to exclude extracellular Ca^{2+} and/or cooling induced damage to intracellular calcium regulatory mechanisms. It has been demonstrated during investigation of other aspects of sperm function (the acrosome reaction) that intracellular calcium concentrations in spermatozoa are maintained by a Ca^{2+}-ATPase (Vijayasarathy *et al.*, 1980; Breitbart and Rubenstein, 1983; Breitbart *et al.*, 1984) and a Na^+/Ca^{2+} antiporter (Babcock *et al.*, 1979; Rufo *et al.*, 1984) which extrude $[Ca^{2+}]$ from the cells and maintain low intracellular concentrations (nM concentrations compared with μM or mM extracellular concentrations). The Ca^{2+}-ATPase function is highly dependent on the physico-chemical properties of the lipid environment surrounding the ATPase. A reduction in temperature would be likely to change the nature of the lipid environment and reduce the efficiency of $[Ca^{2+}]$ extrusion. Function of the ATPase is said to be entirely reversed by hydrolysis of membrane phospholipids (Garrahan and Rega, 1990). The hydrolysis of phospholipids has been implicated in cold induced membrane damage by other workers (Robertson *et al.*, 1990; Das *et al.*, 1991). Together these factors would result in inappropriately high $[Ca^{2+}]_i$ which would affect fertilising ability.

In conclusion, the work in the Chapter indicates that irreversible damage is initiated during cooling to 5°C. This damage results in inappropriately high $[Ca^{2+}]_i$ levels, a decline in motility and a decline in viability. It also appears, from single cell

studies, that despite cooling and storage a discrete sub-population of cells is resistant to thermal damage and retains full ability to regulate$[Ca^{2+}]_i$.

CHAPTER 5

CHARACTERISATION OF CAPACITATION

5.1 Introduction

Sperm capacitation is relatively poorly studied in the equine species when compared to human, bovine and certain laboratory species for which *in vitro* fertilisation systems are in common use. It is said to be difficult to reliably and repeatedly capacitate equine spermatozoa *in vitro* (Ellington *et al.*, 1993b), although successes have been reported (Blue *et al.*, 1989; Palmer *et al.*, 1991). The principal problem with capacitation studies is lack of morphological evidence that the spermatozoon is undergoing the process. To circumvent this problem studies have evaluated chemical induction of the acrosome reaction, using the rationale that capacitation must be complete to allow the acrosome reaction to proceed (Graham *et al.*, 1987; Samper *et al.*, 1989; Zhang *et al.*, 1991). Alternatively, fertilisation, studied *in vitro*, has been used to confirm that capacitation has taken place. Neither approach overcomes the problem of failing to monitor the process of capacitation, only the end point. In addition, it is well recognised that only a proportion, or subpopulation, of any studied population is undergoing capacitation at any one time even under ideal conditions. The magnitude of this subpopulation appears to vary between species, with 60-80% of rodent sperm cultured under capacitating conditions showing capacitation synchronously (Yanagimachi, 1970; Katz and Yanagimachi, 1980) or as little as 20% of human spermatozoa (Burkman, 1984). Certain *in vitro* laboratory techniques have been used to try to identify capacitating subpopulations e.g. identifying and quantifying hyperactivated motility, monitoring surface membrane changes with CTC and using pharmacological substances to accelerate or synchronise capacitation (Ward and Storey, 1984; Byrd and Wolf, 1986; Lee *et al.*, 1987; Parrish *et al.*, 1988; Tesarik *et al.*, 1990; Fraser and McDermott, 1992; Das Gupta *et al.*, 1993; Ellington *et al.*, 1993a; Kervancioglu *et al.*, 1994; Mattioli *et al.*, 1996; Perez *et al.*, 1996). This would seem to be the best approach given the current technology.

This chapter describes a series of experiments conducted to characterise the capacitation of stallion spermatozoa. Because the capacitation process required prolonged incubation *in vitro* it was be necessary to establish a system that would support sperm viability during incubation. In Experiment 1, a pilot study was carried

out to evaluate the ability of different culture media and containers to maintain motility and viability of stallion sperm during incubation for up to 8 hours. Further experiments were directed towards identifying capacitating subpopulations. In Experiment 2, sperm plasma membrane changes were monitored using a combined chlortetracycline (CTC) and Hoechst 33258 fluorescent staining protocol designed to differentiate between uncapacitated, capacitated and acrosome reacted cells. In Experiment 3, the movement characteristics of spermatozoa incubated under capacitating conditions were examined in detail, with particular attention to hyperactivated motility. A preliminary study was performed using human samples because hyperactivated motility is well characterised in this species. A study of motility under incubation conditions used in Experiment 2 was also conducted.

5.2 Materials and Methods

Semen collection and preparation, media preparation, staining procedures and motility assessment are as described in Materials and Methods in Chapter 2.

5.3 Experiments

5.3.1 Experiment 1 - Selection of an incubation system

After initial preparation, one ejaculate from each of three stallions was extended in three different media, Graham TALP, Bavister TALP and Bavister-Milk medium, to a concentration of 50 million per ml. Five hundred microlitre of each treatment was aliquoted into 14 ml Falcon grade and 16 mm flat bottom tissue culture wells and incubated at 38.5°C in 5% CO_2 and humidified air over a period of 8 hours.

Aliquots from each treatment were evaluated for gross motility, percentage motile and percentage live using a double blind procedure and scoring system as described in Section 2.3.1 of Chapter 2.

5.3.1.1 Results

The results of the experiment are summarised in Table 5.1. A significant decline in percentage motile when compared to the initial assessment, was seen by the second hour of incubation for Grahams and Bavister TALP in both tubes and wells, but this decline was significant with Bavister-Milk medium only after 4 hours of incubation. Gross motility declined significantly after 6 hours for all treatments. Spermatozoa extended in Bavister-Milk medium, however, exhibited a more vigorous motility at the start of the incubation. The decline in percentage live was significant after 8 hours of incubation in Bavister-Milk medium as opposed to between 4 and 6 hours of incubation in the other two media. By the eighth hour of incubation, gross motility had declined below 1 for all treatments, percentage motile ranged from 2 to 8% and percentage live from between 23 and 29%. No significant difference ($p=0.354$ for gross motility; $p=0.82$ for percentage motile and $p=0.083$ for percentage live) was detected in the measured parameters, whether incubation was carried out in tubes or wells.

Table 5.1. Mean ± SD of percent motile, gross motility and percent live of stallion spermatoza extended under different incubation systems and evaluated at 0, 2, 4, 6 and 8 hours,respectively.

Incubation system	Parameter evaluated	0 hr	2 hr	4 hr	6 hr	8 hr
	Percent motile	51.7 ± 4.1	36.7 ± 4.1 ***	20.0±3.2 ***	12.5±2.7 ***	2.5±4.2 ***
Tube Graham	Gross motility	1.9 ± 0.4	1.3 ± 0.3	1.0 ± 0.5 *	0.8 ± 0.3 **	0.2 ± 0.2 ***
	Percent live	53.8 ± 5.8	45.0 ± 9.7	43.2 ± 6.2	35.7 ± 13.7 *	27.0 ± 6.6 ***
	Percent motile	51.7 ± 5.2	30.0 ***	25.0 ± 4.5 ***	12.5 ± 2.7 ***	6.7± 5.2 ***
Tube Bavister	Gross motility	1.9 ± 0.4	1.3± 0.3	1.2± 0.3	1.2± 0.4	0.6± 0.6 ***
	Percent live	58.0 ± 5.6	46.2± 8.1	36.7± 9.1**	40.2± 7.8 **	28.5± 10.1***
	Percent motile	50.0	43.3 ± 6.1	25.8 ± 5.9 ***	16.7± 6.1 ***	3.3± 2.6 ***
Tube Bavister Milk	Gross motility	2.3± 0.3	1.9 ± 0.2	1.3 ± 0.3 ***	1.1± 0.4 ***	0.3 ± 0.3 ***
	Percent live	57.8 ± 10.3	51.0 ± 21.4	48.8 ± 12.4	42.5 ± 8.7	29.0 ± 16.0 *
	Percent motile	50.0 ± 3.2	35.0 ± 5.5 ***	21.7± 4.1***	10.8 ± 4.9 ***	4.2 ± 3.8 ***
Well Graham	Gross motility	1.9 ± 0.5	1.3 ± 0.3	1.5 ± 0.4	0.8± 0.3 ***	0.3 ± 0.3 ***
	Percent live	50.5 ± 6.3	45.2± ± 13.7	39.5 ± 6.3 **	40.0 ± 11**	27.67 ± 6.41***
	Percent motile	50.0 ± 3.2	36.7± 8.2 **	22.5 ± 2.7***	12.2± 6.3 ***	5.8 ± 2.0 ***
Well Bavister	Gross motility	1.75 ± 0.27	1.33 ± 0.60	0.9 ± 0.2 **	0.7± 0.3 ***	0.4 ± 0.4 ***
	Percent live	48.33 ± 3.56	43.17 ± 10.19	29.8 ± 7.0 ***	28.5 ± 6.3 ***	26.3 ± 6.4 ***
	Percent motile	50.00	45.00 ± 5.5	33.3 ± 8.8 **	13.3 ± 9.3 ***	8.33 ± 8.16 ***
Well Bavister Milk	Gross motility	2.2± 0.26	2.1± 0.2	1.6± 0.4	0.8 ± 0.5 ***	0.58 ± 0.49 ***
	Percent live	50.5 ± 7.7	44.3 ± 13.5	46.8 ± 19.3	46.5 ± 13.1	23.33 ± 7.12 *

significant difference; * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) in comparison to initial (time 0) observation.

5.3.2 Experiment 2 - Chlortetracycline fluorescent patterns under different incubation conditions

This experiment was conducted to examine the functional state of equine spermatozoa during incubation using the dual CTC/Hoechst staining protocol described in Section 2.9.2 of Chapter 2. One ejaculate from each of three stallions was used in this experiment. After determination of initial concentration, the ejaculate was centrifuged, the supernatant discarded and the pellet re-extended into six different treatments. Treatment 1, comprised spermatozoa diluted in homologous seminal plasma, selected to give an uncapacitated control. Treatments 2-5, consisted of spermatozoa centrifuged to remove seminal plasma and diluted in Bavister-Milk medium supplemented with 0, 0.01, 0.1 or 1 mg/ml pentoxifylline, selected to promote capacitation, and treatment 6, to which 10 nm ionophore A23187 was added for 1 minute prior to assessment. Treatment 6 was selected to promote acrosomal loss. Five hundred microlitre of each treatment (concentration 50 million/ml) was placed into 16 mm flat bottom tissue culture wells and incubated at 38.5°C in 5% CO_2 in humidified air for 5 hours. Samples were taken for percentage live estimation and dual staining after 0, 120 and 300 minutes of incubation.

5.3.2.1 Results

There was a decline in the percentage of live spermatozoa with time in all Treatments (Table 5.2). This decline was greatest in Treatment 1, semen in seminal plasma, by 300 minutes of incubation.

Table 5.2: The percentage of live cells (Mean ± S.D) for 100 spermatozoa in six different treatments, evaluated at specific time points 0, 120 and 300 minutes of incubation.

Treatment	State	0	120 min	300 min
Fresh ejaculate	% live	74 ± 9	53 ± 14	7 ± 6**
Bavister-Milk	% live	78 ± 10	55 ± 10	27 ± 8**
BM(.01 mg/ml PF)	% live	76 ± 5	47 ± 16*	33 ± 2**
BM(.1 mg/ml PF)	% live	83 ± 12	61 ± 2*	27 ± 9**
BM(1.0 mg/ml PF)	% live	81 ± 14	58 ± 7	26 ± 5**
Ionophore (10 nM)	% live	79 ± 9	59 ± 9	34 ± 7**

* - significant difference ($p<0.05$) to time 0; ** significant ($p<0.01$) to time 120 min, a significant difference ($p<0.01$)at 300 minutes of incubation is also seen observed between treatment 1 (seminal plasma) and other treatment.

Figure 5.1a-f are graphical representation of staining patterns evaluated after 0, 120 and 300 minutes of incubation. The majority of cells in Treatment 1 showed the 'uncapacitated' (F) pattern throughout the 300 minute incubation. By 300 minutes only 'uncapacitated' (F) cells were detected in the live population. Treatments 2 to 5 showed a decline in 'uncapacitated' (F) pattern and an increase (significant for treatments to which pentoxifylline and ionophore were added) in 'capacitated' (B) pattern with incubation. For Treatments 1-4 only low levels of 'acrosome reacted' (AR) were detected at any time point (less than 5%). Treatment 5, with 1 mg/ml PF, showed the highest percentage of 'capacitated' (B) pattern after 300 minutes of incubation (mean value of 70%) and the highest percentage of 'acrosome reacted' (AR) after 120 minutes of incubation. Acrosome loss was seen in treatment 6 at all three time points. The highest levels were detected at 300 minutes (mean value 10%).

Figures 5.1g-j, show examples of equine spermatozoa stained by the combined CTC/H258 staining technique.

Figure 5.1a - f Percentage (Mean ± SEM, n=3) of 'F'▨, 'B'□, and 'AR'■ pattern of stallion spermatozoa incubated in six different treatment and evaluated at specific time points of incubation. The symbol (*) denotes significant increase in the 'B' pattern in comparisons to time 0. (* at $p<0.05$ and ** at $p<0.01$).

Figure 5.1a - Fresh

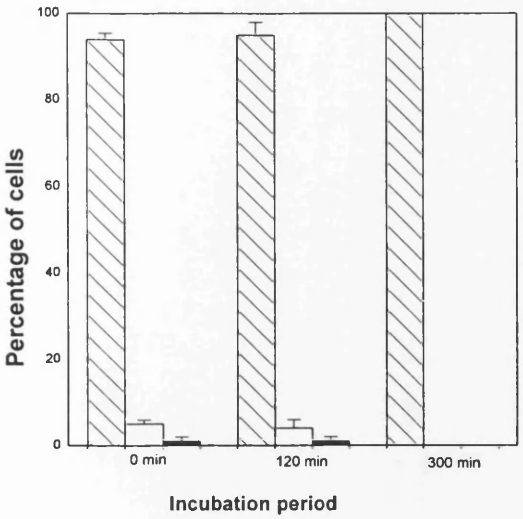


Figure 5.1 b - Bavister-Milk (0 PF)

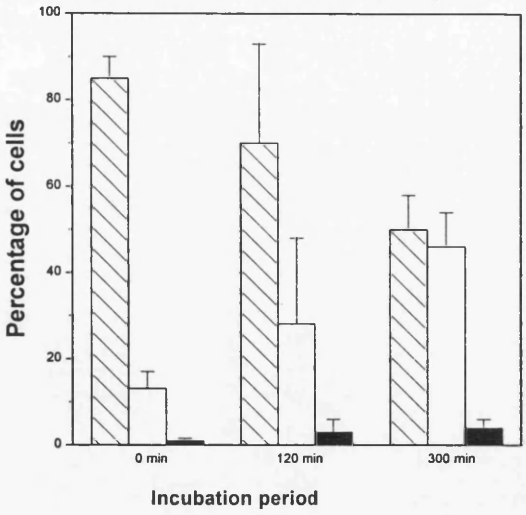


Figure 5.1 c
Bavister-Milk (+0.01PF)

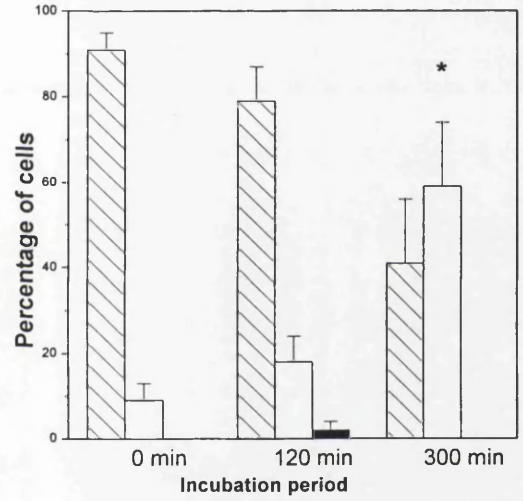


Figure 5.1 d
Bavister-Milk (+0.10 PF)

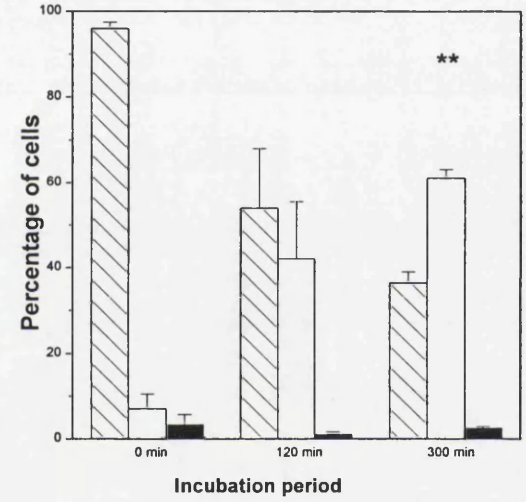


Figure 5.1 e
Bavister-Milk (+1.0 PF)

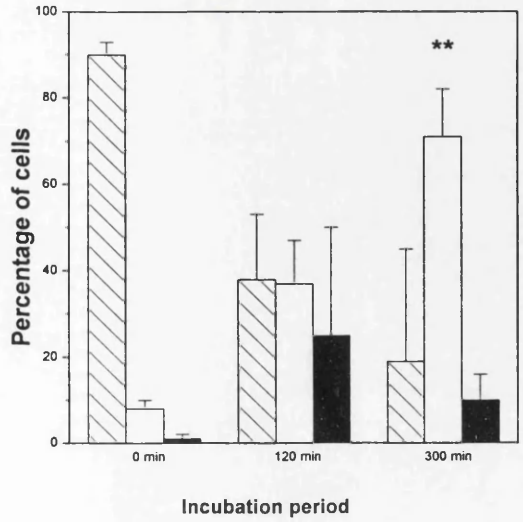


Figure 5.1 f
Bavister-Milk (+A23187)

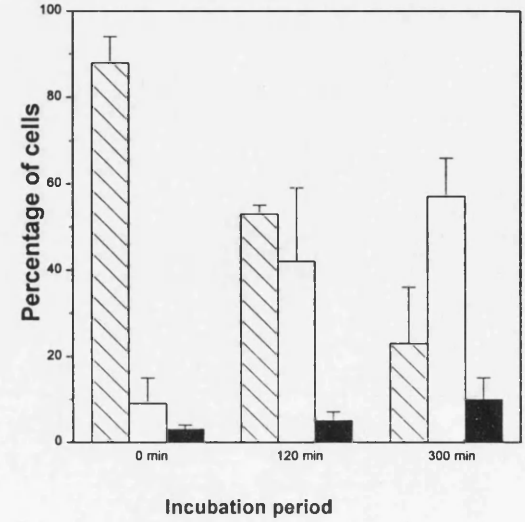


Figure 5.1g Stallion sperm showing uncapacitated 'F' pattern.

Figure 5.1h Decapitated sperm stained by H258, indicating dead sperm.

Figure 5.1i Stallion sperm (enlarged) showing capacitated 'B' pattern. Note the lesser fluorescence band (arrow) in the acrosomal region.

Figure 5.1j (i) 'B' pattern stallion sperm showing exocytosis changes in the acrosome, and (ii) sperm showing acrosome loss.

5.3.3 Experiment 3 - Analysis of movement parameters under different incubation conditions

5.3.3.a Experiment 3a - Analysis of human sperm movement in seminal plasma, post swim-up and after incubation with pentoxifylline

One ejaculate from each of three human donors were collected as described in Material and Methods. Initial samples were prepared by diluting 50 µl of neat semen in homologous seminal plasma (1:1 or 1:2). Seminal plasma was isolated by centrifugation of 400 µl of semen sample at 16,000 rpm for 10 minutes. Concurrently, a swim-up preparation (as described in Section 2.8.2 of Chapter 2) was made using 1 ml of semen and incubated for 45 minutes. The upper interface was then removed and divided into two aliquots. These were centrifuged (300*g*, 5 minutes) and re-extended in 90 µl medium. To one aliquot was added 10 µl of stock pentoxifylline (final concentration 1 mg/ml), and to the control sample 10 µl of medium. Motility analysis of sperm in seminal plasma, post-swim-up and after 15 minutes in pentoxifylline was carried out. Motility parameters (ALH, LIN, VAP, VCL and VSL) were analysed after 15 minutes of incubation at 37°C in 5% CO_2 in humidified air. The number of spermatozoa showing hyperactivated motility was determined using the criteria of Burkman (1991) i.e. ALH $\geq$ 7.5 µm, LIN $\leq$ 65% and VCL $\geq$ 100 μms^{-1}.

5.3.3.b Experiment 3b - Analysis of stallion sperm movement in seminal plasma, post swim-up and after incubation with pentoxifylline

One ejaculate from each of three stallions was collected as described in Chapter 2. Concentration was determined using a hemacytometer and initial samples were extended in homologous seminal plasma to 25 million per ml (a concentration that gives between 5-25 spermatozoa per field for motility analysis). The rest of the sample collected were prepared by swim-up as described previously and the upper interface was harvested and divided into two aliquots. These were centrifuged (300*g*, 5 minutes) and re-extended in 450 µl medium. To one aliquot was added 50 µl

pentoxifylline (final concentration 1 mg/ml), and to the control sample 50 µl medium. Samples were incubated for 15 minutes at 37°C in 5% CO_2 in humidified air. Motility parameters (ALH, LIN, VAP, VCL, VSL) of sperm in semen, post swim-up and after 15 minutes incubation with pentoxifylline was carried out. The number of spermatozoa showing hyperactivated motility was determined using the criteria used for human sperm.

5.3.3.1 Results (Experiment 3a and 3b)

The results for Experiment 3a and 3b are summarised in Table 5.3. For human spermatozoa a significant increase in ALH and VAP and VCL and a significant decline in LIN was observed after the swim-up procedure compared to those parameters in seminal plasma. ALH and VCL increased further after incubation in 1 mg/ml pentoxifylline for 15 minutes. There was no change to VSL with treatment. A mean of 8% of spermatozoa extended in seminal plasma showed hyperactivated activity (range 6-11%). After swim-up, the incidence of hyperactivation increased to 20% (range 16-22%) and after incubation in pentoxifylline increased further to 32% (range 21-47%). For stallion spermatozoa extended in seminal plasma 15% showed hyperactivated activity (range 13-19%). After swim-up, the incidence of hyperactivation did not change significantly (mean 14%; range 10-18%) and incubation in medium supplemented with pentoxifylline did not affect the incidence of hyperactivation (mean 11%; range 8-13%).

Analysis of stallion spermatozoa showed no significant change in ALH, LIN or VSL. However, VCL and VAP increased significantly after swim-up ($p<0.001$). No further change was observed after pentoxifylline treatment. There was no difference in the incidence of hyperactivated spermatozoa between treatments, using the criteria of Burkman (1991).

Table 5.3 : Motion parameters of human and stallion spermatozoa extended in seminal plasma, post swim-up and after addition of pentoxifylline for 15 minutes (Mean ± SD).

		Seminal Plasma n= 431 sperm tracks	Post Swim-up n= 433 sperm tracks	1 mg/ml PF; 15 min n= 424 sperm tracks
	ALH	4.99 ±2.92 [a]	7.08±4.01 [b]	8.57±4.41 [c]
	LIN	80.54±18.10 [a]	70.74±25.65 [b]	65.09±24.05 [c]*
Human	VAP	76.77±22.90 [a]	82.51±22.91 [b]	85.57±20.34 [b]
	VCL	89.35±23.65 [a]	101.78±26.22 [b]	112.46±28.30 [c]
	VSL	71.80±24.5	70.59±27.93	70.48±26.42
	%HA	**8**	**20**	**32**
		Seminal Plasma n= 426 sperm tracks	Post swim-up n= 376 sperm tracks	1 mg/ml PF; 15 min n= 335 sperm tracks
	ALH	7.12±3.51	7.07±3.29	6.74±3.30
	LIN	58.33±20.53	56.28±20.74	57.78±21.09
Stallion	VAP	79.87±31.66 [a]	92.60±29.69 [b]	94.46±28.21 [b]
	VCL	99.58±35.94 [a]	109.67±35.21 [b]	110.31±31.78 [b]
	VSL	57.76±30.41	59.89±27.97	62.17±28.29
	%HA	**15**	**14**	**11**

abc Values with different letters between columns differ ($p<0.001$) and *bc** denote differ($p<.01$)

5.3.3.c Experiment 3c - Analysis of stallion sperm movement during incubation for up to 300 minutes

One ejaculate from each of three stallion was extended after semen preparation as described previously in Bavister-Milk medium containing 0, 0.01, 0.1 or 1 mg/ml pentoxifylline. Motility parameters (ALH, LIN, VAP, VCL and VSL) were analysed after 15, 45, 90, 120, 180 and 300 minutes of incubation. Because it was noted in Experiment 5.2.2 that stallion spermatozoa in medium supplemented with pentoxifylline had a tendency to clump together, a subjective assessment of this phenomenon was also performed (as described in Section 2.3.2) in addition to the motility analysis.

5.3.3.2 Results (Experiment 3c)

The results of movement analysis for Experiment 3c summarised in Table 5.5, indicated that stallion spermatozoa motility was maintained for up to 300 minutes in Bavister-Milk medium. The addition of pentoxifylline did not stimulate motility significantly. In fact, spermatozoa were observed to undergo head to head agglutination ("clumping") after 90 minutes of incubation in Bavister-Milk medium (Table 5.4), which became more obvious with time. This "clumping" was accelerated by the addition of pentoxifylline in a dose dependent manner. The addition of 1 mg/ml of pentoxifylline to the medium made it impossible to evaluated motility on the Hamilton Thorn motility analyser because of the severe clumping seen with this treatment. The incidence of hyperactivation using the detection criteria of Burkman (1991) was high throughout the incubation period. The incidence was numerically highest at 90 minutes of incubation for all treatments. The numbers of spermatozoa showing hyperactivated motility declined after 90 minutes of incubation.

Table 5.4: Subjective assessment of occurrence of head to head agglutination (clumping) of stallion spermatozoa incubated in Bavister-Milk medium containing 0, 0.01, 0.1 or 1 mg/ml Pentoxifylline after 15, 45, 90, 120, 180 and 300 minutes of incubation.

PF mg/ml	15 min	45 min	90 min	120 min	180 min	300 min
BM (0)	-	-	+	+	++	++
BM (0.01)	+	+	++	++	++	++
BM (0.10)	+	++	+++	++	++	++
BM (1.00)	+++	+++	+++	+++	++	++

- no clumping.
+ majority with two sperm heads attached to one another.
++ majority between 2 to 5 sperm heads clumped together.
+++ majority with more than 5 sperm heads clumped together.

Table 5.5 : Motility parameters (Mean ± SD) of stallion spermatozoa incubated in Bavister-Milk medium supplemented with different levels of PF analysed at specific timepoints over a 300 minute incubation period.

		15min	45 min	90 min	120 min	180 min	300 min
	ALH	9.8 ± 4.5	10.2 ± 4.8	10.1 ± 5.3	9.6 ± 4.8	9.6 ± 4.5	8.0 ± 4.3
	LIN	63.8 ± 20.9	67.1 ± 18.8	62.9 ± 22.3	65.0 ± 19.4	63.1 ± 20.6	66.8 ± 20.1
BM only	VAP	145.0 ± 47.7	149.2 ± 52.7	137.4 ± 51.7	136.2 ± 48.8	135.2 ± 51.9	123.8 ± 46.4
	VCL	167.5 ± 51.4	173.6 ± 59.3	162.0 ± 57.5	159.7 ± 55.4	100.0 ± 59.4	141.4 ± 52.3
	VSL	105.1 ± 47.1	116.1 ± 52.0	100.5 ± 50.7	102.5 ± 45.2	100.4 ± 50.0	91.3 ± 41.0
Burkman criteria	% HA	32	36	33	30	25	20
Derived criteria	%HA	14	16	16	13	10	9
	ALH	10.4 ± 5.2	8.9 ± 4.6	9.7 ± 5.5	10.3± 5.6	9.6 ± 4.9	7.4 ± 3.6
	LIN	69.3 ± 18.1	70.2 ± 19.6	65.0 ± 19.9	64.0 ± 19.9	68.9 ± 20.9	66.7 ± 21.0
BM + PF	VAP	144.3 ± 50.7	137.7 ± 52.1	128.3 ± 54.3	133.4 ± 53.7	132.0 ± 56.4	114.0 ± 48.2
(0.01mg/ml)	VCL	170.5 ± 58.5	158.0 ± 57.5	153.6 ± 65.2	158.9 ± 62.2	154.4 ± 65.3	130.5 ± 53.3
	VSL	118.0 ± 51.1	101.2 ± 50.8	97.3 ± 50.9	101.2 ± 50.8	101.6 ± 58.5	83.7 ± 40.8
Burkman criteria	%HA	23	21	30	30	20	22
Derived criteria	%HA	13	8	14	13	9	3
	ALH	10.6 ± 5.2	9.7 ± 5.0	9.7 ± 5.0			
	LIN	71.9 ± 16.6	66.0 ± 19.6	60.9 ± 21.2			
BM + PF	VAP	142.8 ± 49.9	130.0 ± 52.7	126.6 ± 52.6			
(0.10mg/ml)	VCL	169.9 ± 59.4	154.3 ± 62.3	150.9 ± 61.2			
	VSL	121.3 ± 50.4	100.0 ± 50.0	87.6 ± 44.4			
Burkman criteria	%HA	18	26	40			
Derived criteria	%HA	12	12	18			

5.3.3.d Experiment 3d - Hyperactivation of stallion spermatozoa - re-analysis of the data

The results for movement analysis and percentage hyperactivation presented in Table 5.3 for human spermatozoa were similar to results from previous work in this laboratory (Kay *et al.*, 1993; Morris *et al*, 1996) and from other workers using similar approaches (Burkman, 1984; Robertson *et al.*, 1988; Burkman, 1991). The criteria selecting hyperactivated cells, when applied to stallion spermatozoa detected high proportions in seminal plasma, a medium widely considered to be non-capacitating, with no apparent increase in hyperactivation during incubation under conditions believed to support capacitation. For these reasons videotapes were re-examined visually, utilising the playback facility on the analyser. Two hundred sperm tracks were examined and categorised into (1) non-hyperactivated trajectory, (2) transitional phase and (3) star spin trajectory. The procedure was first carried out on human spermatozoa. Subsequently the process was repeated with approximately 400 stallion sperm tracks.

5.3.3.3 Results (Experiment 3d)

The results for human spermatozoa are shown in Table 5.6. and for stallion spermatozoa in Table 5.7.

Table 5.6: Numbers of hyperactivated cells detected in human samples (observed versus computer sorted).

Human Spermatozoa	Seminal Plasma	Post Swim-up	PF 1 mg/ml
Total counted	200	200	200
(1). Non Hyperactivated	181	158	143
(2). Transition Phase	13	21	41
(3). Star-Spin Pattern	5	17	16
%HA (total observed)	9	19	28
%HA(computer sorted)	8	19	25
Difference	-1%	+0%	+3%

Table 5.7: Numbers of hyperactivated cells detected in stallion samples (observed versus computer sorted).

Stallion Spermatozoa	Seminal Plasma	Post Swim-up	PF 1 mg/ml
Total counted	405	347	382
(1). Non Hyperactivated	295	238	265
(2). Transition Phase	9	7	13
(3). Star-Spin Pattern	1	3	4
%HA (total observed) [a]	3	3	5
%HA (computer sorted) [b]	15	14	11
Difference	+12%	+10%	+5%

ab different letters between rows (observed values versus computer sorted) differ ($p<0.003$)

On re-analysis of videotaped human spermatozoa there was no significant difference between numbers of hyperactivated trajectories identified visually and hyperactivated trajectories sorted by computer according to the criteria of Burkman (1991); results are shown in Table 5.6. In contrast, there was a significantly lower visual count of hyperactivated trajectories in stallion semen, in comparison to counts sorted by computer using the criteria of Burkman; results shown in Table 5.7. On re-analysis, it was possible to identify 29 transitional phase trajectories and 8 star spin trajectories from a total of 1,134 trajectories. Values for the ALH, LIN and VCL of these individual cells are summarised in Table 5.8.

Table 5.8: Characteristic of sperm trajectories for stallion semen; Mean ± SD (Range).

Stallion Spermatozoa	Transitional phase (N=29)	Star spin (N=8)
ALH (μm)	17.1 ± 3.5 (12.4 - 25.2)	14.5 ± 3.6 (12.2 - 20.0)
LIN (%)	53.7 ± 7.5 (41.9 - 65.0)	18.0 ± 7.5 (13.1 - 32.7)
VCL (μm/sec)	188.8 ± 24.6 (156 - 235)	173.2 ± 28.2 (141 - 217)

Based on these results a new definition of hyperactivation was derived for stallion spermatozoa (termed derived criteria) which was ALH ≥ 12.5 μm, LIN ≤ 65% and

VCL ≥ 140 μm/sec. These values encompassed the lowest VCL and ALH and the highest LIN of the observed hyperactivated cells. These new parameters were programmed into the "Sort Facility" of the computer, and the track data of Experiment 3c (summarised in Table 5.7) was reanalysed using these criteria. The results are shown in Table 5.9.

Table 5.9: A comparison of numbers of hyperactivated cells selected using the criteria of Burkman (1991), visual observation and the newly derived criteria for stallion spermatozoa. The percentage of hyperactivated spermatozoa (%HA) was calculated using the criteria of Burkman (1991) or the criteria derived for stallion spermatozoa in Experiment 3d.

Stallion Spermatozoa	Seminal Plasma	Post Swim-up	PF 1 mg/ml
Total counted	405	347	382
%HA (Burkman) [a]	15	14	11
%HA (observed) [b]	3	3	5
%HA (derived criteria) [b]	4	5	2

ab different letters between rows differ ($p<0.001$)

The derived criteria (ALH ≥ 12.5 μm, LIN ≤ 65% and VCL ≥ 140 μm/sec) were also used to reanalyse tracks recorded for experiment 5.2.3c. The number of spermatozoa showing hyperactivated motility declined by 50% as a result of incorporating the new criteria. The data is shown in Table 5.5.

5.4 Discussion

The optimal requirements for the capacitation of stallion spermatozoa are unknown, and developing reliable methods for capacitation has been a major obstacle in the advancement of equine *in vitro* fertilisation. The most commonly used media are modified Tyrode solutions (TALP) which have been used by many authors (Blue *et al.*, 1989; Samper *et al.*, 1989; Padilla and Foote, 1991; Zhang *et al.*, 1991; Ellington *et al.*, 1993a; Hochi *et al.*, 1996). The composition of TALP used by these authors varies and is presented in Appendix 1. Because of poor sperm viability, recent interest has turned to co-culturing spermatozoa with uterine tube epithelial cells (Ellington *et al.*, 1993b), uterine tube explants (Pollard *et al.*, 1991) or medium conditioned with oviduct epithelial cells (Ellington *et al.*, 1992). These methods are reported to support viability, capacitation and the acrosome reaction. An alternative approach has been supporting viability by mixing milk media with capacitating media, in different proportions. A mixture of 35% skim-milk media and 65% capacitating media preserved the motility of centrifuged stallion spermatozoa well for 72 hours, despite cooling (Padilla and Foote, 1991; Webb and Arns, 1995).

In this study several media were examined prior to the reported experiments. The unsuccessful results achieved using three types of TALP (Parrish *et al*, 1988; Blue *et al.*, 1989; Padilla and Foote, 1991) were not repeated in this laboratory. Promising preliminary results were achieved using Graham TALP (Dr Graham, Boulder Colorado; personal communication) and Bavister TALP (Zhang *et al.*, 1991). Promising preliminary results were also achieved by mixing Bavister TALP 1: 1 with Kenney's medium. These media were tested further in Experiment 1.

The data from this study suggested that the media examined supported stallion sperm viability reasonably well, in comparison to starting values, for between 4 and 6 hours. It was noted that the time 0 values (50% motile, mean 55% live) were lower than would be expected for domestic species such as ram and bull. Motility was maintained less well but this must be considered together with the information from later motility studies. Media containing milk maintained the highest percentage motile for the longest period. From 4 hours onwards the percentage live greatly

exceeded the percentage motile. The observation has been made by other workers (Robertson and Middleton, 1992) and explained as the presence of viable but immotile spermatozoa. No difference was detected between tubes and tissue culture wells in this study, although other workers reported improved sperm longevity (Ellington *et al.*, 1993b).

Sperm functional state during incubation in Bavister-Milk medium was assessed using a dual chlortetracycline (CTC) and Hoechst 33258 stain. The use of CTC to assess capacitation in stallion sperm had been evaluated by a number of authors (Varner *et al.*, 1987b; Ward *et al.*, 1988). The major criticism of the technique is that membranes are stained regardless of viability and it has long been recognised that one change which can occur during sperm degeneration is acrosome loss (Cross *et al.*, 1986) which cannot be distinguished from acrosomal loss due to physiological reasons using histological dyes (Talbot and Chacon, 1981), lectins (Talbot and Chacon, 1980; Cross *et al.*, 1986; Mortimer *et al.*, 1987) or monoclonal antibodies (Byrd and Wolf, 1986; Moore *et al.*, 1987). In this study, a dual staining technique developed for use with human spermatozoa (Kay *et al.*, 1994), was used to evaluate stallion sperm functional state for the first time. The important point about dual staining is that the Hoechst 33258 dye stains dead sperm; this dye is excluded by live sperm. Fluorescent CTC patterns are then assessed only for live spermatozoa.

Three distinct CTC patterns similar to those described in mouse (Fraser, 1987) and human (Das Gupta *et al.*, 1993; Kay *et al.*, 1994) were observed. Hence the same nomenclature: 'F' or fresh, a uniform fluorescence over the head, characteristic of uncapacitated, acrosome intact cells; 'B' or banded, a fluorescence free band in the post acrosomal region, characteristic of capacitated, acrosome-intact cells; and 'AR', with dull or no fluorescence on the head, characteristic of acrosome reacted cells, has been used. For spermatozoa incubated in seminal plasma the majority of cells showed the 'F' pattern. By 300 minutes only the 'F' pattern was present. This was concurrent with a drop from 70 to 7% live. This decline in percentage live far exceeded that seen in any culture medium but since it is well recognised that prolonged contact with seminal plasma is detrimental to sperm survival this finding was not unexpected. What is more difficult to explain is why there were 'B' and 'AR' patterns in seminal plasma.

Incubation in Bavister-Milk medium without additives showed an increase in 'B' pattern with time, increasing from 10% at time 0 to 60% by 300 minutes, with a corresponding decline in the percentage of 'F' pattern. Negligible levels of 'AR' pattern were detected. This is consistent with other work indicating that spontaneous acrosome loss occurs at a low level (5%) (Stock and Fraser, 1989). The shift from the uncapacitated to the capacitated state increased with the addition of pentoxifylline in a dose dependent manner, with the highest levels of capacitated pattern seen after 300 minutes of incubation in medium supplemented with 1 mg/ml pentoxifylline. A high proportion of acrosome loss was observed after 120 minutes with this treatment (up to 50%). The occurrence of acrosome loss had declined by 300 minutes (mean 10%). This pattern suggests that cells die rapidly after acrosome loss. The sixth treatment in this experiment, addition of ionophore, was a positive control, designed to induce the acrosome reaction. Essentially this treatment was similar to treatment 2, incubation with 0 μg/ml pentoxifylline, with the late addition of ionophore to induce acrosomal loss. The proportion of induced acrosome reactions increased with incubation time. While it is tempting to believe A23187 selectively induces acrosome reactions in capacitated cells the volume of literature does not support this. It was believed at one point that A23187 selectively induced acrosomal exocytosis in capacitated cells (Byrd *et al.*, 1989), however, it is now commonly believed that A23187 induces acrosomal exocytosis regardless of the capacitation status of the cell (Shams-Borhan and Harrison, 1981; Varner *et al.*, 1987b; Magistrini and Palmer, 1991).

To further test the hypothesis that capacitated sub-populations were present within the whole population, an attempt was made to identify stallion sperm hyperactivation using the Hamilton Thorn motility analyser. Because studies of hyperactivated motility are well established in human spermatozoa (Burkman, 1984; Robertson *et al.*, 1988; Zhu *et al.*, 1994), initial experiments looked at quantifying human sperm hyperactivation under noncapacitating and capacitating conditions. The results were as expected (Table 5.3). There was an overall increase in ALH, VAP and VCL, with a decline in linearity (LIN) after swim-up. The numbers of hyperactivated spermatozoa identified, according to the criteria of Burkman (1991), increased two-fold after swim-up and increased further to four-fold that of the starting value, when extended in medium supplemented with 1 mg/ml pentoxifylline.

The criteria defined by Burkman (1991) were also used to automatically identify hyperactivated stallion spermatozoa. It was observed that stallion spermatozoa typically exhibited a wide amplitude of lateral head displacement and were less linear than human sperm even when extended in homologous seminal plasma. Velocities (VAP and VCL; μms^{-1}), increased after swim-up but ALH and LIN, already high, remained unchanged. A relatively high number of allegedly hyperactivated spermatozoa were identified in seminal plasma (Table 5.3). Re-analysis of the videotapes showed that the criteria used to identify human sperm hyperactivation, ALH > 7.5 μm, LIN of < 65% and VCL of >100 μms^{-1}, were insufficiently discriminating for stallion spermatozoa and caused misidentification of a large number of spermatozoa fitting the criteria though not necessarily hyperactivated (i.e. not exhibiting a transitional phase or star spin trajectory as described for human spermatozoa).

Using the playback facility it was noted that the number of stallion spermatozoa showing hyperactivated motility (as determined by visual inspection) was significantly different from the number generated using the computerised sort. From a total of 1134 trajectories visually examined, and classified into non hyperactivated, transition phase and star-spin pattern as described in Robertson *et al.*, (1988) only 29 transitional phase and 8 star spin trajectories were identified. Based on this data, new criteria of ALH > 12.5 μm, LIN < 65% and VCL > 140 μms^{-1} were derived. The number of stallion spermatozoa showing hyperactivated motility, sorted by computer using these new criteria, was significantly lower than for the Human criteria and not different from the numbers determined visually. This approach to determining the criteria for defining hyperactivation has been used before with other species (Robertson *et al.*, 1988; Burkman, 1991). However, because of the small number of cells forming the stallion hyperactivation database this study must be considered preliminary rather than definitive. A problem was encountered which contributed to the identification of low numbers of hyperactivated cells. This is discussed more fully below.

A further experiment was conducted to examine sperm motility in detail, using the computerised analyser, during a 300 minute incubation in the Bavister-Milk medium, under the same conditions as studied by the CTC/ dual staining technique.

Two treatments were dropped from the experiment. Because of the poor viability of spermatozoa incubated in seminal plasma (7% live by 300 minutes) this treatment was excluded. Ionophore treatment (A21837) was included as a positive control for acrosomal exocytosis in the earlier experiment. Since this was not relevance to motility this treatment was also excluded. The initial time point was 15 minutes because of the time required to videotape 3-4 minutes of each treatment sample.

Initial analysis of hyperactivated activity using the criteria of Burkman (1991) showed a higher incidence of hyperactivation for stallion sperm. This may be attributed to larger ALH and VCL and less linear trajectories of initial stallion sperm suspensions. The application of the criteria derived from stallion sperm, reduced the occurrence of hyperactivation, as sorted by computer, by at least 50%. Incubation in medium supplemented with pentoxifylline did not significantly increase the occurrence of hyperactivation.

During this experiment it was observed that stallion sperm exhibited 'head to head' agglutination and clumping after 120 minutes of incubation in Bavister-Milk medium. This phenomenon was exacerbated by the addition of pentoxifylline, and occurred in a dose-dependent manner. This phenomenon is possibly an indication of changes to the sperm plasma membrane. Whether these changes are a component of capacitation is not known. After 300 minutes of incubation however, the extent of clumping declined a little, with live spermatozoa (identified by the continuous flagella beat) still clumped together and individual unclumped sperm completely immotile.

Pentoxifylline is a phosphodiesterase inhibitor which causes an increase in intracellular cAMP. Its actions are not fully understood but various effects on sperm function have been demonstrated. A number of authors have shown effects of pentoxifylline on human sperm motility. It has been shown to increase VCL and ALH of spermatozoa in suspension (Kay *et al.*, 1993; Lewis *et al.*, 1993). It has been shown to stimulate hyperactivation in fresh and cryopreserved human spermatozoa (Kay *et al.*, 1993; Morris *et al.*, 1996). It has been shown to accelerate capacitation of human spermatozoa in the presence (Kay *et al.*, 1994) and absence of progesterone (Das Gupta *et al.*, 1994).

Yanagamachi, (1982) reported this phenomena of agglutination in hamster spermatozoa after between 20 to 60 minutes of incubation in a TALP medium. He

reported that the number of spermatozoa in each clump varied between two and one hundred, and that the agglutinated clumps began to disperse about two hours after incubation. Concomitant with the dispersion was the appearance of hyperactivated spermatozoa. This dispersion from the 'clumped' state to a hyperactivated state however was not observed in the present study.

In conclusion, fluorescent membrane patterns corresponding to those seen during capacitation of other species were detected during incubation of stallion and increased in the presence of pentoxifylline, an agent believed to accelerate capacitation. Pentoxifylline caused severe sperm clumping, another surface membrane change associated with capacitation. Pentoxifylline did not stimulate hyperactivation of stallion sperm as it did for human. It is impossible to say whether this is a real observation or an artefact of clumping, removing these cells from motility analysis.

CHAPTER 6

CALCUIM REGULATION DURING CAPACITATION

6.1 Introduction

Fluxes in intracellular calcium are a component of the regulatory mechanisms of sperm capacitation, hyperactivation and the acrosome reaction. Other workers have shown a requirement for micromolar levels of extracellular calcium to support capacitation (Fraser, 1987; Adeoya-Osiguwa and Fraser, 1993; Das Gupta *et al.*, 1993) and millimolar levels to support the acrosome reaction (Yanagimachi and Usui, 1974; Shams-Borhan and Harrison, 1981; Yanagimachi, 1982; Fraser, 1987; Thomas and Meizel, 1988). Similar requirements for calcium have been demonstrated for hyperactivated motility (Yanagimachi, 1982; White and Aitken, 1989). The presence of calcium pumps and channels have been demonstrated in the sperm plasma membrane of many species (Blackmore *et al.*, 1990; Guerrero and Darszon, 1990; Lievana and Darszon, 1995) and it has been hypothesised that during the process of capacitation the function of Ca^{2+}ATPases responsible for extruding $[Ca^{2+}]_i$ is modified allowing $[Ca^{2+}]_i$ to reach a critical threshold affecting other components of capacitation such as hyperactivation (Adeoya-Osigawa and Fraser, 1996). Other workers have shown that a gradual increase in $[Ca^{2+}]_i$ does occur during prolonged incubation and have speculated that this is a component of capacitation (Canvin and Buhr, 1989; Buhr *et al.*, 1989; Zhou *et al.*, 1990; Zhao and Buhr, 1995).

Recently, the steroids progesterone and 17∝-hydroxyprogesterone have been shown to stimulate a rapid influx of calcium in human spermatozoa (Thomas and Meizel, 1989; Blackmore *et al.*, 1990; Baldi *et al.*, 1991). It has since been proposed that progesterone present in the ovulatory milieu in the fallopian tubes after ovulation plays a physiological role by facilitating or triggering acrosomal exocytosis when the oocyte reaches the site of fertilisation (Blackmore and Lattanzio, 1991). This view was supported by Roldan *et al.*, (1994). In their studies with mouse spermatozoa, they showed that when spermatozoa were exposed first to progesterone and then to zona pellucida, exocytosis was enhanced over that seen when the agonists were not presented together or in the inverse order, suggesting that the steroid exerts a priming effect.

With this information, and in light of the importance of maintaining the

ability to regulate intracellular calcium, as demonstrated in Chapter 4, a series of experiments were carried out to examine calcium regulation under capacitating conditions. The incubation conditions used were those tested in Chapter 5 and found, using the CTC stain, to induce capacitation. The first experiment was designed to examine intracellular calcium concentrations during incubation for 300 minutes. The second experiment was designed to establish whether a calcium transient could be induced by progesterone, as demonstrated by others for human spermatozoa (Thomas and Meizel, 1989; Blackmore *et al.*, 1990; Baldi *et al.*, 1991; Foresta *et al.*, 1993). This experiment was initially carried out with human spermatozoa. Finally, experiments were carried out to identify progesterone receptors in both human and stallion spermatozoa under different incubation conditions.

6.2 Materials and Methods

Semen collection, preparation of media and details of intracellular calcium measurement are as described in Chapter 2.

6.3 Calcium Transients Experiments

6.3.1 Experiment 1 - Intracellular calcium changes during incubation under capacitating and non-capacitating conditions

One ejaculate from each of three stallions was extended in homologous seminal plasma, a non-capacitating medium, and Bavister-Milk medium and Bavister-

Milk medium supplemented with 1 mg/ml pentoxifylline, treatments shown to support capacitation. Two 4 ml aliquots from each treatment were incubated in capped Falcon tubes in 5% CO_2 in air at 38.5°C. After 0 and 300 minutes of incubation subsamples were removed, loaded with Fura-2AM as described previously, and extended in a cuvette at a final concentration of 50 million/ml. Intracellular calcium measurements were recorded for each treatment after 0 and 300 minutes of incubation.

6.3.1.1 Results

There was no significant increase in intracellular calcium for spermatozoa incubated in homologous seminal plasma after 300 minutes of incubation. There was a significant increase in intracellular calcium after 300 minutes of incubation for stallion spermatozoa incubated in Bavister-Milk medium containing 0 or 1 mg/ml pentoxifylline. Mean intracellular calcium was numerically higher when the medium was supplemented with pentoxifylline. However, this difference was not statistically significant. Results are summarised in Table 6.1.

Table 6.1: Intracellular calcium concentration (Mean ± SD; n = 3 ejaculates) of stallion spermatozoa measured after 0 and 300 minutes of incubation.

Time Medium	0 minute	300 minutes
Seminal Plasma	87 ± 16 nM ^{a}	92 ± 5 nM ^{a}
Bavister-Milk	50 ± 21 nM ^{a}	189 ± 60 nM ^{b*}
Bavister-Milk (+PF)	82 ± 31 nM ^{a}	202 ± 86 nM ^{b*}

ab Values with different letters within column differ (P<0.001) and * values differ (P<0.001) from start (time 0)

6.3.2 Experiment 2 - The induction of calcium transients in human spermatozoa using progesterone

The response of human sperm to progesterone is well documented (Blackmore *et al.*, 1990; Baldi *et al.*, 1991; Foresta *et al.*, 1993; Krauscz *et al.*, 1995). Therefore preliminary experiments using human spermatozoa were carried out to validate the technique. Three ejaculates from each of two individuals were prepared by swim-up as described previously. At the end of the swim-up migration, and without further incubation, spermatozoa were loaded with Fura-2AM as previously described, and re-extended in Medicult medium in a cuvette for fluorescence measurements. Fluorescence intensities were recorded for approximately 40 seconds to establish a baseline reading, then progesterone at a final concentration of 1 μg/ml or DMSO, the solvent for progesterone, at a final concentration of 0.1% was added and measurement of fluorescence intensities continued for up to a further 120 seconds.

6.3.2.1 Results

There was a significant increase in intracellular calcium in response to the addition of 1 μg/ml progesterone. The mean value for the initial basal concentration was 49 nM. This increased to peak at 120 nM within 40 seconds of progesterone addition. This value was transient and a new basal concentration was established at 71 nM. No such response was observed on the addition of 0.1% DMSO. Mean values are summarised in Table 6.2. Responses are shown graphically in Figure 6.1a and b.

Table 6.2: Intracellular calcium measured prior to addition of progesterone, for 0-60 seconds after addition of 1 μg/ml progesterone or 0.1% DMSO and new plateau level for human spermatozoa (Mean ± SD; n=6 ejaculates).

Human Spermatozoa	Initial concentration	0-60 sec after addition	Plateau concentration
Progesterone (1 μg/ml)	49 ± 16 nM	93 ± 46 nM *	71 ± 26 nM **
DMSO 0.1%	50 ± 12 nM	49 ± 11 nM	49 ± 11 nM

values differ * ($P<0.001$) and ** ($P< 0.01$)

6.3.3 Experiment 3 - The induction of calcium transients in stallion spermatozoa using progesterone

Experiment 6.3.2 was repeated using stallion spermatozoa. One ejaculate from each of three stallions was used in this experiment. Because of the problems encountered previously when using swim-up migration for separating stallion spermatozoa from seminal plasma, samples were prepared both by swim-up migration and centrifugation. Fura-2AM loading and intracellular calcium measurement was carried out as described previously.

Fluorescence intensities were recorded for approximately 40 seconds, to give a baseline reading, then progesterone, at a final concentration of 1 μg/ml, was added and the measurement of fluorescence intensities continued for up to a further 120 seconds.

6.3.3.1 Results

The results of the experiment are as summarised in Table 6.3. The fluorescence intensities of loaded sample prepared by swim-up were as low as background intensity, hence deduction of background intensity and measurement of intracellular calcium were not possible. In contrast, after centrifugation, the fluorescence intensities at both wavelengths were several-fold higher than

background. When progesterone (1 μg/ml) was added to these samples, no transient rise in intracellular calcium was observed. No response was also observed on the addition of DMSO (Figure 6.2a-b).

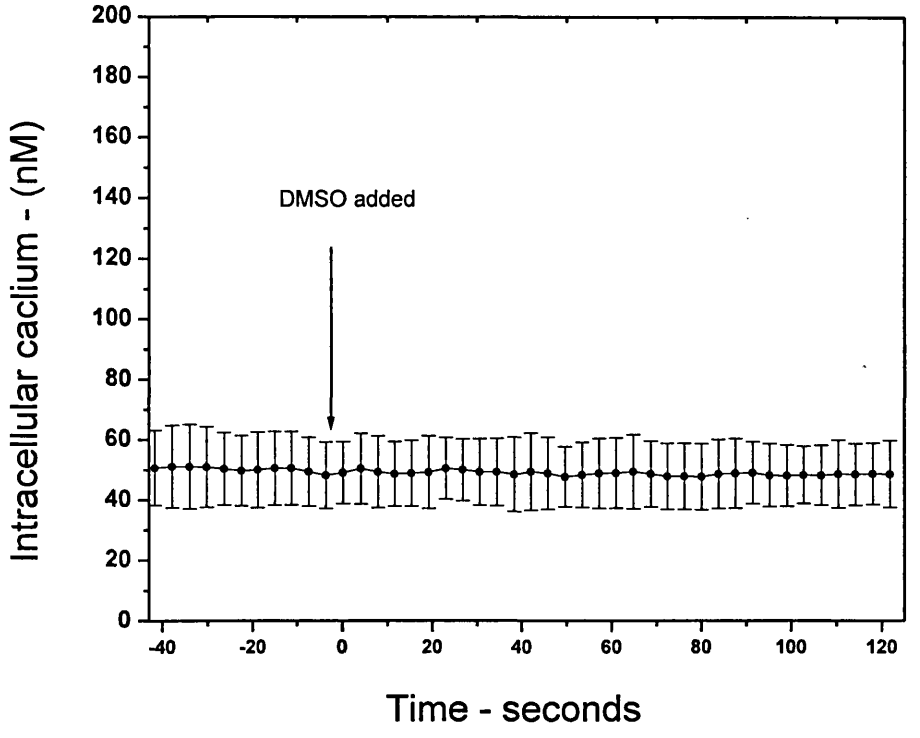
Table 6.3: Intracellular calcium measured prior to addition of progesterone, for 0-60 seconds after addition of 1 μg / ml progesterone or 0.1% DMSO and new plateau level for stallion spermatozoa (Mean ± SD; n=3 ejaculates).

Stallion Spermatozoa	Initial concentration	0-60 sec after addition	Plateau concentration
Progesterone (1 μg/ml)	87 ± 21 nM	88 ± 21 nM	89 ± 22 nM
DMSO 0.1%	87 ± 20 nM	88 ± 21 nM	89 ± 22 nM

Figure 6.1a Graph showing intracellular calcium changes (nM; Mean ± SEM, n = 6) in human sperm suspension in response to DMSO. DMSO was added at time 0.

Figure 6.1b Graph depicting typical changes in intracellular calcium (nM; Mean ± SEM, n = 6) seen in human sperm suspension in response to progesterone. Progesterone (1 μg/ml final concentration) was added at time 0.

6.1a


Intracellular caclium - (nM)
DMSO added
Time - seconds

6.1b

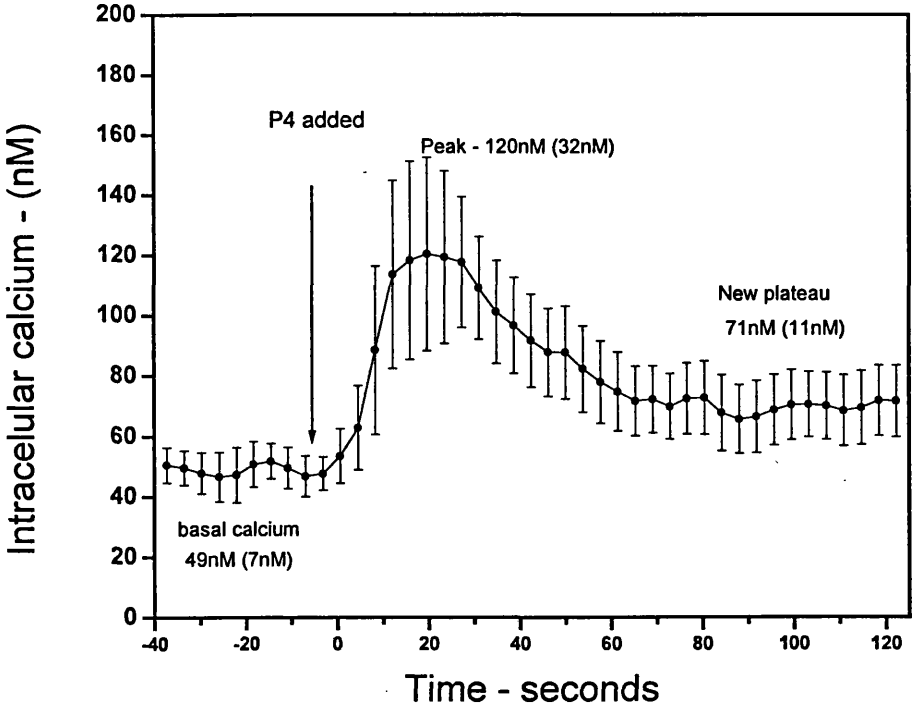
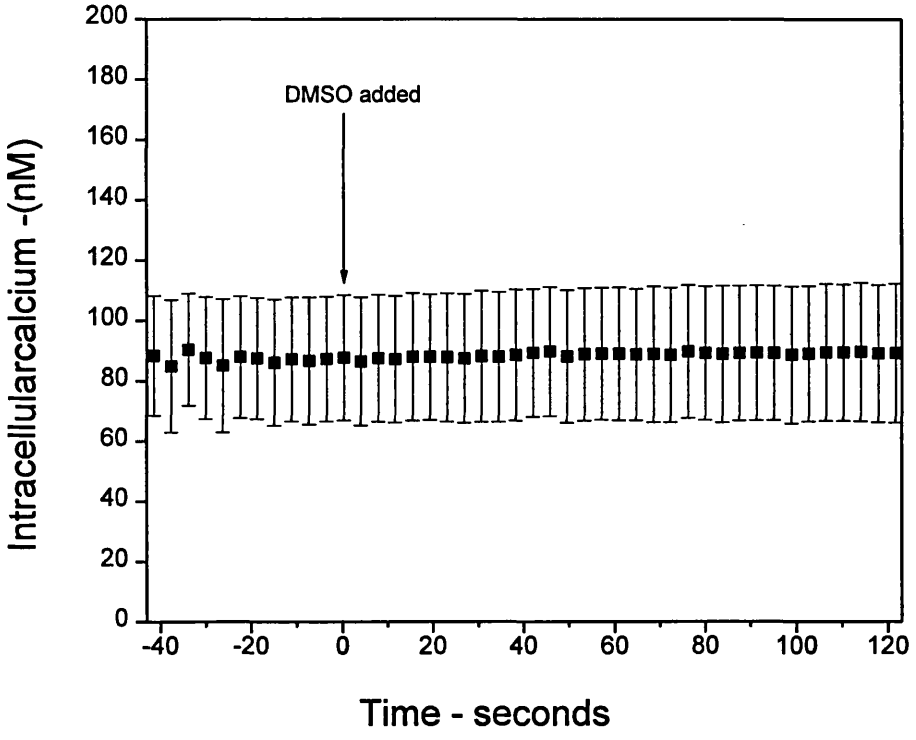

Intracelular calcium - (nM)
P4 added
Peak - 120nM (32nM)
New plateau
71nM (11nM)
basal calcium
49nM (7nM)
Time - seconds

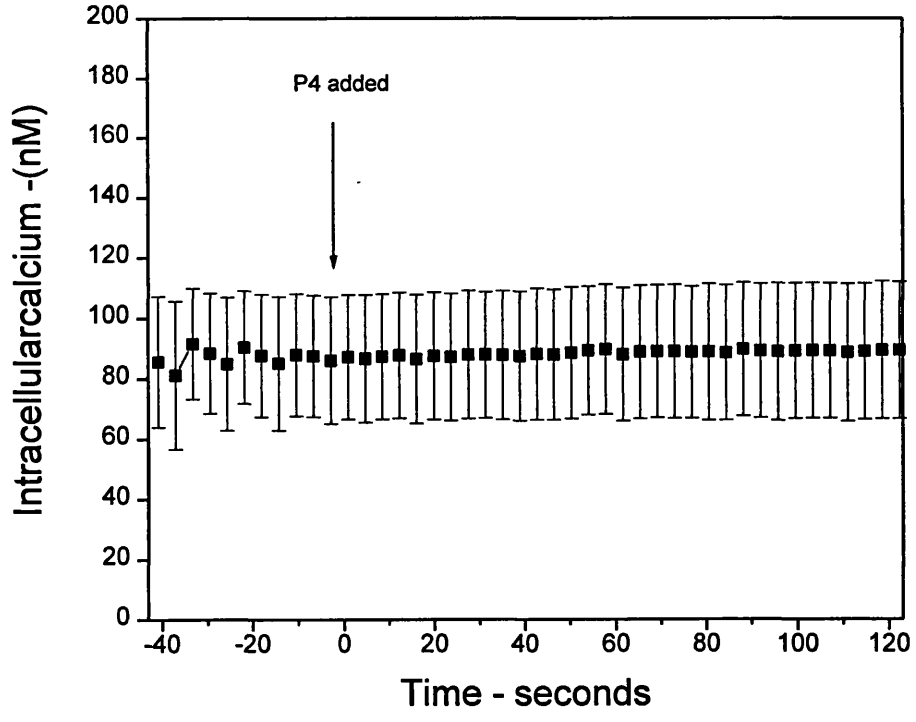
Figure 6.2a Graph showing intracellular calcium changes (nM; Mean ± SEM, n = 3) in stallion sperm suspension in response to DMSO. DMSO was added at time 0.

Figure 6.2b Graph depicting typical changes in intracellular calcium (nM; Mean ± SEM, n = 3) seen in stallion sperm suspension in response to progesterone. Progesterone (1 μg/ml final concentration) was added at time 0.

6.2a


DMSO added
Intracellularcalcium -(nM)
Time - seconds
200
180
160
140
120
100
80
60
40
20
0
-40
-20
0
20
40
60
80
100
120

6.2b


P4 added
Intracellularcalcium -(nM)
Time - seconds
200
180
160
140
120
100
80
60
40
20
0
-40
-20
0
20
40
60
80
100
120

6.3.4 Experiment 4 - The induction of calcium transients in stallion spermatozoa using progesterone, after incubation

One ejaculate from each of three stallions was centrifuged to remove seminal plasma and extended in Bavister-Milk medium containing 0 or 1 mg/ml pentoxifylline and incubated for 300 minutes. Subsamples were removed after 0 and 300 minutes of incubation and loaded with Fura-2AM. After removal of extracellular dye, spermatozoa were suspended in a cuvette at a final concentration of 50 million/ml. Fluorescence intensities were recorded for 40 seconds to establish baseline measurements. Progesterone at a final concentration 1 μg/ml or DMSO at a final concentration of 0.1% was added and recording of the fluorescence intensities was continued for a further 80 seconds.

6.3.4.1 Results

A significant increase in basal intracellular calcium was seen after 300 minutes of incubation for each treatment ($p<0.001$) prior to the addition of progesterone. No response was induced by the addition of progesterone or DMSO to samples after 0 incubation. A calcium transient was observed in response to the addition of progesterone after 300 minutes of incubation in Bavister-Milk medium supplemented with pentoxifylline. No response was detected after the addition of progesterone to samples incubated in the absence of pentoxyifylline and on the addition of DMSO. The mean values are summarised in Table 6.4 and the response to the addition of substances are presented in Figure 6.3 a-f.

Table 6.4: Intracellular calcium concentration (nM) of stallion spermatozoa incubated in Bavister-Milk medium with the addition of 0 or 1 mg/ml pentoxifylline (PF) and challenged by the addition of 1 μg/ml progesterone (P4) or 0.1% DMSO after 0 or 300 minutes of incubation (Mean ± SEM, n = 3 ejaculates).

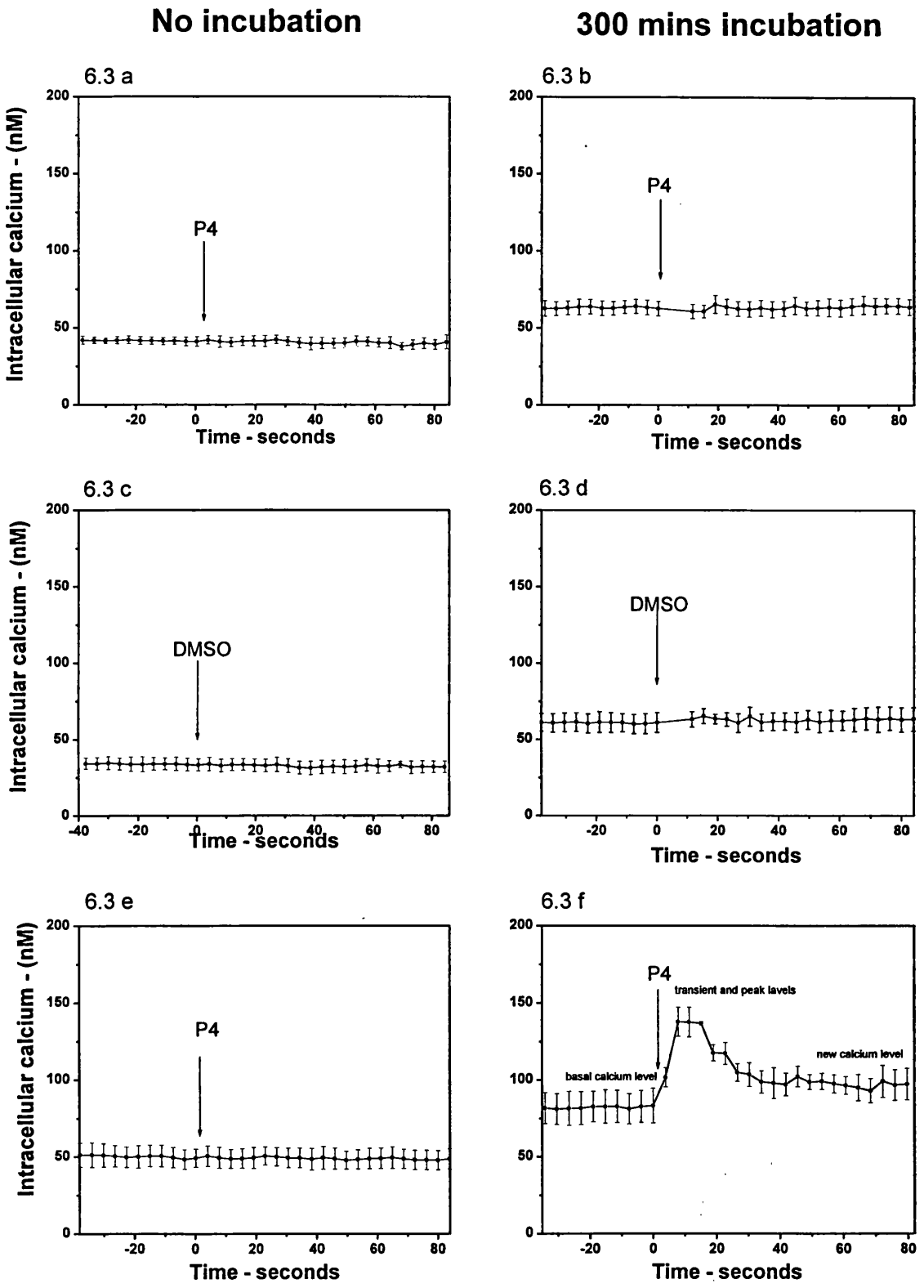
Incubation in Bavister-Milk		0 min	300 min
	Initial basal	34 ± 7	61 ± 11 ^{a}*
0 mg/ml PF	0-60 secs after addition	33 ± 7	63 ± 10 ^{a}
P4 added	Peak values	no response	no response
	Plateau	32 ± 7	63 ± 13
	Initial basal	42 ± 4	63 ± 8 ^{a}*
0 mg/ml PF	0-60 secs after addition	41 ± 6	63 ± 9 ^{a}
DMSO added	Peak values	no response	no response
	Plateau	40 ± 6	64 ± 9
	Initial basal	51 ± 12	82 ± 15 ^{b}*
1 mg/ml PF	0-60 sec after addition	49 ± 11	111 ± 18 ^{c}
P4 added	Peak values	-	137 ± 9.6
	Plateau	47 ± 20	97 ± .7 ^{b}

abc Values with different letters within column differ ($p<0.01$) and * values differ ($p<0.0001$) from start (time 0)

Figure 6.3a - b Spermatozoa incubated in Bavister-Milk. Intracellular calcium (nM; Mean ± SEM) in response to progesterone, assessed after 0 and 300 minutes of incubation.

Figure 6.3c - d Spermatozoa incubated in Bavister-Milk. Intracellular calcium(nM; Mean ± SEM) in response to DMSO, assessed after 0 and 300 minutes of incubation.

Figure 6.3e - f Spermatozoa incubated in Bavister-Milk supplemented with 1 mg/ml pentoxifylline. Intracellular calcium (nM; Mean ± SEM)in response to progesterone, assessed after 0 and 300 minutes of incubation.


No incubation
300 mins incubation
6.3 a
6.3 b
6.3 c
6.3 d
6.3 e
6.3 f
Intracellular calcium - (nM)
Time - seconds
P4
DMSO
transient and peak lavels
basal calcium level
new calcium level

6.3.5 Experiment 5 - The induction of calcium transients in stallion spermatozoa using progesterone after incubation under capacitating and non-capacitating conditions

Experiment 6.3.4 was repeated; for this experiment spermatozoa were suspended in homologous seminal plasma, a non-capacitating treatment, Bavister TALP medium and Bavister-Milk medium supplemented with 1 mg/ml pentoxifylline.

6.3.5.1 Results

A significant increase in basal intracellular calcium was observed after 300 minutes of incubation in Bavister TALP and Bavister-Milk supplemented with pentoxifylline ($p<0.001$). This was not observed for spermatozoa incubated in homologous seminal plasma. No response was induced by the addition of progesterone to samples after 0 minutes of incubation. A calcium transient was induced in response to the addition of progesterone after 300 minutes of incubation in Bavister-Milk medium supplemented with pentoxifylline. No response was detected on the addition of progesterone to samples suspended seminal plasma. A small but non-significant response was detected on addition of progesterone to samples suspended in Bavister TALP after 300 minutes. Mean values are summarised in Table 6.4. Response to the addition of progesterone is presented in Figure 6.4 a-f.

Table 6.5: Intracellular calcium concentration (nM) of stallion spermatozoa incubated in three different medium and evaluated after 0 or 300 minutes of incubation after the addition of 1 μg/ml progesterone (Mean ± SEM, n = 3 ejaculates).

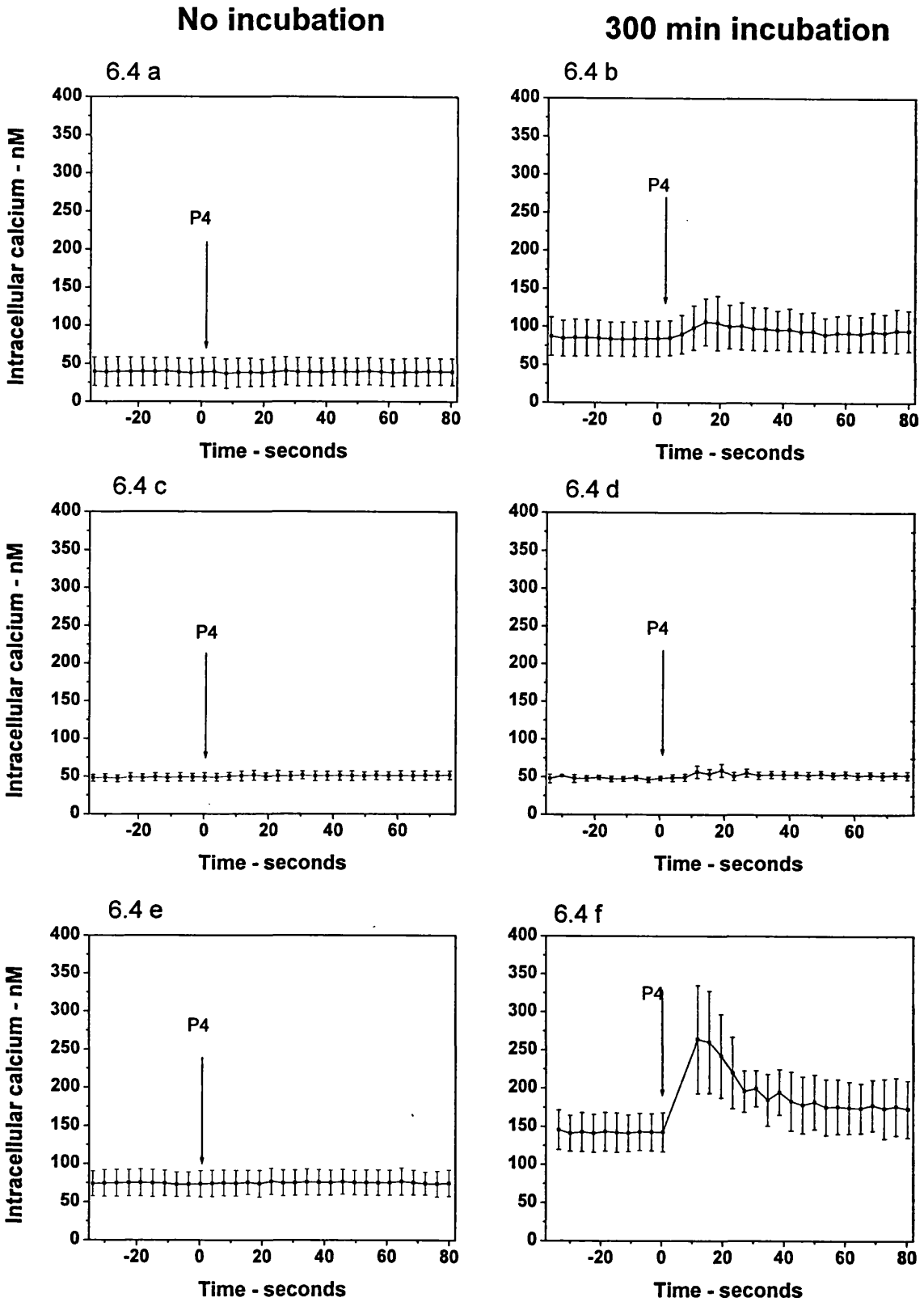
Medium		0 min	300 min
Seminal plasma **P4 added**	Initial basal	48 ± 9	48 ± 6
	0-60 secs after addition	50 ± 10	53 ± 10
	Peak values	no response	no response
	Plateau	51 ± 10	53 ± 8
Bavister TALP **P4 added**	Initial basal	39 ± 18	85 ± 40 *
	0-60 secs after addition	39 ± 19	97 ± 48
	Peak values	no response	106 ± 31
	Plateau	39 ± 18	93 ± 43
Bavister Milk + PF **P4 added**	Initial basal	74 ± 30	143 ± 43 [a]*
	0-60 secs after adding P4	75 ± 29	209 ± 73 [b]
	Peak values	no response	264 ± 122
	Plateau	75 ± 29	176 ± 62 [b]

ab Values with different letters within column differ (P<.030) and * values differ (P<.0001) from start (time 0)

Figure 6.4a - b Spermatozoa incubated in Bavister-Milk. Intracellular calcium (nM; Mean ± SEM; n = 3) in response to progesterone, assessed after 0 and 300 minutes of incubation.

Figure 6.4c - d Spermatozoa incubated in homologous seminal plasma. Intracellular calcium (nM; Mean ± SEM; n = 3) in response to progesterone, assessed after 0 and 300 minutes of incubation.

Figure 6.4e-f Spermatozoa incubated in Bavister-Milk supplemented with 1 mg/ml pentoxifylline. Intracellular calcium (nM; Mean ± SEM; n = 3) in response to progesterone, assessed after 0 and 300 minutes of incubation.


No incubation
300 min incubation
6.4 a
6.4 b
6.4 c
6.4 d
6.4 e
6.4 f
Intracellular calcium - nM
Time - seconds
P4
0
50
100
150
200
250
300
350
400
-20
0
20
40
60
80

6.4 Progesterone Binding Site Experiments

The transient rise in intracellular calcium shown by human spermatozoa in response to exposure to progesterone is well documented (Thomas and Meizel, 1989; Blackmore *et al.*, 1990; Baldi *et al.*, 1991; Foresta *et al.*, 1993; Krausz *et al.*,1995) and has been reproduced in Experiment 1 of this chapter. Other studies have shown that this response is mediated by non-genomic progesterone receptors (Blackmore and Lattanzio, 1991; Revelli *et al.*, 1994; Baldi *et al.*, 1995; Alexander *et al.*, 1996). Experiments were carried out in this series to identify binding sites and characterise receptor site numbers on the membranes of human and stallion spermatozoa, under different incubation conditions. The principle underlying radioligand binding studies is that a radioligand (in this case tritiated progesterone) will bind to receptors in a saturable manner, whereas attachment to non-receptor components is less saturable. Therefore unlabelled progesterone will selectively inhibit radioligand binding to the receptors, but not influence binding to other membrane constituents. The difference between the amount of radioligand bound in the absence and presence of unlabelled progesterone is taken as a measure of the amount of isotope, attaching to the receptor.

Two preliminary experiments were carried out to establish firstly, that specific binding sites existed and, secondly, that binding to these sites was saturable. This information was used to optimise assay conditions and further experiments were conducted to examine numbers of specific binding sites under capacitating and non-capacitating conditions.

6.4.1 Experiment 1 - Identification of specific binding sites on human spermatozoa

Preliminary work was carried out using one ejaculate from three human donors. Spermatozoa were separated from seminal plasma by swim-up migration and the concentration was adjusted to 50 million/ml. To a 100 µl aliquot of sperm suspension was added 20 µl of tritium labelled progesterone and 10 µl of 0, 10, 100

and 1000 μg/ml unlabelled progesterone. The tubes were mixed well and incubated at 37°C for 45 minutes. At the end of the incubation period, the suspension was centrifuged, the supernatant discarded to remove excess isotope and the pellet re-extended in 50 μl medium which was topped up to 4 ml with scintillation medium (Ecoscint A, National Diagnostics, Atlanta, Georgia, 30336). The amount of isotope adhering to the spermatozoa was determined using a Liquid Scintillation Analyser (Model 1600TR, Packard Instrument Company, Meriden).

6.4.1.1 Results

A logarithmic plot representing the percentage displacement of total binding plotted against unlabelled [progesterone] is presented graphically in Figure 6.5a. Numbers of specific binding sites on human spermatozoa declined as the concentration of unlabelled progesterone added to the suspension was increased, suggesting the presence of specific receptor sites for progesterone.

6.4.2 Experiment 2 - Identification of specific binding sites on stallion spermatozoa

The radioligand binding assay was repeated for stallion spermatozoa using one ejaculate from each of three stallions. Seminal plasma was removed by centrifugation and the pellet formed after centrifugation re-extended in Bavister TALP medium to a spermatozoa concentration of 50 million/ml. To a 100 μl of sperm suspension was added 20 μl of tritium labelled progesterone and 10 μl of 0, 10, 100 and 1000 μg/ml unlabelled progesterone as before. After removing excess isotope, bound isotope was counted as before.

6.4.2.1 Results

The results showed that numbers of specific binding sites on stallion spermatozoa declined as the concentration of unlabelled progesterone was increased, giving evidence of the presence of specific progesterone receptors. The percentage displacement of total binding plotted using logarithmic scale against unlabelled [progesterone] is presented graphically in Figure 6.5b. The magnitude of the decline in specific binding was less than that seen with human spermatozoa.

6.4.3 Experiment 3 - Effect of varying the concentration of tritiated progesterone

A further preliminary experiment was carried out to monitor the effect of increasing the concentration of radioligand. Derived from the first two experiments the concentration of unlabelled progesterone was fixed at 1000 μg/ml. The concentration of tritium-labelled progesterone, added in counts per minute, was increased from 0.33 pmoles up to 2.05 pmoles in steps of 5 μl volume. The radioligand binding assay was then repeated as before for stallion spermatozoa from one ejaculate.

6.4.3.1 Results

The results are summarised in Table 6.6. Figure 6.6a is a graphical presentation of total binding, non specific binding (blank) and specific binding (displaceable) over the range of concentrations of tritiated progesterone.

Figure 6.6b represents specific binding, as a percentage of total radioligand added. The percentage displaced or specific binding was saturated by the addition of 0.73 pmoles tritiate progesterone. Specific binding was relatively stable over the range of 0.73 - 2.06 pmoles.

Based on these preliminary experiments, subsequent assays for radioligand binding site studies were carried out using 20 µl tritiated progesterone (1.46 pmoles) and 10 µl (1000 µg/ml) unlabelled progesterone in 100 µl of sperm suspension at a concentration of 50 million/ml.

Table 6.6: Radioligand binding results of one ejaculate from one stallion using fixed unlabelled progesterone (1 mg/ml) and increasing radioligand concentrations.

	5 µl	10 µl	15 µl	20 µl	25 µl	30 µl
Total added (cpm)	61756	135849	204279	272710	328057	383400
Total added (pico moles)	0.33	0.73	1.09	1.46	1.76	2.06
Total bound (cpm)	14627	55928	96073	128762	147908	158989
Blank (cpm)	10661	14178	22280	30440	37653	30627
Displaced	3965	41750	73793	98332	110255	128362
Displaced (% of total)	6	31	36	36	40	33

6.4.4 Experiment 4 - Identification of specific progesterone binding sites on stallion spermatozoa after 0 or 300 minutes of incubation

To test the hypothesis that specific progesterone receptor sites were induced during capacitation stallion spermatozoa from one ejaculate from three stallions were extended in homologous seminal plasma, Bavister-Milk medium or Bavister-Milk medium supplemented with 1 mg/ml pentoxifylline to a final concentration of 50 million/ml and incubated at 38.5°C in 5% CO_2 for up to 300 minutes. After 0 and 300 minutes of incubation, duplicate 100 ml aliquots of each treatment were removed for progesterone receptor radioassay as described in the previous experiments.

6.4.4.1 Results

The results are summarised in Table 6.7. Specific binding was detected for all treatments, confirming the presence of receptor sites. The specific binding, expressed as a percentage of total isotope added, declined significantly ($p<0.05$) for all treatments evaluated, from an initial range of 12-15% to between 4-7% after incubation for 300 minutes.

Table 6.7: Radioligand binding assay results for stallion spermatozoa (n=3 ejaculates) incubated in three different media for 0 and 300 minutes.

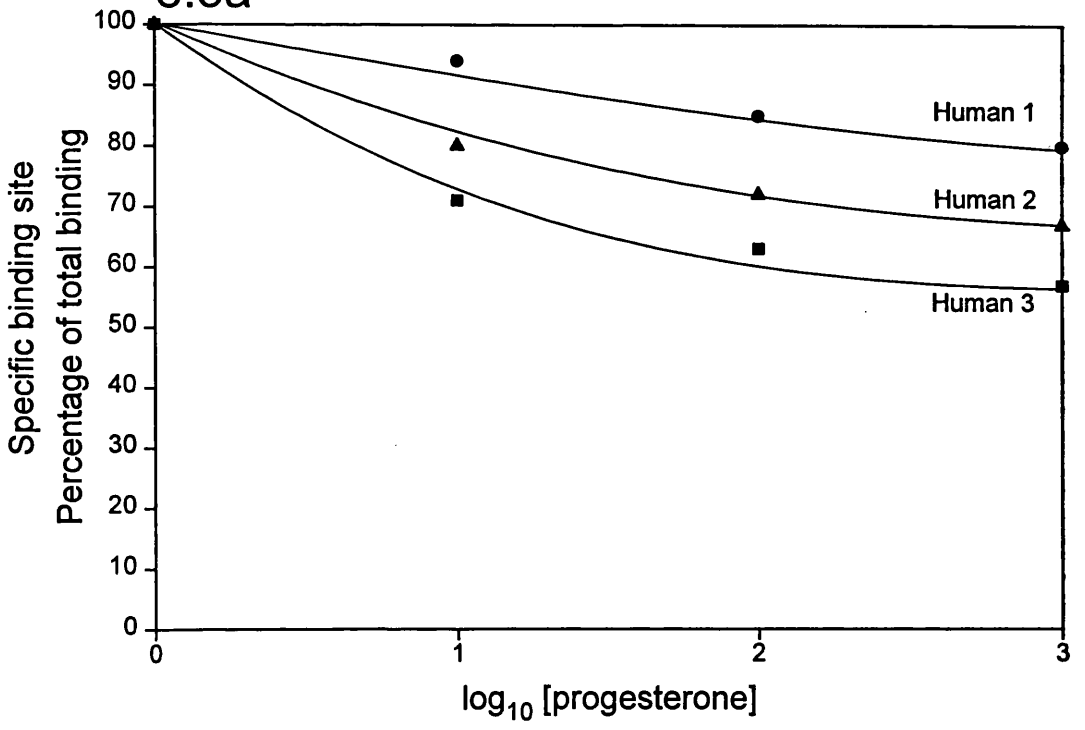
Medium	Incubation time		Total added (cpm)	Total binding (cpm)	Non specific binding (cpm)	Specific binding (cpm)	as a % of total cpm added
	0 min	Horse 1	272710	77693	77995	*	*
		Horse 2	272710	66363	33766	32579	12
		Horse 3	272710	83176	34957	48219	18
		Mean (SEM)					**15 (3.0)^a**
seminal							
plasma	300 min	Horse 1	272710	24546	14336	10210	4
		Horse 2	272710	21252	14212	7040	3
		Horse 3	272710	49308	33904	15404	6
		Mean (SEM)					**4.3 (0.8)^b**
	0 min	Horse 1	272710	72628	37768	34860	13
		Horse 2	272710	66901	38872	27219	10
		Horse 3	272710	71529	31474	40055	15
		Mean (SEM)					**12.7 (1.5)^a**
Bavister							
milk	300 min	Horse 1	272710	38850	26673	12717	5
		Horse 2	272710	35357	28263	7094	3
		Horse 3	272710	46357	29691	16666	6
		Mean (SEM)					**4.7 (0.9)^b**
	0 min	Horse 1	272710	72530	39828	34860	12
		Horse 2	272710	72932	37408	27219	13
		Horse 3	272710	53869	20236	40055	12
Bavister		**Mean (SEM)**					**12 (0.3)^a**
Milk							
+ PF	300 min	Horse 1	272710	38850	14659	13451	5
		Horse 2	272710	35357	14212	21792	8
		Horse 3	272710	46357	33904	21107	8
		Mean (SEM)					**7.0 (1.0)^b**

* - non specific binding value higher than total binding, hence no specific binding, while different letters *ab* within column shows values differ ($p<0.05$).

Figure 6.5a A logarithmic plot. % of total binding against increasing concentration of labelled progesterone; human spermatozoa.

Figure 6.5b A logarithmic plot. % of total binding against increasing concentration of labelled progesterone; stallion spermatozoa.

6.5a


Specific binding site
Percentage of total binding
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
log10 [progesterone]
Human 1
Human 2
Human 3

6.5b

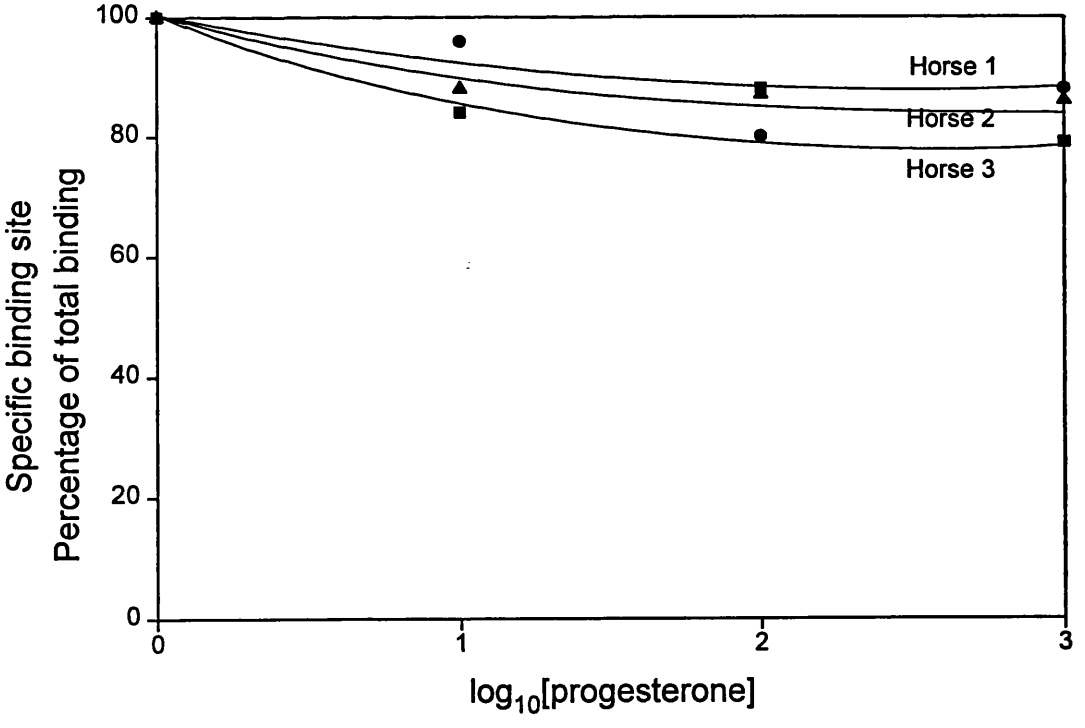
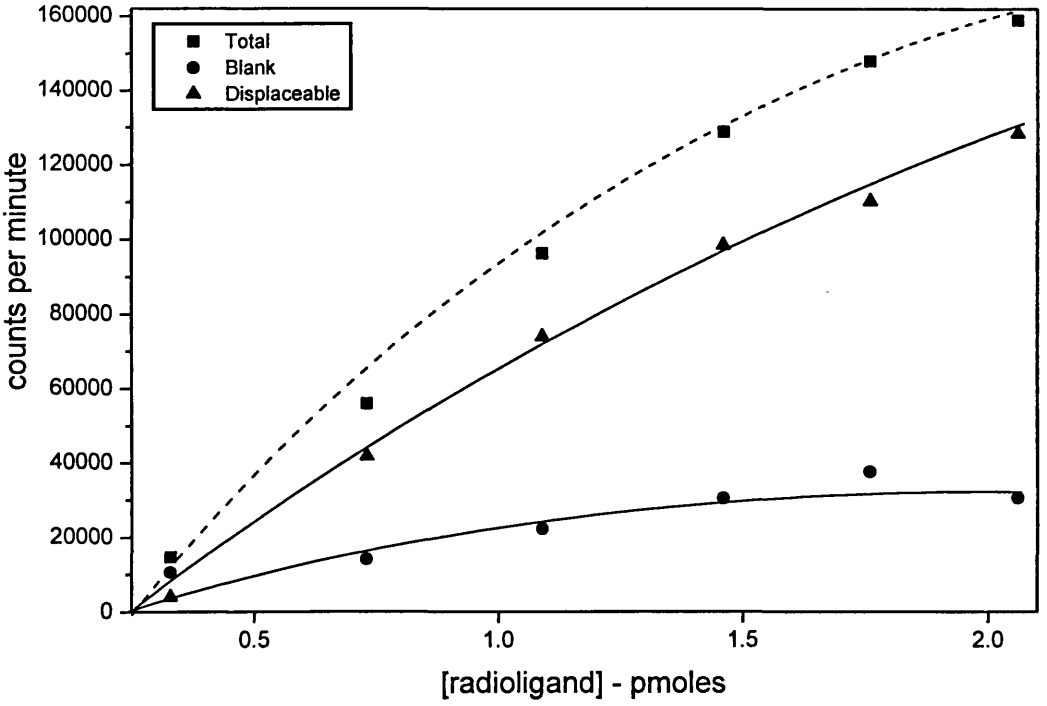

Specific binding site
Percentage of total binding
100
80
60
40
20
0
0
1
2
3
log10[progesterone]
Horse 1
Horse 2
Horse 3

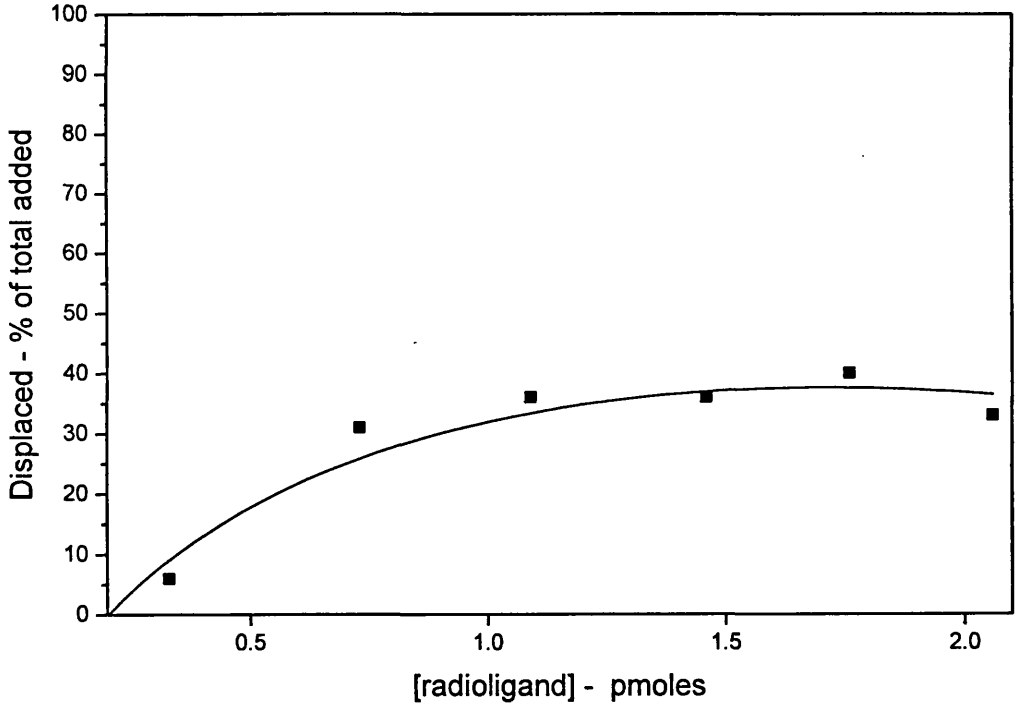
Figure 6.6a Total binding, non-specific binding and specific binding (counts per minute; cpm) against increasing concentration of labelled progesterone.

Figure 6.6b Specific binding as calculated percentage of total labelled progesterone added, against increasing concentration of labelled progesterone.

6.6a


Total
Blank
Displaceable
counts per minute
160000
140000
120000
100000
80000
60000
40000
20000
0
0.5
1.0
1.5
2.0
[radioligand] - pmoles

6.6b


Displaced - % of total added
100
90
80
70
60
50
40
30
20
10
0
0.5
1.0
1.5
2.0
[radioligand] - pmoles

6.4.5 Experiment 5 - Relationship between specific progesterone binding sites on human and stallion spermatozoa and numbers of live spermatozoa

Results of Experiment 4 indicated the presence of progesterone receptors but did not support the hypothesis that specific progesterone receptor sites were induced during capacitation. It was hypothesised that a higher binding activity would be seen as more receptor sites becomes available after incubation in a medium capable of supporting capacitation. It was reasoned that this may not have been evident because of the decline in percentage of live sperm after 300 minutes of incubation.

Hence, Experiment 3 was repeated using both human and stallion spermatozoa as before and the percentage of live spermatozoa was determined before and after treatment.

The procedure for human spermatozoa, one ejaculate from three donors, was as described in Experiment 2 in Section 6.3.2; in this experiment a calcium transient was consistently induced by the addition of progesterone to sperm suspensions after swim-up migration and Fura-2AM loading. The procedure for stallion sperm was as described in Experiment 3 in 6.3.3, for one ejaculate from each of three stallions. Percentage live spermatozoa was estimated using eosin-nigrosin staining as described previously.

6.4.5.1 Results

The percentage of live spermatozoa did not differ significantly before and after swim-up in human samples with a mean value of 81% and 85%, respectively ($p=0.2$). Specific binding, indicating the presence of progesterone receptors, was identified. However, no significant increase in percentage of specific binding was seen in post swim-up samples, with a mean percentage binding of 10% before and 11% after swim-up.

The percentage of stallion spermatozoa declined during incubation for all treatments ($p<0.001$). The mean percentage specific binding for samples incubated in

seminal plasma declined during incubation; this decline was not significant (p=0.7). There was a higher mean percentage specific binding for stallion sperm extended in Bavister Milk with or without pentoxifylline after 300 minutes of incubation than at) minutes. However, differences between time or treatments were not statistically significant. The results for human and stallion spermatozoa are summarised in Table 6.8 and 6.9 respectively.

Table 6.8: Radioligand binding (cpm) and percentage live [Mean % (SD)]for human spermatozoa (n=3 ejaculates) before and after swim-up migration.

		Total added (cpm)	Total bound (cpm)	Non-specific binding (cpm)	Specific binding (cpm)	Specific binding as a % of total added	Mean % live (SD)
Before	Human 1	272710	48915	21278	27637	10	81 (3.4)
Swim-up	Human 2	272710	45659	20267	25392	9	
	Human 3	272710	50872	22254	28618	10	
	Mean (SEM)					9.7 (0.3)	81 (1.9)
After	Human 1	272710	39601	12264	27337	10	85 (3.0)
Swim-up	Human 2	272710	41425	12464	28961	11	
	Human 3	272710	44931	10562	34346	13	
	Mean (SEM)					11.3 (0.8)	85 (1.7)

Table 6.9: Radioligand binding assay results for stallion spermatozoa (n=3 ejaculates) incubated in three different media for 0 and 300 minutes.

Medium	Incubation time		Total added (cpm)	Total binding (cpm)	Non specific binding (cpm)	Specific binding (cpm)	as a % of total cpm added	% live (mean)
Seminal plasma	0 min	Horse 1	272710	81867	72410	9457	3	57
		Horse 2	272710	83362	64653	18709	8	63
		Horse 3	272710	76744	63836	12908	5	70
		Mean(SEM)					**5.3(1.5)^a**	**63.3(3.8)^a**
	300 min	Horse 1	272710	85348	83102	2264	1	17
		Horse 2	272710	109990	84530	25460	9	13
		Horse 3	272710	81867	72409	9458	3	21
		Mean(SEM)					**4.3(2.4)^a**	**17(2.3)^c**
Bavister milk	0 min	Horse 1	272710	72490	67740	4750	2	53
		Horse 2	272710	66792	64673	2119	1	66
		Horse 3	272710	88030	73686	14345	5	73
		Mean(SEM)					**2.7(1.2)^a**	**64(5.9)^a**
	300 min	Horse 1	272710	104042	86156	17886	7	34
		Horse 2	272710	115919	88853	27066	10	43
		Horse 3	272710	63284	45874	17410	6	48
		Mean(SEM)					**7.3(1.6)^a**	**41.7(4.1)^b**
Bavister milk + PF	0 min	Horse 1	272710	81159	71346	9813	4	53
		Horse 2	272710	74366	64364	10002	4	65
		Horse 3	272710	63322	56055	7267	3	70
		Mean(SEM)					**3.4(0.3)^a**	**62.7(5.0)^a**
	300 min	Horse 1	272710	103470	83620	19850	7	44
		Horse 2	272710	119052	89539	29459	11	45
		Horse 3	272710	77033	72742	4291	2	57
		Mean(SEM)					**6.7(2.6)^a**	**47.0(2.5)^b**

abc - Different letters within column differ, ***ab*** ($p<0.05$) and ***ac*** ($p<0.001$)

6.5 Discussion

Experiments in this Chapter were designed to examine two facets of capacitation-related $[Ca^{2+}]_i$ regulation, not previously characterised for equine sperm; a gradual increase in intracellular calcium during incubation and a rapid calcium transient induced by exposure to progesterone. In these experiments it was shown that intracellular calcium increased during incubation in Bavister-Milk medium with or without pentoxifylline. The same phenomenon was observed during incubation in Bavister TALP. In contrast, the intracellular calcium concentration of stallion spermatozoa extended in homologous seminal plasma remained unchanged after 300 minutes of incubation. This intracellular calcium rise observed in sperm populations incubated in medium devoid of seminal plasma is consistent with findings reported for other species (Buhr *et al.*, 1989) and may reflect gradual changes in the efficacy of calcium extrusion mechanisms (Fraser *et al.*, 1995).

A progesterone-induced calcium transit was readily produced in human spermatozoa. This reproduced the work of others (Blackmore *et al.*, 1990; Baldi *et al.*, 1991; Forresta *et al.*, 1993; Krausz *et al.*, 1995) and indicated that the measurement system within this laboratory was sufficiently sensitive to detect this transient peak of calcium. It should be noted that the human sperm preparation method, a swim-up of 45 minutes in Medicult, is not considered a sufficient period of incubation to capacitate a large population of cells, although hyperactivated cells and acrosomal exocytosis have been detected immediately post swim-up (Robertson *et al.*, 1988).

This experiment could not be reproduced exactly for equine spermatozoa as the swim-up preparation yielded such a small population of spermatozoa that no reliable base-line signal for Fura-2 was established and no response to the addition of progesterone addition was detected. Hence, this approach was abandoned in favour of preparation by centrifugation and incubation for up to 300 minutes, using media tested in Chapter 5 and shown to induce capacitation using the CTC/ dual staining method. No progesterone-related response was stimulated after 0 minutes incubation in any medium. Nor was a response induced after 300 minutes in seminal plasma, a

treatment deliberately selected as a non-capacitating treatment. A progesterone-induced calcium transient was stimulated after 300 minutes of incubation in Bavister-Milk medium in the presence of 1 mg/ml pentoxifylline. But the same response could not be elicited after incubation in Bavister-Milk without pentoxifylline, a treatment for which CTC staining indicated capacitation. Nor could it be induced after incubation in Bavister TALP, a treatment proven to support capacitation in other species and which it was thought might accelerate capacitation of stallion spermatozoa over that of Bavister-Milk medium.

A radioassay was set up to try to detect specific progesterone binding which would indicate the presence of progesterone receptors. The results of the progesterone receptor binding studies suggested that progesterone binding site were present on both human and stallion spermatozoa. For human spermatozoa these receptors were detected on both non-capacitated, prior to swim-up and still in the presence of seminal plasma, and after swim-up where a proportion, albeit small, are likely to be capacitated. The specific binding as a percentage of total binding was very similar before and after swim-up, indicating similar numbers of binding sites.

Stallion spermatozoa were examined under the same incubation conditions used in previous experiments, of 300 minutes incubation in seminal plasma, Bavister-milk and Bavister-milk with pentoxifylline; the latter treatment representing the conditions under which a response to progesterone was elicited. Binding sites were detected for all treatments after 0 and 300 minutes of incubation. Percentage binding was stable or increased after 300 minutes of incubation despite a fall in percentage live spermatozoa. However, there was no significant difference between treatment, and no difference between the presence or absence of pentoxifylline. It was concluded from these experiments that progesterone receptors were present on the membrane of capacitated and uncapacitated human and equine spermatozoa.

Despite the presence of receptors stallion spermatozoa only responded to progesterone after 300 minutes of incubation in Bavister-Milk medium supplemented with pentoxifylline. Other workers have suggested that the majority of spermatozoa are inherently incapable of expressing the receptor or that the receptor function is under the control of some yet unknown factor (s) switching on the receptor only when the cells are ready to fulfil a role in fertilisation. Mendoza and Tesarik (1993) suggest

that the expression of the active receptor on the sperm cell surface membrane is a dynamic process controlled by the basal level of intracellular free [Ca^{2+}]. This suggestion was supported by other work (Shimuzu *et al.*, 1993).

As discussed previously, pentoxifylline is a phosphodiesterase inhibitor which will increase intracellular cAMP levels. Several reports indicate that an increase in cAMP levels is a stage in the capacitation process (Stein and Fraser, 1984; Fraser and Monks, 1990; De Jonge *et al.* 1991). It can be speculated that the presence of pentoxifylline during incubation has accelerated some cAMP mediated component of capacitation, rendering the stallion spermatozoa responsive to progesterone.

While the mechanisms of progesterone mediated stimulation is still unclear, the action of progesterone on the human sperm is known to operate at least three distinct events: opening of a progesterone-activated channel on the plasma membrane permeable to both [Ca^{2+}] and Na^{+} hence also promoting plasma membrane depolarisation (Foresta *et al.*, 1993), opening of a plasma membrane Cl-channel (Turner *et al.*, 1994), and activation of a protein tyrosine kinase (Mendoza *et al.*, 1995). Phosphorylation is a widespread mechanisms for ion channel modulation (Levitan, 1994; Chen and Huang, 1992). An abolition of $[Ca^{2+}]_i$ influx by PKC inhibitors suggests that the phosphorylation state of the progesterone receptor/channel modulates $[Ca^{2+}]_i$ influx, showing high permeability in the phosphorylated state and low permeability in the dephosphorylated state (Foresta *et al.*, 1995). Since the phosphorylation state of receptors is cAMP mediated it can be speculated that the effect of pentoxifylline during incubation is upon the phosphorylation state of the progesterone receptor.

In conclusion, progesterone receptors were detected on equine spermatozoa under conditions for which it was not possible to induce a progesterone-mediated calcium flux. It was only possible to induce a progesterone-mediated calcium response after incubation for 300 minutes under conditions which supported capacitation and resulted in an increase in basal intracellular calcium. Moreover, the presence of the phosphodiesterase inhibitor pentoxifylline was required. This evidence suggests that raised basal intracellular calcium and raised cAMP levels are components of a system regulating the capacitation of equine spermatozoa.

CHAPTER 7

GENERAL DISCUSSIONS

7.1 General Discussions

As discussed in the Objectives of the Study (1.9) there has been relatively little basic research carried out with stallion spermatozoa in comparison to other species. This is due to a combination of factors, with one factor being lack of impetus and funding from a large artificial insemination and cryopreservation industry such as there is for the bull. Another factor hindering basic research is that the horse is an expensive and impractical experimental model. Nor has an easily repeatable *in vitro* fertilisation system been established. For these reasons this study was undertaken to assess basic aspects of sperm function after cooling and storage at 5°C and under incubation conditions designed to support capacitation. The functional parameters examined in detail were motility, using a computerised motility analyser to undertake detailed analysis of movement patterns, and the ability to regulate $[Ca^{2+}]_i$, a reflection of plasma membrane integrity. Further, studies were designed to test the hypothesis that the ejaculate is composed of a heterogeneous cell population, within which there are discrete subpopulations of different physiological importance.

During the study several different techniques were used to assess independent aspects of sperm function. Computerised movement analysis was used because this technology can analyse large numbers of motile cells objectively and give data on specific characteristics of the motility pattern, such as progressive velocity (VSL) and amplitude of lateral head deviation from the mean path (ALH), which are thought to be of physiological importance. The reliability of the data depends on the settings of the equipment so a series of experiments were undertaken to validate this machine for the equine species. A great deal of the work utilised the intracellular fluorescent calcium probe Fura-2AM. Again a series of experiments were run to test the effect of sperm concentration, extending medium and the effect of the presence of dead cells upon the ability of the fluorescent marker to enter the intracellular compartments of the living sperm cells in suspension. The marker Fura-2AM was also used to examine calcium concentrations within individual cells; again preliminary experiments were required to establish the optimal protocol for immobilising spermatozoa during ratiometric readings. Changes to the sperm plasma

membrane during incubation under capacitating conditions were examined using a surface membrane fluorescent marker, chlortetracycline. Since it was critical that only data from living cells was considered and it is known that degenerating cells show a variety of surface membrane patterns, this fluorescent marker was used in conjunction with a nuclear stain H33258, which identified dead cells. Further assessment of surface membrane properties was carried out using a radioligand binding assay, targeted, in these experiments, at putative progesterone receptors. Several novel observations were made during the study and these are discussed below.

A comparison of sperm function was made after cooling to 5°C using two different cooling protocols. Protocol A involved more rapid cooling (0.5°C/min) from 37°C to 22°C, then cooling at 0.05°C/min from 22°C to 5°C. Protocol B maintained the lower cooling rate from 37°C to 5°C. It might have been expected that prolonged slow cooling would be beneficial in terms of preserving sperm function. However, all of the markers used to assess sperm function suggested that the slower rate of cooling had a deleterious effect. In 2 out of 5 experiments using Protocol B, all sperm were immotile by 72 hours. It was also observed that velocity parameters declined to a greater degree after cooling by Protocol B. Two other effects were noted; (1) a decline in VSL, because sperm assumed non-progressive motility and were circling and (2) that ALH was high after cooling; high ALH has been related to high intracellular calcium in other studies (Bailey *et al.*, 1994). Measurement of intracellular calcium, indeed, showed that the prolonged slow cooling of Protocol B resulted in a highly significant increase in intracellular Ca^{2+} after cooling and a greater increase than that seen for Protocol A.

The question arises of whether the high intracellular calcium is simply a marker of loss of regulatory ability or whether it is causal of declining sperm function. In this study high intracellular calcium can be clearly linked to a decline in motility. High ALH and low VSL occurred concurrently with very high intracellular calcium. A marked decline in ALH and maintained low VSL occurred thereafter. Subsequently, 100% cell death was the most marked feature of Protocol B. This would tend to suggest that high intracellular calcium caused the decline in ALH, if not also the decline in velocity parameters. Comparison of cooling protocols A and B suggests that the greater the increase in intracellular calcium, the greater the decline in

sperm velocities and reduction in sperm survival. One importance of this observation is that efforts to improve cooling methods would be best directed towards controlling Ca^{2+} influx during the cooling process.

There are very few studies of calcium regulation by individual sperm cells and they have focused on either examining cell response to progesterone (Plant *et* al., 1995) or changes in intracellular Ca^{2+} during the development of hyperactivation (Suarez *et al.*, 1993; Suarez and Dai, 1995). Single cell Ca^{2+} studies have not been carried out before with stallion spermatozoa nor with any spermatozoa exposed to cooling and low temperature storage. The single cell studies in this work supplied two pieces of information. Firstly, the mean data for all the individual cells confirmed the evidence of the population studies, that intracellular Ca^{2+} increased during cooling and storage. Secondly, and more importantly, the single cell studies indicated that despite cooling and storage there still existed a discrete sub-population with plasma membranes resistant to cooling and still able, even after 48 hours of storage at 5°C, to regulate $[Ca^{2+}]_i$ at precooled levels. It can be speculated that it is this sub-population which retains the ability to fertilise despite cooling and storage and it would be interesting to determine exactly what characteristics render this small sub-population resistant to an environment which has so severely damaged the majority of the population.

Subsequent experimental work was directed towards examining calcium-related event during capacitation. Increases in intracellular calcium are a component of the regulatory mechanisms of the pre-fertilisation events of capacitation, hyperactivation and the acrosome reaction. It has been suggested that modification of the function of Ca^{2+}ATPases responsible for extruding intracellular calcium, to allow $[Ca^{2+}]_i$ to reach threshold levels required to trigger other events, is a component of capacitation (Adeoya-Osigawa and Fraser, 1996). Work in other species has indicated that progesterone triggers a rapid influx of calcium which, in turn, regulates acrosomal exocytosis (Thomas and Meizel, 1989; Blackmore *et al.*, 1990; Baldi *et al.*, 1991; Blackmore and Lattanzio, 1991; Roldan *et al.*, 1994). This experimental work examined calcium regulation in equine spermatozoa incubated under conditions capable of supporting capacitation. However, before this work could be carried out conditions which supported capacitation had to be defined.

Published reports have indicated that it is difficult to capacitate stallion spermatozoa (Ellington *et al.*, 1993a). It is not clear how long capacitation takes *in vivo* takes and studies of *in vitro* capacitation have acknowledged the difficulty of maintaining stallion spermatozoa in long term cultures (Ellington *et al.*, 1993b). A number of different media formulations were tested in preliminary experiments (see Appendix 1) before three media were tested in Chapter 5. The system which best maintained motility during incubation in these studies was a 1:1 mixture of TALP and Kenney's medium. The inclusion of a preservative (milk), which is by definition non-capacitating, opened to question the effectiveness of the mixture for supporting capacitation. Evidence from CTC staining, however, indicated that about 46% of the population showed the capacitated pattern by 300 minutes of incubation. Addition of pentoxifylline to the medium hastened the appearance of the surface membrane capacitation-related changes detected by CTC staining.

Attempts to corroborate the CTC evidence of successful capacitation by measuring the capacitation-related motility pattern of hyperactivation were not very successful for equine sperm. In Chapters 5 and 6 certain techniques were used for human sperm prior to use with equine sperm because the successful use of these techniques had been reported for the former. Two such techniques were the detection of hyperactivated human sperm using an algorithm incorporated in the Sort Function of the computerised motility analyser, and the stimulation of hyperactivation by the use of the phosphodiesterase inhibitor pentoxifylline. Attempts to apply the first technique to equine sperm were confounded by the finding that parameters used to measure hyperactivated motility in human spermatozoa were not sufficiently discriminating for stallion spermatozoa. Based on visual observation of hyperactivated stallion spermatozoa alternative, more discriminating, parameters were formulated. However, the new algorithm could not be tested extensively because pentoxifylline caused rapid and severe agglutination of equine sperm, unlike human spermatozoa in which pentoxifylline stimulated hyperactivation. No correlations could be made between CTC and hyperactivation because agglutinated spermatozoa could not be measured. Whether all or only some component parts of capacitation were actually achieved is not clear. Definitive proof would require successful fertilisation and even within an *in vitro* fertilisation system the nature of the individual

fertilising spermatozoan is unknown. However, the CTC staining response was consistent with capacitation in other species (Fraser, 1987; Das Gupta *et al.*, 1993; Kay *et al.*, 1994).

As a further step towards elucidating regulatory mechanisms, experiments were carried out to examine calcium dynamics under these incubation conditions. It was found that mean $[Ca^{2+}]_i$ increased during a 300 minute incubation in capacitating medium; this increase was statistically significant in the presence of pentoxifylline. No increase was seen during incubation in seminal plasma. This is consistent with the hypothesis that Ca^{2+}ATPases become less efficient at extruding Ca^{2+} during capacitation resulting in a gradual increase in intracellular Ca^{2+} (Fraser *et al.*, 1995). A second Ca^{2+} mechanism was examined; the activation of non-genomic progesterone receptors resulting in an intracellular Ca^{2+} influx. A progesterone-induced Ca^{2+} influx was readily induced in washed human spermatozoa. The same Ca^{2+} influx could only be induced in stallion spermatozoa after a 300 minute incubation in the presence of pentoxifylline.

Radioligand binding studies using tritiated progesterone were carried out to identify progesterone receptors. Progesterone binding studies suggested that progesterone receptors were present on both human and stallion spermatozoa. For both human and equine spermatozoa progesterone binding sites were identified under non-capacitating and capacitating conditions. With equine spermatozoa, numbers of binding sites were maintained or increased during 300 minutes of incubation despite falling numbers of live spermatozoa. Thus there were more receptors present on fewer live spermatozoa after incubation. It would be necessary to show definitively that binding to dead spermatozoa was not greater than binding to live but incubation in seminal plasma, which resulted in greater numbers of dead spermatozoa than incubation in media, did not result greater numbers binding sites. This observation may be evidence that further binding sites are unmasked during incubation or capacitation. For many years workers have believed that removal of 'decapacitation factors' and unmasking of binding sites were a critical component of capacitation (Austin, 1951; Chang, 1951; Bedford, 1983).

As stated above, after incubation in capacitating medium and in the presence of pentoxifylline, the cell population showed a progesterone-induced calcium

transient. Other workers (Baldi *et al.*, 1991; Mendoza and Tesarik, 1993) have shown that progesterone-induced $[Ca^{2+}]_i$ transients were more pronounced in human cell suspensions with higher basal $[Ca^{2+}]_i$ levels. This current data suggests that the non-genomic progesterone receptor, at least of equine spermatozoa, is only activated when 1) intracellular Ca^{2+} reaches a certain threshold and 2) and/or some other mechanism, linked to cAMP and the phosphodiesterase inhibiting action of pentoxifylline, is triggered. As was discussed in Chapter 6 this may be cAMP-mediated phosphorylation of the progesterone receptor. A recent review (Meizel, 1997) suggests that progesterone acts on a sperm gamma-aminobutyric acid (GABA) receptor-like chloride channel, causing membrane depolarisation and consequent activation of voltage-sensitive Ca^{2+} channels and that tyrosine phosphorylation of the sperm GABA receptor-like chloride channel is an important component of the progesterone-initiated acrosome reaction.

It was beyond the limits of the current study to determine whether progesterone responsiveness was a feature of the whole cell population or whether a functionally distinct subpopulation existed. Mendozoa and Tesarik (1993) showed that the increased responsiveness to progesterone of human spermatozoa was explained by an increase in the size of the subpopulation capable of responding, rather than an increase in the responsiveness of the whole population. They suggested that many spermatozoa possess the receptor in a functionally inactive form and that receptor function is switched on asynchronously during capacitation to supply a steady stream of spermatozoa primed for acrosomal exocytosis and fertilisation. This contrasted with the evidence of Plant et al. (1995) who determined that the majority (93%) of human spermatozoa showed a increased $[Ca^{2+}]$i in response to progesterone. Obviously further work is required to clarify this issue.

It has been suggested by others that cryopreserved or cooled spermatozoa undergo changes resembling capacitation (Watson, 1995; Fuller and Whittingham, 1997). The work carried out for this study has certainly helped to draw parallels between the two situations. This work showed that increased $[Ca^{2+}]_i$ is common to both cooled and capacitated cells. It is possible to speculate that the increase in intracellular Ca^{2+} which occurs during cooling may trigger components of the capacitation sequence. Certainly there is evidence from other species that

cryopreserved spermatozoa can penetrate eggs without further incubation (Watson, 1995). It has also been suggested that a premature increase in intracellular calcium is deleterious to sperm function (Robertson and Watson, 1986; Bailey *et al.*, 1994) which is consistent with the findings of this study, where a marked decline in motility and viability followed high intracellular Ca^{2+}.

The objectives of the study were fulfilled in that uncontrolled $[Ca^{2+}]_i$ levels were related to a decline in sperm function during cooling and storage. It was shown that very specific mechanisms exist to regulate $[Ca^{2+}]_i$ during capacitation. Several pieces of evidence supported the hypothesis of the existence of functionally distinct subpopulations of cells. Analysis of individual cooled cells revealed the existence of a subpopulation still capable of $[Ca^{2+}]_i$ regulation after cooling and storage. Chlortetracycline staining showed the presence of a capacitated subpopulation. A hyperactivated subpopulation was observed but could not be characterised objectively. Further work is required to clarify whether all or a subpopulation of equine spermatozoa are able to respond to progesterone. In conclusion, it is clear from this study that the ability to control $[Ca^{2+}]_i$ is absolutely critical to survival of cooling and low temperature storage and critical, again, for capacitation and, ultimately, fertilisation.

APPENDIX

APPENDIX 1

Different composition of TALP medium used by different authors in stallion sperm function studies.

Medium / Composition	1	2	3	4	5
	g/100 ml	g/100 ml	g/100 ml	g/100 ml	g/100 ml
PVA	0.100	-	-		
Penicillin (sodium salt)					0.003
Penicillin G	10,000IU	-			
Phenol red	0.001				
HEPES	0.384*	0.240 [a]	0.240	0.238	
NaCl	0.666	0.216 [a]	0.637	0.420	0.655
KCl	0.024	0.075 [a]	0.024	0.187	0.030
KH_2PO_4	-	0.016 [a]	-	-	-
$CaCl_2.2H_2O$	0.029	0.025 [a]	0.029	0.029	0.033
$MgCl_2.6H_2O$	0.010	-	0.010	0008	0.011
$MgSO_4.7H_2O$		0.029 [a]	-	-	-
Na lactate	185 µl	370 µl [b]	85µl	31µl	-
$NaH_2PO_4.H_2O$	0.005	-	0.005	0.005	0.011
Glucose	0.090	0.100 [b]	-	-	0.250
Fructose	-	1.520 [b]	-	-	
NaHCO3	0.210	0.300 [a]	0.017	0.210	
Na Pyruvate		0.002 [b]	0.002	0.011	0.011
BSA	1.000 g*	0.300 [b]	0.400g	0.600g	0.300g
pH adjusted to					7.800
Osmolality					305

Medium 1 - described in Bavister, (1989) and used by Zhang *et al.*, (1991) with some modifications (*).

Medium 2 - suggested by Dr J.K.Graham (personal communication) was made up by mixing Stallion Tyrodes (a) and Stallion TALP (b) at a ratio of 1:1 (v/v). In Bedford *et al.*, (1995) this medium was used at a ratio of 1:1 (v/v) with milk medium [Dry skim milk, 9.46g; Glucose, 0.5g deionize water 90 ml]

Medium 3 - used by Blue *et al.*, (1989).

Medium 4 - TALP, composition described in Padilla and Foote, (1991) and used at 65%:35% (TALP: Kenney's medium) as extender for long term low temperature storage. The ability of Medium 4 to maintain stallion sperm motility after cooling and storage at 5°C was also evaluated by Webb and Arns (1995).

Medium 5 - described by Brackett and Oliphant, (1975) and used to resuspend and incubate stallion spermatozoa by Hochi *et al.*, (1996)

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