AN INVESTIGATION OF BOAR SPERM MOTILITY USING A NOVEL COMPUTERIZED ANALYSIS SYSTEM

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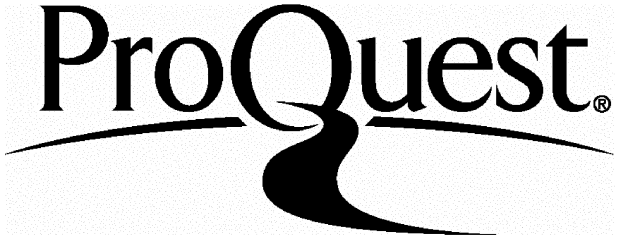

ABSTRACT

A novel computer-assisted sperm analysis (CASA) system was validated and used to investigate the value of sperm motility in predicting the fertility of semen samples. Appropriate Hobson Sperm Tracker set-up parameters were derived for the analysis of boar spermatozoa and techniques developed to facilitate CASA analysis of this species.

The accuracy and precision of the system were evaluated and minimum sperm number and minimum velocity criteria were developed to ensure the quality of the analyses performed.

Relationships between the CASA measurements were investigated and ejaculates compared before and after storage. Cluster analysis of the track data was performed to investigate whether sub-populations of sperm could be distinguished. Using this methodology, hyperactivated boar spermatozoa could be identified from the quantitative analysis of motility data.

The motility characteristics of semen samples during incubation at 39°C were compared to data from two artificial insemination trials. Each employed a controlled sperm dose (1.5×10^9 spermatozoa) and standardized semen preparation and insemination protocols. The results revealed a significant relationship between sperm motility measurements and the fertility data (maximum correlation with average litter size $P=0.007$; maximum correlation with non-return rate $P=0.00002$). Overall, sperm velocity over a two hour incubation period proved to be the most informative motility characteristic.

The quality of sperm motility is altered on interaction with the zona pellucida and objective analysis of this response might be useful for fertility estimation. Zonae ghosts were collected and solubilized; the addition of this preparation to boar sperm samples severely impaired motility and prevented analysis using the Hobson Sperm Tracker. Further experiments indicated that this was a calcium-dependent effect.

This research supports the hypothesis that sperm movement characteristics are indicative of fertility.

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To my father

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LIST OF ABBREVIATIONS

AI	Artificial Insemination
ANOVA	Analysis of Variance
AR	Acrosome Reaction
ATP	Adenosine Triphosphate
AVL	Average Litter
AVL0	Average Litter (incorporating conception failures)
BSA	Bovine Serum Albumin
BTS	Beltsville Thawing Solution
cAMP	Cyclic Adenosine 3' 5' Monophosphate
CASA	Computer Assisted Semen Analysis
CFDA	Carboxyfluorescein Diacetate
CFI	Competitive Fertility Index
DMSO	Dimethylsulphoxide
EGTA	Ethylene Glycol-bis(β-amino-ethyl ether) N,N,N',N',-Tetraacetic Acid
FITC	Fluorescein Isothiocyanate
HST	Hobson Sperm Tracker
IVF	In Vitro Fertilization
LSD	Least Significant Difference
MFN	Minimum Frame Number
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
PSA	Pisum Sativum
s.d.	Standard deviation
SDS	Semen Delivery Service
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
s.e.m	Standard error of the mean
SIP	Standard Isotonic Percoll
SR	Search Radius
SZP	Solubilized Zonae Pellucidae
TBM	Tris Buffered Medium
VDCC	Voltage Dependent Calcium Channel
ZP	Zona Pellucida

CHAPTER 1. - INTRODUCTION AND LITERATURE REVIEW

1.0 INTRODUCTION

A method of predicting ejaculate fertility would benefit both the human fertility clinics and the agricultural artificial insemination (AI) industry. A variety of techniques have been developed for this purpose (for a review see Amann and Hammerstedt, 1993; Aitken, 1988) but an accurate and reliable indicator of fertility remains elusive. One approach has been to assess sperm motility and to evaluate the relationship between sample motility and subsequent fertility. This has led to the development of automated motility analyzers to provide objective measurements of motion parameters.

Most of the research involving computerized sperm assessment has examined human sperm (e.g. in conjunction with assisted reproduction - Holt *et al.*, 1989; Marshburn *et al.*, 1992; or *in vitro* fertilization - Holt *et al.*, 1985; Jeulin *et al.*, 1986; Barlow *et al.*, 1991; Liu *et al.*, 1991; Oehninger *et al.*, 1992) or rodent sperm (e.g. in reproductive toxicology - Toth *et al.*, 1989; Slott *et al.*, 1991; Yeung *et al.*, 1992). Far fewer studies have assessed ejaculates from domestic species (see later).

Increased use of pig AI has led the Semen Delivery Services that produce bottled semen to investigate methods of predicting ejaculate fertility. This thesis contains basic data on boar sperm motility collected using a novel computerized semen analysis system. The changes in sperm motility over time in capacitating media have been monitored and the boar and ejaculate variation examined. Some of these parameters have then been compared to the fertility results available.

The computerized semen analysis system used for this research was developed in conjunction with Sense and Vision Electronic Systems Ltd. (Sheffield). The research for this thesis has involved advising on software improvements and calibrating new version of the system as they appeared. In contrast to other commercially available systems, the Hobson Sperm Tracker can perform continual assessment of sperm in real-time, and so allows a new approach to sperm assessment. The next chapter describes the evaluation of the systems capabilities and the determination of the optimal set-up of the system for the experimental work conducted. Chapters three and four then report how this system was used for the analysis of boar sperm movement.

In this chapter, the sperm cell, the mechanisms of sperm motility and factors

that affect cell movement are described. The role of sperm motility in fertilization is then discussed, followed by an overview of computerized sperm motility analysis and its application.

1.1 MAMMALIAN SPERMATOZOA - STRUCTURE AND FUNCTION

1.1.1 Introduction

The role of the spermatozoon is to transport genetic material from the male to the oocyte. To achieve this the cell must be able to reach, penetrate and fuse with the oocyte and convey the information necessary for correct development of the zygote. Sperm structure reflects these requirements, each cell consisting of a head region containing a fusogenic area and the genetic information, and a tail region that generates the motility of the cell. These are contained within a plasma membrane which is differentiated into specialized regions. Detailed reviews of these sperm structures and their function can be found in Eddy (1988); Bedford and Hoskins (1990) and Yanagimachi (1994). The following description concentrates on the sperm flagellum, sperm motility and fertilization, and applies to mammalian sperm in general.

1.1.2 The sperm flagellum

The shapes and lengths of spermatozoa vary between species (see Cummins and Woodall, 1985 for a review): in sperm from Sus domesticus, the combined length of the head and the flagellum is approximately 47μm (Bonet and Briz, 1991). The sperm flagellum is sub-divided into four sections: the neck, the middle piece, the principal piece and the end piece (See figure 1.).

The neck region contains the proximal centriole attached to which are the microtubules of the axoneme, the area of the sperm tail responsible for generating motility. The microtubules are organized in the 9+2 arrangement common to eukaryotic cilia and flagella. Each of the nine peripheral doublets is composed of the structural protein tubulin and joined to the adjacent doublet by two dynein arms, (dynein is a heterogeneous protein with ATP-ase activity in the presence of Magnesium - see Johnson, (1985) for a review). Also extending between adjacent doublets are nexin links, proteinaceous filaments thought to be responsible for maintenance of the cylindrical shape of the axoneme (Eddy, 1988).

Throughout the middle piece and the initial section of the principal piece, the axonemal microtubules are surrounded by a ring of nine longitudinal outer dense fibres. These are composed of keratinous proteins highly cross-linked by disulphide bonds which protect the axoneme and function as a stiffening structure to confer elastic rigidity to the flagellum (Cornwall *et al.*, 1988). The dense fibres are surrounded by mitochondria in a helical arrangement which produce the ATP necessary for sperm motility (see below). The junction between the middle and principal pieces is characterized by the annulus, a membrane specialization resembling a tight junction. Here the axoneme and outer dense fibres become surrounded by a fibrous polypeptide sheath which forms a flexible, ribbed structure that allows for the beating of the flagellum (Bedford and Hoskins, 1990).

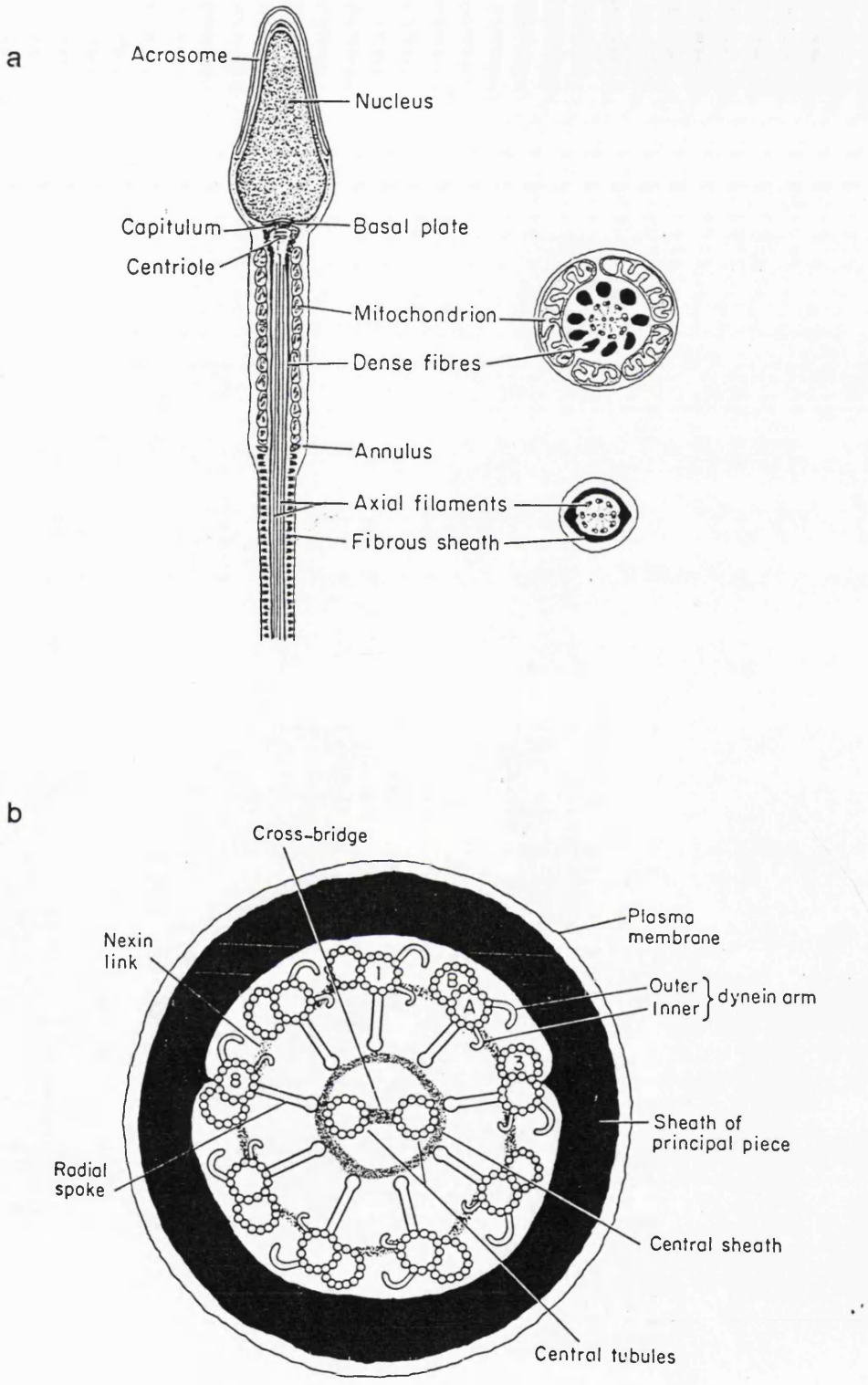

Figure 1. Diagram of a mammalian (eutherian) spermatozoon (from Bedford and Hoskins, 1990). Figure (a) shows a sagittal section through the spermatozoon whilst figure (b) describes a cross section through the principal piece.

1.1.3 Generation of sperm motility

Spermatozoa can generate ATP from the glycolysis of sugars and from mitochondrial respiration. Boar sperm rely primarily on the latter to provide the energy required for motility. Glycolytic rates are much lower than in most other species, too low to support motility, hence boar sperm become poorly motile under anaerobic conditions. This may account for the low levels of glycolytic substrates found in boar semen compared to other species (Ford and Rees, 1990).

In the presence of Mg^{2+} and Ca^{2+}, dynein ATP-ases catalyze the hydrolysis of ATP, effecting the movement of the microtubules. The dynein arms of A sub-units are ATP labile and in the presence of ATP the dynein arms detach from sub-unit B, shorten and tilt approximately 40°. The arms then elongate when ATP is hydrolyzed and reattach at a new site, followed by the return of the arm to the original orientation. In this way the doublets are moved relative to each other. This model of motility is called the 'Sliding Filament Model' (Afzelius 1959). Conversion of the microtubule sliding to the bending of the flagellum probably occurs due to the attachment of the microtubules to the base of the sperm tail, and by the resistance to displacement afforded by the nexin links. Microtubule sliding is only active in one direction suggesting that the antagonistic tubule sliding occurring on the other side of the axoneme (resulting in a planar beat) is passive. Although propagation of the flagellar beat is understood, it is unclear where initiation of the wave occurs (Eddy, 1988).

Sperm motility is influenced by many factors. The biochemical environment of the sperm (such as substrate availability and ionic composition; see Ford and Rees, 1990; Gatti *et al.*, 1993) and the physical properties of the fluid environment (such as temperature and viscosity; see Katz *et al.*, 1989; Suarez *et al.*, 1991a) all modify the resultant movement patterns. Sperm populations are highly heterogenous and motility patterns vary both within ejaculates, and between and within species. Each individual cell may itself exhibit a variety of motility types which vary in response to environmental changes. In some mammals epididymal sperm are immotile and only gain motility on ejaculation. This may be due to the suppression of motility rather than a sudden stimulation of sperm movement (e.g. in the rat where immotility is due to the presence of a high molecular weight glycoprotein 'immobilin' (Usselman and Cone, 1983)). Boar sperm display increasing motility as they pass through the epididymis, and are motile prior to

ejaculation (Holtz and Smidt, 1976). Sperm motility may also be inhibited during sperm storage in the female tract, followed by resumption of movement due to, as yet, unknown cue(s) (Suarez, 1987; Overstreet and Cooper, 1975). Upon further maturation in the female tract, sperm of several species will display a qualitative change in movement pattern to 'hyperactivated' motility - a convulsive, high curvature, non-progressive motility (for a review of hyperactivation see Katz *et al.*, 1993).

1.1.4 Biochemical control of sperm motility

The control mechanisms regulating sperm motility are unclear, but require ATP, Ca^{2+}, and cyclic AMP (Tash and Means, 1983; Ford and Rees, 1990). Incubation of sperm in Ca^{2+}-free media causes loss of motility, a state that is initially reversible but becomes permanent after a species-specific length of time (Feng *et al.*, 1988), probably as a result of the inactivation of Ca^{2+}-dependent adenylate cyclase. The addition of cAMP to sperm samples, or substances that indirectly increase cAMP levels, increases motility. Methyl xanthines such as caffeine and pentoxifylline stimulate velocity and/or the proportion of motile cells (Aitken *et al.*, 1983; Rees *et al.*, 1990; De Lamirande and Gagnon, 1991). The stimulatory action is probably due to increases in intracellular cAMP concentrations, resulting from the inhibition of cAMP phosphodiesterase. However, different methyl xanthines have different effects on sperm function. Addition of caffeine to human sperm results in increased lateral displacement of the head whilst pentoxifylline addition increases sperm velocity but only effects a small change in sperm head displacement (Rees *et al.*, 1990). This indicates either additional or alternative influences on sperm processes besides pure phosphodiesterase inhibition.

An increase in the intracellular cAMP concentration promotes the cAMP-dependent phosphorylation of proteins responsible for the initiation and maintenance of sperm motility via adenylate cyclase (sites for which are found in both the head and tail regions of the sperm plasma membrane). Adenylate cyclase can be activated directly by the addition of compounds such as bicarbonate (Okamura *et al.*, 1985), calcium (Hyne and Lopata, 1982) or egg yolk (Okamura *et al.*, 1991) resulting in increased sperm motility (percentage of motile cells).

Sperm movement has been shown to change in response to a variety of external cues that may be encountered *in vivo*, in the male and female secretions. Both stimulatory and inhibitory factors have been isolated from boar seminal plasma (Iwamoto *et al* 1992). The addition of follicular fluid can enhance sperm velocity, promote sperm motility and induce changes in movement patterns *in vitro* (Revelli *et al*, 1992, Mackenna *et al.*, 1993). Lee *et al* (1992) isolated a 52 kD protein from porcine follicular fluid capable of maintaining sperm motility in nutrient free media (PBS) and concluded that this may act to give sperm a final 'push' in the vicinity of the oocyte. Sperm motility (and fertilizing capacity) have been shown to be maintained *in vivo* by binding of the sperm to epithelial cells of the oviduct (Bovine - Pollard *et al.*, 1991; Porcine - Suarez *et al.*, 1991b). This may be due to secretions from the epithelial cells, or a result of the physical interaction of sperm binding. Additionally, the oviductal explants also promoted hyperactivated motility in these species.

1.1.5 Physical effects on sperm motility

The motility patterns and velocities displayed by an individual sperm are partly a function of its physical environment. For fertilization *in vivo*, sperm must pass through substances of varying structure and viscosity, such as the seminal plasma, the mucus secretions of the female tract and the vestments of the oocyte (for reviews see Katz *et al.*, 1989, Suarez *et al.*, 1990). Sperm motility varies in response to these obstacles. Suarez and co-workers (1991a) showed that hamster sperm responded to increased viscosity in the surrounding medium with decreased flagellar amplitude, wavelength, beat frequency and velocity. Hyperactivated sperm maintained a greater degree of forward motility than non-hyperactivated sperm due to increased flagellar forces.

Sperm moving through the cumulus and zona-pellucida have been observed to use 'hatchet-like' and 'rocking' movements to penetrate these viscoelastic microstructures (Drobnis *et al.*, 1988a,b,c; Bedford, 1991). The motility of hamster sperm penetrating these layers was found to be bimodal - high amplitude, low frequency lever strokes alternated with low-amplitude, high frequency sinusoidal flagellar movement (Drobnis *et al.*, 1988a,b). These movements increase the forces exerted against the egg vestments and facilitate penetration by the spermatozoon.

1.1.6 Hyperactivation

The ability of some sperm to display a qualitatively different type of motility from the majority of the sperm population was first noted in the Golden Hamster (Yanagimachi, 1969; Gwatkin and Anderson, 1969; for a review see Katz *et al.*, 1993; Yanagimachi 1994). This type of movement, called hyperactivated motility, has since been observed in several mammalian species such as mouse, human, rabbit and ram (Fraser, 1977; Morales *et al.*, 1988; Cooper *et al.*, 1979 and Shams-Borhan and Harrison, 1981 respectively). The acquisition of this vigorous, erratic, non-progressive motility may be part of 'capacitation', the maturational process that renders the sperm capable of fertilizing ova (see figure 2.).

The functional significance of hyperactivation is unclear, though several possibilities have been proposed. *In vivo* the increased thrust of the hyperactivated sperm may aid detachment of the sperm from oviductal epithelial cells. Sperm have been noted to swim away from oviductal explants in a hyperactivated manner *in vitro* (Pollard *et al.*, 1991). Alternatively, the increased forces on the sperm head may facilitate penetration of oviductal fluid (Suarez *et al.*, 1991a) or the cumulus and zona pellucida (Drobnis *et al.*, 1988a,b). Several clinical studies have reported correlations between the incidence of hyperactivation in human sperm and the results of sperm penetration assays (Morales *et al.*, 1988; Wang *et al.*, 1991a; Boatman and Robbins, 1991), IVF (Wang *et al.*, 1993) and donor inseminations (Mackenna *et al.*, 1993). As yet there is no evidence that this motility change is a pre-requisite for successful fertilization either *in vivo* or *in vitro*. Whilst hyperactivated sperm may progress more rapidly through the female tract, both hyperactivated and non-hyperactivated sperm are able to penetrate between the cumulus cells of the oocyte (Drobnis *et al.*, 1988a). In addition, although both sperm hyperactivation and zona penetration involve extreme flagellar bends, the direction of these bends are different for the two processes (see Introduction, Chapter 4). Therefore sperm may require hyperactivation *in vivo* to reach the oocyte, but not for fertilization *per se*.

In the golden hamster, Shalgi and co-workers (1992) found that hyperactivation resulted in poor sperm transport through the utero-tubal junction, and concluded that *in vivo*, hyperactivation onset occurred after sperm entered the oviduct. Most authors have reported hyperactivated sperm in the oviduct, although reports of uterine hyperactivated sperm do exist (Suarez *et al.*, 1983) and the site

of hyperactivation onset may be species-dependent. Hyperactivation is an episodic process (Katz and Yanagimachi, 1980) and presumably sperm hyperactivated in the uterus could still proceed to the oviduct.

The relationship between hyperactivation, capacitation and the acrosome reaction is unclear (Katz *et al.*, 1989). Using the calcium-sensitive fluorescent dye indo-1 and video-imaging, the intracellular calcium concentration ($[Ca^{2+}]i$) of hyperactivated and non-hyperactivated but motile (termed 'activated') hamster sperm have been evaluated (Suarez *et al.*, 1993). This report indicated that $[Ca^{2+}]i$ concentrations in the head and tail regions of hyperactivated sperm are higher than for activated sperm, and that peak frequency $[Ca^{2+}]i$ oscillations in the proximal flagellar mid-piece of hyperactivated sperm appear identical to the frequencies of flagellar beat cycles. In addition, greater $[Ca^{2+}]i$ levels were attained in hyperactivated sperm which had been induced to acrosome react, than in hyperactivated but non-reacted sperm. As hyperactivated, acrosome reacted sperm also show increased flagellar bending, the authors of this study concluded that the increase in $[Ca^{2+}]i$ associated with the acrosome reaction also affects flagellar function, but not vice-versa.

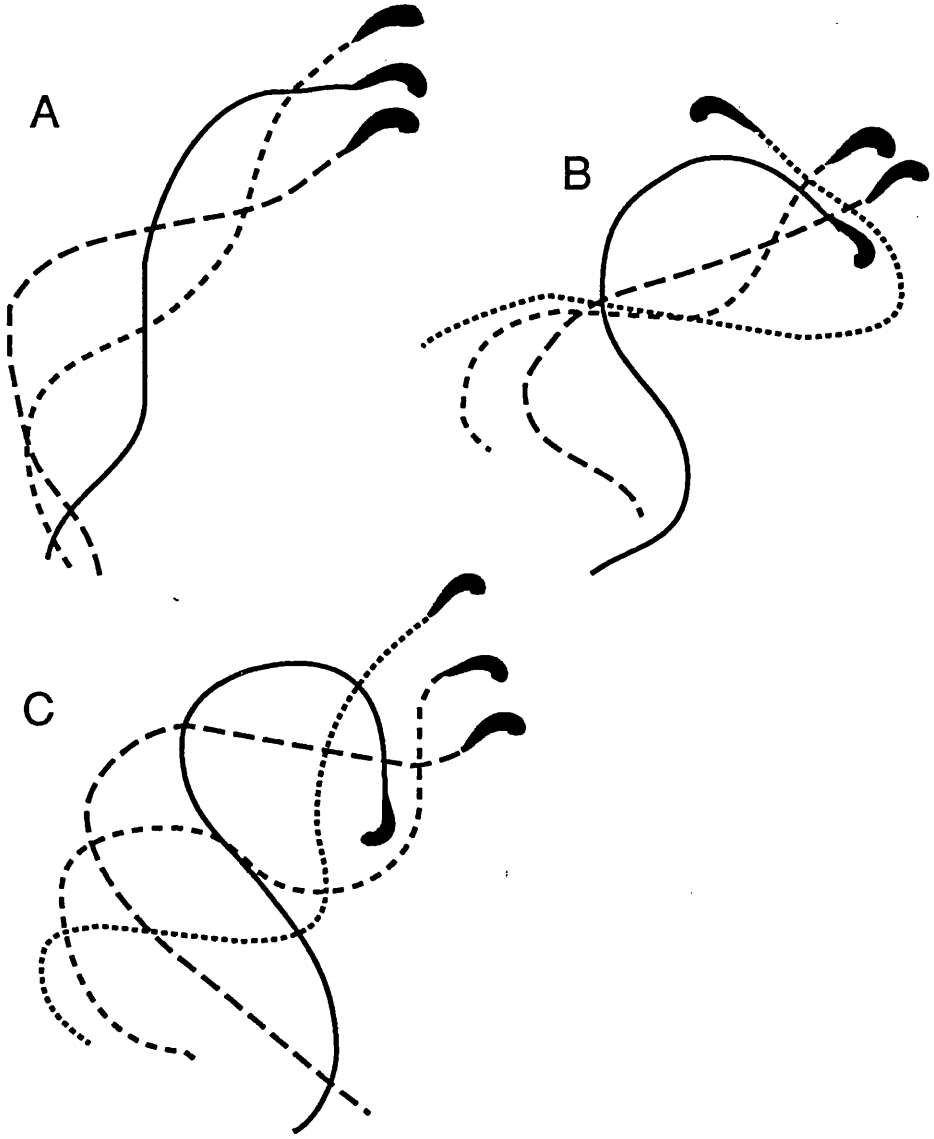

Figure 2. Movement patterns of hamster sperm during capacitation (from Suarez *et al.*, 1991). Patterns have been traced from individual frames of a video-recording, the tracing of the first frame is a solid line and subsequent frames are indicated by line fragments of decreasing length.

(A) Activated sperm. Flagellar beating is rapid, planar and of moderate amplitude.

(B) Transitional sperm. Three dimensional flagellar beats of increased asymmetry and amplitude that produce helical trajectories.

(C) Hyperactivated sperm. Asymmetrical, two dimensional, high amplitude flagellar beating.

During hyperactivation, the asymmetry and curvature of flagellar bending is increased in the midpiece and proximal regions, resulting in sperm movements that are non-progressive and highly erratic in low viscosity fluid such as culture media (See figure 2.). In some species, the motility has been reported to be phasic, with periods of progressive motility intermittent with the erratic motion (Katz *et al.*, 1989). The general characteristics are increased lateral head movement, a reduction in movement linearity, and a decrease in the beat frequency of the flagellum (Morales *et al.*, 1988; Burkman, 1991). The majority of studies rely on visual recognition of hyperactivated motility and few have attempted to define objectively the relevant motility values. Where CASA has been employed, researchers have used either criteria developed originally from sperm visually identified as hyperactivated, or used criteria suggested by the manufacturers of the CASA systems. Some authors identify more than one type of movement pattern for hyperactivated sperm (e.g. Circling, Thrashing, Star-spin and Helical (Burkman *et al.*, 1991)), whilst others describe a mid-way stage between activation and hyperactivation termed 'transitional' hyperactivated motility (Robertson *et al.*, 1988a). (For a review of the evaluation of hyperactivation using CASA see Katz *et al.*, 1993).

Few reports of hyperactivated boar sperm exist (Saxena *et al* 1986., Hamano *et al.*, 1989). Suarez and co-workers (1992) attempted to observe hyperactivated boar sperm by flushing the surgically removed oviducts of mated gilts at the approximate time of ovulation. A total of five hyperactivated sperm were recovered from thirteen oviducts, the majority of the remaining sperm being immotile (81-97%). This suggests that either the experimental conditions were not able to sustain hyperactivation, or that boar sperm do not hyperactivate in a manner consistent with sperm from other species. Subsequently, Suarez and co-workers applied a 4 µmol/L pulse of calcium ionophore to samples *in vitro* which resulted in approximately 30% of sperm showing motility designated as hyperactivated. However, whether this method of generating hyperactivation accurately mimics the physiological process is unclear.

1.2. THE PASSAGE OF SPERM IN THE FEMALE TRACT.

For fertilization to occur *in vivo* the spermatozoa must travel through the female tract to the site of the ovulated oocyte, a journey that requires survival by the spermatozoa within a variable environment. The following description applies to this process in the pig.

1.2.1 Insemination

Insemination normally occurs at the onset of oestrus, approximately 40-42 hours before ovulation. Spermatozoa are deposited in the uterus (whether insemination is natural or artificial), a typical ejaculate having a volume of between 150 and 400 mL and containing 50 to 100 x 10^9 spermatozoa (Moore, 1975). Spermatozoa pass through the uterotubal junction by self-propulsion and into the isthmus region of the oviduct. Within two hours of mating at the onset of oestrus, a population of functional spermatozoa is established in the isthmus (Hunter, 1984). This is a rapid progression by the spermatozoa; within 15 - 30 minutes of coitus enough sperm are present to fertilize the majority of oocytes that will be ovulated (Hunter, 1990). A similar pattern of reservoir formation has been observed in other species such as the mouse (Zamboni, 1972), hamster (Yanagimachi and Mahi, 1976) and sheep (Hunter and Nichol, 1983).

1.2.2 Sperm reservoir formation

Hunter (1984) using gilts mated at the onset of oestrus found that ligation and transection of the distal oviduct up to 38 hours after coitus prevented fertilization. This indicates a pre-ovulatory arrest of spermatozoa in the isthmus region for approximately 36 hours and suggests coordination of the passage of the male and female gametes. Maintenance of the sperm in this region is probably due in part to binding of the sperm head to cells of the isthmus mucosa. This binding is specific, sperm binding to only the ciliated cells of the tissue (Suarez *et al.*, 1991b), although *in vitro* the association occurs with samples from both isthmus and ampullar regions of the oviduct. Confinement of the spermatozoa to the isthmus region *in vivo* is probably due to physical factors, with spermatozoa reaching the isthmus but then being prevented from reaching the ampullar region due to their interaction with the isthmic ciliated cells. The oviductal mucus may

also have a retaining effect, porcine oviductal mucus is not penetrated by sperm *in vitro* (Suarez *et al.*, 1991b).

After 36 hours the sperm detach from the epithelia and proceed up the oviduct. The reason for this release of the sperm is unclear. Changes may occur within the mucosal epithelium at the time of ovulation or alternatively there may be alterations in the sperm as a result of the capacitation process. A link between capacitation and the mucosal binding was suggested by work with the hamster where introduction of non-capacitated spermatozoa to the oviduct resulted in attachment to the mucosa, whilst capacitated sperm were found not to bind (Smith and Yanagimachi, 1991). A change in sperm motility to the hyperactivated state and the resulting vigorous patterns of movement observed may generate enough force to release the sperm. Bovine sperm have been observed to swim in a hyperactivated manner on detachment from bovine oviductal explants (Pollard, 1991). However, hyperactivated motility is also observed in species that do not form oviductal reservoirs.

The reason for the evolution of the sperm reservoir is unclear but various suggestions have been made. It may act to coordinate the maturation of the gametes, delaying capacitation of the sperm until the onset of ovulation. Capacitated spermatozoa tend to be unstable and fragile and remain viable for only a short period of time. Hunter (1984) proposed that retention of the sperm in the reservoir delayed capacitation until nearer ovulation whilst preventing metabolic exhaustion. Binding to the oviductal mucosa has been shown to promote the maintenance of sperm motility; 90% of boar sperm attached to epithelial surfaces *in vitro* retained motility over 24 hours compared to 50% motility in sperm incubated in media alone (Suarez, 1991). Pollard (1991) noted a similar effect in bovine spermatozoa incubated with bovine oviductal epithelial cells. The sperm bound to the endosalpingeal cells by attachment of the head region and maintained their motility and fertilizing capacity throughout a 30 hour period compared to a more rapid loss of both in populations incubated with media alone. In addition, in this study only sperm incubated with oviductal cells developed hyperactivated motility.

1.2.3 Fertilization

From the site of the reservoir, sperm reach the environs of the ovulated

oocytes, possibly aided by peristaltic contractions of the oviduct that occur at ovulation, (Battalia and Yanagimachi, 1980). The sperm penetrate between the cumulus cells surrounding the oocytes and bind to the zona pellucida (ZP) (see below). Binding involves the association of specific complementary receptors on both gametes, the identities of which remain controversial (for a review see Yanagimachi, 1994). Once bound, sperm undergo the acrosome reaction and use a combination of enzyme hydrolysis and physical thrust to penetrate the zona pellucida. Fusion of the sperm and oolemma is followed by decondensation of the sperm chromatin and the formation of pronuclei.

1.2.4 The zona pellucida

The zona pellucida is an extracelluar glycoprotein layer that surrounds the mammalian oocyte, overlying the vitellus membrane. The width of the eutherian zona pellucida varies from 7μm in rodent species to 27μm in the cow and is approximately 16μm in the pig (for a review of zona pellucida structure see Dunbar *et al.* (1991)). During fertilization, sperm bind to the ZP, undergo the acrosome reaction and penetrate the matrix. More than one sperm may bind to the ZP but few will penetrate, and the zona is thought to be involved in the blocking of polyspermy. In some species only capacitated sperm will bind to this layer, (mouse - Saling *et al.* 1978), whilst the ZP of other species do not show such selective properties, (pig - Peterson 1981).

The pig is a polytocous species and ovaries are readily available from abattoir material, greatly facilitating research on the porcine ZP (for a review of the glycoprotein families found in the ZP of other species see Nakano, 1989). As in other species, the solubilized proteins have been shown to induce acrosome reactions in homologous sperm (Berger *et al.*, 1989a) and to block sperm-egg-binding (Berger *et al.*, 1989b). Chemically, the porcine ZP is composed of three families of glycoproteins. The molecular weights assigned to these three groups vary between reports as a result of the different evaluation methods used and heterogeneity in the glycosylation of the core proteins (Dunbar and Raynor, 1980; Hedrick and Wardrip, 1986; Henderson *et al.*, 1987; Yurewicz *et al.*, 1987). Unfortunately the nomenclature used to describe the glycoprotein families has not yet been standardized. A brief summary is given below.

Under non-reducing conditions, two glycoprotein families are apparent using

1-dimensional gel electrophoresis. These have molecular weights of 55 kD (termed ZP3) and 82-90 kD (termed ZP1). A glycoprotein family with a molecular weight of 164 kD has been reported by some groups but its existence remains to be verified (see Nakano *et al.*, 1989). Under reducing conditions the 82-90 kD glycoprotein splits into 61-65 kD and 21-25 kD components. De-glycosylation of the 55 kD family results in two sub-groups, one of molecular weight 32-36 kD (designated ZP3β by Yurewicz *et al.*, (1987) and 55-kDα by Hedrick and Wardrip, (1986)) and one of 37-40 kD (designated ZP3α by Yurewicz's group and 55-kDβ by Hedrick and Wardrip). Research indicates that the ZP3 family acts as the sperm receptor and stimulates the acrosome reaction, whilst ZP1 is thought to be structural and the preferred substrate for acrosin (Dunbar *et al.*, 1985). No specific role has been described for the 164 kD macromolecule.

1.2.5 Penetration of the zona pellucida

Capacitated sperm bound to the porcine zona pellucida undergo the acrosome reaction and subsequently penetrate the glycoprotein matrix. This penetration step involves proteolytic enzymes contained within the sperm acrosome and an element of thrust generated by the sperm flagella. Vesiculation of the acrosome releases enzymes, such as acrosin and hyaluronidase, which have been shown to break down the glycoproteins of the zona pellucida. From electron microscopic studies of sperm-oocyte interactions it appears that this lysis is highly localized as the penetration slit formed by the sperm head is narrow (see Bedford 1991). However, ZP1 has been shown to be more sensitive to proteolysis by acrosin than the ZP3 family (Dunbar *et al.*, 1985). This could allow for some proteolysis of the matrix without complete dissolution of the 3-dimensional structure. Evidence exists that the loss of acrosomal enzymes is rapid and these may not reach the inner regions of the zona pellucida (Kopecny and Flechon, 1987). If this is the case there would be a clear need for sperm thrust in the penetration of the zona pellucida matrix.

1.3 COMPUTER ASSISTED SEMEN ANALYSIS

With the advances in image analysis and computer technology in recent years, much interest has been shown in Computer Assisted Semen Analysis

(CASA) and the relationships of various motion parameters with other *in vitro* tests of semen quality, and fertility *in vitro* and *in vivo*. The term computer assisted semen analysis is a misnomer, as the analyses made are of sperm morphology or motility patterns rather than semen profiles. Sperm motility is more commonly examined by CASA than morphology, perhaps because motility analyzers are more readily available commercially. However several studies of sperm morphology by CASA do exist (Wang *et al.*, 1991b,c; Kruger *et al.*, 1993).

1.3.1 The principles of CASA

Early motility analysis took the form of tracing the paths of individual sperm heads across a video screen by hand, or employed long-exposure photographs of spermatozoa (Aitken *et al.*, 1982). This developed into tracking systems using graphics tablets linked to video-cameras, (Holt *et al.*, 1985; Tessler and Olds-Clarke, 1985), and later to computer systems that could record small numbers of sperm in real-time over a few seconds (Katz and Davis, 1987). More recently, systems have progressed to become capable of processing information at higher speeds and for greater numbers of sperm. A variety of commercially available systems exist, all of which are based around the following framework (See figure 3.).

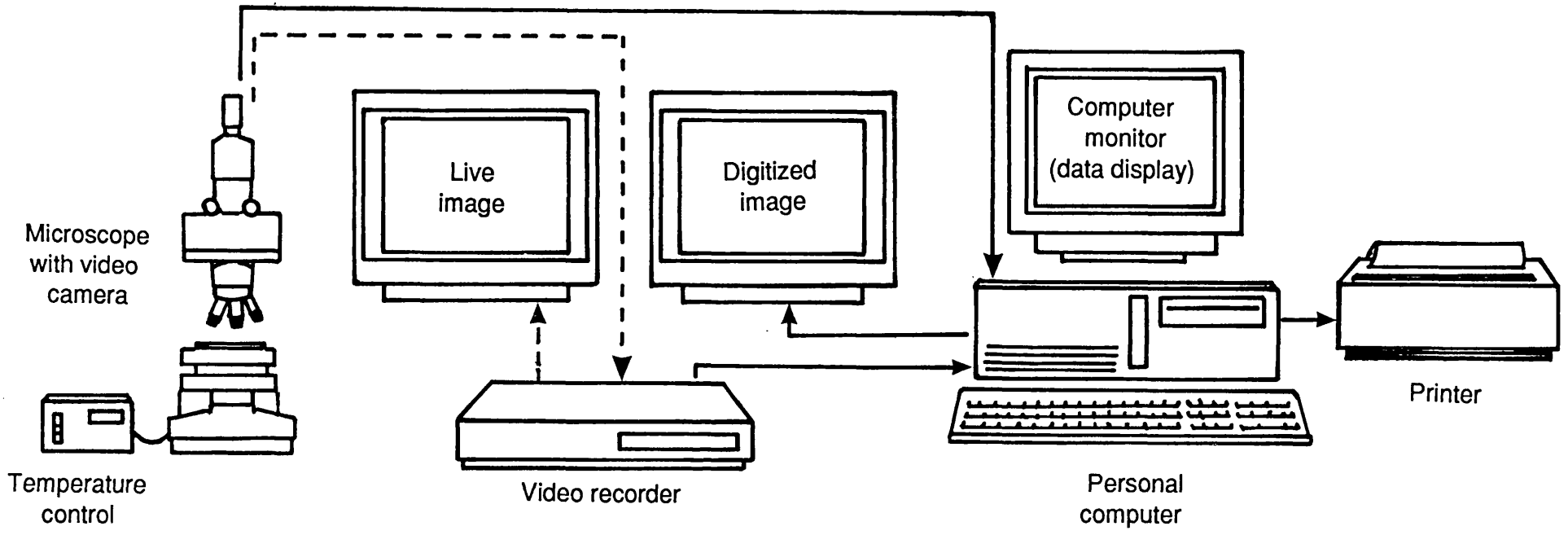

Figure 3. A typical CASA system. The assessment system comprises a phase-contrast microscope with temperature controlled stage; a video-system; monitors on which the video-recorded image and digitized image are shown; a computer system with appropriate image analysis hardware and software; and a printer and computer screen on which the analysis data is displayed (after Boyers et al., 1989). The figure represents a generalized CASA set-up; components vary between the different systems commercially available.

An image of the sperm sample is produced by a phase-contrast microscope (usually equipped with negative-high phase optics) connected to a video camera. The analogue video signal is converted to digitized information using a numerical scale for the grey tones of the sample image - this is the image analysis step, the conversion of the picture into numbers. Objects that conform to user-defined shape and size values are detected as sperm heads in the video-frame (for a review of CASA see Boyers et al., 1989). The computer establishes the position of the sperm head in consecutive video frames (for a clearer description of this process see section 2.0.1), and various algorithms are applied to calculate the movement of the sperm across the microscope field. (For example the velocity of the sperm from one position to the next can be estimated, and identification of the centroid in a third frame will establish a directional component.) The head of the sperm is detected rather than the flagellum which beats at frequencies above the most commonly used video frame-rates (25 and 30 Hz). Recently, systems capable of determining characteristics such as the flagellar curvature ratio have been developed (Suarez *et al.*, 1991a).

By specifying a minimum velocity gate to the computer, a user defined value, motile spermatozoa can be distinguished from immotile individuals and debris. Similarly, the number of frames for which a sperm is tracked, the minimum/maximum sperm size and particular motility criteria can all be specified to the system.

1.3.2 Motility parameters recorded during CASA

The types of motility recorded by CASA can be split into assessments of vigour and assessments of pattern (Katz and Davis, 1987). The former is characterized by velocity measurements whilst the latter includes the measurements of angular change in the sperm movement. The parameters of motility and the nomenclature employed depends on the CASA system that is used. In the last few years there has been a move towards standardization of CASA terminology; a description of the most commonly used abbreviations and definitions is shown below (and in figure 4) following the system suggested by Boyers, Davis and Katz from the University of California at Davis (Boyers *et al.*, 1989).

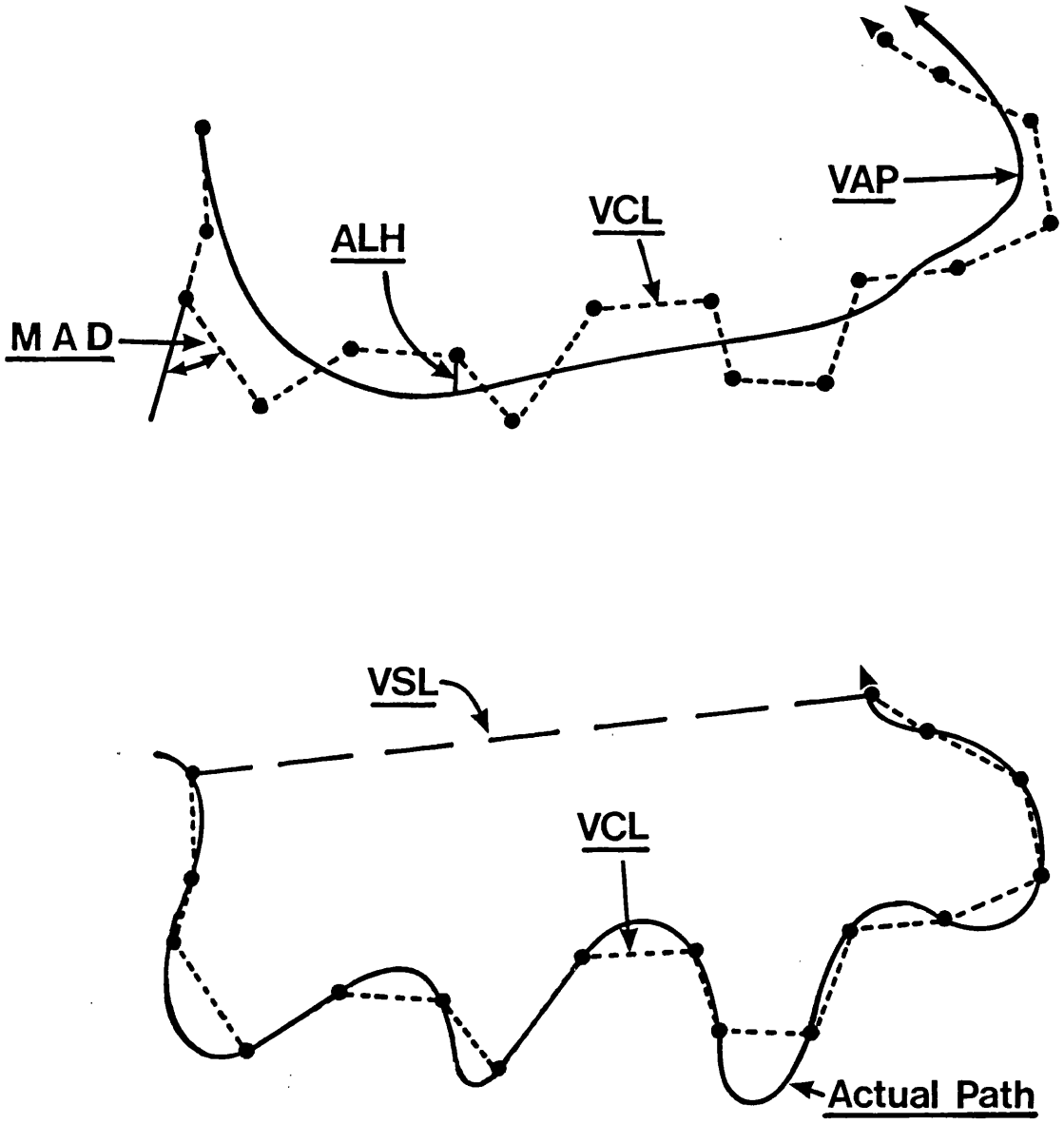

Figure 4. The kinematic parameters of a sperm trajectory. Each solid circle represents the head of the spermatozoa in adjacent frames of the analysis. See text for an explanation of the CASA measurements.

CURVILINEAR VELOCITY (VCL) - The time average velocity of the sperm head along its actual trajectory (or the closest approximation to this that the computer can make).

STRAIGHT LINE VELOCITY (VSL) - The time average velocity of the sperm head along a straight line from its first detected position to its last detected position.

AVERAGE PATH VELOCITY (VAP) - The time average velocity of the sperm head along its average trajectory. The average trajectory is computed by smoothing the actual trajectory using either an unweighted or weighted fixed-length running average, or by using an unweighted variable length running average.

AMPLITUDE OF LATERAL HEAD DISPLACEMENT (ALH) - the amplitude of variations of the actual sperm-head trajectory along its average trajectory. Previously called LHA or AH.

MEAN ANGULAR DISPLACEMENT (MAD) - the time average of absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory.

BEAT CROSS FREQUENCY (BCF) - The time average rate at which the actual sperm trajectory crosses its average path trajectory.

LINEARITY (LIN) - the linearity of the curvilinear trajectory (VSL/VCL). Previously called the progressiveness ratio (PR).

WOBBLE (WOB) - the degree of oscillation of the actual sperm trajectory around its average path (VAP/VCL). Previously called curvilinear progressiveness ratio (PRC).

STRAIGHTNESS (STR) - the straightness of the average path (VSL/VAP). Previously called the linear index (LI).

1.3.3 Advantages and disadvantages of CASA

CASA systems remove the need for time consuming, manual assessments and produce highly reproducible results for a given sample (Mack *et al.*, 1988). They also enable data for large numbers of individual spermatozoa to be gathered rapidly and accurately and remove the between-technician variation inherent in trials using subjective evaluations (Dunphy *et al.*, 1989; Tuli *et al.*, 1992). However sources of error are still present, and new ones arise from the use of CASA. For example, sampling error may be introduced initially by the use of inappropriate sperm concentrations (Vantman *et al.*, 1988). In addition, the CASA systems

employ user defined set-up parameters and the values chosen for these (often arbitrarily) can exert a significant effect on the results obtained (Knuth *et al.*, 1987). More recently, computer simulations of sperm tracks have been employed to study these parameter effects (Owen and Katz, 1993).

At present, the commercially available CASA systems do not produce comparable sets of data, and discrepancies occur between the results obtained from different CASA models, both for live split samples and video-tapes of samples (Mahony *et al.*, 1988; Olds-Clarke *et al.*, 1990; Davis *et al.*, 1992b). For results from different centres to be readily comparable there must be standardization of the algorithms used to determine motility parameters and of the nomenclature employed to describe them. In addition, centres must attempt to establish standardized set-up values for each make of CASA machine, and try to calibrate the different systems to each other (Davis and Katz, 1993a,b).

1.3.4 Clinical significance of CASA

"While their potential as clinical and research tools is undeniable, CASA systems today provide information, the biological and clinical relevance of which is still unclear. In light of this , the rapid proliferation of CASA systems is a triumph as much of marketing as of science, and reflects an uncritical acceptance of new technology" (Boyers *et al.*, 1989).

The automated systems have been used extensively in clinical situations in an attempt to discern a relationship between the sperm motility variables and subsequent sample fertility. The majority of research performed has involved assessing samples from fertile (donor) and infertile (patient) men participating in IVF programs. The motility variables have either been compared between these two groups, or with the results of sperm penetration assays or IVF outcome. (The following discussion therefore is based on clinical studies in the human; domestic species are discussed later.) A variety of CASA systems and experimental protocols have been employed, in light of which, the consistency of results is promising. Variations in motility algorithms and nomenclature mean that any comparisons between the results of these studies must be treated cautiously.

Several studies have reported significant differences in the motility patterns

of sperm from patients and donors. CASA has been used to investigate hyperactivated sperm in the two groups, with reports indicating a higher incidence of hyperactivation in donors than in patients (Morales *et al.*, 1988; Wang *et al.*, 1993; Mackenna *et al.*, 1993). Elsewhere, studies have not highlighted the hyperactivated sub-populations, instead investigating population means for the motility variables.

In the human, sperm swimming speeds tend to be stable over time for a given individual (Holt *et al.*, 1985). This makes such measurements attractive as potential predictors of fertility, as intra-individual variation is low. Several studies have correlated sperm velocity with fertility criteria (Fetterolf and Rogers, 1990; Barlow *et al.*, 1991; DeGeyter *et al.*, 1992; Oehninger *et al.*, 1992; Zouari *et al.*, 1993). Holt and co-workers (1985) used a semi-automatic computerized technique for the analysis of human spermatozoa and were able to correlate sperm velocity with the results of human IVF. This group, and others, found that the proportion of slow swimming cells, e.g. below 10 or 20 μm/sec, was an important indicator of fertility (Milligan *et al.*, 1980; Aitken *et al.*, 1982; Badenoch *et al.*, 1990; Liu *et al.*, 1991). Similar findings are reported for cryopreserved sperm after artificial insemination (Holt *et al.*, 1989; Marshburn *et al.*, 1992). The results of the former study indicated that samples showing poor maintenance of sperm velocity over time (>40% decline in velocity over 3.5 hours) had a significantly lower pregnancy rate than sample groups with better survival (<40% decline). Green (1988) suggested that spermatozoa with low swimming speed probably lack the minimum thrust required for penetrating cervical mucus and the zona-pellucida.

Significant differences in the values for ALH have been found between samples from donor and patient groups (Jeulin *et al.*, 1986) and from IVF success and failure groups (Barlow *et al.*, 1991; Zouari *et al.*, 1993). In all cases, the poor fertility samples produced a lower mean ALH value. However, only the latter two studies also detected a difference in mean sperm velocities between the two groups.

1.3.5 CASA of domestic species

One of the difficulties in determining the clinical significance of CASA for any species is the need for extensive *in vivo* fertility data for a single ejaculate. The outcome of insemination *in vivo* depends on several factors besides sperm

fertilizing ability including female fertility, animal management, detection of oestrus (for a review see Amann, 1989). The statistical effects of these can be ameliorated in trials using domestic species by multiple inseminations of a single, split ejaculate. Although there are reports of the application of CASA to sperm from many species (Rabbit - Farrell *et al.*, 1993; Dog - Ellington *et al.*, 1993; Stallion - Jasko *et al.*, 1990; Goat - Tuli *et al.*, 1992; Bull - Anzar *et al.*, 1991; Ram - Leidl *et al.*, 1993; Hamster - Suarez et al., 1991a, Rat - Slott et al., 1993), few comparisons of CASA measurements to fertility have been made.

An early attempt to relate objective measurements of sperm motility to 75-day non-return rates in the cow produced non-significant correlations in a trial using cryopreserved bull semen (O'Connor *et al.*, 1981). This study also used a competitive fertility index (C.F.I.) constructed using heterospermic inseminations of ejaculates from nine bulls, and found that the objective velocity measurements were significantly correlated to the C.F.I. results. Analysis of frozen straws from the same ejaculates was performed eight years later using a more modern CASA system (Amann, 1989) and produced comparable results, with VCL, VSL and LIN being significantly correlated to the C.F.I. results, but not return rates.

Further authors have suggested, cautiously, that CASA measurements are useful indicators of fertility, either used alone (stallion - Andersson and Katila, 1992) or in conjunction with other assays of sperm (rabbit - Farrell *et al.*, 1993). However, a recent study using bull sperm found no association between CASA variables and fertility (Bailey et al., 1994).

1.3.6 CASA of boar sperm

Few examples exist of CASA of boar sperm. Extended boar semen has been assessed by CASA in an attempt to relate motility (%) and/or velocity to litter size (Aumüller and Willeke, 1988; Rath *et al.*, 1988; Riebenwein, 1989) but these studies could not establish any relationship between the fertility and motility information. Tuli and co-workers (1992) analyzed the motility of frozen/thawed and fresh boar sperm and concluded that the computerized assessments were comparable to the assessments made by experienced technicians, but that the latter were more time-consuming and were subject to between technician variation. The incidence of hyperactivation in boar sperm has been investigated using criteria originally developed for the rabbit (Suarez *et al.*, 1992). Recently, Grant and co-

workers have used CASA to examine the effect of washing protocols on the outcome of porcine IVF (Grant *et al.*, 1994).

These CASA studies highlight certain characteristics of boar sperm which make it difficult to assess for motility. Washed boar sperm demonstrate 'stickiness' to each other, to debris and to glass surfaces such as microscope slides (Suarez *et al.*, 1991b; Harrison and Vickers, 1990; Dacheux, 1983). These problems can be overcome by the assessment of washed sperm in specially prepared slide chambers. In addition, isolation of cells from semen/extenders by methods such as washing or swim-up, followed by incubation in media results in cells forming head to head agglutinations (described in the next chapter).

1.4 PIG ARTIFICIAL INSEMINATION.

1.4.1 Introduction

AI in pig breeding now accounts for 11% of total matings in Britain (Reed, 1990a). Although this is still low compared to parts of the European community, (e.g. 70% of matings in Norway and 57% in the Netherlands), there has been a rapid increase in demand over the last few years. Use of an AI service allows a breeder access to high quality, performance tested boars; minimises the health risks to herds from the movement of animals and can overcome "insufficient boar power" where a farm needs a large number of boars, but cannot meet the associated costs.

Semen Delivery Services (SDS's) maintain nucleus herds of boars with important phenotypic traits, such as rapid growth rate or high lean meat to fat ratio. These animals are trained to mount 'dummy sows', and the ejaculated semen collected into pre-warmed containers. The gel-fraction of the ejaculate is removed and the sperm-rich fraction passed immediately for semen assessment. Subsequently the semen is diluted with liquid extender to the desired concentration (based on the semen evaluation) and bottled. Packaging ensures semen temperature is maintained above 16°C, below which boar semen suffers cold-shock (Pursel *et al.*, 1972).

1.4.2 The economics of AI

Breeding experiments with pig nucleus herds have shown many

economically important characteristics to be highly heritable (e.g. carcass traits - Bryner *et al.*, 1992). The commercial advantages of access to genetically superior stock can be large. Meat producers use assessment indices to score individual pig carcasses for merit - the average score being approximately 200. Boars with superior carcass traits (and hence higher index scores) pass on these genetic benefits to their offspring. The Meat and Livestock Commission estimates that an increase of 10 points in the index score is worth about 23 pence in each of the boar's progeny. A boar at an AI centre may sire about 6,500 pigs a year (Meat and Livestock Commission, 1988), hence the value of identifying top quality boars and maximizing the availability of their semen is obvious.

1.4.3 Semen assessment techniques in pig AI

Farmers prefer to use young boars for AI, and SDS centres attempt to maintain their studs as young as possible. A prospective evaluation of semen quality would be highly advantageous for the SDS companies. This would eliminate the need to accumulate non-return rate results to estimate boar fertility and could take account of the day to day variation in the ejaculates from a boar.

The response to long term liquid storage of semen also varies between boars (Waberski *et al.*, 1991). The 'quality' of a semen sample on collection, as assessed by present methods, is not indicative of its response to storage in liquid extenders. A means of predicting this response, enabling accurate estimations of ejaculate shelf-life would be valuable.

Present methods used to assess quality of boar semen are crude and cannot be used to predict accurately ejaculate fertility (Woelders, 1990). Ejaculates are assessed subjectively with the light microscope and an estimation of the motility made on the basis of the "swirl" of the spermatozoon in the ejaculate, resulting in a qualitative evaluation ranked from 0 to 5. The concentration of the spermatozoa, and the different morphologies present are also ascertained. These examinations can identify the more severe cases of reproductive failure (such as oligo- or asthenozoospermia) but cannot distinguish idiopathic infertility or more subtle differences in fertilizing potential from one ejaculate to the next (Reed, 1988).

Whilst a variety of assessment methods may be available to clinicians working with human infertility problems, the number of tests suitable for use in the

SDS centres is more limited. Only a short time is available between semen collection and despatch, and any appraisal must be rapid and technically easy to perform. The next chapter introduces a novel system for the analysis of sperm motility and evaluates optimal set-up conditions for the assessment of boar ejaculates.

CHAPTER 2 - VALIDATION OF THE HOBSON SPERM TRACKER FOR USE WITH BOAR SPERM.

2.0 INTRODUCTION

The purpose of the experiments described in this chapter was to establish a system for the analysis of boar sperm using the Hobson Sperm Tracker (HST). The motility data obtained by CASA machines is not truly objective, even though it is often represented as such (Comhaire *et al.*, 1992). Subjectivity arises because the results of any CASA analysis are dependent on the computer software/hardware, the system set-up parameters, and sample preparation (for a review of these considerations see Boyers *et al* 1989; Owen and Katz, 1993). An evaluation of how these factors influence the motility data was required prior to choosing the conditions for future analyses.

A further aim of this chapter is to introduce the HST and to explain some of the principal differences between this and other CASA machines. This is followed by a description of the set-up parameters required for the HST and a brief introduction to the methods of specimen preparation used for the boar sperm samples. The experiments performed to optimize the system are then detailed and the final choice of parameters discussed.

2.0.1 CASA software and hardware

CASA systems use digital image analysis to detect sperm movement (for a review of the processes involved in CASA see Boyers *et al.*, 1989). This is the conversion of an image into a framework of numbers, based on a grey scale such that any shade in the image is given a value ranging from black (0) to white (255). Objects such as sperm appear a different shade of grey to the background, and therefore produce a change in this framework of numbers. This assumes that a shade contrast exists, hence the use of phase-contrast optics in CASA microscopy. Originally CASA systems used positive phase-contrast optics where the sperm appear dark against a light background. Systems using negative phase contrast optics have been introduced recently; the IVOS system from Hamilton-Thorn Research Inc., and the SM-CMA from Strömberg-Mika Medical Equipment. The HST system at the Institute of Zoology also uses a negative-high phase contrast microscope. Yeung and Nieschlag (1993) have compared the two types of phase-contrast optics and concluded that negative phase-contrast optics improves the analysis of motile sperm compared to positive, but may be inferior at detecting

immotile sperm in the samples.

The digitization-step transforms the image into numeric form after which the computer employs user-defined criteria to identify the sperm heads in each video frame. The centre of the sperm head (often termed the 'centroid') is calculated by averaging the cartesian coordinates of the pixels making up the sperm image[1]. The system then moves to the next frame and locates the second position of the sperm head by searching in a defined area around the site of the original centroid. Subsequent positions of the sperm head are established, usually a minimum of five, and algorithms applied to evaluate the paths and speeds of the sperm. The capabilities of most systems are such that they can analyze up to 30 video frames in one run. The systems store the information from each of the frames and must then pause to process the information, a method known as 'frame-storing'. Framing rates for video systems are dependent on the frequency of the electricity supply, hence in the U.K. and Europe 25 frames/sec are used whilst in America, a frame rate of 30 frames/sec is more common. This results in sperm being tracked for approximately one second in either case. This time period can be extended by omitting to sample every consecutive frame, e.g. analyzing every third frame, but the resulting virtual path approximates less to the actual track with implications for the resulting data (Katz and Davis, 1987). Even using this technique, tracking time cannot be extended to more than a few seconds.

2.0.2 The Hobson Sperm Tracker

The Hobson Sperm Tracker has been developed over the past four years in conjunction with a Sheffield based electronics company, Sense and Vision Electronics Systems Limited.. Originally developed for the tracking of motor vehicles, the system could in theory be applied to the tracking of any moving object. The HST is similar to other CASA models but has the unique feature of a 'frame grabber' board - a high speed processor board capable of multiplying the host personal-computer microprocessor speed by 100 fold. The result is a

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[1] The Cell-Trak system uses 'edge detection' to determine the position of the sperm centroid, i.e. only the pixels defining the perimeter of the sperm head are digitized and sent to the computer (Boyers et al., 1989). The HST identifies the position of a sperm head by placing a minimum enclosing rectangle around the sperm head. A centroid coordinate is determined by taking the midpoint of the two sides of the rectangle (G.Hobson *pers comm*).

prototype CASA system which performs image analysis of video input in real time with no restrictions on the period of tracking. The movement data is continually up-dated, with histograms displaying the current distribution of the motility variable. Once the user-defined tracking period has elapsed population means for the motility variables are calculated and the data for each individual spermatozoon is down-loaded to the P.C. hard disk.

Several versions of software have been developed in the past three years, incorporating improvements suggested as a result of experimental work performed at the Institute of Zoology. As the software evolved it became obvious that results obtained using different versions of the software were not comparable. Therefore video-tapes of the sperm samples (made routinely as a part of all experiments) were re-analyzed using version seven software. This ensures that for this thesis the results from different experiments are comparable.

2.1 SAMPLE PREPARATION

2.1.1 Introduction

Sample preparation will alter both the motility produced by the sperm and the image received by the video-camera (See Davis and Katz, 1993a). Motility assessments are dependent on the choice of media, viscosity and temperature (tests of sperm motility are usually performed at the species body-temperature - 39°C in the pig). In this way, the preparation of samples for CASA is no different from the preparation of samples for visual assessment. The latter tend to use a broad scale of motility vigour (e.g. 1-5; W.H.O., 1987) whilst the measurements made by CASA are more detailed, (velocities in µm/sec). Hence small alterations in motility may not alter the outcome of visual assessments whilst significantly affecting CASA results.

Two considerations in particular affect CASA - the type of chamber used, and the presence of dead cells and/or debris in the sample. A deep specimen chamber creates more than one microscopic field of view and sperm can swim in and out of focus, breaking up the CASA tracking (Le Lannou *et al.*, 1992). The converse, using a small chamber depth, creates drag effects on the sperm which swim slower and tend to move only laterally (Amann *et al.*, 1988; Slott and Perreault, 1993). The second consideration arises because the contents of an

ejaculate often includes immature sperm, cellular debris (e.g. leucocytes), particulate matter, dead cells, and separated sperm heads and tails. These may be detected as moving objects if they are knocked by motile sperm or caught in currents within the slide preparation.

Initial attempts at CASA analysis of boar sperm highlighted two further considerations. The first was the tendency of sperm to stick head down to the surface of glass slides. This occurred immediately and although vigorous flagellar motion continued, there was no forward progression of the sperm and only a few were seen to detach from the glass. There are no references to this sticking in previous reports of boar CASA analysis (Tuli *et al.*, 1992, Rath *et al.*, 1988), however Suarez and co-workers used agar-coated slides when examining washed and re-suspended boar sperm (Suarez *et al.*, 1992) suggesting similar problems.

The second observation was the tendency of dead sperm and debris to act as sites for the aggregation of both dead and live sperm cells. Large, slow moving groups resulted and many of the free swimming sperm were rapidly removed from the samples. The elimination of dead cells and debris delayed the onset of this process, which meant that a washing step was required that simultaneously removed the sperm from the extender and produced a debris-free sample. These groupings of sperm could easily be distinguished from the more specific head to head agglutinations that developed during incubation, (discussed further in the next chapter).

2.1.2 Materials and methods

The following practices were adopted for the preparation of samples.

Preparation of slides - A solution of 0.4% agar (Sigma, Poole U.K.) was prepared in distilled water by heating to nearly boiling. The solution was then allowed to cool to 65°C, and racks of glass coverslips (22 x 22 mm; BDH, Poole, U.K.) and glass slides, (76 mm x 26 mm) were then submerged, removed and allowed to dry. In the original reference (Suarez *et al.*, 1991a) one side of the slide was wiped clean, however this second layer of agar did not appear to affect the video-image and the step was therefore omitted. 25 µL of the sperm sample was placed on the slide and a coverslip added creating a specimen preparation approximately 50 µm deep.

Washing protocol - A variety of washing protocols were investigated. Normal

centrifugal washing allowed separation of the sperm from extender, but could not split the live sperm from dead cells and debris. Swim-up techniques removed dead cells, but resulted in large numbers of cytoplasmic droplets in the sample. A modification of the Percoll washing technique used for boar sperm by Berger and Horton (1988) produced a very clean sample and was therefore adopted.
Saline at x 10 the isotonic strength was prepared, and then 1 mL was diluted with 9 mL Percoll (Sigma, Poole, U.K.) to make an isotonic solution (SIP). This solution was then added to isotonic media in two ratios:-

40% Percoll solution - 4 mL SIP to 6 mL media.
80% Percoll solution - 8 mL SIP to 2 mL media.

2 mL of 40% Percoll solution were placed in a 13 mL conical bottom tube, and a 2 mL syringe with 4 cm needle used to underlay 2 mL of 80% Percoll solution. This created a visible interface. 2 ml of semen in extender were then layered onto the surface. The tubes were centrifuged at 300g for 20 minutes, which produced a loose pellet of sperm in the bottom of the tube and two distinct bands of sperm/debris at the interfaces of the three solutions. The top 5.5 mL of liquid were removed, and the pellet washed twice by resuspension in 2 mL of media (pre-warmed to 39°C) followed by centrifugation at 300g for 5 minutes. Finally, the pellet was resuspended in media to the required sperm concentration. The resulting sperm sample was free from contaminants and contained no immotile cells and very little debris.
Sample concentration - Washed sperm samples were diluted 1:1 with formol saline and drops of the mixture transferred to an Improved Neubauer Haemocytometer. Duplicate counts were made and the average used to calculate sperm concentration, and only complete sperm were included in the count.
Sample handling - Unless otherwise stated, all sperm samples prepared for CASA experiments had been Percoll washed and were analyzed on agar coated slides. Slides, coverslips and pipette tips were pre-warmed to 39°C, and a heated stage at 39°C used on the Olympus BH-2 negative-high phase-contrast microscope (Olympus, Tokyo, Japan). Prepared slides were transferred to the heated stage, allowed to settle for 30 seconds and then video-recorded at x200 magnification using a JVC Super-VHS recorder and Phillips S-VHS videotapes.

2.2 SET-UP PARAMETERS

2.2.1 Introduction

The parameters that are user-defined depend on the system employed and typically include grey threshold level, minimum number of track points, and threshold cell velocities. The assigned values influence the motility values obtained and ultimately, the results of any experimental work (Knuth *et al.*, 1987; Mack *et al.*, 1988; Owen and Katz, 1993; Slott *et al.*, 1993). As demonstrated in the following experiments, the amount of error that can potentially be introduced by using inappropriate set-up values is large and could easily overshadow any changes in cell motility.

Many researchers have treated this as a black box area choosing to use 'manufacturers recommendations' for their set-up values. These may not be optimal as different experimental situations require distinct system set-ups (Anzar *et al.* 1991). Mack and co-workers (1988) reported that grey-threshold values need to be altered depending on whether the sperm sample is washed or remains in seminal plasma. Similarly sperm morphologies and motility patterns are species dependent, requiring re-setting of the system when different species are studied (Rath *et al.*, 1988).

2.2.2 Sperm concentration

The results of any CASA analysis are related to the concentration of the sperm in the sample (Vantman *et al.*, 1988). At high concentrations (or if a spermatozoon is tracked for a long time) the probability of one sperm colliding with another is increased (Jasko *et al.* 1990; Yeung and Nieschlag, 1993). However, as concentration decreases, fewer sperm pass the video-camera's field of view within the analysis time, reducing the numbers of sperm for which data is collected. A concentration of 20×10^6 sperm/ml produced a satisfactory compromise between these two effects and therefore experiments were performed to evaluate the optimal set-up parameters for this concentration of sperm.

2.2.3 Grey threshold

Altering grey-threshold levels (also called digitization thresholds) affects the identification of individual sperm heads by the CASA machines, (Davis and Katz,

1993a; Mack *et al.*, 1988; Knuth *et al.*, 1987). This parameter sets the amount of shade contrast that must exist for an object to be discerned from the background. As yet there is no objective way of establishing correct grey-threshold levels for any type of CASA machine (Davis and Katz, 1993a), therefore the grey-threshold level was set subjectively and all other set-up parameters optimized for this value. Previously it has been shown that the values obtained for motility parameters (VCL, VSL, LIN, ALH and BCF) are not significantly different over a wide range of grey levels (Mack *et al.*, 1988) indicating that this is a fairly robust parameter. Knuth and co-workers reported that concentration and percentage motile measurements were greatly affected by this parameter but velocity and linearity were not (Knuth *et al.*, 1987).

Setting the grey threshold on the HST is achieved by watching the computer track sperm on the screen and simultaneously adjusting the threshold value. The sperm heads appear to be covered by a 'blob' or 'halo' of blue colour which indicates that the system has distinguished the object from the background. Lowering the grey-threshold creates larger halos and eventually spurious blobs appear on the screen. The value is adjusted until the blue blobs cover the sperm heads, but do not create shadows of blue around the sperm. The digitization of the image is strongly affected by changes to the microscope light source. This must remain at a constant setting for the chosen grey-threshold levels.

2.2.4 Search radius

The identification of an object as a sperm head is the first step towards establishing the path of a cell. The position of the sperm head in the digital framework will shift from frame to frame as the cell moves through its trajectory. In order to locate successive positions of the sperm-head, the system searches a circular area in the next video-frame centred upon the site of the previous sperm head. The 'search radius' is the set-up parameter that defines the size of this circle. As the value is increased the area scrutinized becomes larger, until the tracking becomes highly erratic as separate sperm are incorrectly linked. Decreasing the search radius causes the sperm tracks to break up as faster sperm move out of the range of the defined area.

2.2.5 Minimum frame number

Several papers have been published which examine the minimum number of frames for which a sperm must be tracked to obtain an accurate representation of the sperm trajectory (Davis and Katz, 1993a; Mack *et al.*, 1988; Knuth *et al.*, 1987). In systems using frame-store technology, the maximum number of frames for which a sperm can possibly be tracked is limited by the processing power of the computer to approximately 30 frames. (The Cell Trak increases the number of frames stored by using edge detection methods to minimise the information retained per frame). The HST does not suffer this constraint and can track a cell for as long as it remains in the area being viewed. Increasing the number of frames over which a sperm is tracked increases the accuracy of the data (Owen and Katz, 1993) as more sample points are obtained. Linearity in particular has been shown to be strongly influenced by the number of frames upon which it is calculated (Knuth *et al.*, 1987; Vantman *et al.*, 1988). A minimum number of frames, above which the margins of error occurring are acceptable can be evaluated, most studies agree on a minimum value of 15 frames.

The Minimum Frame Number (MFN) and Search Radius (SR) set-up values are not independent, and the effect of one on the motility data is influenced by the other. Therefore the interaction of these two factors must be examined in tandem. In this study this was achieved by evaluating the results obtained for a standard section of video-tape using combinations of SR and MFN values.

2.2.6 Experimental design

All combinations of 10 search radii values (SR= 5.94 µm to 16.63 µm) and 10 minimum frame values (MFN= 10 to 28) were evaluated. To overcome any systematic effects on the data due to stretching of the videotape, the search radii/minimum frame combinations were randomized and evaluated in a 10 x 10 Latin Square.

Choice of SR and MFN ranges - At an SR<5 µm, the tracking of the sperm is almost absent and at a SR>17 µm the tracking is confused by extensive mis-matching of tracks. The range of MFN's was from the lowest possible value (MFN = 10) to a point almost twice the value generally accepted as sufficient.

Procedure - Bottled boar sperm was prepared as described previously using a Tyrodes-based media developed for pig IVF (see appendix). Sperm concentration

was adjusted to 20 x 10^6 sperm/ml and the sample allowed to equilibrate in a 39°C, 5% CO_2, 100% humidity incubator for 10 minutes. Subsequently an aliquot was removed, mounted and then video-taped for a period of four minutes, at which point the sperm showed rapid, progressive motility. Greater than 95% of the sperm remained motile throughout the period of taping. This section of tape was copied ten times onto a second video tape to provide ten consecutive repeats of the original four minute section.

HST grey-threshold levels were determined subjectively by varying the threshold values and evaluating the effect on the digital image of the sperm produced by the computer. The recordings were then analyzed using the MFN and SR combinations.

2.2.7 Results and decisions

It is possible to determine theoretically the approximate sample size (n) required to obtain results within predefined tolerances using the following equation (from Snedecor, 1956):-

$$n = \frac{4\sigma^2}{L^2}$$

σ = st. dev. of a motility variable

L = allowable error, 5% in this instance.

The number of sperm recorded from the specimen tape section exceeded the estimated value (n) for most of the experimental combinations of variables. The sample sizes for cases where SR=5 μm with MFN ≥16 were below the value (n) due to the detrimental effect of the small SR on numbers of sperm tracked.

Each combination of variables produced a mean and distribution for the six motility values VCL, VAP, VSL, MAD, BCF and ALH, and a total number of sperm tracks recognized for the tape sequence. Two other parameters available as an option on the HST - LIN and STR - were not examined as these are ratios calculated using the motility parameters mentioned previously. The SR and MFN combinations produced various effects on the motility measurements. In the following description, the motility variables are discussed separately to avoid

confusion. Reference is made throughout to an identical experiment performed using sperm from the common marmoset, Callithrix jacchus. These sperm swim with very straight paths in contrast to the boar sperm which appear to have more erratic trajectories with wide lateral head movement.

Number of sperm tracked - SR was positively correlated to sperm number ($r=0.489$, $P<0.001$) whilst a negative relationship was found between M.F.N and sperm number ($r=-0.718$, $P<0.001$; see figure 5). The range of sperm numbers recorded was between n=46 (for the combination of maximum M.F.N and minimum SR) and n=2291 (for the minimum MFN and maximum SR combination), although the numbers detected were very similar for any given MFN with SR≥9. As MFN increases, fewer tracks are within the analysis window for the defined length of time and the number of sperm successfully recorded falls. Increasing SR values results in an expanded area available for sperm detection, raising the number of tracks successfully followed.

Velocities - The ranges of the mean values for the three velocity variables were:- VCL 77-148 µm/sec, VAP 66-124 µm/sec, VSL 64-105 µm/sec. The scatter of the data was similar for all three variables (figure 6a); mean values varying less than 10 µm/sec for a given SR greater than 8µm. Mean VAP and VSL were significantly correlated to MFN (VAP $r=-0.329$, $P<0.001$; VSL $r=-0.421$, $P<0.001$) whilst mean VCL was not ($r=-0.173$, $P<0.086$). This pattern had been predicted based on the premise that at higher MFN's, fast-swimming sperm would be more likely to pass out of the analysis window without fulfilling the MFN requirement. This removes rapid sperm from the resulting velocity distributions, producing lower mean velocities. This also proved to be the case for the marmoset sperm, which produced correlation curves strikingly similar to those of the boar, but with higher mean velocities.

The VCL and VAP of the boar sperm were positively correlated to SR ($r=0.848$, $P<0.001$ and $r=0.582$, $P<0.001$ respectively). This is because with smaller SR's there is a greater probability that a fast sperm will move out of the detection area in the time between consecutive video frames. Mean VSL decreases as SR increases due to an accompanying increase in error tracks which could be seen on the HST screen. As described previously, to establish a sperm

trajectory the HST must be able to locate a particular sperm in consecutive video frames. The method used is to define 'search areas' in successive frames within which any sperm present are assumed to be the original sperm. The sizes and positions of these areas are defined by the search radius and the location of the sperm head in the preceding video-frame. Error tracks occur when sperm other than the original one are present in the 'search area'; and the probability of this happening increases as search radius increases. When the trajectory of one sperm is erroneously aligned with that of another sperm in this manner the result is a track with a sudden bend where the path of the second sperm is picked up. This was not be reflected in mean VCL or VAP, but reduced the linearity of the path, as the sperm appeared to veer to one side or even travel back on themselves, and therefore reduced the VSL.

ALH - Mean ALH values were positively correlated to SR ($r=.943$, $P<0.001$, range of 1.93 - 8.41 µm). This may have been due to the higher search radii detecting wider ALH movements, or may mean that more rapid sperm (increased detection at higher SR's - see above) have wider head movements. At low SR values, mean ALH was consistent across all MFN combinations whilst at higher SR values ALH increased as MFN increased (see figure 6b). ALH was positively correlated with MFN values ($r=0.262$, $P<0.001$).

An almost identical pattern was derived from the marmoset data, means occurring within the same range of ALH values.

The positive correlation of mean ALH and SR was further investigated by plotting the distributions for the combinations of MFN=18 (MFN mid-point) with SR values 5.94 to 16.63 µm. As the SR increases, the distribution of ALH values moves to the right, a result of the larger search area aiding the detection of wider ALH movements. Simultaneously unexpectedly large ALH values appear and a combination of these two events results in the positive correlation of mean ALH and SR. The few large ALH values that occur, e.g. ALH >30 µm, are associated with small BCF values, and arise where the HST tracks a straight swimming (or drifting) sperm which suddenly changes direction. This is perceived by the computer as a single artificially large movement of the head across the averaged path, rather than a redirection of the sperm path.

BCF - Mean values for this variable ranged from 5.89 to 7.32 Hz, and demonstrated a negative correlation with MFN ($r=-0.757$, $P<0.001$; see figure 6b). Checking the distributions yielded by this variable (using SR = 10 µm and all MFN values) revealed that higher MFN values produced more normally distributed BCF values, each with a maximum recorded BCF=13 Hz. In theory, the video framing rate of 25 Hz that is used dictates a maximum BCF evaluation of 12.5 Hz, due to the process of aliasing (see discussion) but the tracker numerically rounds these values to 13 Hz. At lower MFN values the distributions were skewed towards higher BCF measurements, however, the number of cases with BCF=0 was also increased).

Means from the marmoset sperm data were different to those for the boar sperm. The range of mean values was much smaller, (3.5 to 5.5 Hz) with no obvious relationship to either MFN or SR. This is possibly a reflection of the smooth pattern of marmoset sperm movement, where the lateral movement of the head is less striking than that found in boar sperm.

MAD - The distribution for MAD revealed a negative correlation with MFN ($r=-0.366$, $P<0.001$, range of means = 77.2 to 88.3°) whilst SR was positively correlated to MAD, ($r=0.661$, $P<0.001$; see figure 6b). For all combinations with SR≥9 µm, mean MAD values were within two units of each other for any given MFN; the results for SR=<9 µm were up to six units lower. These results were similar to those from the marmoset sperm data which also fell within the same range (80°≤M.A.D.≤90°). The marmoset data generated using SR/MFN combinations where SR=5.94 µm were up to 40 units lower than SR≥7 µm combinations.

From this data a minimum frame number of 24 and a search radius of 13 µm were chosen for use in future CASA experiments. All the distributions contained a subset of cases in which BCF and ALH measurements were zero, and the proportion of these error cases decreased as MFN increased. In addition, at SR≤13 µm the distributions contained few cases with large ALH values. The choice of SR=13 and MFN=24 represents the best compromise between error-free analyses of sperm samples and the decrease in numbers of sperm tracked that occurs as these set-up values are increased. The results of analyzing the

specimen tape section using these set-up values are shown in figure 7. All the histograms except ALH, LIN and STR approximate to a normal distribution, whilst VAP and ALH both show the presence of outliers. Cases where VAP>VCL or VSL>VAP can be identified as error tracks and removed from the distributions on the basis that it is not possible for a track to have a VAP value greater than the corresponding VCL value, or a VSL value greater than the corresponding VAP value. An ALH>30 was subjectively chosen as the point above which tracks were considered as erroneous, and these too were removed from the distribution. The mean motility values generated using these criteria were not significantly different from those determined previously containing error track data (data not shown).

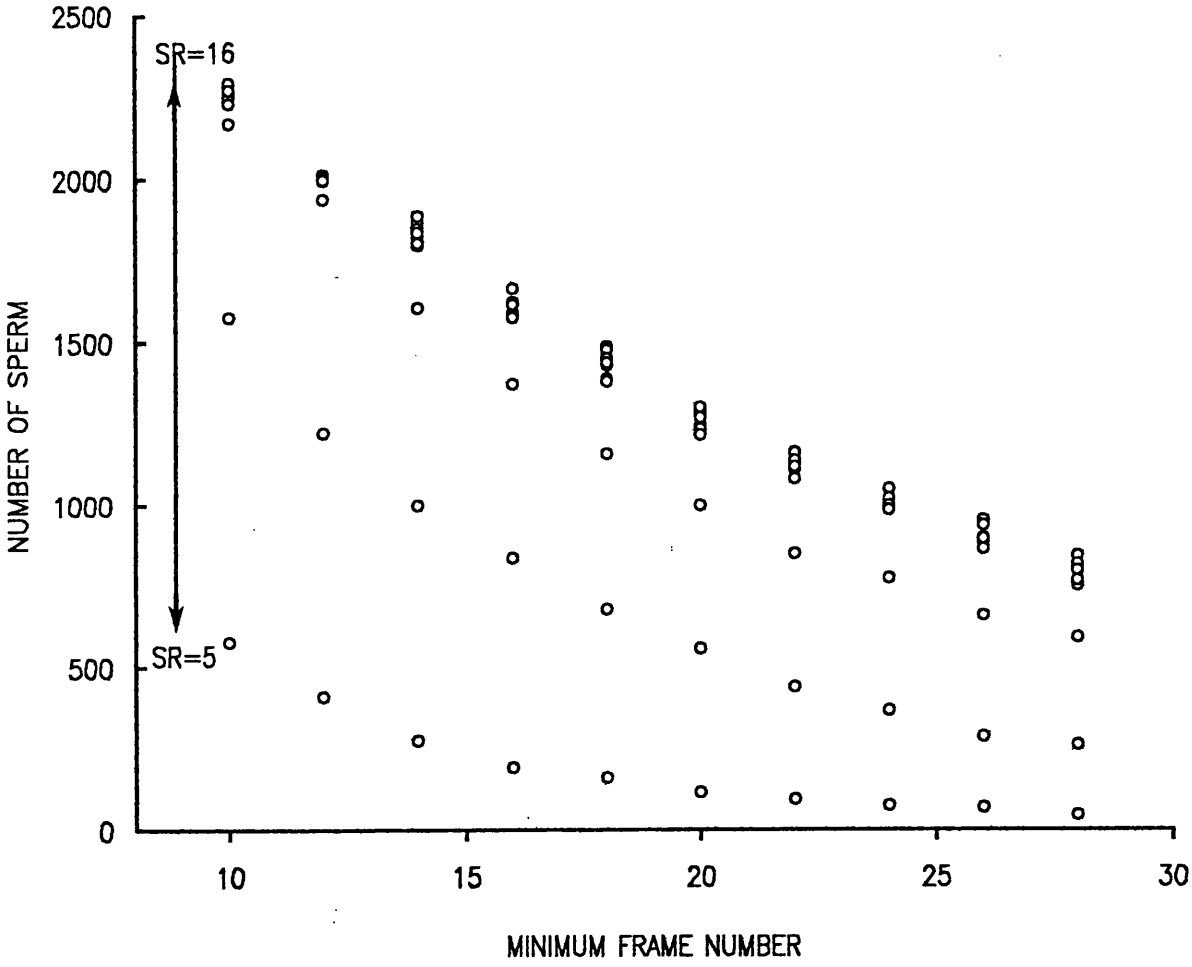


Figure 5. The effect of varying the parameters 'search radius' and 'minimum frame number' on the number of sperm recorded by the HST. A four minute video-recording of sperm movement was analyzed using combinations of SR = 5 to 16 and MFN = 10 to 28 (see text). The number of sperm recorded by the CASA system for each of these SR/MFN combinations, shown above, varied 46 and 2291.

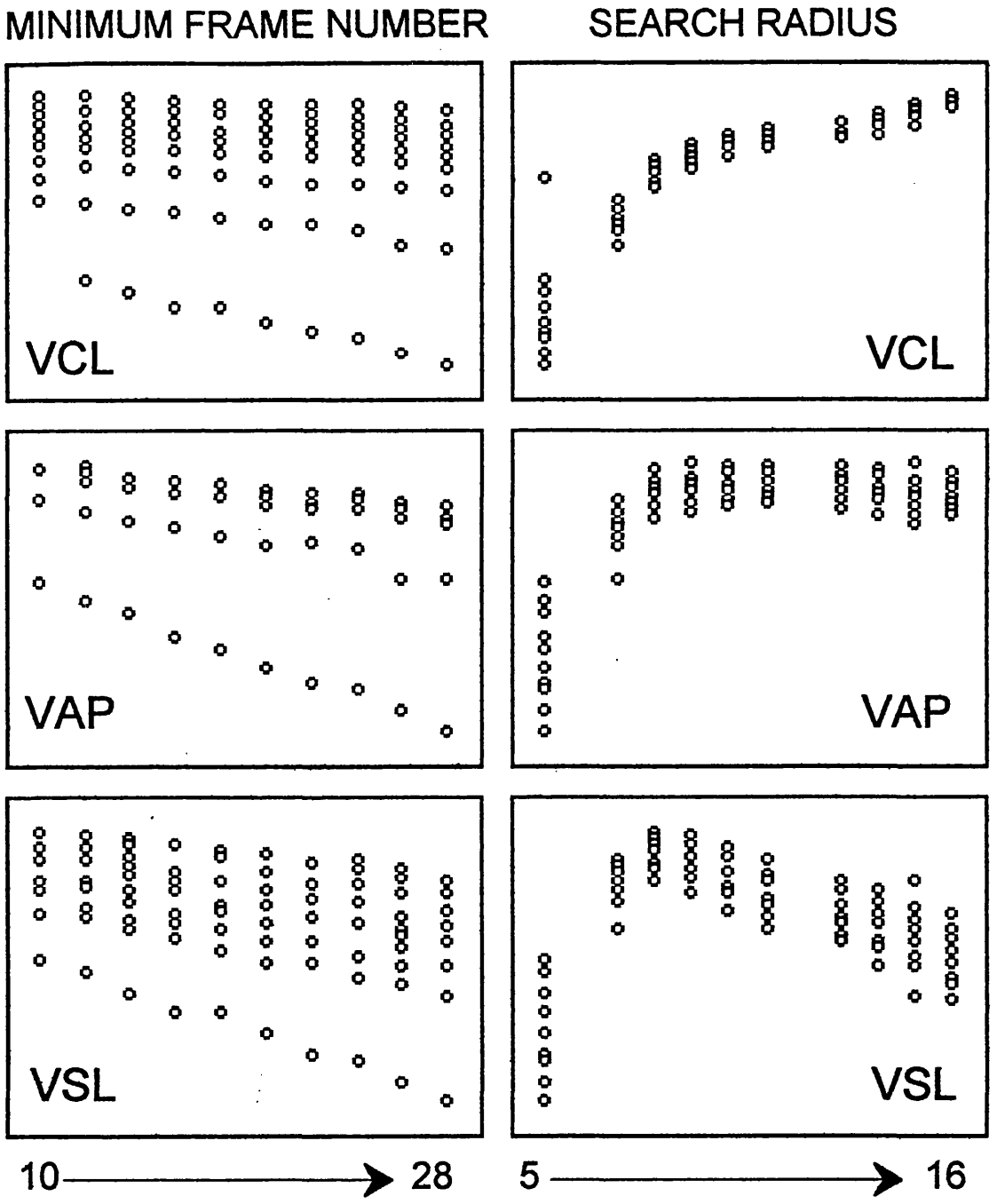


Figure 6a and 6b. The relationship between CASA measurements and the set-up parameters - search radius and minimum frame number. The figure illustrates diagrammatically the effect of increasing MFN and SR on CASA results. Absolute measurements are not shown (to aid clarity). Each point represents the mean CASA measurement for a standard video-recording of sperm, derived using varying combinations of MFN and SR values (see text).

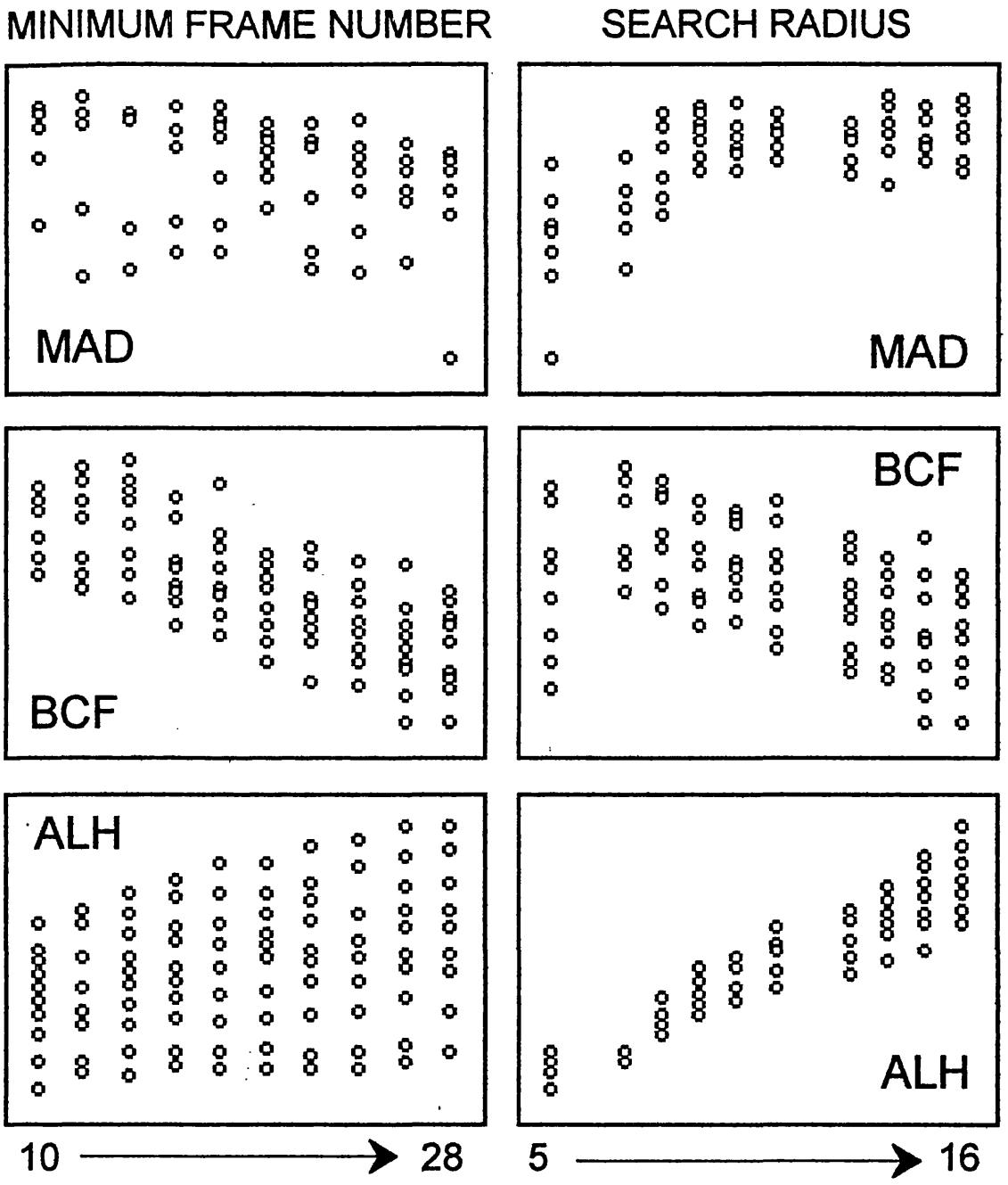

MINIMUM FRAME NUMBER
SEARCH RADIUS
MAD
MAD
BCF
BCF
ALH
ALH
10
28
5
16

Figure 6b.

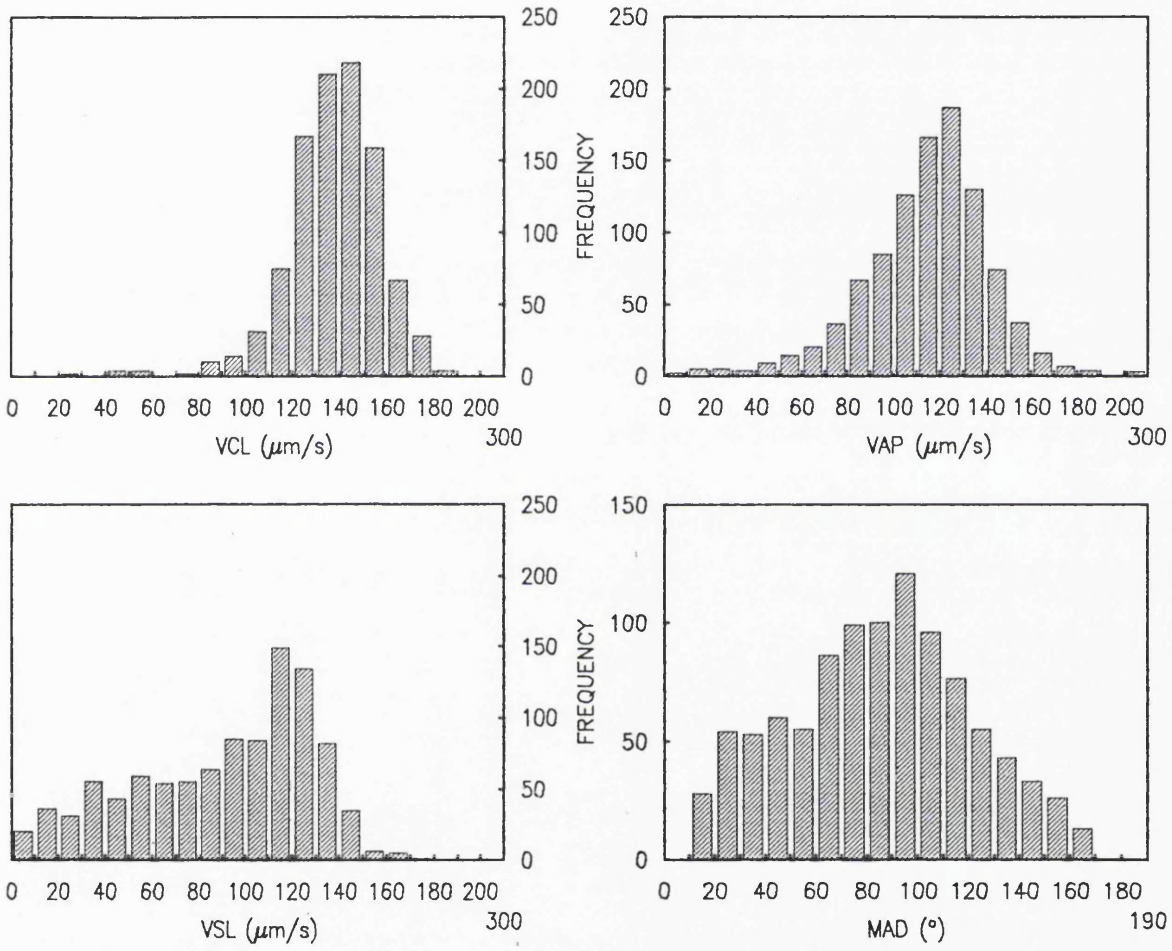


Figure 7a and 7b. CASA measurements of boar sperm obtained using MFN = 24 and SR = 13. A four-minute video-recording of activated sperm was analyzed using MFN = 24 and SR = 13. The distributions obtained for the eight CASA measurements are shown. A total of 998 sperm were recorded.

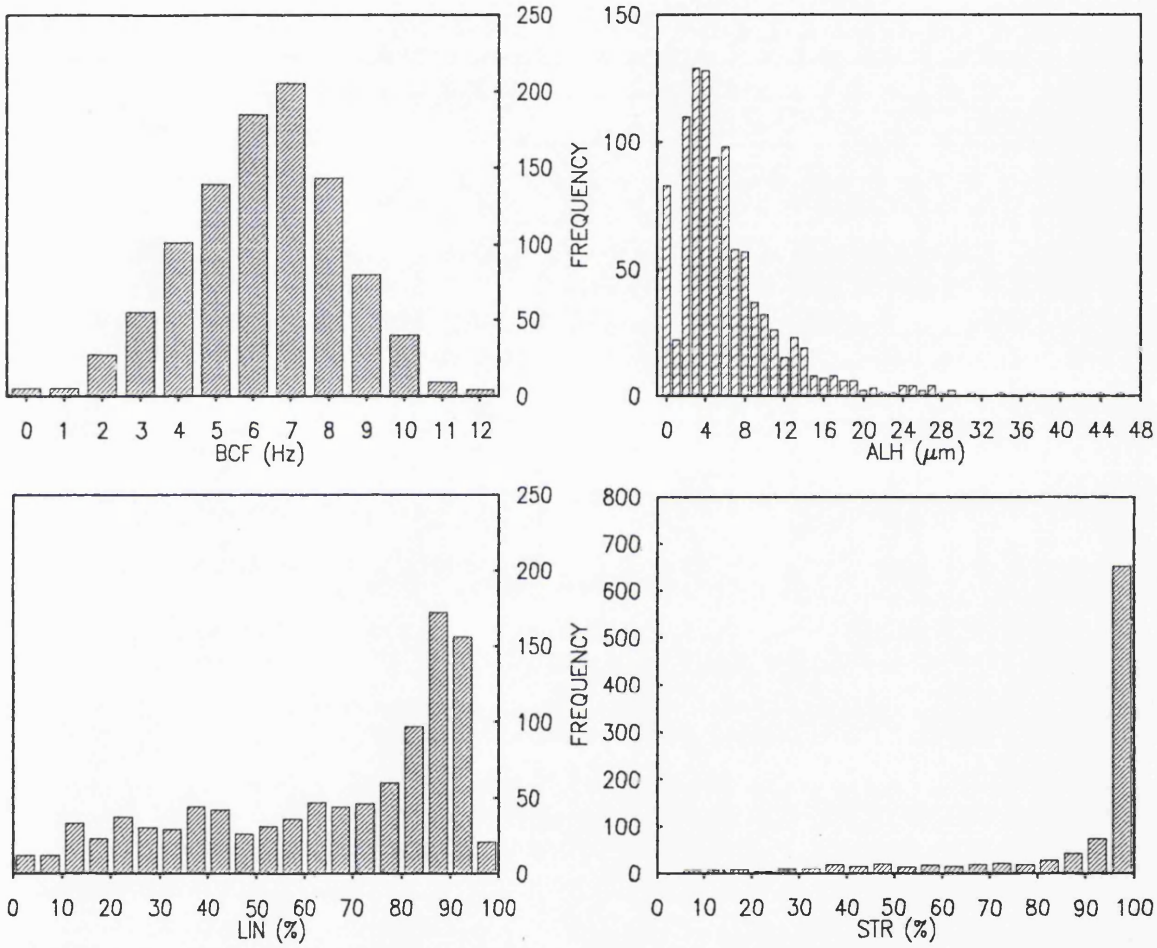


Figure 7b

2.3 DETERMINATION OF THE MINIMUM VELOCITY THRESHOLD

Immotile sperm within a slide preparation will appear to move if caught in currents within the sample chamber. This drift causes the CASA systems to register these cells as slow moving sperm, skewing sample data by the inclusion of low velocity tracks. Defining a threshold and excluding unacceptable tracks will remove this effect, but may eliminate the trajectories of slow but truly motile sperm. The following experiment was an attempt to estimate the effect of drift in a normal sample, and then determine a minimum threshold velocity.

2.3.1 Experimental design

Boar sperm was prepared as described above and the concentration adjusted to 40 x 10^6 sperm/mL. The cells were fixed by 1:1 dilution with 3.5% paraformaldehyde in phosphate buffer, and a slide preparation made in the usual manner. Five replicates were video recorded and analyzed for a total of two minutes each. For each of the replicates the ranges of the motility measurements was evaluated and the inter-replicate variation evaluated.

2.3.2 Results

The number of sperm tracked in the two minute period varied between 0 and 49, dependent on the amount of drift within the sample. The maximum values obtained for each of the parameters were within the normal measurements expected in a sperm population (See table 1).

Approximately 99% of the VAP measurements and 100% of the VSL measurements were below 20 µm/sec, and therefore this was the threshold value adopted in future experiments.

TABLE 1. The determination of minimum velocity thresholds

MEASUREMENT	MEAN ± S.D.	MAXIMUM VALUE
VCL (µm/sec)	19.96 ± 8.01	52
VAP (µm/sec)	7.54 ± 3.87	24
VSL (µm/sec)	5.16 ± 3.17	17
MAD (°)	68.16 ± 18.74	119
BCF (Hz)	4.58 ± 1.51	8
ALH (µm)	2.06 ± 1.19	10
LIN (%)	28.82 ± 15.16	77
STR (%)	81.06 ± 20.65	100

Fixed sperm samples (n=5) were recorded for two minutes at 39°C at a sperm concentration of 20 x 10^6 sperm/mL and the video-tapes then analyzed using the HST. The mean number of sperm recorded per sample was 19 ± 18.9.

2.4 THE EFFECT OF SPERM NUMBERS ON MEAN MOTILITY RESULTS

The precision of a sample mean improves as the number of sperm upon which it is calculated increases. Several reports have suggested that the minimum number of sperm needed to be analyzed for a given tolerance is between 100 and 200 cells for a degree of error of 3% or below (Rath *et al.*, 1988; Furnus *et al.*, 1992). In practice, instances may occur where motile sperm numbers are low and it is not possible to record these numbers of sperm. This could be due to a low sperm concentration, or the result of a motility decline due to specific experimental conditions or prolonged incubations. In the latter instances, it may then become more appropriate to consider this absence of suitable sperm numbers as a treatment effect in itself (Slott and Perreault, 1993).

Many of the experiments described in the following chapters employed a long incubation period during which the proportion of motile sperm declined to very low levels. It was useful to examine the effect of reduced sperm numbers on the mean motility results, and so approximate a cut-off point below which mean motility data should not be used.

2.4.1 Experimental design

The following statistical treatment used data from a single semen sample. Track data was obtained using the set-up parameters derived in the experiments above (data were screened to remove cases where VAP>VCL, VSL>VAP and ALH>30) and then varying numbers of cases within the data set were used to generate the motility means and confidence limits, (n= 500, 200, 100, 50, 25 and 10).

2.4.2 Results

Table 2. The effect of sperm numbers on motility data.

SPERM NUMBER	MEASUREMENT							
	VCL (µm/sec)	VAP (µm/sec)	VSL (µm/sec)	MAD (°)	BCF (Hz)	ALH (µm)	LIN (%)	STR (%)
500	142.1 ± 1.9	117 ± 2.3	94.8 ± 3.4	88.4 ± 3.2	6.5 ± 0.2	5.9 ± 0.4	67.4 ± 2.2	89.4 ± 1.7
200	143.0 ± 3.5	116.2 ± 4.3	96.4 ± 5.5	92.2 ± 5.0	6.6 ± 0.3	6.0 ± 0.6	67.5 ± 3.3	91.5 ± 2.3
100	145.9 ± 4.3	117.6 ± 6.0	100.1 ± 7.4	91.9 ± 7.0	6.4 ± 0.4	6.2 ± 1.0	69.6 ± 4.3	91.2 ± 3.3
50	144.0 ± 7.9	113.0 ± 9.7	94.5 ± 12.2	93.2 ± 9.8	6.4 ± 0.5	5.8 ± 1.1	67.2 ± 6.8	90.9 ± 5.3
25	139.0 ± 12.1	112.7 ± 14.9	100.5 ± 16.8	99.6 ± 15.2	6.6 ± 0.7	4.9 ± 1.1	73.6 ± 8.5	95.3 ± 3.2
10	143.7 ± 20.0	110.4 ± 29.0	93.6 ± 30.8	109.6 ± 20.5	6.8 ± 1.1	5.8 ± 2.0	64.1 ± 17.6	93.3 ± 7.5

The motility results from a single section of video-tape were calculated using decreasing numbers of sperm. Figures are means ± 95% confidence limits.

Table 2. shows the motility results obtained; as expected, as the number of sperm in the analysis was decreased the spread of the 95% confidence limits increased. The means for the motility values were consistent over the range n=500 to n=50, and only varied slightly when n=25. In preliminary trials, total

motile sperm numbers (n) in experimental samples had proved to be small even after short incubations. This meant that a low cut-off point was favoured. The slight loss of confidence in the sample means that arises at n=25 can be offset when analyzing results statistically by ensuring that a high level of significance is used ($P<0.01$). On this basis, a sample size cut-off point of $n \geq 25$ was chosen.

2.5 PRECISION OF THE HST

CASA machines were developed in an attempt to improve the accuracy and precision of semen analysis. Both precision and accuracy are a reflection of the methodologies used with the CASA machine. The precision of a system is relatively easy to determine, usually by repeated analysis of one section of tape. Accuracy is much harder to evaluate; the methods used to determine the accuracy of the HST are described in section 2.6 of this chapter.

2.5.1 Experimental design

One two-minute segment of the tape used in section 2.2.6 of this chapter was analyzed ten times. The video tape was electronically marked and analysis started at this mark. The coefficient of variation for each motility measurement was determined across the ten repeated measurements.

2.5.2 Results

The C.V.'s for the measurements (shown below) were all below 3%, indicating that the precision of the machine is high. Previously, Davis and co-workers, (1992b) performed a similar experiment using the CellTrak system and obtained comparable results (coefficient of variation values $\leq 3\%$ for trajectory data).

Table 3. Evaluation of the precision of the HST

MEASUREMENT	MEAN ± S.E. (n=10)	C.V. (%)
VCL (µm/sec)	138.97 ± 0.14	0.32
VAP (µm/sec)	118.43 ± 0.36	0.95
VSL (µm/sec)	93.99 ± 0.46	1.56
MAD (°)	85.46 ± 0.14	0.52
BCF (Hz)	6.38 ± 0.02	0.89
ALH (µm)	5.96 ± 0.05	2.76
LIN	67.35 ± 0.33	1.53
STR	88.70 ± 0.23	0.83

One section of video-taped sperm was repeatedly analyzed using the HST (n=10). The coefficient of variation for each measurement was calculated using the means of the ten repeated measurements.

2.6 ESTIMATION OF HST ACCURACY USING CALIBRATION TAPES

2.6.1 Introduction

Any attempt to optimise the performance of a CASA machine encounters the problem that one cannot know the true motility values of the sperm samples being studied. Manual analysis - hand-tracing the paths of sperm from video-tapes or time-lapse photographs - has frequently been used to produce standard values for comparison with CASA results (Olds-Clarke *et al.*, 1990; Mack *et al.*, 1988). This process is laborious and therefore the numbers of sperm that can be analyzed is small (typically <50 sperm per sample). In addition, there is no proof that the values produced this way are any more accurate than those established using the computer systems.

Recently, Owen and Katz (1993) investigated the effects of sampling factors using computer-generated simulations of sperm tracks. This report is the only published research using artificially created analogues of sperm motility - such tapes are not available commercially and are difficult to obtain. The calibration tape used in the following experiment was generated from a computer program written by Sense and Vision Electronic Systems Ltd.

2.6.2 Experimental design

Assessment of the tape employed the set-up parameters derived above, but without minimum velocity restrictions or screening of the motility data for ALH or velocity considerations.

The calibration tape showed two bright spots (each similar to the image of a sperm head obtained using negative-high phase-contrast microscopy) moving across the screen at a variety of prescribed speeds. The recreated movements were simple comprising straight paths and sinusoidal waves, but allowed an estimation of the accuracy of the HST. The sections of tape showed the spots moving either horizontally, vertically or diagonally, firstly with straight paths and secondly with sinusoidal paths.

2.6.3 Results

The quality of the calibration tape was poor which caused flickering of the video image. This interfered with the image analysis causing some tracks to fragment and meant that the total number of artificial sperm recorded was slightly larger than the true figure. The speeds of the dots were written on the background of the video image in light lettering. Where text was present, the contrast of the moving object with the background was reduced, hampering the accurate detection of the spots and occasionally causing erroneous measurements.

The results for horizontal and vertical motion showed few differences and have therefore been combined. Table 4. shows the expected values for each of the sections of tape alongside the actual modes and ranges for the measurements. Some of the distributions were bimodal (e.g. Velocities - see figure 8.) as the two dots had differing properties. Typically, the modes contained most of the cases, with only a few tracks creating the ranges shown. The modes were less prominent in the data from the diagonal paths, indicating less accurate tracking of the spots. The results from all sections contained a small number of tracks with every measurement equal to zero, possibly a result of video playback instability. In a practical situation these cases would be removed by the minimum velocity criteria employed; where this restriction is not used it may be necessary to screen for the zero measurements.

The HST set-up calibration procedure involves the positioning of the cursor over two points which are a known distance apart. This process is usually

performed with a microscope graticule marked at 10 μm intervals, and two points are marked 100 μm apart. Therefore accurate calibration also depends on the positioning of the cursor which could slightly affect the absolute values calculated by the computer.

Overall, the accuracy of the HST was good but the results obtained were not all exact. Actual modal values tended to be close to the expected modes - non-velocity measurements of straight trajectories were the most accurate measurements. Velocity measurements were generally underestimated, possibly as a result of the calibration step described above. The distributions of the measurements varied greatly and in some instances (e.g. figure 8.b.) the spread of the points was wide. Future software updates should aim to reduce this variation whilst maintaining or improving the level of accuracy obtained in this experiment.

Table 4. Estimation of the accuracy of the HST using calibration tapes.

Each table shows the expected values for the eight CASA measurements and the actual values obtained. Results for HST analysis of straight (A and B) and sinusoidal (C and D - next page) paths are shown. The frequency histograms for VAP in these tables are shown in figure 8.

SECTION A. - Straight (horizontal and vertical) movement at 50 and 25µm/s.

	EXPECTED	ACTUAL MODE	RANGE
VCL	50, 25	48, 24	42-51, 19-28
VAP	50, 25	48, 24	41-51, 19-30
VSL	50, 25	48, 24	38-48, 15-24
MAD	0, 90, 180	0, 90, 180	0-4, 83-90, 173-180
BCF	0	0	0-8
ALH	0	0	0-6
LIN	100	98	70-100
STR	100	98	70-100

SECTION B. - Straight (diagonal) movement at 50 and 25 µm/s

	EXPECTED	ACTUAL MODE	RANGE
VCL	50, 25	46, 22	37-52, 11-23
VAP	50, 25	48, 24	37-58, 11-34
VSL	50, 25	47, 24	30-48, 14-25
MAD	45, 135	45, 135	41-56, 126-135
BCF	0	3	0-10
ALH	0	0	0-19
LIN	100	100	86-100
STR	100	100	89-100

SECTION C. -Sinusoidal (horizontal and vertical) movement.

	EXPECTED	ACTUAL MODE	RANGE
VCL	*168, *47	152, 43	144-157, 38-49
VAP	50, 25	50, 24	45-57, 19-26
VSL	50, 25	48, 24	40-49, 17-24
MAD	N.C.	56, 66, 89, 113, 123	55-58, 64-69, 75-94 109-114, 121-125
BCF	4, 2	4, 2	0-4
ALH	20, 10	17, 8	15-18, 6-10
LIN	*30, *53	31, 55	25-33, 44-57
STR	100	100	90-100

SECTION D. - Sinusoidal (diagonal) movement.

	EXPECTED	ACTUAL MODE	RANGE
VCL	*168, *47	212/213, 60	205-216, 49-71
VAP	50, 25	52, 24	50-56, 23-26
VSL	50, 25	47, 24	44-49, 20-25
MAD	N.C.	104, 120	99-106, 115-126
BCF	4, 2	4, 2	0-4
ALH	20, 10	25, 12/13	20-26, 11-13,
LIN	*30, *53	22, 40	21-25, 34-43
STR	100	100	85-100

*denotes approximate value calculated by considering the curved sinusoidal track to be a trapezium.

N.C.= not calculated.

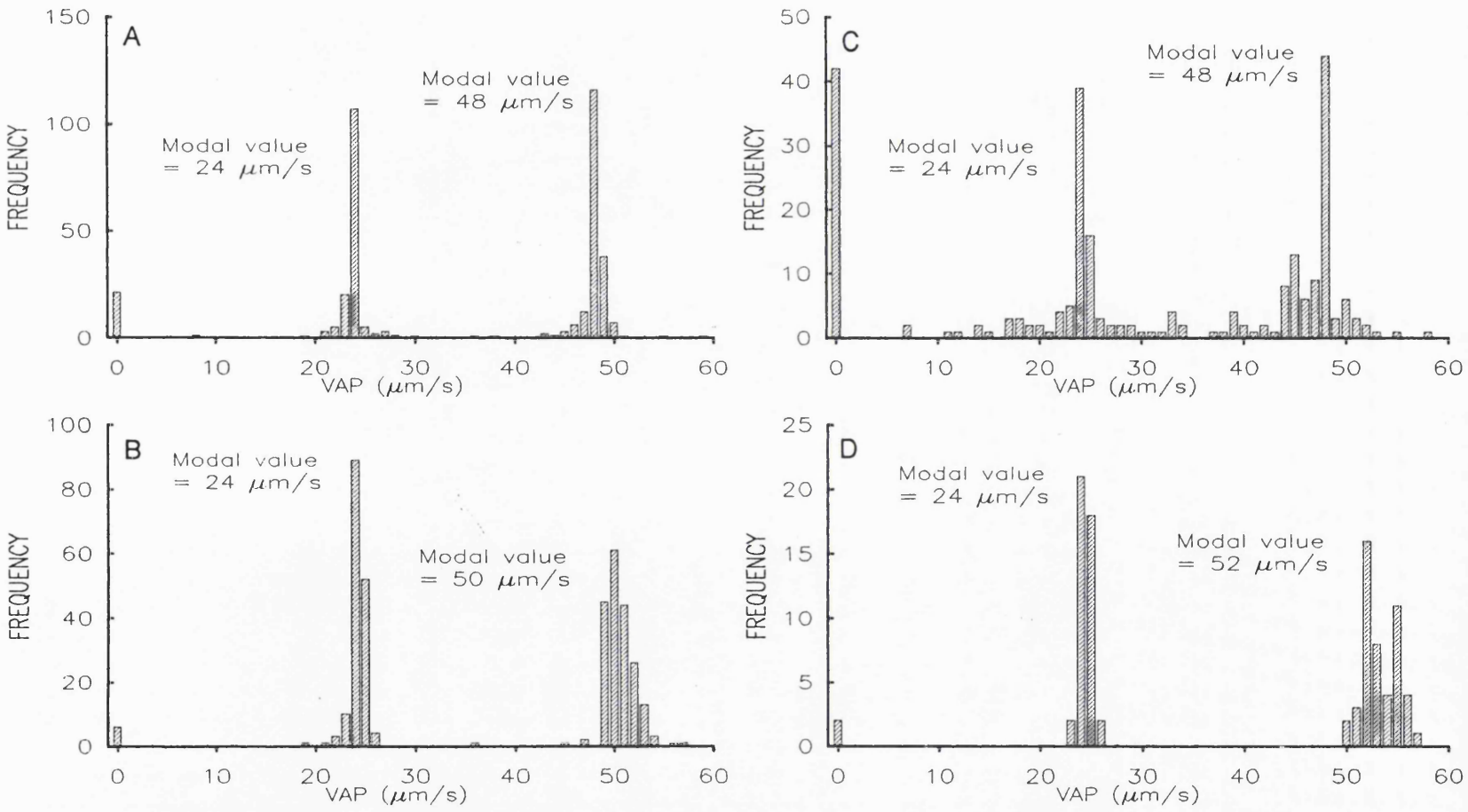


Figure 8. HST analysis of the calibration tape. The four frequency histograms describe the VAP data from Table 4 and illustrate the distributions of the measurements made by the HST. A) Straight (horizontal and vertical) movement at 50 and 25μm/s; B) Straight (diagonal) movement at 50 and 25 μm/s; C) Sinusoidal (horizontal and vertical) movement; D) Sinusoidal (diagonal) movement.

2.7 IMPLICATIONS OF THE HST FIXED FIELD OF VIEW

2.7.1 Introduction

To obtain information about sperm populations, the frame-store CASA systems use short tracks of movement gathered from three or more fields of view. This approach is used to take account of possible spatial heterogeneity within the sample chamber. The method used with the HST is to collect data from one field of view only, and therefore could be criticized for not evaluating truly representative sperm populations. However, as the HST tracking period is so long relative to the speed of a typical sperm, there is a turnover of sperm within the field of view. Spatial heterogeneity is therefore overcome by the movement of sperm throughout the preparation. In addition, movement of the slide to another field often created drift within the sample, requiring a further settling period. It is preferable to minimize the time the slide is kept on the heated stage to prevent evaporation of the media and the associated increase in sample osmolarity.

The fixed field of view does create one theoretical consideration. The probability of a sperm passing through the camera field of view will be related to the speed of the cell. A fast-swimming sperm travels further within a sample preparation than a slow-swimming sperm, and is therefore more likely to pass through the analysis window. Theoretically this should skew the distributions of the velocities towards higher speeds. (In practice, the initial VCL and VAP distributions were normally distributed, the VSL distribution however is skewed to the higher speeds.)

The following experiment assumes that the correlation between 'sperm speed' and 'chance of entering the field of view' is linear. Using this assumption it is possible to obtain a true representation of the sperm population by dividing the number of sperm with a certain speed bracket by that speed. This process was performed for a real sperm populations and the resulting distributions of sperm velocities compared to the original distributions. The influence of this phenomena on the motility information collected was then evaluated.

2.7.2 Experimental design

For this experiment the distribution obtained for the sample tape was used, with the data restrictions applied. The VCL,VAP or VSL data was classified into

10 µm/sec groups (<10 µm/sec, 10-19 µm/sec, etc) and the total number in each group recorded. This figure was then divided by the speed of the group (for convenience the median point was used; i.e. 4.5 µm/sec, 14.5 µm/sec etc). The data from the transformations was plotted as histograms using the 10 µm/sec groupings and compared to the original distributions.

2.7.3 Results

The VCL and VAP distributions retained their normal characteristics after transformation and in both cases the shape of the new distribution closely resembled that of the generic measurement. The VSL distribution which previously had been skewed to the higher speeds, now displayed the opposite skew. Figure 9. shows the histograms for each measurement before and after transformation.

It is probable that the velocity measurements of sperm are correlated to other measurements of motility. If this is the case, then any skew of the velocity data arising from the HST method of analysis will also skew the results for these variables. However, unless the correlation between the motility measurements is very high, it is difficult to adjust the non-velocity data to compensate for this consideration. (The correlations between measurements are discussed in the next chapter, but proved to be insufficiently high to justify the use of the transformation for non-velocity measurements.)

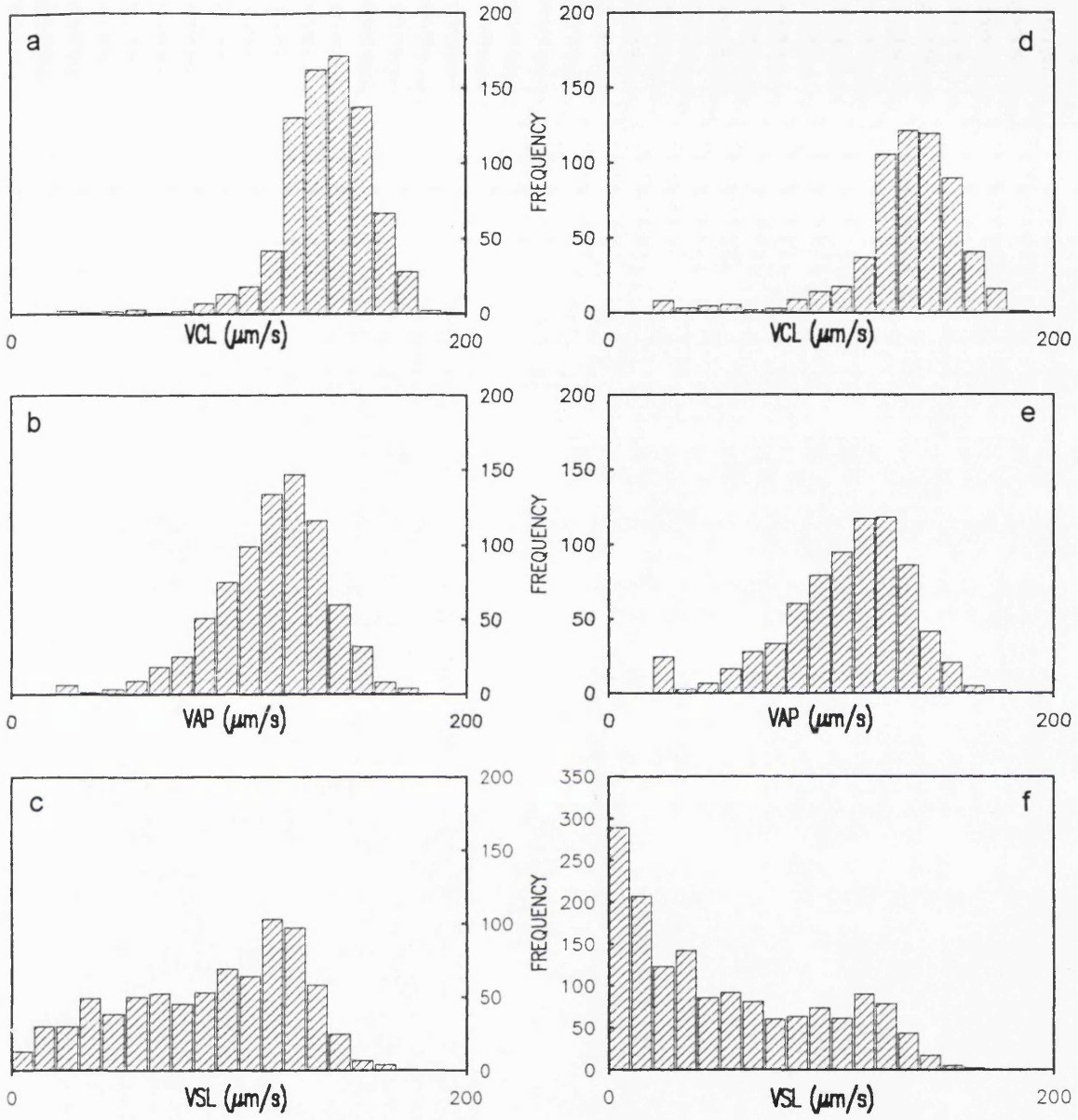


Figure 9. The effect of the HST fixed field of view on velocity measurements. The figure shows frequency histograms of sperm velocity measurements before and after application of the transformation described in section 2.7 (see text). VCL, VAP and VSL measurements were classified into 10 μm/sec groups and the total number in each group divided by the speed of the group. The distributions a-c show the velocity data prior to transformation, distributions d-f show the corresponding data after transformation.

2.8 DISCUSSION

The studies described in this chapter served to establish the experimental conditions for subsequent analysis of boar sperm using the Hobson Sperm Tracker. It is interesting to compare the set-up verifications needed for the Hobson Sperm Tracker with those reported in the literature for other CASA systems. Whilst some questions are similar, such as those regarding accuracy and precision, other considerations are unique.

For CASA systems using frame-store technology, there has necessarily been a trade off between framing-rate and duration of analysis. The benefits of increased accuracy gained from a high sampling frequency are countered by a shortened period of analysis. The HST uses real-time image analysis and therefore the period of analysis is not constrained. However, the framing rate is fixed at 25 Hz. This situation will be remedied when HST systems working at 50 frames/sec (and higher) become available.

Recently, Owen and Katz (1993) investigated the effects of sampling factors using computer-generated simulations of sperm tracks. These had known motility characteristics and were created to mimic the paths of actual sperm. Their research demonstrated that interactions between sampling frequency (frames/sec) and the beat frequency of the sperm can impair motion assessment. Where the video-framing rate is equal to, or a multiple of, the beat frequency of a sperm, the resulting apparent trajectory is a straight line, and aberrant motility data is obtained (e.g. ALH and BCF equal to 0). Human sperm have a beat frequency range of 5-30 Hz, and most CASA systems use comparable framing rates (European-25 Hz, U.S.A.-30 Hz), therefore it is possible that this situation may arise within an analysis. This factor cannot explain the 'error tracks' observed in the present experiments as these also had velocity values of zero. Using a framing rate equal to the beat frequency of the sperm should not prevent evaluation of the sperm displacement, though the accuracy of this measurement may be impaired.

Using the artificial sperm tracks, this group also demonstrated that the results obtained for several characteristics were, to a varying degree, affected by a process known as 'aliasing' (Owen and Katz, 1993; Davis and Katz, 1993a). Aliasing occurs when signal (beat) frequencies greater than half the sampling frequency cause the perceived signal frequency to be incorrectly moved into the

range below the sampling frequency (see figure 10 for further explanation). Previously it had been suggested that unreliable data would be derived when the sampling frequency was less than double the signal frequency (Mack *et al.*, 1988; Boyers *et al.*, 1989); however, Owen and Katz (1993) suggest that a sampling rate of 10 times the highest inherent frequency should be used to overcome these effects.

Systems that use framing rates of 60 Hz are available and research using 200 Hz framing-rates has been published (Mortimer *et al.*, 1988a; Davis *et al.*, 1992a). These systems will fulfil the suggested criteria for only the lowest beat frequencies. Increasing the duration for which a sperm is tracked may alleviate some of these problems by including more points representative of the sperm beat, albeit in separate cycles (Davis and Katz, 1993a). This method of averaging the beat cycle would be more suitable for trajectories where the cell motion is regularly repeated (non-hyperactivated motility).

The novel method of sperm tracking used by the HST favours the inclusion of rapid sperm within the data set obtained for a sample. This effect can be compensated by the use of a crude method of transformation. The evidence from section 2.7. suggests that VSL data collected for real sperm populations is skewed towards higher velocities due to this effect. A decision was made not to incorporate this step into the HST protocols as the transformation process is lengthy and cannot be used for the non-velocity measurements.

Initial practical problems included that of the washed boar sperm adhering to glass surfaces. This was overcome by coating slides in 0.4% agar and ensuring that all implements in contact with the sperm (e.g. test-tubes, Pasteur pipettes) were made of plastic. A more persistent problem was the formation of aggregations of sperm during incubation. Two types of groupings could be identified; in the first the sperm were orientated randomly, apparently bound to dead cells and debris. In the second, sperm were specifically agglutinated in a head to head fashion, with binding occurring over the acrosomal region. This latter type of agglutination is discussed further in the next chapter. The tendency of boar sperm to bind to debris has previously been reported, (Suarez *et al.*, 1991b). It was found that the onset of this process could be delayed by washing the sperm using a Percoll gradient, and thereby removing most of the aggregation points.

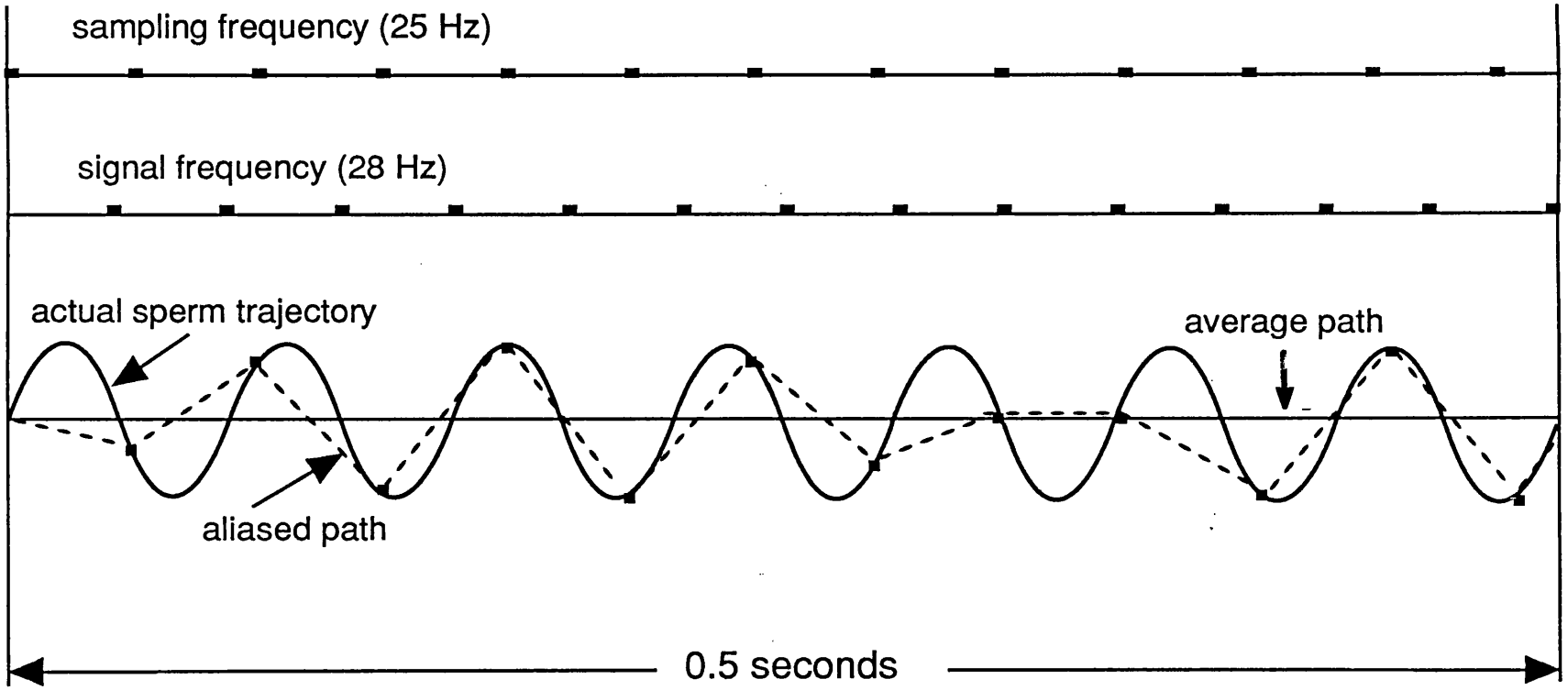


Figure 10. The process of aliasing. The above diagram shows how sampling frequency affects the measurement of a signal frequency. The actual sperm trajectory results in a signal frequency (BCF) of 28 Hz, however, a sampling frequency (framing rate) of 25 Hz results in an aliased value of 22 Hz. To avoid aliasing, sampling frequency must be ≥ twice the signal frequency (Nyquist sampling theorem).

Boar sperm samples prepared in this way were used to investigate the interplay between minimum frame number and search radius. The relationships that emerged did not always comply with expectations. In these instances it was useful to compare the distributions for different SR/MFN combinations to identify the sperm populations being lost or gained. Inclusion of the marmoset data enabled comparisons of the erratic tracks of boar sperm with a very smooth, regular pattern of movement, the latter producing more predictable results. Analysis of the two types of sperm revealed that the HST was fragmenting the tracks of the boar sperm, although encountering no difficulties with the marmoset sperm. Based on this information software updates will be directed towards overcoming this problem. Using sperm with different properties is a useful method of investigating the weaknesses of any system. Toxicology studies using the Hamilton-Thorn HTM analyzer found this system unable to discern accurately the falciform shape of rat sperm, resulting in the generation of rodent specific software (Slott *et al.*, 1993).

The optimal set-up parameters appeared to be a search radius of 13 µm and a minimum frame number of 24. The fact that these values are slightly higher than previously determined (Vantman *et al.*, 1988; Mack *et al.*, 1988) may reflect the erratic motility of boar sperm, or the type of system used. Minimum sperm number was defined as 25, which is a comparatively low value for this threshold (Furnus *et al.*, 1992). Sperm motility declined rapidly in preliminary work and therefore a low figure was chosen for minimum sperm number (25) although this meant an increase in the allowable error. The results from statistical analyses of future data must therefore be judged using stringent significance levels.

Screening of the motility data generated using these set-up values indicated that machine error was creating a sub-set of tracks with impossible values (VAP>VCL, VSL>VAP). Selection criteria can be applied to the data during statistical analysis to remove these tracks. Further screening enabled improbable ALH data (ALH>30 µm) to be eliminated and a minimum velocity threshold (VAP>20 µm/sec) was imposed to counter the effects of sample drift. These steps were included to improve the reliability of the data obtained.

The calibration tape provided a quick and technically simple method of testing system accuracy. The results from this section indicate that the HST is not completely accurate, but produces motility estimations close to the real values;

accuracy was comparable to other CASA systems previously tested (Mack *et al.*, 1988; Slott *et al.*, 1993). The moving spots in the recording only mimicked a few of the possible sperm movements and it is likely that the accuracy of the HST is poorer outside the ranges covered. A more comprehensive set of artificial tracks would improve the calibration. The precision of the machine was tested using real sperm images and proved to be as good (Davis and Katz., 1992) or better (Davis *et al.*, 1992; Slott *et al.*, 1993) than previously tested CASA instruments. Slight inaccuracies will arise due to the onset of the analysis being mistimed. The tape was marked electronically to overcome this, but human error may still have influenced the results.

2.9 SUMMARY

The aim of the research reported in this chapter was to derive set-up values for the HST in a methodical manner. Evidence of the effects of set-up parameters on motility data was obtained empirically, and used to identify appropriate threshold values. The result is an overview of the capabilities of the HST and a framework of set-up values for the analysis of boar sperm. The next chapter describes how the HST has been used to evaluate boar sperm motility and the relationship of sperm movement characteristics to fertility.

CHAPTER 3 - CASA OF BOAR SPERMATOZOA USING THE HOBSON SPERM TRACKER

The research described in this chapter was performed in order to obtain basic data on the motility of boar sperm and its relationship to ejaculate fertility. Assessments were made using the Hobson Sperm Tracker employing the parameters derived in the previous chapter. Initial experiments used a small group of ejaculates to derive a sensible basis for future sampling regimes. The motility characteristics of ejaculates on collection and after storage and transportation were compared. Subsequently motility data from bottled semen was correlated with fertility data from artificial inseminations performed using liquid stored semen from the same ejaculate. Alternative approaches to examining sperm movement characteristics were then explored using the motility data collected.

3.0 INTRODUCTION

CASA technology has become widespread only relatively recently and the different systems are at present not equivalent (Jasko *et al.*, 1990; Davis and Katz, 1992; Yeung and Nieschlag, 1993; Holt *et al.*, 1994). This fact, combined with the sensitivity of sperm motility to media composition and temperature, means that sperm velocity and pattern of movement are highly influenced by the experimental conditions employed (Davis and Katz., 1993). A small amount of CASA information is available from previously published studies of boar sperm motion (see Chapter 1.). The results obtained in this chapter illustrate sperm assessment with one particular system and have been compared as far as possible to these reports of boar sperm motility.

The experiments described below were performed to provide CASA measurements of boar sperm movement patterns in a capacitating medium, and to establish how these changed during incubation. A prolonged assessment period was used based on the hypothesis that measurements of motility changes over several hours would be more informative than single CASA measurements. Initial experiments used a small number of ejaculates to investigate how motility alters over time and to establish realistic sampling patterns for future use.

Most of the experimental work in this thesis was performed using the bottles of extended semen available from the SDS at Thorpe Willoughby. Semen was used for motility assessment approximately twenty-four hours after ejaculation. As liquid storage is known to have deleterious effects on sperm function (Clarke and

Johnson, 1987; Weitze, 1990), experiments were performed to compare the motility data collected for extended sperm with that obtained from the fresh ejaculate.

Subsequent experiments correlated the results of motility assessments with fertility data from the Pyramid herds of the Meat and Livestock Commission, (later taken over by JSR Healthbred Ltd). Sperm samples from split ejaculates were analyzed at the Institute of Zoology and bottles of semen from the same ejaculates simultaneously used for AI within the company herds. (Additional AI results were obtained for the bottles of semen from clients of the SDS). The sperm movement characteristics of the semen sample were then compared to two measures of the fertility of the ejaculate. These were: (1) the success/failure rate of inseminations performed with extended semen from the ejaculate and (2) the average litter size resulting from the inseminations.

Parallel experiments examined whether analyzing sperm movement in high relative viscosity media improved the correlation of sperm motility with ejaculate fertility. Spermatozoa were assessed in media containing the high molecular weight hyaluronic acid polymer - 'Sperm Select®' (Pharmacia, Uppsala, Sweden). This substance was developed initially as a substitute for the aqueous humour of the eye and is now also used as a cervical mucus substitute for the collection and assessment of sperm samples for IVF (Wikland *et al.*, 1987; Mortimer, 1990). Viscous secretions, such as cervical mucus, may modulate sperm transport in vivo by impeding morphologically abnormal spermatozoa (Hanson and Overstreet, 1981) whilst promoting the movement of hyperactivated sperm (Suarez *et al.*, 1991) and sperm movement through cervical mucus is commonly used as an assessment of functional competence (WHO, 1987). To determine if sperm analyzed in a more viscous medium produced more informative CASA results, sperm samples were evaluated in low relative viscosity media and the same media with Sperm Select® added. The motility information from this section was then compared to fertility information from the AI trials.

Previously, several studies have concluded that spontaneous and induced acrosome reactions are useful indicators of fertility (Marshburn *et al.*, 1991; Cummins *et al.*, 1991; Fénichel *et al.*, 1991; Pilikian *et al.*, 1992). Samples from more fertile individuals contained fewer spontaneously acrosome reacted cells but a greater proportion of the sperm could be induced to acrosome react (using techniques such as addition of calcium ionophore or progesterone and follicular

fluid). This chapter includes experiments performed to test this hypothesis, using solubilized zona pellucida proteins or calcium ionophore A23187 to induce exocytosis (Berger *et al.*, 1989a; Töpfer-Petersen *et al.*, 1988).

The semen samples used in this study were from a highly selected population of boars and it is possible that ejaculate variation no longer exists between the different individuals. The results from these experiments were used to investigate whether motility and acrosomal changes are dependent on the boar from which the ejaculate is collected. Animal-dependent sperm characteristics, if related to fertility, would enable boar-quality to be established rapidly. This would facilitate the early removal of poorly fertile animals from the stud and improve the efficiency of the SDS companies.

The SDS boars kept at Thorpe Willoughby are all known to be highly fertile. Animals that do not match certain fertility criteria (78% fertilization rate, average litter size $\geq$ 10.8) are removed from the stud, therefore the range of fertility being examined is small. This is a problem common to fertility trials performed in a commercial environment as, for obvious economic reasons, breeders want to remove males with suspected low fertility. To maximise the range of fertilities being examined motility information was collected from five boars classified as sub-fertile by the SDS stud managers. These results were compared to the data obtained for ejaculates from a random subset of animals in the Thorpe Willoughby stud.

Several studies have examined the extensive heterogeneity of sperm populations (Bonet and Briz, 1991; Burkman, 1991; Gadella *et al.*, 1991). Only a small proportion of the sperm inseminated into the female tract (whether naturally or during AI) will ultimately reach and fertilize the oocyte. Therefore when attempting to identify markers of sperm fertilizing ability, studying the properties of sperm sub-populations may prove to be more informative than considering mean characteristics of sperm samples (Amann and Hammerstedt, 1993). One method of grouping together entities with similar characteristics is to use the statistical technique 'cluster analysis' (for an introduction to cluster analysis see Afifi and Clark, 1990). The partitioning of sperm samples by cluster analysis of the motility data is described in section 3.7. Artificially created sperm populations were used to verify that discrete sub-populations of sperm could be identified in this manner should they exist. Subsequently sperm motility data collected in the previous

experiments was analyzed using this technique. The characteristics of the resulting sperm sub-groups were evaluated and the data used to identify hyperactivated sperm without recourse to subjective methods.

The HST criteria developed in chapter 2 were used throughout this research. The media predominantly used is the high-calcium TRIS-buffered media (abbreviated to TBM in this thesis) developed by Berger for the capacitation of boar sperm (Clarke and Johnson, 1987; Berger and Horton, 1988; Berger and Parker, 1989). Considerable effort has been invested in the development of protocols for the *in vitro* capacitation of sperm from species such as the mouse or hamster. This has resulted in a variety of methodologies for the promotion of hyperactivation or IVF success in these species (Fraser, 1992). Establishing such protocols for boar sperm has proved difficult as until recently few reports of porcine IVF existed (Cheng et al., 1986; Rath, 1990). The high calcium TBM media was originally designed to capacitate large proportions of the sperm population and was chosen in order to improve the probability of detecting motility changes associated with capacitation.

3.1 MATERIALS AND METHODS

3.1.1 Collection of ejaculates

Fresh ejaculates were collected at the Thorpe Willoughby JSR Healthbred Pig Breeding Centre, Selby, Yorkshire from boars trained to mount dummy sows. The sperm rich fraction was collected into a pre-warmed plastic container and maintained in a water bath at 35°C whilst sperm assessments were made. Sperm concentration was analyzed using a colorimeter and sperm motility estimated subjectively; both assessments were performed less than 10 minutes after collection of the ejaculate.

The raw semen was then cooled to room temperature and the semen mixed with BTS diluent also at room temperature. The volume of BTS added to the semen was dependent on the initial assessments of sperm motility and concentration. 75 mL bottles were filled with semen in extender; sperm doses within the 75 mL bottles varied between 2×10^9 and 4×10^9 sperm. Sperm dose was held at 1.5×10^9 for the fertility trials (see section 3.4).

Bottles of the split ejaculates were packaged in polystyrene boxes to

maintain semen temperature >16°C (below this temperature boar sperm are known to suffer cold-shock - Pursel *et al.*, 1972) and transported to the SDS customers via the postal service.

3.1.2 Sperm preparation prior to CASA

Bottles of boar sperm extended in BTS diluent were obtained from the Thorpe Willoughby Semen Delivery Service, Selby. Bottles were delivered by post, arriving the morning after collection. This is the delivery method used for farmers receiving the semen for AI, the majority of which is performed on the day the semen is received. Sperm cells were washed from the diluent prior to CASA using a Percoll gradient (see section 2.1.2). The washed sperm samples were resuspended to a concentration of 20 x 10^6 sperm/mL in TBM media and allowed to equilibrate for 10 minutes at 39°C, in an atmosphere of 5% CO_2, 100% humidity.

3.1.3 CASA assessment

Individual sperm samples were removed from the incubator and resuspended by gentle shaking. A 25 µL aliquot was removed (using pre-warmed pipette tips), mounted on pre-warmed agar-coated slides and covered with an agar-coated coverslip (See section 2.1.2). The centre of the slide was positioned under the microscope objective and the image focused. The slide preparation was allowed to settle for approximately 30 seconds and then video-recorded for 2 minutes for analysis at a later time.

For each time point the individual sperm track data was collected and used to generate mean VCL, VAP, VSL, MAD, BCF, ALH, LIN and STR measurements, and the total number of sperm in the analysis was recorded.

3.1.4 Assessment of acrosomal status

This was performed using the lectin Pisum sativum conjugated to a fluorescent label (PSA-FITC) (Cross *et al.*, 1986). A 20µL aliquot of the sample was smeared onto a pre-warmed slide, allowed to dry and then fixed and permeabilized in absolute alcohol for 10 minutes. After fixation the smears were covered with a 20µL drop of 100µg/mL PSA-FITC (Sigma Chemical Company, Poole, U.K.) in isotonic saline. Slides were placed in moist boxes in the dark at room temperature for 10 minutes. After this period, the slides were washed by

gentle agitation in de-ionized water, blotted dry and mounted under a coverslip in a glycerol based compound which inhibits fading of fluorescent dyes (Citifluor; City University, London). Slides were stored in the dark until examination; sperm counts were always performed within three days of the slide being made to minimize degeneration of the sample or fading of the fluorescence. The green fluorescence was excited using a Nikon Diaphot-TMD microscope (Nikon, Tokyo, Japan) equipped with epifluorescent optics and samples were observed at x 1000 magnification. Duplicate smears were made for each sample; these were counted 'blind' and at least 100 sperm from each slide were assessed.

The types of acrosomal staining patterns observed presented a continuum (see figure 11) from the solid fluorescence over the apical ridge and acrosome, denoting intact cells (figure a), to a complete absence of fluorescence where exocytosis had occurred (figure d). Sperm with faint or patchy fluorescence over the acrosome (figure 11, b and c) were classified as acrosome-reacted. This classification resulted in the higher numbers of acrosome-reacted sperm reported here in comparison with previous studies (Berger *et al.*, 1989a). The post acrosomal region of all cells remained unstained, however, labelling over the equatorial segment varied between spermatozoa. The majority of cells (both acrosome-reacted and acrosome-intact) had faint fluorescence over the equatorial segment. A sub-population (approximately 20% of sperm) showed more intense labelling of this region (see figure 11, d).

3.1.5 Sperm viability

Sperm viability was assessed using the eosin/nigrosin stain (W.H.O. 1987). 50µL of sample were mixed with 50µL of stain in an Eppendorf tube and allowed to stand for 30 seconds. One drop was placed on a pre-warmed glass slide and a smear prepared. The air-dried smear was stored in a desiccator until evaluation to delay the penetration of viable sperm by the eosin. In all cases slides were examined within 24 hours of preparation. Slides were counted blind and at least 100 sperm were counted for each slide.

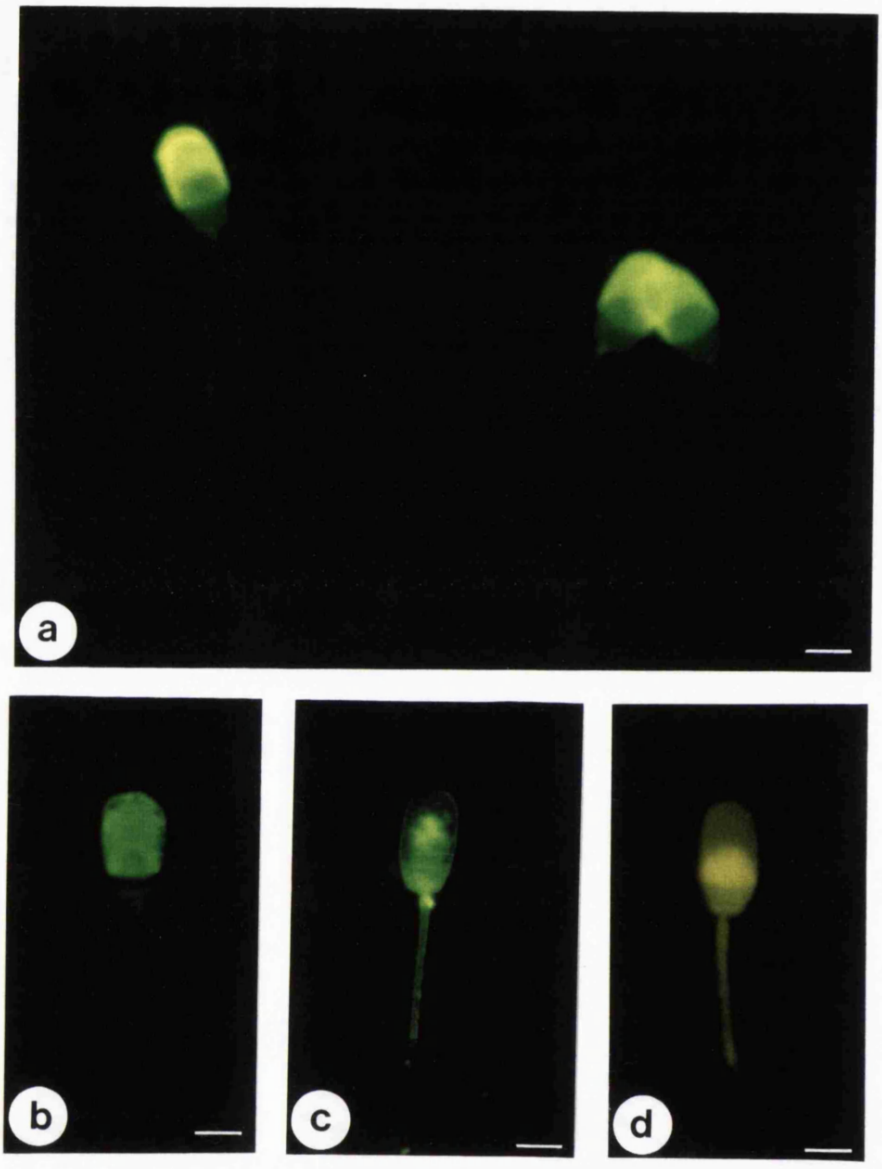


Figure 11. PSA-FITC labelling of the sperm acrosome. Washed semen samples were smeared onto a pre-warmed slide, allowed to dry and then fixed and permeabilized in absolute alcohol for 10 minutes. After addition of the PSA-FITC in isotonic saline, slides were placed in moist boxes in the dark at room temperature for 10 minutes. The slides were washed by gentle agitation in de-ionized water, blotted dry and mounted under a coverslip. The green fluorescence was excited using a Nikon Diaphot-TMD microscope equipped with epifluorescent optics (see text). Bars represent 3 μm.

Figure (a) - solid fluorescence denoting acrosome intact cells

(b, c) sperm with patchy fluorescence over the acrosome

(d) - absence of fluorescence where exocytosis has occurred

3.1.6 Ultrastructural characterization of sperm agglutinations

Samples were fixed by the addition of an equal volume of 4% glutaraldehyde and 4% sucrose in 100 mM cacodylate buffer (pH 7.3) for 2 hours. After fixation, the cells were rinsed in cacodylate buffer, centrifuged and the pellet osmicated in 1% osmium tetroxide overnight at 4°C. After washing to remove excess osmium, dehydration was performed with ascending grades of ethanol, transferred to propylene oxide then the pellet embedded in araldite. Sections, prepared using a Reichert ultramicrotome, were mounted on copper grids and stained in uranyl acetate and lead citrate (Reynolds., 1963) and viewed using a JEM-1200EX electron microscope (JEOL, Tokyo, Japan).

3.1.7 Fertility trials

For the period of this experiment, sperm dose in the 75 mL bottles was standardized to 1.5 x 10^9 sperm (2 x 10^7 sperm/mL). Fresh ejaculates were collected on Monday mornings and processed as described in section 3.1.1. Bottles of semen were sent to Pyramid herds at four locations in the U.K. - Pigwood (Northwich); Baveney (Kidderminster); Eastburn (Driffield) and Newbottle (Banbury). These were received through the post on Tuesday morning and AI conducted on the same day. Inseminations were performed at the onset of oestrus and again approximately 12 hours later; extended semen from the same ejaculate was used at both times. Each of the two inseminations used one 75 mL bottle of semen.

The details of the inseminations in the Pyramid herds (including date, ejaculate used, success/failure to achieve pregnancy and litter size) were collated at the JSR Healthbred central office. Bottles sent to SDS customers were accompanied by a printed card requesting details of the insemination (including date, number of bottles used, pure or mixed semen, success/failure of insemination and litter size). Customers were asked to return cards to the Thorpe Willoughby Centre. Fertility information from both sources became available approximately five months after the AI was performed.

3.1.8 Calcium ionophore A23187

Stock solutions of 500µM calcium ionophore (free acid; Sigma Chemical Company, Poole, U.K.) in dimethylsulphoxide (DMSO) were diluted to a working

concentration of 20μM with pre-warmed TBM media. This solution was added to 1 mL of the sperm samples to a final ionophore concentration of 1μM and a sperm concentration of 5 x 10^6 sperm/mL (Shams-Borhan and Harrison, 1981; Töpfer-Petersen *et al.*, 1988). The samples were immediately returned to the incubator for a further 15 minutes after which exocytosis was assessed using PSA-FITC.

3.1.9 Solubilized zona pellucida proteins

The isolation and solubilization of porcine zona pellucidae is described in chapter 4. Pre-warmed TBM media was added to lyophilized solubilized zona pellucidae (SZP) preparations to produce a protein concentration of 250 μg/mL. This SZP preparation was added to sperm samples to a final sperm concentration of 10 x 10^6 sperm/mL and a SZP protein concentration of 125μg/mL, (a concentration known to increase significantly the proportion of acrosome reacted sperm (see section 4.3.). After a one hour co-incubation the proportion of acrosome-reacted sperm was assessed using the PSA technique (see section 3.1.4).

3.1.10 Statistical analysis

Only the results from samples where greater than 25 motile sperm were recorded were used in any statistical analyses (see section 2.4.). Nevertheless, some of the measurement means are based on a small number of sperm and the associated confidence limits around the mean are relatively wide. With this in mind, results which are significant with $0.01<P<0.05$ are considered with caution.

Analyses were made using the CSS:STATISTICA software package (StatSoft Inc., Tulsa, U.S.A.). Data for the percentage of intact acrosomes and sperm viability were all subjected to arc sine transformation before use in the analyses of variance (ANOVA).

In the previous chapter, analysis of the specimen tape revealed that the VCL, VAP, VSL, MAD and BCF variables produced normal distributions whilst ALH, LIN, STR did not. However, when this observation was checked using the results obtained from this section, the distributions produced for each motility variable were not consistently normal. Therefore the non-parametric Spearman Rank Correlation was employed for the analysis of the variables VCL, VAP, VSL, MAD, BCF, ALH, LIN and STR when the individual track data was used. The

distributions of the sample means were normal and in these instances parametric ANOVA was used.

Normality of distributions was assessed using the Kolmogorov-Smirnov test and the Chi-square of the observed and expected frequencies.

The non-parametric Kruskal-Wallis ANOVA was used to compare two sets of data when the number of ejaculates available for comparison was small. Using the Kruskal-Wallis test, each time point has to be considered separately therefore it was not possible to calculate the interaction of time with other measurements.

3.2 A PRELIMINARY STUDY OF BOAR SPERM MOTILITY DURING INCUBATION IN A CAPACITATING MEDIA.

The objective of this experiment was to establish how incubation in the TBM media affected boar sperm samples. CASA measurements and the proportions of acrosome reacted and viable cells were examined over a six hour period. The information collected was used to determine future sampling protocols for experiments involving larger numbers of semen samples. The track data from individual sperm was subsequently used to evaluate the correlation between motility variables. A strong correlation between two variables would mean that one of the pair could be excluded from statistical tests without significantly reducing the power of the analysis. (i.e. it would be possible to identify redundancy). Conversely, highly correlated variables could distort the significance of statistical tests.

Preliminary observations indicated that boar sperm formed agglutinations during incubation in the TBM media. Transmission electron microscopy of the sperm samples was performed to investigate

(a) whether the agglutination reaction was a physiologically relevant process or simply an artefact and

(b) the condition of the acrosome of sperm bound in agglutinations, specifically whether cells were able to acrosome react whilst bound to other sperm.

3.2.1 Experimental design

Washed extended semen samples (n=8 boars, one sample from each boar) were analyzed after 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360

minutes incubation in the TBM media. At each of these times sperm viability, the proportion of acrosome reacted sperm and sperm movement were evaluated. For each sample, means of the CASA measurements were calculated from the individual sperm tracks recorded by the HST.

The CASA results from the first analysis point (immediately after sample preparation) were used to correlate the motility variables with each other (e.g VCL with VAP). The total number of sperm in each data set was adjusted to n=100 (one sample contained only 87) to produce homogeneous variance. Correlations between pairs of measurements were evaluated for each of the eight samples individually to enable between-sample comparisons. Correlations between variables were judged to be significant if $P<0.01$.

For assessment of the sperm agglutinations using transmission electron microscopy, two 10 µL and one 1 mL aliquots were removed from the washed, extended sperm samples after 60 and 300 minutes incubation in the TBM media. The 10 µL aliquots were immediately smeared onto a glass slide, air dried and processed for PSA-FITC assessment. The 1 mL aliquots were fixed and prepared for transmission electron microscopy as described in section 3.1.6. Sperm were classified based on the appearance of the acrosomal membrane (acrosome reacted or acrosome intact) and on their position relative to other cells (individual or agglutinated). This produced the four categories shown in table 5.

Table 5. The classification of sperm agglutinations using electron microscopy.

TYPE	DESCRIPTION
A	Single, acrosome intact.
B	Single, acrosome reacted.
C	Agglutinated, acrosome intact.
D	Agglutinated, acrosome reacted.

Using transmission electron microscopy sperm were categorized as single or agglutinated and the presence or absence of the acrosome ascertained. The proportion of sperm in each category was estimated after incubation of samples for one and five hours.

3.2.2 Results

Summary - The number of motile sperm recorded in the samples decreased over the incubation period and the ejaculates showed considerable variation in maintenance of motility over the six hours. All eight samples satisfied the 'more than 25 motile sperm' criteria at time 0. After 90 minutes, samples from two of the eight ejaculates did not satisfy this criteria. By the end of the incubation period (360 minutes) only one sample still recorded greater than 25 motile sperm. The loss of motility appeared to be the result of an increase in non-motile cells and of sperm agglutination.

Sperm formed agglutinations in two ways; sperm were either orientated randomly and apparently bound to dead cells and debris, or specifically agglutinated in a head to head manner with binding occurring over the post acrosomal region. The majority of head to head (H:H) agglutinations consisted of two to eight sperm, although some larger groupings were seen (<5% of samples). The H:H agglutinations tended to remain motile, however, the proportion of bound cells with flagellar motion was difficult to discern.

Owing to the decrease in the proportion of the samples recorded with >25 sperm, only the data for the first 150 minutes were used for the correlation of the motility variables with time. At each time point up to and including 150 minutes four or more samples remained eligible for inclusion in the analysis. A Spearman Rank test was used for this part of the analysis as the number of cases included was small. ANOVA was used to assess the effect of time on sperm numbers, acrosomal status and sperm viability. For clarity, the results are described in sections and reference made throughout to figure 12 and 13.

Number of sperm analyzed over the two minute period - Although sperm concentrations had been adjusted to a constant value, the number of sperm recorded at the initial time point varied from 51 to 281. The number of sperm recorded was significantly correlated with the duration of incubation ($r=-0.664$, $P<0.001$; figure 12). Every sample contained some motile sperm after the six hour incubation, usually including a few highly motile cells. Occasionally a sample which had contained fewer than the 25 sperm limit at one time point, later recorded >25 sperm probably as a result of the observed break-up of the agglutinations.

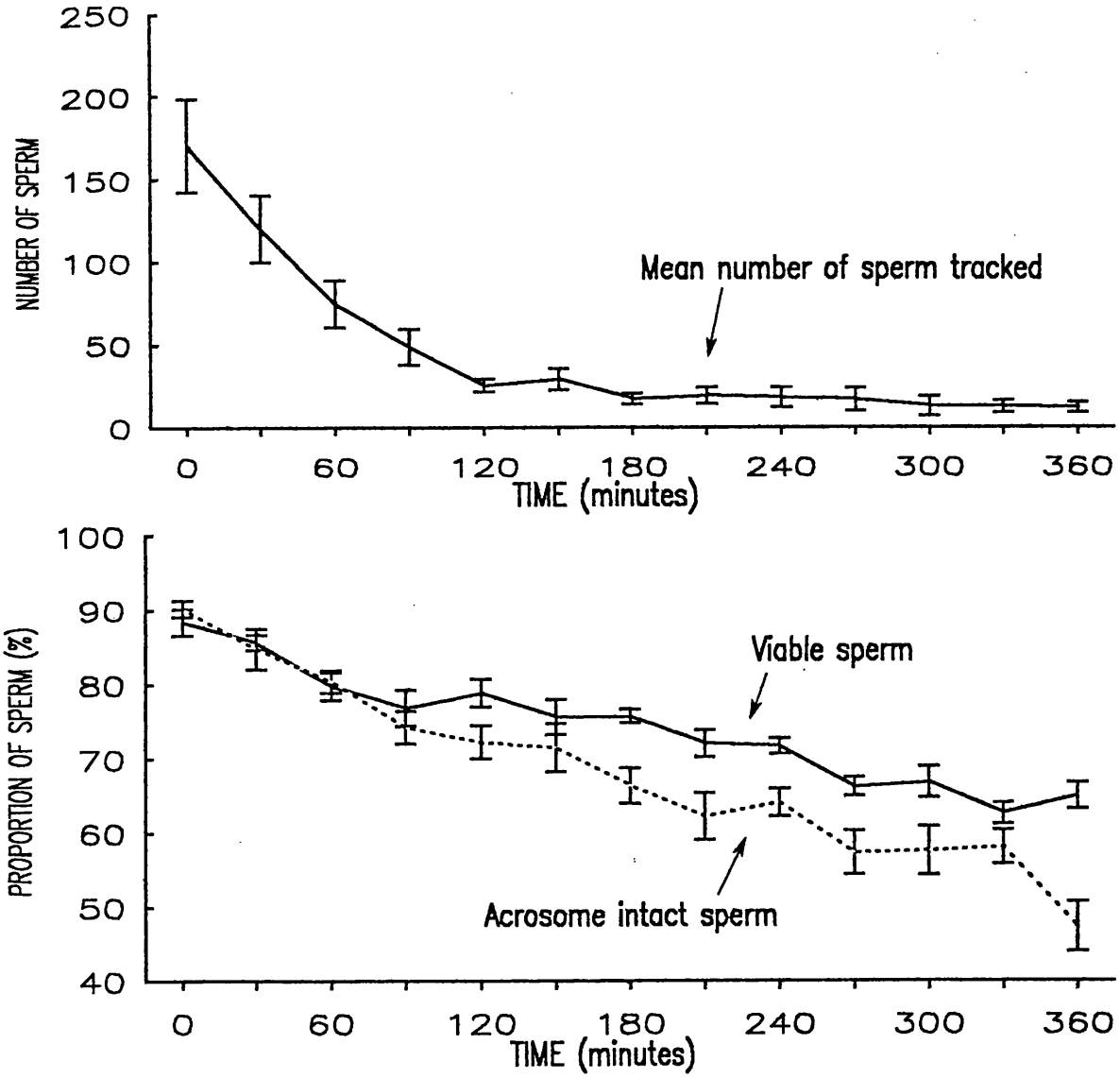


Figure 12. The effect of incubation on sperm motility, viability and acrosome reactions. The upper graph shows the decline in the mean number of sperm recorded by the HST over a 6 hour incubation period. The lower graphs illustrate the effect of incubation on the proportion of acrosome reacted sperm and the decline in sperm viability. Values shown are the mean and s.e.m calculated from eight samples.

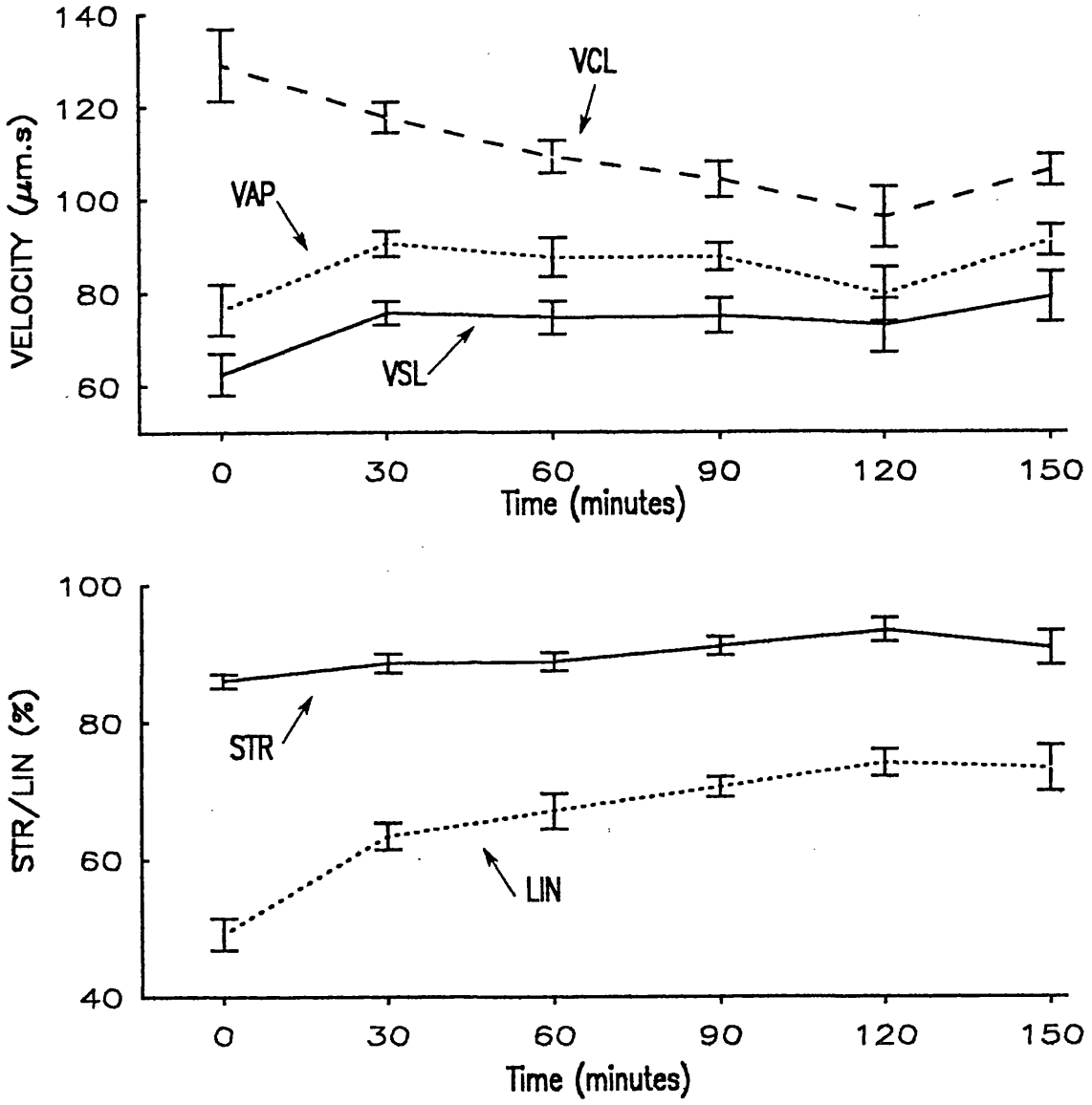


Figure 13a and b. Sperm motility changes during incubation in a capacitating media. The four graphs illustrate changes in the CASA measurements of boar sperm populations over a 150 minute incubation period. Washed, liquid-stored semen samples (n=8) were incubated in a high calcium medium at 39°C, in an atmosphere of 5% CO_2, 100% humidity, and sub-samples analyzed every 30 minutes using the HST. After 150 minutes, only two of the eight samples recorded >25 sperm in a two minute period. Therefore only results for the first 150 minutes are shown; values are the mean and s.e.m. calculated from n≥4 samples.

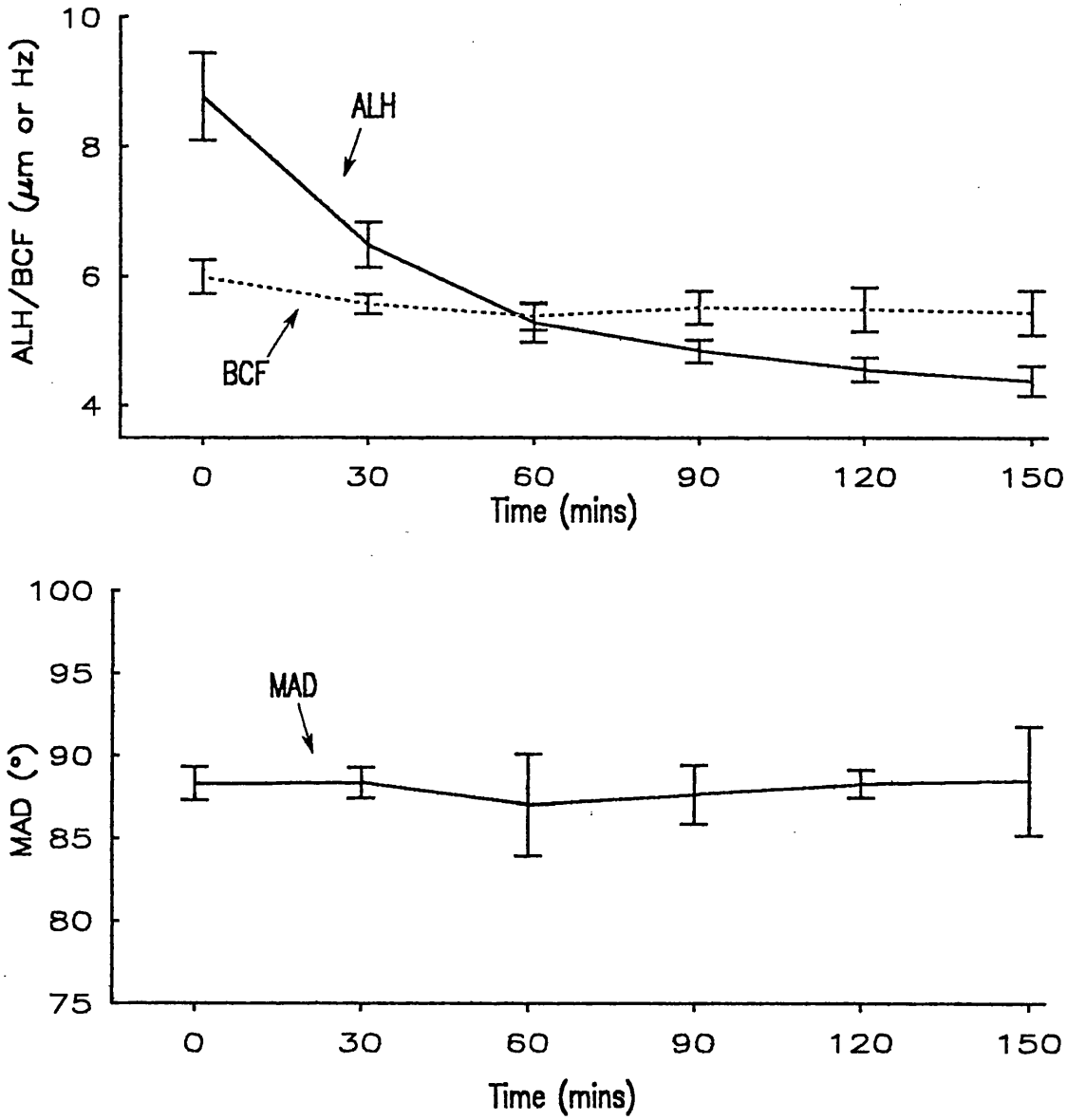

ALH/BCF (μm or Hz)
10
8
6
4
ALH
BCF
0
30
60
90
120
150
Time (mins)
MAD (°)
100
95
90
85
80
75
MAD
0
30
60
90
120
150
Time (mins)

Figure 13b

Sperm acrosome reaction and viability - Figure 11. shows the different patterns of fluorescent staining found within a population of sperm assessed with the PSA-FITC. The proportion of cells with an intact acrosome (figure 11, a) declined over the six hours from an initial mean of 90.14% to a mean of 47.38% (correlation with incubation period: $r=-0.807$, $P<0.001$; figure 12) concomitant with an increased incidence of partially and fully acrosome-reacted sperm (figure 11, b-d). Over the six hours the mean proportion of live cells declined from 88.4% to 65.0% in a linear manner. Although the mean loss of viability was <25%, the range amongst the ejaculates was 10% to 34%. The graphs for the proportion of acrosome intact sperm and for viable sperm are highly correlated ($r=0.733$, $P<0.0001$). This could indicate either that the sperm are dying soon after acrosome reacting, or that the acrosome reactions measured are degenerative. The fact that at all sampling times a greater proportion of cells were acrosome reacted rather than non-viable suggests the former, in agreement with previous studies (Harrison and Vickers, 1990).

Motility measurements - The curvilinear velocity of the motile sperm population declined over the six hour period ($r=-0.526$, $P<0.001$; figure 13). Whilst maximum mean VCL was recorded at the initial time point, VAP and VSL measurements increased over the first 30 minutes to a maximum and then maintained a constant velocity for the next 120 minutes. Neither VAP or VSL were significantly correlated with time (VAP - $r=0.158$, $P=0.344$; VSL - $r=0.292$, $P=0.075$). Over the first six time points, both mean LIN values and mean STR values increased (LIN - $r=0.762$, $P<0.001$ and STR - $r=0.457$, $P<0.01$; figure 13). Mean MAD values remained constant over the 150 minute period, staying within a range of 87.00 to 88.49 degrees, and were not significantly correlated with the duration of incubation ($r=0.039$, $P=0.816$; figure 13). Mean BCF values remained constant throughout the 150 minute period ranging from 5.44 Hz to 5.98 Hz and were not significantly correlated with the incubation period ($r=-0.185$, $P=0.266$; figure 13). Mean ALH values fell from 8.77 µm to 4.38 µm and this decline was significantly correlated with the duration of incubation ($r=-0.810$, $P<0.001$).

Correlations of the measurements from individual tracks - Table 6. shows the combinations of variables possible and the number of samples in which the

correlation was significant (i.e.P<0.01) out of a total of eight. The three velocity measurements were all highly and consistently correlated (VCL:VAP 8/8, VCL:VSL 7/8 and VAP:VSL 8/8 significant correlations). This relationship is predictable given that VAP and VSL measurements are reflections of the speed of the sperm (VCL) and the trajectory of the sperm path (measured by LIN and STR; see chapter 1). The consistent correlation of VAP and VSL measurements with LIN and STR values further reflects this relationship.

LIN is a measurement of the curvilinear path whilst STR describes the average path. As the latter is derived from the curvilinear path the strong correlation between LIN and STR is unsurprising (LIN:STR 8/8 significant correlations). BCF and ALH were also significantly correlated in all eight samples. This is a negative correlation and arises because the frequency with which the sperm head crosses the average path will be lower if the displacement either side is greater for a given VCL.

Table 6. The correlation of CASA measurements of sperm motility.

COMBINATIONS OF VARIABLES							
	VCL	n=8 EJACULATES					
VAP	8	VAP					
VSL	7	8	VSL				
MAD	1	1	0	MAD			
BCF	6	4	5	1	BCF		
ALH	5	6	3	0	8	ALH	
LIN	1	8	8	1	3	5	LIN
STR	1	8	8	0	3	3	8

The motility measurements from individual sperm tracks at time 0 (n=~100) were examined using a Spearman Rank Test. The process was repeated for the eight ejaculates; the number of times a variable pair was significantly correlated, out of a maximum of eight, is shown (e.g. VCL:ALH were significantly correlated in five out of the eight samples). Correlations between variables were judged to be significant if P<0.01.

These results suggested that it would be possible to omit one of the velocity measurements; either LIN or STR; and possibly one of either ALH or BCF. However, the correlations fluctuated in significance between different ejaculates and therefore made it unsafe to extrapolate these results to all ejaculates. Instead, the HST software was modified to down-load motility data into CSS:STATISTICA files, greatly facilitating the processing of the information.

Ultrastructural characterization of sperm agglutinations - Electron micrographs of the four categories of spermatozoa found in the samples are shown in figure 14. The samples removed after 1 hour contained mostly type (A) and (C) sperm, although a few type (B) were also present. Approximately 4% of the sperm were acrosome reacted at this time as assessed using the PSA-FITC staining. By five hours, type (D) sperm were visible as well as examples of the other three sperm types, and 43% of the sperm population appeared to be acrosome reacted using the lectin staining.

Type (C) sperm demonstrated a close apposition of the plasma membranes overlying the acrosome. The acrosomal membranes and overlying plasma membranes appear to remain closely associated although in figure 14c, slight disruption of the plasma membrane is visible. The distances between the plasma membranes of juxtaposed sperm varied; in some instances this interval was comparable to the distance between the plasma and acrosomal membranes of a single spermatozoon.

Quantitative assessment of the proportion of each sperm formation was not undertaken, but <10% of the total sperm were judged to be of type (D). Sperm were classified as type (D) if the plasma membranes surrounding the agglutinated sperm were disrupted in a manner consistent with exocytosis, but the sperm heads remained aligned and in close approximation. These sperm tended to be further apart than type (C) sperm, and it was difficult to distinguish genuine agglutinations from single sperm in close proximity.

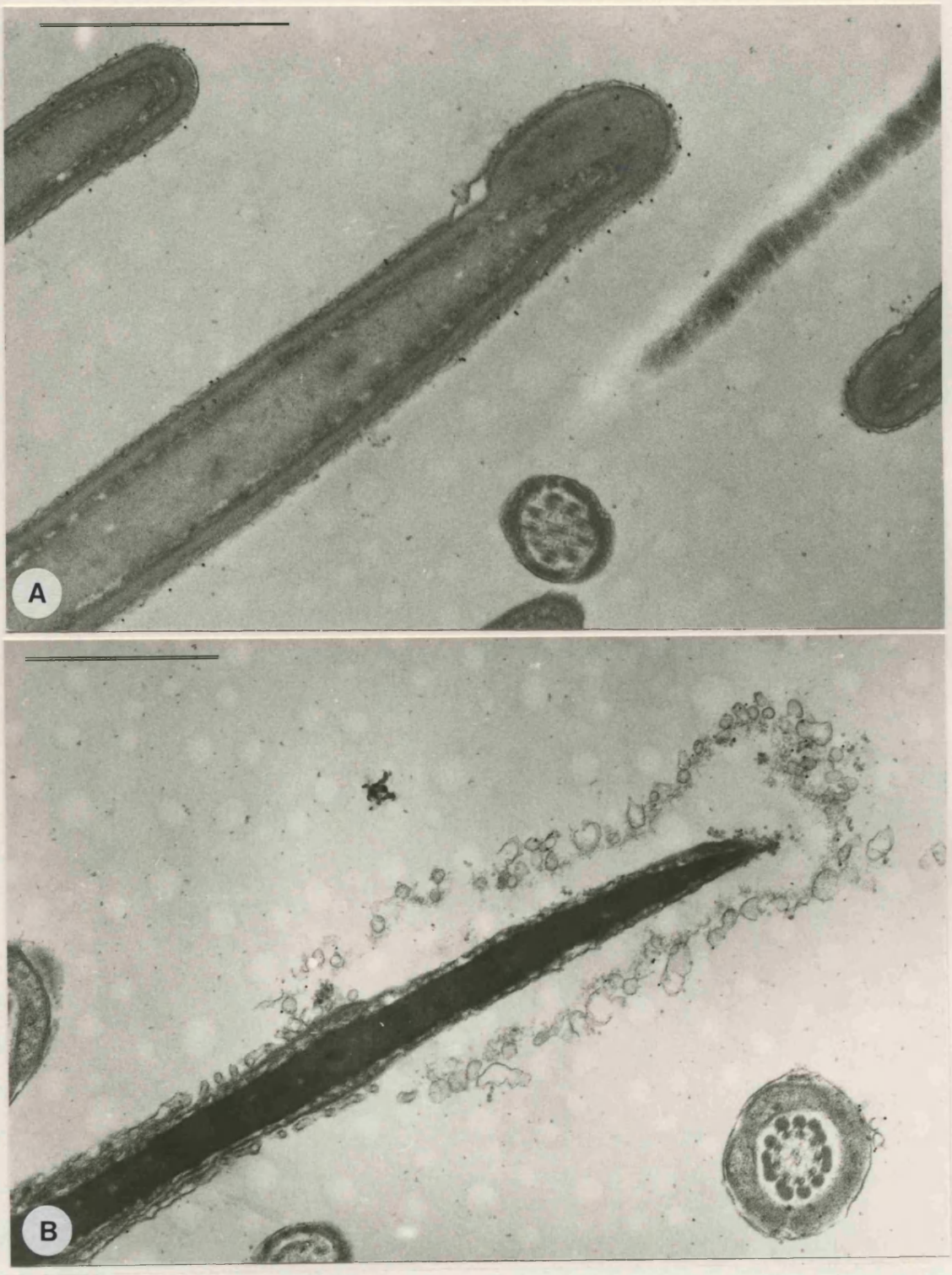
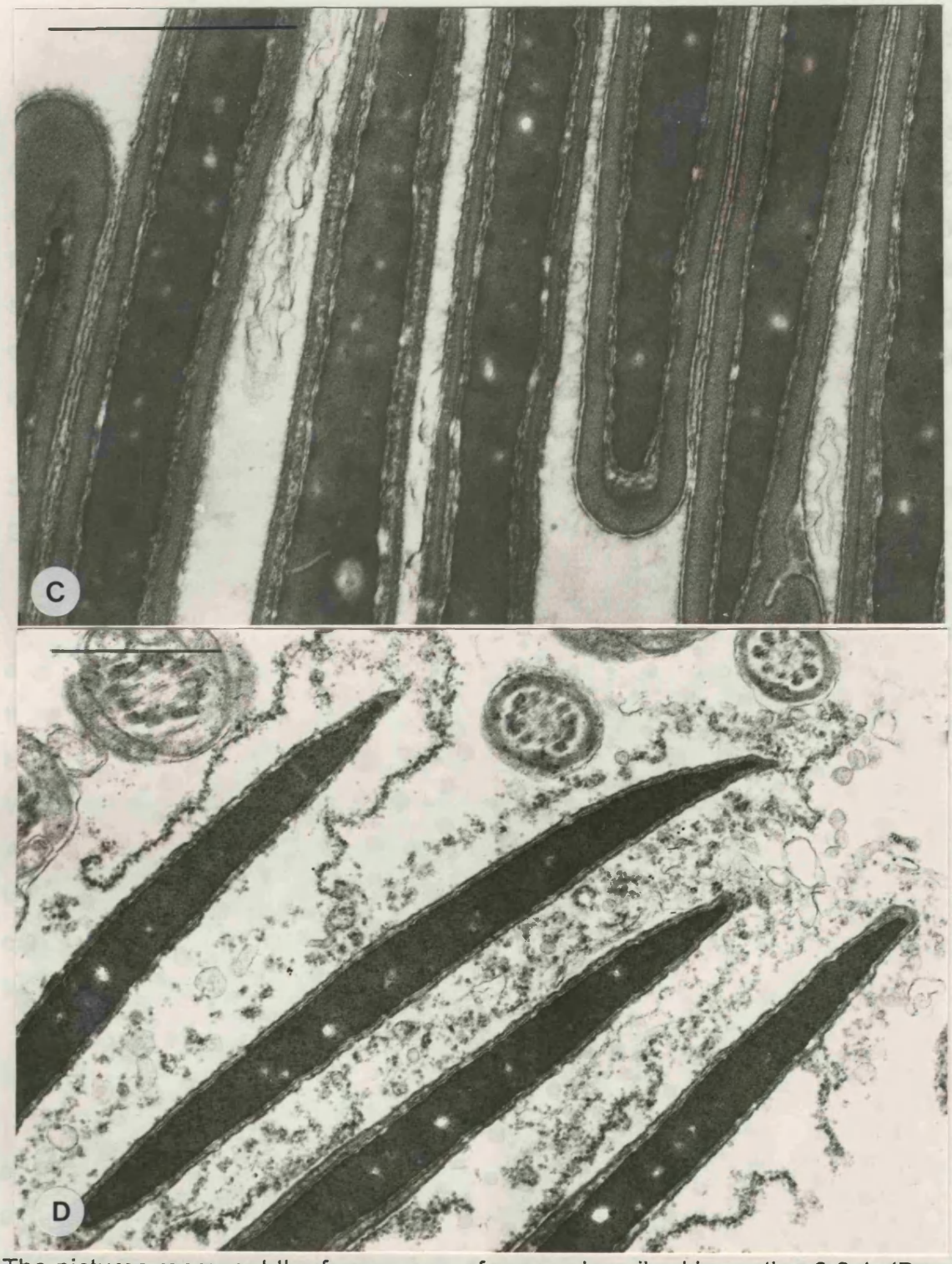


Figure 14. Electron micrographs of boar spermatozoa. Washed sperm samples were resuspended to a concentration of 20 x 10^6 sperm/mL in TBM media and incubated at 39°C, in an atmosphere of 5% CO_2, 100% humidity. Samples were fixed for transmission electron microscopy after 60 and 300 minutes incubation.



The pictures represent the four groups of sperm described in section 3.2.1 (Bars represent 1 μm).

Fig. A. Single, acrosome intact

Fig. B. Single, acrosome reacted

Fig. C. Agglutinated, acrosome intact and

Fig. D. Agglutinated, acrosome reacted.

3.2.3 Summary

Sperm viability decreased over time as did the proportion of cells with intact acrosomes. Mean sperm velocity and ALH decreased over the incubation period, whilst sperm trajectories straightened. Many of the motility measurements were correlated to each other in one or more instances, some consistently so. **Based on the results from this experiment, a protocol of sampling initially and then every two hours for a period of six hours was used in subsequent experiments.** This allowed changes in sperm motility to be examined thoroughly without needing extensive sampling regimes which would have severely reduced the number of ejaculates that could be processed on one day.

3.3 COMPARISON OF THE MOTILITY DATA OBTAINED FROM FRESH AND EXTENDED SEMEN.

Technicians at the Thorpe Willoughby SDS centre perform semen assessments immediately after collection of the fresh ejaculate, prior to addition of the extender. This is the time at which semen assessment using the HST could be most conveniently performed. However, the majority of semen samples used in this research had been liquid stored for 24 hours before analysis with the HST. As liquid storage is known to affect sperm motility and fertility (for a review, see Weitze 1990), it was important to establish whether the mean motility characteristics of the semen sample after 24 hours liquid extension were correlated to those of the fresh ejaculate.

3.3.1 Experimental design

A total of twenty-eight ejaculates from eight boars were assessed. For each ejaculate assessments were made of the fresh ejaculate prior to extension ('fresh') and after 24 hours liquid storage ('extended'). CASA assessments of fresh and extended semen were made after 0, 2, 4 and 6 hours incubation and mean VCL, VAP, VSL, MAD, BCF, ALH, LIN, STR recorded. At the end of the incubation period the proportion of sperm that had undergone the acrosome reaction was assessed in both fresh and extended samples using the PSA-FITC technique.

The data collected was used to establish:

(a) whether the motility characteristics derived for a sample before and after liquid storage were significantly correlated and

(b) whether the results from the fresh and extended samples were significantly different.

Assessments of the semen prior to liquid storage were performed at the Thorpe Willoughby Pig Breeding Centre. Aliquots (200µL) were removed from the sperm-rich fraction of freshly collected ejaculates maintained at 35°C (see section 3.1.1. for a description of the collection and processing of ejaculates at the Thorpe Willoughby centre). The raw semen was layered onto a Percoll gradient and the sperm washed using the protocol described in section 2.1.2. Video-recordings of the sperm movement were made at the Thorpe Willoughby centre and analyzed at the Institute of Zoology. The sperm-rich fraction of the ejaculates were processed by the Thorpe Willoughby technical staff using the standard protocols employed at the centre and the semen transported to London for assessment the next day. At this time the extended samples were prepared and assessed using the protocol described in section 3.1.2 and 3.1.3.

Nomenclature

To facilitate describing the results, the following nomenclature has been adopted; a measurement and the time point (i.e. zero, two, four or six hours) are represented together such that curvilinear velocity after two hours would be termed VCL_2.

3.3.2 Results

Data describing the individual sperm tracks measured within a specimen after 0, 2, 4 or 6 hours incubation were combined to produce means. Table 7 summarises the correlations between these means for the fresh and extended samples. At the first analysis point (immediately after sample preparation) five of the mean motility measurements were highly correlated between fresh and extended samples ($P<0.01$). Subsequently the data became less closely associated, and none of the measurements were significantly correlated after 6 hours. This may result from the smaller number of samples in the analysis at this point when the loss of motility reduces the number of samples fulfilling the '>25

motile sperm' criteria.

The variable pairs were then compared using the non-parametric Sign test to establish whether mean values of the motility characteristics changed significantly over 24 hours storage (see table 8). Only the VAP and STR (and consequently VSL) variable pairs at time 0 were significantly different (VAP P=0.0005; VSL P=0.0088; STR P=0.0088). These velocity measurements, and STR, were greater in the extended samples probably indicating that sperm in the stored samples had straighter trajectories.

When the acrosome reaction data from the fresh and extended samples were compared a weak correlation was found (P=0.024); overall the proportions of exocytosed sperm were found to be similar in the two sample groups (P=0.307).

Table 7. The correlation of motility measurements before and after 24 hours liquid storage.

VARIABLE PAIR	TIME 0 n=21	TIME 2 n=20	TIME 4 n=20	TIME 6 n=17
AR_0 & AR_24				r=0.434 P=0.024
VCL_0 & VCL_24	N.S.	N.S.	N.S.	N.S.
VAP_0 & VAP_24	r=0.76 P=0.0001	r=0.53 P=0.015	r=0.753 P=0.0001	N.S.
VSL_0 & VSL_24	r=0.66 P=0.001	N.S.	r=0.712 P=0.001	N.S.
MAD_0 & MAD_24	N.S.	N.S.	N.S.	N.S.
BCF_0 & BCF_24	r=0.55 P=0.009	r=0.61 P=0.004	r=0.52 P=0.016	N.S.
ALH_0 & ALH_24	r=0.58 P=0.005	r=0.45 P=0.046	N.S.	N.S.
LIN_0 & LIN_24	r=0.59 P=0.005	N.S.	r=0.54 P=0.012	N.S.
STR_0 & STR_24	r=0.49 P=0.023	N.S.	r=0.48 P=0.028	N.S.

The relationships between pairs of measurements after 0, 2, 4 and 6 hours were examined using Spearman Rank Test. Correlations with P<0.05 are shown.

3.3.3 Summary

The motility data obtained for the fresh samples at the first time point correlated well with the data for the extended samples. Subsequently the correspondence between measurements diminished. However, mean values for

fresh and extended samples did not diverge significantly.

These results suggest that any relationship between measures of fertility and the motility traits within extended semen samples may not be reproduced when fresh samples are analyzed.

Table 8. CASA measurements before and after 24 hours liquid storage.

VARIABLE PAIR	TIME 0 n=21	TIME 2 n=20	TIME 4 n=20	TIME 6 n=17
VCL_0	99.44 ± 3.16	89.35 ± 3.09	81.25 ± 2.59	82.97 ± 2.22
VCL_24 (µm/s)	103.75 ± 3.53	91.14 ± 2.50	87.04 ± 2.52	81.26 ± 2.44
VAP_0	47.19 ± 2.52*	50.92 ± 3.56	46.47 ± 2.66	52.76 ± 3.03
VAP_24 (µm/s)	58.58 ± 3.71	59.17 ± 3.41	53.86 ± 3.33	54.36 ± 3.74
VSL_0	24.04 ± 2.93*	33.59 ± 3.86	31.29 ± 2.90	38.22 ± 3.43
VSL_24 (µm/s)	33.47 ± 3.13	40.63 ± 3.13	38.74 ± 3.46	40.15 ± 3.70
MAD_0	86.35 ± 1.02	87.46 ± 1.30	88.94 ± 1.27	89.80 ± 0.82
MAD_24 (°)	84.96 ± 1.43	89.60 ± 0.73	84.93 ± 1.37	88.54 ± 1.35
BCF_0	5.54 ± 0.25	5.57 ± 0.21	5.65 ± 0.21	5.46 ± 0.19
BCF_24 (Hz)	5.12 ± 0.27	5.23 ± 0.21	5.45 ± 0.23	5.40 ± 0.17
ALH_0	7.86 ± 0.41	6.57 ± 0.35	5.88 ± 0.31	5.88 ± 0.29
ALH_24 (µm)	7.93 ± 0.34	6.08 ± 0.31	5.99 ± 0.29	5.22 ± 0.16
LIN_0	24.48 ± 2.34	36.54 ± 3.43	37.72 ± 3.03	44.53 ± 3.25
LIN_24 (%)	32.16 ± 2.13	43.00 ± 2.41	42.98 ± 2.95	47.39 ± 2.98
STR_0	54.12 ± 2.83*	64.02 ± 3.40	65.89 ± 3.07	71.25 ± 3.54
STR_24 (%)	61.75 ± 2.42	69.96 ± 1.97	70.78 ± 2.33	73.65 ± 2.41
AR_0 (n=28) AR_24 (%) (n=27)				32.51 ± 1.42 32.65 ± 1.57

CASA measurements for fresh and liquid stored samples after 0, 2, 4, and 6 hours incubation in TBM media and acrosome reaction data after 6 hours incubation are shown. Values are means and s.e.m's from 28 samples. Variable pairs were compared using the non-parametric Sign test, * indicates significant differences between the variable pair ($P<0.01$).

3.4 THE RELATIONSHIP OF MOTILITY CHARACTERISTICS TO FERTILITY DATA

The aim of the following experiments was to evaluate the correlation between the motility characteristics of sperm populations and their fertilizing ability *in vivo*. Artificial inseminations were performed using bottled split ejaculates and the fertility results compared to CASA measurements of extended semen from the same ejaculate. In addition the relationship of spontaneous and induced acrosome reactions with ejaculate fertility was also examined (see chapter introduction). Two fertility trials were performed (see below); in trial 1, an ionophore challenge was used to induce exocytosis, in trial 2 solubilized zona pellucida (Z.P.) proteins were employed for this purpose. Information about the *in vivo* fertilizing ability of sperm was obtained through multiple artificial inseminations of split extended ejaculates from the Thorpe Willoughby SDS. Bottles of semen from split ejaculates were sent to the Pyramid herds of JSR Healthbred and to customers of the SDS. The majority of the insemination data included in the analysis were provided by the Pyramid herds (however, the results from the SDS customers were useful for augmenting this data).

The fertility results collected in such trials reflect the ability of sperm to move and survive within the female tract and to bind to and fertilise the oocyte. The information will also reflect features such as female fertility and management practice. The issues involved in planning and performing field fertility trials have been discussed previously (Amann, 1989; Den Daas, 1992; Amann and Hammerstedt, 1993). Measures were taken to ameliorate the confounding effects highlighted in these reports.

Research using AI of domestic species has shown that the relationship between fertilization success and insemination dose is described by an exponential curve, (for a review see Den Daas, 1992). Increasing the number of spermatozoa inseminated enhances the probability of conception; however, a threshold point exists above which further addition of sperm does not affect the outcome of the insemination. The asymptotic point of the curve (ie maximum fertility), and the rate at which it was approached were found to vary between bulls (Den Daas, 1992). This meant that bull ranking was dependent on the insemination dose used (for doses below the threshold level). Adjusting sperm doses to account for this

observation may produce more consistent relationships between sperm characteristics and fertility. However, an enormous amount of inseminations are required to determine this ranking for any set of individuals and is therefore impossible for the majority of field trials.

At insemination doses above the threshold value maximum fertility results would be achieved, and the range of fertilities encompassed in the study would be severely reduced. The insemination dose chosen here (1.5×10^9 sperm/bottle, a concentration below the doses normally employed) was considered to represent the best compromise between the desire for test sensitivity and the need for company efficiency.

Different methods of semen preparation also affect the apparent fertility of an ejaculate (e.g. Reed *et al.*, 1990) and strenuous efforts were made to standardize the procedures used. All semen samples collected at the Thorpe Willoughby centre conform to British Standard BS5750 which requires quality control of the preparation procedures and adherence to standard laboratory practices. This ensures that all ejaculates are treated in a reproducible manner. Insemination methods and sow management procedures are more difficult to standardize. The majority of A.I data came from three centres (the Pyramid herds of Pigwood, Baveney and Newbottle) and the inseminations at these sites are performed by company trained managers following routine practices. Further standardization of herd management and AI procedures was not possible even though it was recognized that this would be an important source of variation.

The experiments described below took place between May and September. Performing both trials within the summer months ensured that seasonal environmental factors were similar throughout the two trials. Seasonal effects are known to influence the fertility of both boars and sows (Wettemann and Bazer, 1985; Claus and Weiler, 1985). However, fluctuations in farrowing rate and the average number of pigs born over these months are known to be small (Reed, 1987).

Both inherent male and female fertility influence the outcome of an insemination. The former is the effect under consideration whilst the latter is a confounding factor. Variations in fertility between sows can be overcome by the insemination of numerous females with extended semen from each ejaculate. When planning the trial it was expected that, as each ejaculate produces

approximately 30 bottles of semen, the maximum number of inseminations per ejaculate would be approximately 15. It was foreseen that some of these bottles would not be used in 'pure' AI matings (i.e. either natural service or pooled semen would be employed) and that not all 'results' cards would be returned. On this basis, results for eight or more inseminations were anticipated for each ejaculate. However, changes in farming practice during the study (i.e. increased use of mixed semen samples or AI combined with natural service) meant that this target was not actually attained (see discussion).

The effect of high viscosity media on sperm movement characteristics

In the course of the first fertility trial it became clear that sperm velocity was the factor most significantly related to ejaculate fertility. In an attempt to discriminate more clearly between sperm populations with different amounts of thrust, a high molecular weight hyaluronic acid polymer (Sperm Select®, Pharmacia, Uppsala, Sweden) was used to impede the motion of the sperm. Semen samples were analyzed in TBM media alone and in TBM media containing Sperm Select® and the motility results compared to fertility data. These results were used to investigate whether the addition of Sperm Select® prior to CASA analysis of samples improved the correlation of the motility measurements with fertility.

3.4.1 Experimental design

Bottles of split, extended ejaculates (total sperm dose = 1.5×10^9) were sent to four JSR Healthbred Pyramid herds for use in their AI programme. Bottles of semen from these ejaculates were also sent to the Institute of Zoology for assessment. On advice from statisticians at the Meat and Livestock Commission, two trials were conducted and the results retained as separate data sets. This meant that any significant effects arising out of the first trial could be checked using independent data from the second trial.

TRIAL 1 - June to September, 1992.

Each semen sample was assessed using the HST after 0, 2, 4 and 6 hours incubation and mean VCL, VAP, VSL, MAD, BCF, ALH, LIN, STR recorded. The proportion of acrosome reacted sperm in the sample was assessed after 6 hours

incubation and again after induction of exocytosis using calcium ionophore A23187.

TRIAL 2 - May to June, 1993.

Each semen sample was assessed using the HST after 0 and 2 hours incubation and mean VCL, VAP, VSL, MAD, BCF, ALH, LIN, STR recorded. Motility assessments of sperm were made in TBM media alone and in TBM media containing Sperm Select®[2]. The proportion of acrosome reacted sperm was assessed after 3.5 hours incubation and again after induction of exocytosis using solubilized zona pellucida proteins.

Statistical analysis

The fertility data was quantified in two ways:

(1) the success or failure of an insemination,
(2) the average litter size produced by an ejaculate.

Different statistical tests were appropriate for the two measures of fertility. The litter size data is quantal and therefore linear regression techniques can be used (Pearson Correlation and multiple regression). Conception outcome is binary and therefore logistic regression was used to relate the dichotomous success/failure data to the continuous motility variables (Altman, 1991). The different combinations of motility and acrosome reaction variables used in the statistical analyses are referred to as 'models'. As a large number of models could theoretically be constructed it was not feasible to compare every one with the fertility data.

Two methods were used to calculate the average litter size for each ejaculate. In the first (termed AVL) the average was constructed using only the data from the successful inseminations. In the second (termed AVL0) the unsuccessful inseminations were included in the calculation as a litter size of 0.

In addition to the eight motility measurements at each time point, two further

[2] At each sampling time, a 25µL aliquot was removed from the semen sample and used for motility analysis with the HST A second 25µL aliquot was then mixed with an equal volume of pre-warmed Sperm Select® and 25µL of this mixture used for analysis with the HST

variables were derived:

(1) the difference in velocity measurements between time 0 and time 2 (e.g. [VCL initially - VCL after two hours] and

(2) the mean velocity measurements for over this period of time (i.e. [VCL initially + VCL after two hours] ÷ 2).

Sperm Select® Experiments

Correlations between the motility measurements of sperm in media alone and media with Sperm Select® were evaluated using the Pearson Correlation test (except for those involving the STR measurements which were tested using the Spearman Rank non-parametric test because of the non-normality of the STR distribution). The two sets of results were compared using the t-test for dependent samples (STR measurements were compared using the non-parametric Sign test).

3.4.2 Results

The fertility data collected during both trials are summarized in table 9. Initially it had been hoped to obtain at least eight results for each ejaculate. In practice the number of results available per ejaculate was far lower (Trial 1, mean = 3.5, mode = 2; Trial 2, mean = 2.2, mode = 2) owing to a change in farming practice (see discussion).

The results for both trials indicated that correlations of motility variables with AVL0 tended to be more significant than correlations with AVL. This could be because inclusion of the litter sizes of 0 broadens the range of litter sizes in the analysis.

Linear regression

The results for trial 1 indicated that **sperm velocity after 2 hours** was the characteristic most related to measurements of average litter size (AVL0). Linear regression indicated a significant correlation of the variable VSL_2 with AVL0, ($P<0.019$, $r=0.259$; see table 10.) and of the multiple regression model [VAP_2+VSL_2+STR_2] with AVL0 ($P=0.007$). Eight further models were significant at $P<0.02$, all of which included the variables VAP_2 and VSL_2 (see table 11 and 12).

The results from section 3.2. indicated that VAP, VSL and STR

measurements are highly correlated. Therefore it was important to establish whether the observed significance was due to repeated measuring of the same variation. The multiple regression module for CSS:STATISTICA provides an estimate of the variation explained by the model. This was 3.40% for VAP_2 alone, 6.68% for VSL_2 alone, 2.26% for STR_2 alone, 8.79% for VAP_2+VSL_2 and 14.25% for VAP_2+VSL_2+STR_2. This indicates that the overlap between each of the measurements is not large, and they are contributing independent information to the regression line.

The results from trial 2 confirmed the relevance of the motility data from the first two hours of incubation to the fertility data. Whilst trial 1 had highlighted sperm velocity after two hours, in trial 2 the **change in velocity** over the two hours appeared to be the most important factor. The variable [VAP_0-VAP_2] and six multiple regression models were significantly correlated to AVL0 ([VAP_0-VAP_2] = $P<0.05$; multiple regression models = $P<0.02$). Four of these models contained both of the derived variables [VAP_0-VAP_2] and [VSL_0-VSL_2]. The significant multiple regression models also included measurements of sperm head movement made in the first sampling period. The most significant model included the variables BCF_0 and ALH_0, neither of which had been highlighted in the results from trial 1.

Logistic regression

Numerous models were constructed to evaluate the relationship of the motility data to the insemination success/failure rate for trial 1, however, none of these were statistically significant. Using the statistics package it was possible to examine the significance of the component variables of these models, the P-values of which are dependent on the overall model being used. This revealed that ALH_2 was significant (at $P<0.05$) within five models, all of which also included the parameter VCL_2 (N.S.). When ALH _2 alone was used to construct a model, the significance of the logistic regression was $P<0.06$.

Analysis of the results from trial 2 indicated seven highly significant logistic regression models ($P<0.001$) all of which contained the derived variables [VAP_0-VAP_2] and [VSL_0-VSL_2] (see table 13 and 14.). The measurements of sperm head movement at the first sampling time were again present in some of the significant models, though less frequently than the measures of velocity change.

Table 9. Summary of the fertility results available for trials 1 and 2.

	TRIAL 1	TRIAL 2
NUMBER OF ANIMALS	27	26
NUMBER OF EJACULATES	98	72
EJACULATES PER ANIMAL	1 TO 10	1 TO 7
FERTILITY RESULTS PER BOAR	1 TO 43	1 TO 18
FERTILITY RESULTS PER EJACULATE	1 TO 12	1 TO 7
MEAN AND RANGE OF AVERAGE LITTER SIZES (AVL)	11.2 3 TO 25	11.8 5 TO 21
MEAN AND RANGE OF AVERAGE LITTER SIZES (AVL0)	8.2 0 TO 15.3	9.6 0 TO 21

TABLE 10. Linear regression of motility measurements with average litter size (AVL0) - Pearson correlation.

VARIABLE	P VALUE	R VALUE
TRIAL 1		
VSL_2	P=0.019	r=0.259
VAP_4	P=0.048	r=0.256
VSL_4	P=0.026	r=0.287
LIN_4	P=0.049	r=0.255
STR_4	P=0.026	r=0.287
VAP_6	P=0.040	r=0.304
VSL_6	P=0.022	r=0.336
LIN_6	P=0.026	r=0.329
STR_6	P=0.028	r=0.324
(VSL_0+VSL_2)/2	P=0.044	r=0.224
TRIAL 2		
(VAP_0-VAP_2)	P=0.040	r=-0.281

Correlations with $P<0.05$ are shown

TABLE 11. Linear regression of motility measurements with average litter size (AVL0) - Multiple regression.

MODEL	R^2 VALUE	SIGNIFICANCE
TRIAL 1		
VAP_2 + VSL_2 + STR_2†	14.25 %	P=0.007
VAP_2 + VSL_2 + STR_2 + ALH_2†	15.28 %	P=0.012
VAP_2 + VSL_2 + VCL_2	13.17 %	P=0.012
VAP_2 + VSL_2 + LIN_2 + STR_2	14.57 %	P=0.015
VAP_2 + VSL_2 + STR_2 + SPONTANEOUS A.R.	15.37 %	P=0.015
VAP_2 + VSL_2 + LIN_2	12.25 %	P=0.016
VAP_2 + VSL_2 + STR_2 + INDUCED A.R.	14.99 %	P=0.016
VAP_2 + VSL_2 + ALH_2	12.09 %	P=0.018
VAP_2 + VSL_2 + [(VCL_0+VCL_2)/2]	12.04 %	P=0.019
TRIAL 2		
BCF_0 + ALH_0†	12.94 %	P=0.010
BCF_0 + ALH_0 + MAD_0	15.66 %	P=0.010
[VAP_0-VAP_2] + [VSL_0-VSL_2] + MAD_0	20.09 %	P=0.010
[VAP_0-VAP_2] + [VSL_0-VSL_2] + MAD_0 + ALH_0	23.12 %	P=0.011
[VAP_0-VAP_2] + [VSL_0-VSL_2] + ALH_0	19.40 %	P=0.012
[VAP_0-VAP_2] + [VSL_0-VSL_2]†	14.59 %	P=0.018

The linear regression (multiple regression) of mean motility characteristics to average litter size (AVL0); correlations with P<0.02 are shown. The regression coefficients, standard errors, t-values and p-values of models marked † are detailed in table 12.

TABLE 12. Component variables of the linear regression models.

MODELS	B	SE(B)	t	P-LEVEL
CONSTANT	18.518	5.497		
VAP_2	-0.166	0.067	-2.487	0.015
VSL_2	0.276	0.089	3.101	0.003
STR_2	-0.179	0.080	-2.229	0.029
CONSTANT	15.007	6.596		
VAP_2	-0.173	0.067	-2.570	0.012
VSL_2	0.272	0.089	3.050	0.003
STR_2	-0.148	0.087	-1.702	0.093
ALH_2	0.242	0.251	0.964	0.338
CONSTANT	-4.420	4.489		
BCF_0	1.389	0.537	2.587	0.012
ALH_0	0.741	0.278	2.667	0.010
CONSTANT	10.296	0.760		
[VAP_0-VAP_2]	-0.122	0.042	-2.882	0.006
[VSL_0-VSL_2]	0.072	0.036	2.001	0.051

The regression coefficients (B) and associated standard errors, t-values and p-values for the variables within three of the multiple regression models in table 11. are shown.

TABLE 13. Logistic regression of motility measurements with AI results (Success/failure).

MODEL	SIGNIFICANCE
[VAP_0-VAP_2] + [VSL_0-VSL_2] + MAD_0‡	P=0.00002
[VAP_0-VAP_2] + [VSL_0-VSL_2] + MAD_0 + ALH_0 + BCF_0‡	P=0.00008
[VAP_0-VAP_2] + [VSL_0-VSL_2]‡	P=0.0001
[VAP_0-VAP_2] + [VSL_0-VSL_2] + [VCL_0-VCL_2]	P=0.0002
[VAP_0-VAP_2] + [VSL_0-VSL_2] + [ALH_0-ALH_2]	P=0.0003
[VAP_0-VAP_2] + [VSL_0-VSL_2] + [(VCL_0+VCL_2)/2]	P=0.0003
[VAP_0-VAP_2] + MAD_0	P=0.0005

The correlation of mean motility characteristics with insemination outcome (success/failure) using logistic regression. Correlations with P<0.001 are shown. None of the models constructed using the data from trial 1 were significant therefore the results shown are for trial 2 only. Models marked ‡ are described in greater detail in table 14.

TABLE 14. Component variables of the logistic regression models.

MODELS	B	SE(B)	t	P-LEVEL
CONSTANT	-10.197	4.815	-2.118	0.037
[VAP_0-VAP_2]	-0.065	0.017	-3.882	0.0002
[VSL_0-VSL_2]	0.039	0.013	2.951	0.004
MAD_0	0.139	0.056	2.492	0.014
CONSTANT	-10.778	5.100	-2.113	0.037
[VAP_0-VAP_2]	-0.070	0.027	-2.603	0.011
[VSL_0-VSL_2]	0.043	0.027	1.580	0.117
MAD_0	0.127	0.058	2.177	0.032
ALH_0	0.148	0.151	0.982	0.328
BCF_0	0.029	0.460	0.063	0.950
CONSTANT	1.897	0.308	6.154	0.0001
[VAP_0-VAP_2]	-0.063	0.016	-3.911	0.0002
[VSL_0-VSL_2]	0.036	0.012	2.950	0.004

The regression coefficients (B) and associated standard errors, t-values and p-values for the variables within three of the logistic regression models in table 13. are shown.

Predictive equations of ejaculate fertility

The models detailed above could be used to generate prognostic indices of ejaculate fertility.

For example, the multiple regression models can be used to obtain a predicted AVL0 value for any individual semen sample. The generic multiple regression model can be written as:

$$y = \text{constant} + b_1x_1 + b_2x_2 + \ldots\ldots b_nx_n$$

where

y is the dependent variable

$b_1 \ldots b_n$ are regression coefficients

x is the individual value of the variable in the model

constant is the intercept of the regression line.

Using the first model in table 12, the prognostic value (y) of AVL0 would be defined as:

$$y = 18.518 - 0.166(VAP\_2) + 0.276(VSL\_2) - 0.179(STR\_2)$$

A similar expression is used for predicting conception rate using the logistic regression models. In logistic regression the binary variables (success/failure) are recoded to 0 and 1 and the proportion of cases with each value transformed to a probability (between 1 and 0) using the logit transformation below

$$\text{logit}(p) = \ln[p/(1-p)]$$

where

p is the proportion of matings that are successful

[p/(1-p)] is the ratio of the proportion of successful matings to the proportion of unsuccessful matings, the 'odds'.

ln is the natural logarithm (also termed $\log_e$)

Therefore the logistic regression models can be used to predict the logit transform of the dependent variable (y), such that

$$\text{logit}(y) = \text{constant} + b_1x_1 + b_2x_2 + \ldots\ldots b_nx_n$$

The main difference is that the values from the models are used to predict the transformed dependent variable. For example, using the first model in table 13,

$$\text{logit}(y) = \ln[y/(1-y)] = 10.197 - 0.065[\text{VAP\_0-VAP\_2}] + 0.039[\text{VSL\_0-VSL\_2}] + 0.139(\text{MAD\_0})$$

or prob (success) =

$$\frac{1}{1+\exp\{10.197-0.065[\text{VAP\_0-VAP\_2}]+0.039[\text{VSL\_0-VSL\_2}]+0.139(\text{MAD\_0})\}}$$

Sperm Select®

Motility measurements made from samples containing Sperm Select® were not significantly correlated to either average litter size or insemination success/failure rate. A new variable was constructed by calculating the difference between the motility measurements before and after the addition of the hyaluronic acid (i.e. [VCL_0 media only]-[VCL_0 media+Sperm Select®]. However, inclusion of this variable in the linear regression or logistic regression models did not improve the correlation between fertility and motility data.

Acrosome reactions

The data for spontaneous and induced acrosome reactions is shown in figure 15. Both treatments significantly increased the proportion of acrosome reacted sperm ($P<0.0001$), and the proportion of exocytosed sperm before and after induction of the acrosome reaction was highly correlated in each trial (trial 1, $P<0.0001$, $r=0.448$; trial 2, $P<0.0001$, $r=0.521$). A further variable was created from the data by calculating the difference between the percentages of spontaneous and induced acrosome reactions (Induced A.R. - Spontaneous A.R.).

In both trials, the correlations made between the acrosome reaction data and the fertility information were non-significant ($P>0.05$; Pearson correlation). Inclusion of the acrosome reaction data in the logistic regression models did not improve the correlations of the models with conception rate. However, two multiple regression models incorporating the acrosome reaction information were significant, (see table 11.), both derived from data collected in trial 1.

The effect of high viscosity media on sperm movement characteristics

Analyzing sperm in media containing Sperm Select® did not improve the correlations evaluated between sperm motility and fertility (see above). However, addition of the hyaluronic acid did improve the tracking of the most rapidly moving cells. This was indicated by a decrease in the frequency of broken tracks visible on the screen, probably as a result of reduced cell velocity.

Measurement means from the 72 samples were combined and used to produce distributions for each CASA variable. Means of each of the measurements were normally distributed, except for the Sperm Select® STR 0 and 2 hour measurements. These distributions contained a sub-group with reduced STR values. However, the samples comprising it were not the same at both time points.

Table 15. summarises the changes in sperm motility on addition of the hyaluronic acid. All the velocity measurements were reduced except for VSL at time 0, which was significantly increased ($P<0.01$). Both LIN and STR measurements became significantly closer to 100% (the value of a straight trajectory) in the Sperm Select® whilst the width of the head movement decreased and the beat frequency increased.

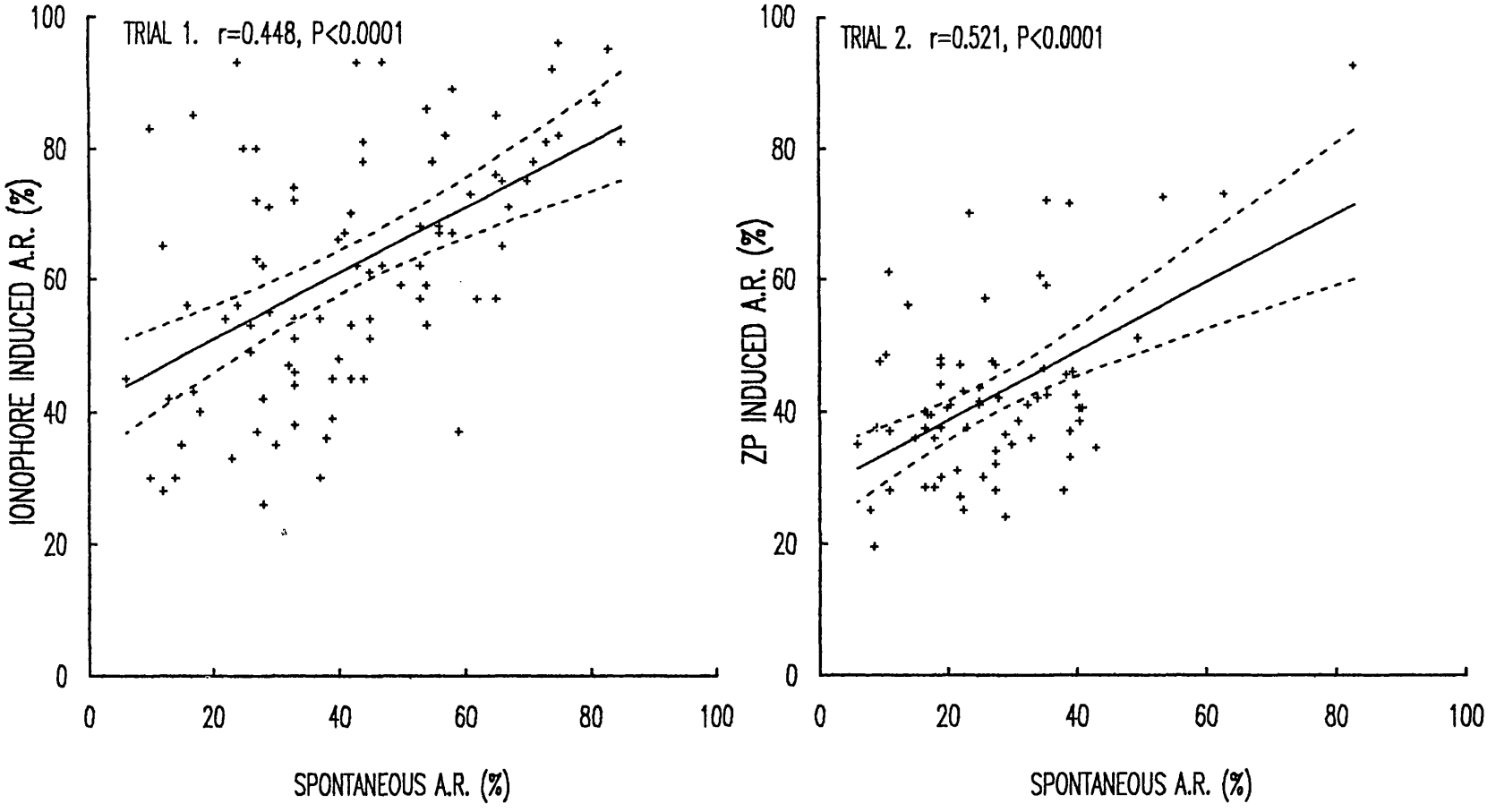


Figure 15. The correlation between spontaneous and induced acrosome reactions. The proportion of acrosome reacted sperm was assessed after incubation in capacitating media (6 hours - trial 1, or 3.5 hours - trial 2) using the FITC-conjugated PSA. Exocytosis was then induced using either 1μM calcium ionophore (trial 1) or 125 μg/mL solubilized zona pellucidae preparation (trial 2). The two scatterplots illustrate the correlation between the proportion of acrosome reacted sperm before and after ionophore/SZP treatment.

Table 15. Sperm motility in the presence and absence of Sperm Select®.

VARIABLE PAIR	MEAN OF SAMPLE MEANS		CORRELATION
	MEDIA SELECT	SPERM-SELECT	COEFFICIENT
VCL_0 (µm/sec)	146.2 ± 2.99	118.5 ± 1.65 ***	+ 0.143
VAP_0 (µm/sec)	95.8 ± 2.66	82.1 ± 1.89 ***	+ 0.266 *
VSL_0 (µm/sec)	58.7 ± 2.95	69.0 ± 1.81 **	+ 0.186
MAD_0 (°)	88.1 ± 0.52	88.9 ± 0.69	- 0.275 *
BCF_0 (Hz)	4.78 ± 0.13	6.76 ± 0.16 ***	+ 0.592 ***
ALH_0 (µm)	10.01 ± 0.26	6.20 ± 0.16 ***	+ 0.407 ***
LIN_0 (%)	39.0 ± 1.62	57.5 ± 1.15 ***	+ 0.227
STR_0 (%) †	66.8 ± 1.83	87.5 ± 0.85 ***	+ 0.328 **
VCL_2	125.4 ± 3.80	97.9 ± 2.34 ***	+ 0.018
VAP_2	99.4 ± 3.63	78.7 ± 2.37 ***	+ 0.368 *
VSL_2	75.4 ± 3.70	65.4 ± 2.62 *	+ 0.186
MAD_2	86.5 ± 1.02	88.4 ± 1.34	- 0.186
BCF_2	4.72 ± 0.11	5.28 ± 0.10 **	+ 0.007
ALH_2	7.48 ± 0.28	5.15 ± 0.16 ***	- 0.278
LIN_2	54.7 ± 1.82	64.4 ± 1.66 ***	- 0.008
STR_2 †	76.2 ± 1.72	86.9 ± 1.42 ***	- 0.031

CASA measurements after 0 and 2 hours incubation in TBM media are shown (means of the sample means, and standard errors). The two sets of results (+/-Sperm Select®) were compared using the t-test for dependent samples and significant differences are indicated. The correlation coefficients for samples before and after addition of Sperm Select®, and their associated significances are also shown.
*P<0.05; **P<0.01; ***;P<0.001

†Non-parametric Spearman Rank Correlation and Sign test used.

The correlations made between samples before and after the addition of Sperm Select® were less significant than expected (however, the head movements were strongly associated at the first time point (BCF_0 r=0.592, P<0.001; ALH_0 r=0.407, P<0.001). The possibility that the effects of Sperm Select® on sperm motility depend on the animal from which the ejaculate originates was examined.

The results for the six boars which had four or more ejaculates assessed were further analyzed using ANOVA. This revealed that changes in sperm LIN and STR on addition of the hyaluronate were significantly animal-dependent ($P<0.002$ and $P<0.005$ respectively). Two further measures (VSL_0 and ALH_2) showed a similar, but less significant, effect (both $P<0.02$). This indicates that the changes in sperm motility on addition of the hyaluronic acid were mediated by the animal from which the ejaculate originated.

3.4.3 Summary

Both fertility trials suggest that a relationship exists between sperm motility over two hours and subsequent fertility. However, in trial 1, **sperm velocity after two hours** incubation was the most significant factor, whilst in trial 2 **the change in sperm velocity over the first two hours** was the more significant. Sperm velocity was decreased and sperm trajectories straightened by addition of the hyaluronic acid, and the use of Sperm Select® improved the tracking of spermatozoa by the HST. The increased viscosity of the media did not improve the correlation of motility results to fertility.

3.5 AN EXAMINATION OF BOAR VARIATION

The previous experiments indicated that sperm motility during incubation in the TBM media varied extensively between samples. This may be due to random variation between samples or the result of true diversity between ejaculates. Additional variation may arise due to differences in the spermatozoa from the individual boars (owing to modifications of spermatogenesis or sperm storage in different individuals). This experiment used the data from section 3.4. to establish whether the motility characteristics of a sample were dependent on the animal from which the ejaculate was collected.

3.5.1 Experimental design

In trial 1, nine of the 27 boars had four or more of their ejaculates assessed. The data for these animals were used to evaluate the boar variation using ANOVA. The data for the last time point (6 hours) was not included in the analysis as the loss of motility by some of the ejaculates meant that few results were available at

this time. The boar effect on the spontaneous and induced acrosome reaction results was also evaluated for both trial 1 and trial 2. The latter contained six animals for which four or more ejaculates were assessed.

3.5.2 Results

ANOVA revealed that there was a strong effect of 'boar' on ALH measurements (see table 16 and figure 16.). This effect was more significant at the 0 and 2 hour time points than after 4 hours. LIN measurements after 2 and 4 hours also varied significantly between boars, but less so at the initial time point. The velocity measurements which appeared to be boar dependent were VCL at time 0 and VSL after 4 hours (both $P<0.02$).

The results from trial 1 indicated that there was no significant effect of 'boar' on the acrosome data gained after prolonged incubation, either before or after induction of the acrosome reaction. The same statistical treatment was applied to the data from trial 2, using the six animals with $n>4$ results. This indicated that the proportions of spontaneous and SZP-induced acrosome reactions both reflected the animal from which the semen sample originated ($P<0.0002$ and $P<0.0001$ respectively).

3.5.3 Summary

Measurements of sperm head movement (ALH) appeared to be dependent on the animal from which the ejaculate was collected. Data describing spontaneous acrosome reactions collected after 3.5 hours also appears to be animal dependent, however, this effect was not apparent when spermatozoa were sampled after 6 hours. Evidence of a 'boar-effect' on the population motility characteristics is encouraging as it indicates that consistent differences between animals exist, and this basic variation may prove to be related to animal fertility.

Table 16. Animal dependent CASA measurements.

MEASUREMENT	INCUBATION TIME	RANGE OF MEANS	P-VALUE
ALH (µm)	0	6.48 - 13.80	P<0.0001
	2	3.93 - 12.24	P=0.003
	4	4.46 - 10.97	P=0.015
LIN (%)	0	18.10 - 57.50	P=0.036
	2	21.90 - 73.90	P=0.013
	4	30.20 - 78.40	P=0.009
VCL (µm/s)	0	87.20 - 172.70	P=0.014
VSL (µm/s)	4	27.00 - 100.70	P=0.015
ACROSOME REACTIONS (%) -			
after 3.5 hours incubation†		8 - 83	P=0.00012
after co-incubation with SZP †		24 - 92.5	P<0.0001
after 6 hours incubation*		6 - 85	P=0.211
after addition of calcium ionophore*		26 - 96	P=0.116

CASA measurements and acrosome reaction data from boars with four or more ejaculates were analyzed using ANOVA. Significantly animal-dependent characteristics and the ranges of the sample means are shown in the table. See also figure 16.

*Acrosome reaction data from trial 1 (n=9 boars)

†Acrosome reaction data from trial 2 (n=6 boars).

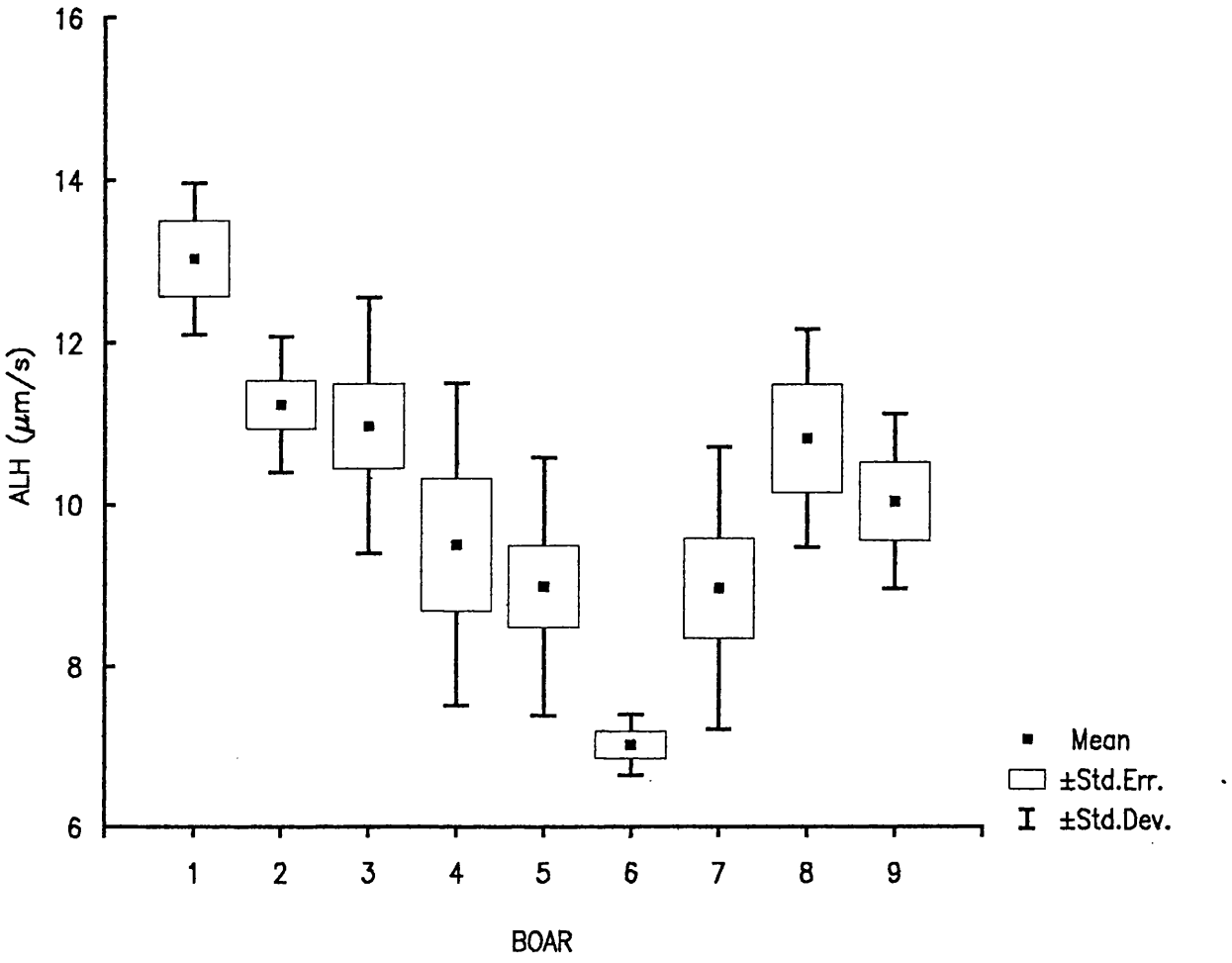


Figure 16. The variation of ALH measurements between boars. The CASA measurements from four or more ejaculates were compared across nine boars. Several measurements were found to be significantly animal-dependent (see table 16). The box-whisker plot above illustrates this variation between boars for one CASA measurement - ALH. Mean ALH and the standard deviation and standard error of the mean are shown for the nine animals.

3.6 DO LOWER FERTILITY BOARS HAVE DIFFERENT MEAN SPERM MOTILITY CHARACTERISTICS FROM THE SDS BOARS?

The boars maintained at Thorpe Willoughby are known to be highly fertile. (The fertility of each animal is estimated from the AI results that accumulate from the Pyramid herds and the SDS customers.) The ejaculates used in all the previous experiments were from boars that have at least a 78% conception rate and an average litter size (AVL) of 10.8 or more. By using ejaculates from less fertile animals it was hoped to extend the breadth of the analysis of sperm motility and fertility. Five boars from the Thorpe Willoughby SDS stud were used in this experiment, all considered to be sub-fertile by managers at the centre. These animals had a conception rate <78% (range 42 to 75%) and an average litter size (AVL) <10.8 (range 8.5 to 9.8) based on between 24 and 54 results. These were assessed using the same protocol employed for the normal ejaculates, and the results compared to the data from section 3.2. the preliminary assessment of boar sperm motility.

3.6.1 Experimental design

Semen samples (n=8 from five boars) were assessed after 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 minutes. At each time point sperm motility and the proportion of acrosome reacted sperm were evaluated. Population means for the CASA measurements were calculated for each of the samples at each of the time points.

3.6.2 Results

Owing to the reduction in sperm motility during incubation, by the fifth time point (120 minutes) only four 'normal' and four 'sub-fertile' ejaculates still contained sufficient numbers of sperm to be included in the analysis. Therefore the two sets of data were examined over this time period only.

The data revealed that sperm head movement may be greater in lower fertility ejaculates, (ALH measurements after 60 and 90 minutes were significantly higher in these ejaculates compared to the ejaculates from the SDS boars; see table 17). Sperm trajectories within the lower fertility samples appeared to be more erratic (reduced LIN and STR values). Velocity measurements were not

significantly different in the two groups, however there was a trend towards higher mean VCL measurements and lower mean VSL measurements in the low fertility samples. The proportions of acrosome reacted sperm were not significantly different in the two groups at any time throughout the incubation period.

TABLE 17. Sperm motility measurements found to be significantly different between SDS-stud and SDS-rejected boars.

MEASUREMENT	INCUBATION TIME	MEAN ± S.E.M. SDS BOARS	LOW FERTILITY	KRUSKAL WALLIS
ALH (µm)	60	5.27 ± 0.32	6.89 ± 0.30	P<0.02
	90	4.83 ± 0.19	7.01 ± 0.78	P<0.02
LIN (%)	90	70.58 ± 1.58	58.47 ± 4.37	P<0.05
	120	74.06 ± 2.29	56.65 ± 8.18	P<0.05
STR (%)	0	86.01 ± 1.10	79.27 ± 2.73	P<0.05
	30	88.59 ± 1.46	84.56 ± 1.52	P<0.05
	90	91.06 ± 1.46	81.72 ± 4.60	P<0.05

Semen samples from eight SDS-stud boars and five SDS-rejected boars were analyzed over a six hour period and the mean motility measurements compared. Owing to the loss of sperm motility during incubation, means and standard errors are constructed from a declining numbers of samples. Therefore the two sets of data were examined over 120 minutes only.

3.6.3 Summary

Mean sperm motility measurements within ejaculates from lower fertility boars and SDS boars were found to differ. The significant characteristics tended to reflect sperm trajectory rather than sperm speed.

3.7 CAN CLUSTER ANALYSIS BE USED TO DETERMINE HYPERACTIVATION CRITERIA ?

The definition of hyperactivation as an erratic, non-progressive motility is useful when sperm populations are being observed subjectively. When objectivity is desirable, specific criteria need to be defined. The following experiment used the motility characteristics of the sperm groups generated by cluster analysis to establish criteria defining hyperactivation. The criteria were then checked by identifying qualifying sperm using the HST.

3.7.1 Introduction

Sperm populations are highly heterogenous. Means of measurements for populations are commonly used in CASA as this is the simplest method of using the data generated. However, this approach may obscure information concerning small sub-groups within the main population, which may be functionally or diagnostically very important. Statistical techniques exist that will divide data into groups of like individuals, regardless of number, based on defined characteristics. These methods, known collectively as 'Cluster analysis', have been used extensively in the discipline of taxonomy to group organisms into classifications based on traits such as colour or morphology (see Afifi and Clark, 1990). Cluster analysis has also been used in medicine to assign patients to diagnostic groups based on their symptoms, and in andrology to group ejaculates or patients (Holt *et al.*, 1985; Drobnis *et al.*, 1988d; Oehninger *et al.*, 1992). The work described below investigated the use of cluster analysis to group the tracks of sperm within an ejaculate rather than the ejaculate *per se* and has been used previously only for the partitioning of human epididymal sperm (Davis *et al.*, 1991).

Cluster analysis produces a hierarchical tree in which similar tracks are brought together in sets, and these sets orientated to each other based on the similarity of the group characteristics. When the data contains a clear "structure" i.e. clusters of objects (sperm) that are similar to each other, then this structure will often be reflected in the hierarchical tree as distinct branches.

An alternative approach to cluster analysis is to force the data into a pre-defined number of groups (K-means clustering). This removes the need for a subjective division of the hierarchical tree into its major component branches, but

may result in the merging of distinct sets of tracks if the pre-defined number of groups is too small. Therefore it was considered more appropriate to use the hierarchical tree method for the splitting of sperm populations.

The cluster analysis program within CSS:STATISTICA allows a large number of variables to be employed in the grouping of the sperm. Previous experiments had shown the close correlation of many of the variables. Therefore only one velocity measurement was chosen to prevent the replication of information (VAP); ALH was used as it is highly correlated with BCF, LIN and STR, whilst MAD was also chosen as few of the variables were correlated with it and therefore provided a distinct source of trajectory information. This enabled 3-D scatterplots to be used to represent the groups produced by the cluster analysis, which greatly aided understanding of the data.

Prior to performing cluster analysis, it is advantageous to standardize variables. In standardization, all values of a selected variable are adjusted so that the sample values have a mean of zero and a standard deviation of one. This ensures that the cluster analysis uses relative rather than absolute differences between objects to establish classifications. Relative values are employed because the different measurements have different magnitudes of scales.

The first experiment was performed to verify that sperm in an ejaculate could be divided into natural groups using cluster analysis. The tracks of three sperm populations with distinct characteristics were combined to create artificially one mixed sperm population. This was then subjected to cluster analysis and the resulting population characteristics examined. Subsequently real sperm populations were split using the cluster analysis to establish whether natural sub-populations were present, and to investigate any changes in the populations that may occur during capacitation.

The analysis of artificial sperm populations was performed before the version 7 software had been written and therefore the data files upon which it is based were generated using version 6 software for the HST. This part of the experiment was performed to verify that cluster analysis could divide sperm populations with the CASA generated motility data; as part of the experimental procedure involves standardizing the data the actual values of the measurement are therefore irrelevant. It was felt that the results generated using version 6 software were a valid demonstration of the power of this statistical technique, and

held for the results generated with the version 7 software. The motility data generated is very similar to that collected using version 7 software, however MAD values appear to be smaller.

3.7.2 Cluster analysis of artificial sperm populations

Three sections of video-tape were used in this preliminary experiment, each of two minutes duration. In the first, approximately half of the sperm were swimming slowly in small circles; in the second, sperm movement was linear and rapid; in the third, the paths of the sperm were erratic and non-progressive. These populations are referred to as CIRC, PROG and ERR respectively. The sections of video tape were processed through the HST and the VAP, MAD and ALH measurements of the individual sperm data from the three sections combined. The artificial data set was then standardized and subjected to cluster analysis.

3.7.3 Results

Four groups could be identified in the hierarchical tree, the mean measurements of which are shown below. For each track, the original section of tape from which the track was derived was compared to the cluster to which it was assigned. Figure 17. shows this comparison of the original and assigned populations. In each of the four groups created, the majority of members come from one of the original tape sections only.

The sperm tracks from the CIRC tape section are the major components of both group A and D. In addition to the sperm with slow circular tracks, this section of tape contained some sperm with the rapid, linear paths typical of activated boar sperm. (Although the trajectories of the sperm in this latter group were progressive, the velocity of these cells was visibly less than that of the sperm recorded in the PROG tape section.) It is reasonable to suppose that groups A and D represent the 'linear' and 'circular' sperm. The data in table 18 suggests that group D contains the faster (>VAP) sperm, i.e. the sperm with rapid, linear tracks. This is supported by the inclusion in this group of many sperm from the other two tape sections, both of which contained typical activated sperm in addition to the more extreme swimming patterns.

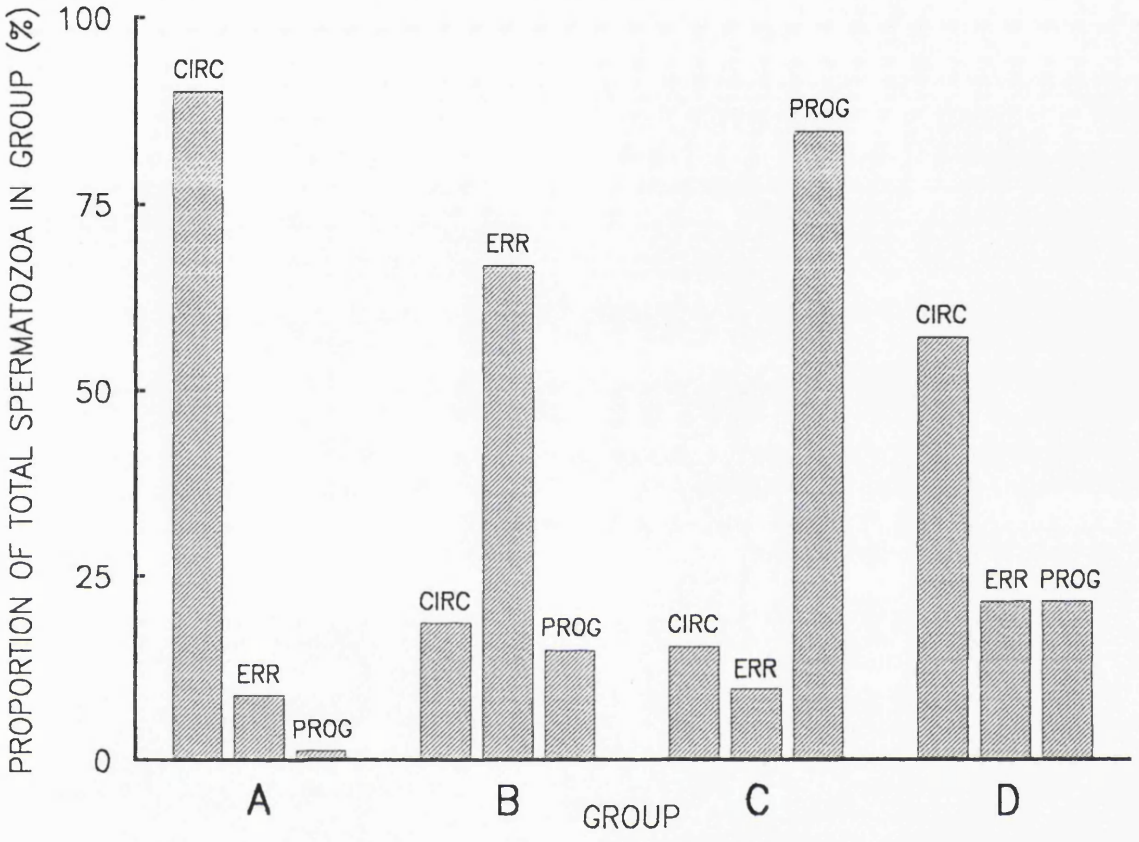


Figure 17. The partitioning of an artificial sperm population using cluster analysis. The four groups A-D were obtained by cluster analysis of data from three video-recordings of sperm populations (CIRC, ERR or PROG, see text). The four groups were then analyzed to reveal from which video-recording each member originated. Sperm tracks in groups A-C came predominately from one each of the three video-recordings whilst group D probably represents activated sperm (see text). The CASA measurements of each of the four groups A-D are described in table 18.

Table 18. Sperm groups identified from the artificially created sperm population using cluster analysis.

GROUP	n*	VAP (μm/s) MEAN and RANGE	MAD (°) MEAN and RANGE	ALH (μm) MEAN and RANGE
A	80	46.03 (26-78)	49.44 (12-99)	3.84 (0-22)
B	27	76.00 (42-131)	122.04 (90-171)	3.44 (0-11)
C	52	97.75 (53-136)	35.54 (9-90)	14.19 (1-27)
D	93	77.95 (53-104)	54.89 (11-96)	5.26 (0-16)

Motility characteristics of the four sperm groups identified using cluster analysis. Sperm were clustered using their VAP, MAD and ALH characteristics (see text) and the tape section from which each sperm originated and the cluster to which it was assigned compared (see figure 17).

* n is the number of sperm within each group.

Therefore it appears that the cluster analysis did indeed split the sperm into four groups A-D, representing the circular, erratic, progressive and activated sperm populations respectively. These classifications are based on the origin of the members of the group and the mean motility characteristics they produce.

The cluster analysis of this artificial population was repeated using the parameters VSL, ALH and LIN on the premise that these variables would be useful indicators of hyperactivated motility in a real population. The members of the groups obtained using these criteria did not tend to originate from one particular tape section, and the groups formed did not match those obtained previously using the cluster analysis (data not shown).

3.7.4 Cluster analysis of real sperm populations

The track results from eight semen samples were chosen at random from the data from trial 1, (see section 3.4.). Cluster analysis was performed on the data from each semen sample at each of the four sampling times (after 0, 2, 4 and 6 hours incubation in the TBM media). The resulting groupings of the sperm were examined to investigate whether new populations emerged during sperm incubation.

The same statistical procedures used to group the artificial data set (described in section 3.7.2 above) were applied to the track data from the eight semen samples. The data for each ejaculate at each time point was standardized

and cluster analysis performed using the variables VAP, ALH and MAD to cluster the sperm data. The resulting hierarchical trees were subjectively split into groups and the mean measurements for each of the clusters recorded.

3.7.5 Results

The number of groups identified for each analysis varied between 3 and 6. The mean measurements of the groups were used to produce 3-d scatterplots for each ejaculate at each time point. Figure 18. shows an example of the four plots for one ejaculate. The percentage of the total sperm population in each group was calculated, and the positions of the sperm groups compared across the ejaculates.

For each ejaculate the number of groups diminished as time progressed. Of the eight original samples, five satisfied the n>25 criteria after 6 hours (each of the scatterplots for this time-point contained four groups). No consistent pattern was seen in the positions of groups in each scatterplot, or the emergence of groups as time progressed.

These scatterplots conveyed only information regarding the VAP, ALH and MAD of the clusters. Subsequently the mean VCL, VSL, ALH and LIN values for each of these cluster from the eight ejaculates were examined. Hyperactivated sperm are characterized by a high VCL, low VSL (as the sperm are non-progressive), low LIN and possibly increased ALH. It became apparent that in the majority of the samples (7/8) a high mean VCL (>100μm/sec), low mean VSL (<42μm/sec), low mean LIN (<38%), high mean ALH (>10μm) group existed at one or more of the time points. Therefore groups with disparate VCL/VSL/LIN means have been identified although the cluster analysis was actually performed with the variables VAP, MAD and ALH.

The track measurements recorded for the individual sperm within the high VCL, low VSL, low LIN clusters were broadly distributed. This suggests that the types of motility displayed by the members within a group were not homogeneous. To establish whether these groups contained a distinct population of sperm, perhaps hyperactivated, one of the tape sections which contained a 'high VCL/low VSL/low LIN' group was re-examined visually. The mean and range of the VCL, VSL, ALH and LIN for the six sperm in this cluster are shown in Table 19.

Table 19. The motility characteristics of sperm identified as hyperactivated through the use of cluster analysis.

VARIABLE	MEAN	RANGE
VCL (μm/sec)	123	104-132
VSL (μm/sec)	32	5-70
ALH (μm)	13.2	9-19
LIN (%)	25	5-54

The six spermatozoa which this data represents were the members of a cluster with high VCL, low VSL, low LIN characteristics. The data was clustered using the measurements VAP, MAD, and ALH but in 7/8 semen samples one or more groups with similar VCL/VSL/LIN/ALH characteristics to the above were produced.

Sperm that conformed to these criteria were identified from the video-tape using the 'Select' facility of the HST. (The 'Select' feature distinguishes spermatozoa complying to user-defined criteria by a red, rather than green track on the image screen of the computer system. The criteria used were VCL $\geq$ 100 μm/sec, VSL $\leq$ 70 μm/sec, ALH $\geq$ 9 μm and LIN $\leq$ 55%). The sperm identified in this manner were (1) non-progressive, with an erratic, thrashing movement and (2) progressive with a very wide lateral head movement. Figure 19. shows parts of the trajectories of these sperm, produced using the 'Trail Draw' facility of the HST. By replaying the section of video-tape it was verified that all the sperm with type (1) trajectories were highlighted by the 'Select' facility. This could not be ascertained for the type (2) motility as it was difficult to distinguish unequivocally sperm with this type of movement.

3.7.6 Summary

The ability of cluster analysis to group spermatozoa according to their motility characteristics was verified using artificially created sperm populations. Cluster analysis was then used successfully to identify hyperactivation in real sperm populations.

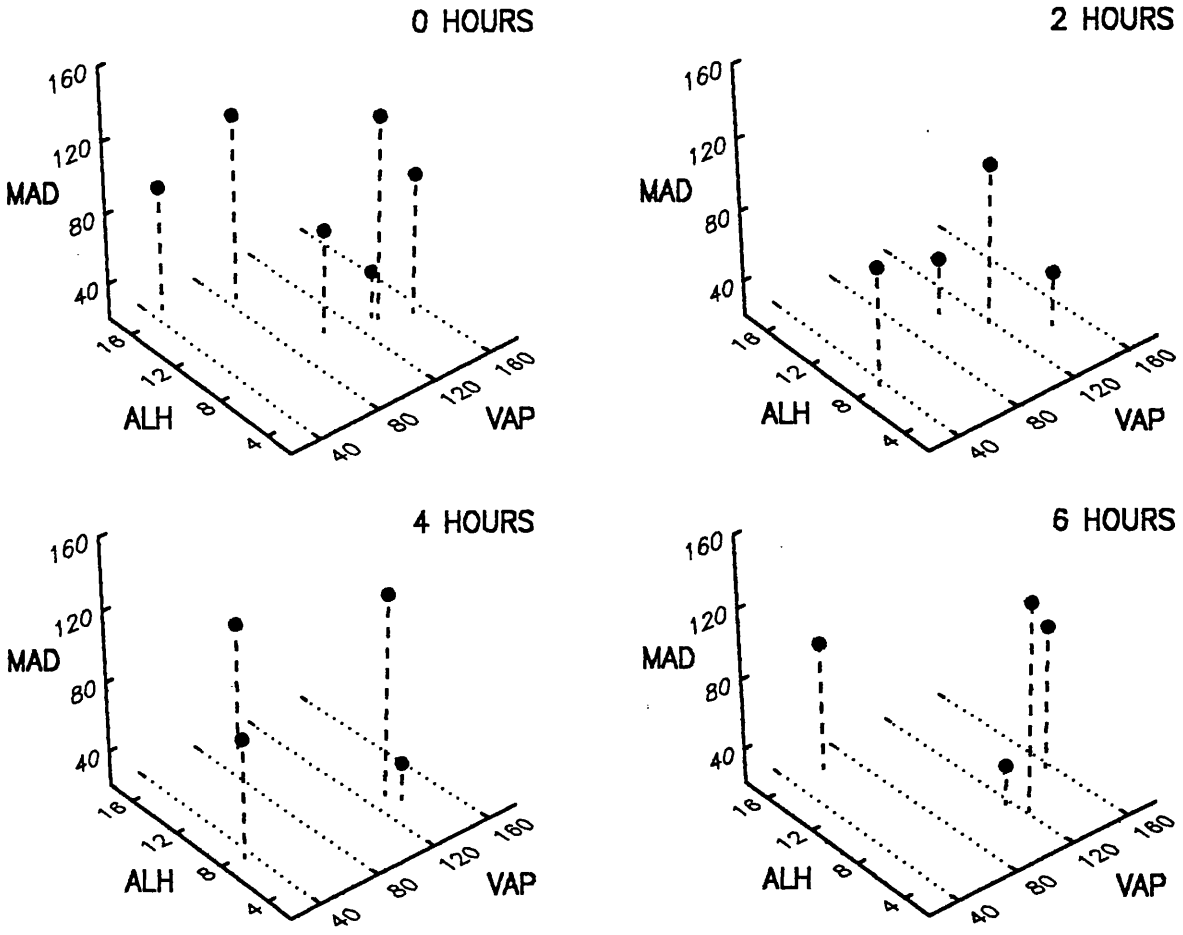


Figure 18. The division of sperm populations using cluster analysis. This figure shows four scatter plots illustrating the sub-populations of sperm present in an ejaculate after 0, 2, 4 and 6 hours. CASA data were partitioned using the variables VAP, MAD and ALH, and the resulting sub-populations compared across eight ejaculates (see text).

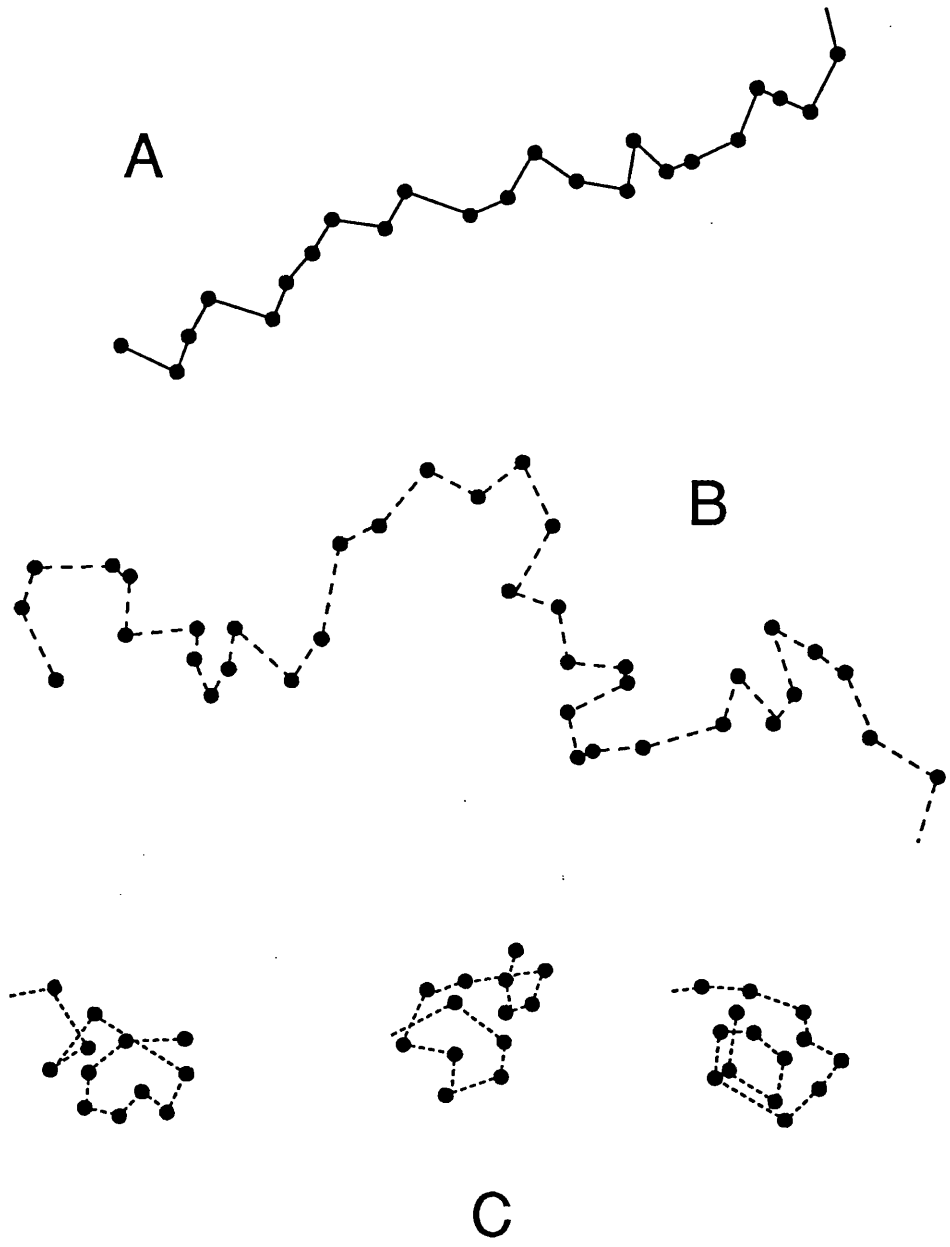


Figure 19. Trajectories of activated and hyperactivated boar spermatozoa. Figure 19a shows the path of an activated boar sperm, produced using the 'Trail Draw' facility of the HST (see text). Sperm trajectories conforming to the hyperactivation criteria derived in section 3.7.5 (VCL ≥ 100 µm/sec, VSL ≤ 70 µm/sec, ALH ≥ 9 µm and LIN ≤ 55%) are illustrated by figures 19 (b and c). Figure 19c represents spermatozoa with non-progressive, erratic, thrashing movement, whilst figure 19b represents progressive spermatozoa with a very wide lateral head movement.

3.8 DISCUSSION

The purpose of the experimental work described in this chapter was to investigate whether the Hobson Sperm Tracker would be a useful tool for the prediction of ejaculate fertility. The hypothesis that measures of sperm motility made over several hours are more informative than a single assessment was examined. Using this approach, the characteristics of the sample at a particular time and the change in sperm motility can be evaluated. The results described above suggest that this is an important consideration when developing prognostic tests of fertility based on sperm motility.

The results from the preliminary experiment indicated that the response of different sperm samples to incubation in the TBM media varied greatly. Experiments described in section 3.5 of this chapter examined this variation between the different boars and found that the animal from which the ejaculate originated affected the motility result obtained. Evidence of a 'boar-effect' on the population motility characteristics is encouraging as it indicates that consistent differences between animals exist, and this basic variation may prove to be related to animal fertility.

COMPARISON OF THE MOTILITY DATA WITH PREVIOUS CASA STUDIES OF BOAR SPERM.

The motility data from this experiment compares well with previous studies. A recent paper included measurements of boar sperm made using a Hamilton Thorn system (Grant *et al.* 1994) and many of the results are similar to those gained here. Percoll washed sperm were analyzed after incubation for 0, 1, 3 and 5 hours in a TALP media at 37°C. The resulting means fell in the following ranges; VCL 107.0 to 122.7 µm/s; VAP 95.8 to 113.4 µm/s; VSL 83.3 to 105.3 µm/s; BCF 7.7 to 11.0 Hz. Mean values for ALH measurements are not stated but the distribution is shown graphically and values are distributed normally between 1 and 10 µm. Unfortunately, there is no indication of the number of samples used in the experiment or the number of sperm tracked.

In contrast to the results of the present study, Grant and co-workers reported that mean velocity measurements are maximal after 3 hours incubation. This could reflect the different media and incubation temperatures used in the two studies. Cell viability data is not available for comparison, however, their data on

the proportion of motile cells shows a decline from 80.0% initially to 67.4% after 5 hours incubation. The proportion of live cells in the TBM media at the same time points are 88.4% and 66.9% respectively. Even if the assumption is made that all immotile sperm are non-viable, these results suggest that TBM and TALP are similar in their ability to support sperm viability.

Other studies have reported a mean VSL of 58.8µm/s and a mean VCL of 70.7µm/s for boar sperm in a HEPES based media (Suarez *et al.*, 1992). Although the VCL values generated in this experiment are far higher than those of the Suarez report, the VSL values compare well. A velocity measurement of 49.5µm/s and a linearity measurement of 7.83 was reported for boar sperm in extender by Aumüller and Willeke (1988), however, the type of velocity measurement was not identified. Boar sperm velocity, in fresh semen was estimated to be between 35.6 and 38.6µm/s (Reibenwein 1989). In the latter case, the relatively low (unspecified) velocity measurement probably reflects the media in which the sperm were recorded. The increased viscosity of fresh semen compared to salt solution media would reduce the recorded cell velocities (Amann and Hammerstedt., 1980).

SPERM MOTILITY DURING CAPACITATION

Boar sperm populations incubated in the TBM media appear to undergo the following changes. Their overall velocity decreases (reduction in VCL); however, owing to alterations of the sperm path, VAP and VSL values do not decline. These changes involve a straightening of the overall sperm trajectory, as indicated by the increase in LIN and STR measurements. Similarly the decrease in mean ALH values demonstrates a reduction of the lateral movement of the sperm. These results contrast with previous studies of sperm motility during capacitation which have indicated an increase in sperm velocity and lateral head movement and a decrease in path linearity (human: Morales *et al.*, 1988; Mortimer *et al.*, 1984; hamster: Suarez *et al.*, 1991a). Such changes are associated with increased sperm hyperactivation within the capacitating population. The disparity between the reports for human and hamster sperm and for boar sperm may reflect the incidence of hyperactivation in these species. Hyperactivation of human and hamster sperm has been extensively characterized (for reviews see Katz *et al.*, 1993; Yanagimachi, 1994) whilst comparatively few accounts exist of boar sperm hyperactivation (Nagai *et al.*, 1984; Saxena *et al.*, 1986; Hamano *et al.*, 1989;

Suarez *et al.*, 1992).

Alternatively, the apparent smoothing of the sperm path may be a reflection of the increased number of agglutinated sperm. Where several motile cells are joined, the individual forces of the sperm appear to reduce the small oscillations typical of an individual cell. However, the distributions of the motility measurements do not appear to be bimodal suggesting that the measurements for single cells and for agglutinations overlap.

SPERM AGGLUTINATIONS

Several authors have reported the binding of boar sperm to debris, and the more specific head to head agglutinations found in these experiments (Dacheux *et al.*, 1983; Suarez *et al.*, 1991b), and similar reports exist for other species (Bull - Pollard *et al.*, 1991; Ram - Dott *et al.*, 1979; Dacheux *et al.*, 1983; Rabbit - Bedford, 1970; Hamster - Robbins and Boatman, 1988). Dacheux and co-workers reported that on dilution epididymal sperm from both boar and ram showed head to head agglutination. This grouping of the sperm appeared after 15 minutes and was maximal after 1 hour incubation. A similar time-course for the agglutination was observed in the experiments in the present study. It was suggested that sperm may be prevented from agglutinating *in vivo* by the presence of an anti-agglutination compound(s) in the cauda epididymidis and ejaculate, and that dilution of this compound precipitates agglutinations. The washing protocol used in this chapter employs a Percoll gradient which would ensure the separation of the sperm from such semen components.

The E.M. results reveal the very close association of agglutinated sperm and confirm that sperm in agglutinations are capable of undergoing the acrosome reaction (as indicated by the PSA-FITC staining). They also suggest that after exocytosis the sperm subsequently become less closely associated. This may be the cause for the observed decrease in the proportion of agglutinated sperm towards the end of the incubation period.

CORRELATIONS BETWEEN THE MOTILITY MEASUREMENTS

The observation that many of the HST measurements are highly correlated is important for future statistical analyses. Several measurements were consistently correlated and in some instances this reflects the use of one

measurement in the derivation of another (for a review see Boyers *et al.*, 1989). For example, the VSL of the sperm path is a reflection of both the curvilinear velocity and the linearity of the trajectory; and the high correlation of VSL to both these variables is consistent with this fact. Similarly, STR describes the straightness of the average path and the high correlation of VAP and STR is therefore unsurprising. VCL is not correlated to the LIN/STR measurements because the curvilinear velocity is independent of the trajectory of the sperm.

Both VAP and VSL are derived from the curvilinear velocity of the sperm and are highly correlated to VCL and to each other. Logically, there are only two situations in which the velocity measurements might not appear correlated:-
(1) if a spermatozoon was swimming in a circle (in which case the VSL becomes a reflection of the position of the sperm when the tracking starts and finishes) or
(2) if most sperm in a population were swimming in an extreme zig-zag motion (the VCL would be associated with a relatively low VSL).

Many of the correlations between CASA measurements were only significant in a sub-set of the samples. This inconsistency makes it unsafe to generalize about sperm populations using data from only one ejaculate (for example - the examination of CASA measurements in Katz and Davis, 1987; Liu *et al.*, 1991). It is unclear why measurements may be highly correlated in one sample but not another. Technical error could be responsible, however, the consistent relationship between the velocity variables and linearity measurements suggests that the preparative techniques used were accurately reproduced for each sample.

Based on the results from this preliminary experiment, successive experiments assessed samples every two hours for a period of six hours. This protocol meant that changes in sperm motility could be examined thoroughly without the use of impractical sampling regimes.

MOTILITY CHARACTERISTICS BEFORE AND AFTER LIQUID STORAGE.

For maximum efficiency, the commercial SDS companies need to assess fresh ejaculates prior to addition of extender. Sperm characteristics are significantly affected by storage procedures and the quality of the fresh ejaculate may correlate poorly with the inseminated sample (Weitze, 1990). The semen samples assessed in the fertility trials were liquid-stored in BTS for approximately 24 hours prior to insemination. Assessing semen samples in a similar condition

as the inseminate (i.e. after the relevant storage procedures) may provide more useful information for predicting sperm fertility than assessment of the fresh ejaculate. This hypothesis is supported by studies of cryopreserved human sperm used for assisted reproduction (Paraskevaides *et al.*, 1991; Marshburn *et al.*, 1992). Therefore in the fertility trials, motility measurements were made of liquid stored semen.

In section 3.3 boar ejaculates were examined before and after liquid storage and the motility data was found to be highly correlated at the first time point only. Many of the pairs of motility measurements were significantly correlated after 0 and 4 hours incubation in the TBM media but not after 2 hours incubation. This may be a reflection of the presence of agglutinations after 2 hours incubation that are subsequently reduced at the 4 hour sampling time. The smoother trajectories of the agglutinations reduce the range of motility patterns recorded and may obscure any significant correlations between the measurements at this time.

The fertility trials described in section 3.4 indicate that sperm velocity characteristics over two hours are significantly correlated to fertility. This observation has been made using extended samples and may not be repeated when samples are assessed prior to liquid storage. It may prove more informative, though less efficient, for SDS companies to examine samples after preservation in extender.

Only the VAP, VSL and STR measurements at the first time point were significantly different in the fresh and extended samples. The stored samples had faster average path velocities and straighter trajectories at this time, a trend which continued throughout the experiment. The reason for this is unclear although it may be a result of poorly motile cells dying during the 24 hours storage and therefore being removed from the subsequent analysis. (Sample purification with Percoll would remove immotile and poorly motile sperm from the fresh specimen but would leave any population that was above a minimal but critical threshold of motility. If the motility of the sperm in this sub-population became poorer during storage, these cells would be removed when the stored samples were subsequently purified. Assuming that the rest of the sperm in the semen sample have retained their original motility, the result would be a faster sperm population.) However, comparison of the distributions for VAP and VSL before and after storage revealed a population shift towards greater velocities, rather than the loss

of a sub-population of sperm. In addition, VCL results were not significantly different in the two sample groups. This suggests that storage affects the trajectories of sperm, straightening the sperm path and so increasing VAP and VSL without an alteration in VCL. Previous reports have indicated that sperm velocity is maintained (Jasko *et al.*, 1991) or reduced (Malmgren *et al.*, 1992) during liquid storage, however, the type of velocity measurement is not specified in either study.

FERTILITY DATA

The number of fertility results supplied by the Pyramid herds was smaller than had been anticipated originally. This was due to an unforeseen and rapid change in farming practices over a three year period from the initiation of this project in 1991. The incidence of 'Pure AI' inseminations (using semen from only one ejaculate) has fallen substantially concomitant with an increase in the use of 'Pooled AI' (inseminations use extended semen from more than one ejaculate or combine AI with natural service). Combining semen from different ejaculates has been shown to increase conception rate by 8-10% (H. Reed, *pers comm*).

Owing to this increased use of pooled semen, the number of fertility results available for this research was severely reduced. The relatively small amount of fertility information should decrease the probability of correlations between sperm motility and fertility appearing significant (as the confounding influences of herd management and sow fertility cannot be partitioned accurately). However, significant correlations between sperm parameters and the available AI results were established. This suggests that a strong relationship exists between sperm motility and fertility which warrants further research. The experiences of the present study suggest that future experiments should ensure that:
(1) specific herds of sows are dedicated to 'Pure AI' matings and
(2) the number of inseminations per ejaculate is monitored.

SPERM MOTILITY AND FERTILITY

In trial 1, sperm velocity after two hours was the variable most significantly correlated to litter size whilst ALH after 2 hours was the measurement most closely correlated to conception rate. In trial 2, derived variables describing changes in sperm velocity over the first two hours of incubation were the factors most indicative of ejaculate fertility. It is encouraging that sperm velocity during the

initial two hour incubation period has been highlighted by separate fertility trials, however, why the emphasis is on different factors within this period is unclear.

Previously, the ALH and velocity of human sperm have been found to correlate significantly with the outcome of IVF or AID. (ALH - Jeulin *et al.*, 1986; Barlow *et al.*, 1991; Davis *et al.*, 1991; Marshburn *et al.*, 1992; Zouari *et al.*, 1993; Velocities - Holt *et al.*, 1985 and 1989; Chan *et al.*, 1989; Barlow *et al.*, 1991; Liu *et al.*, 1991; Davis *et al.*, 1991; Marshburn *et al.*, 1992; Oehninger *et al.*, 1992; Zouari *et al.*, 1993). Fewer studies report the absence of significant correlations between sperm kinematic variables and fertility (Hinting *et al.*, 1989; Grunert *et al.*, 1989).

Surprisingly, few reports exist of comparable studies using species other than the human. Significant correlations of CASA motility measurements with fertility (AI/IVF results or competitive fertility indices) have been described (bull - O'Connor *et al.*, 1981; Amann, 1988 and 1989; stallion - Andersson *et al.*, 1992; rabbit - Farrell *et al.*, 1993), however further studies have found no association between these parameters (bull - Budworth *et al.*, 1988; Bailey *et al.*, 1994).

Sperm velocity and ALH have been consistently highlighted as the most useful kinematic indicators of semen quality, however, the reasons for this are unclear. Both measurements may reflect sperm thrust and hence the ability of the sperm to pass through the female tract and reach the oocyte. Aitken and co-workers (1985) reported that the ALH of human sperm is significantly correlated to progression through cervical mucus, and concluded that sperm require adequate lateral head displacement to push aside the mucus microstructure. Subsequent penetration of the cumulus and zona pellucida requires the expression of specific sperm movement patterns and a significant element of thrust (Drobnis *et al.*, 1988a and b).

It is possible that the motility variables highlighted in these studies do not affect sperm fertility, but are indirectly quantifying secondary characteristics such as morphology or viability. Sperm morphology is known to affect motility (Katz, *et al.*, 1982) and mean sperm velocity may reflect the proportion of morphologically normal cells within an ejaculate. Several studies have highlighted the significant relationship between sperm morphology and fertility (e.g. Kruger *et al.*, 1986; Liu and Baker, 1988). Alternatively, motility measurements made after prolonged incubation may indicate the vigour of sperm samples. Prior to fertilization, sperm

may reside within the female tract for several hours (Hunter 1984). If spermatozoa are unable to remain viable over this period, the probability of conception will be reduced. In the present study, sperm incubation for 2 hours resulted in the significant correlation of sperm motility with fertility. Previous research has also indicated an association between the maintenance of sperm velocity during incubation and the *in vivo* fertility of human sperm (Holt *et al.*, 1989). However, at least one report has indicated that correlations of sperm movement with IVF outcome are more significant after a period of incubation (Zouari *et al.*, 1993). Whilst artificial insemination techniques assess sperm survival in the female tract, *in vitro* fertilization will not. The benefits of measuring sperm motility after prolonged incubation may therefore reflect a different component of sperm fertilizing ability.

SPERM SELECT® AND SPERM MOTILITY

The results from trial 1 indicated that sperm velocity may be associated with sperm fertility, therefore in trial 2 attempts were made to improve the expression of this relationship. It was reasoned that increasing the resistance of the media to sperm movement might help discriminate between sperm with good or poor thrust.

The results did not support this hypothesis; measures of sperm motility in high relative viscosity media proved to be poorly correlated with fertility. However, addition of the Sperm Select® to samples reduced sperm concentration to approximately 10×10^6 sperm/mL and the HST set-up may have been sub-optimal (see chapter 2.).

Addition of the Sperm Select® changed sperm movement, slowing and straightening the path of the sperm. The observed increase in VSL at the initial time point probably arises because straightening of the sperm path increases VSL, outweighing drag effects on the sperm speed. The HST measures of angularity support this hypothesis, both LIN and STR measurements became closer to 100%. ALH values were decreased in media with Sperm Select® added, possibly due to increased resistance against the thrust of the sperm head, whilst the observed increase in BCF values may reflect the inverse relationship of ALH and BCF. Similar effects on sperm velocity, progressiveness (STR) and beat amplitude have been reported for hamster sperm incubated in viscous Ficoll solutions (Suarez *et al.*, 1991).

The motility of sperm samples in the presence and absence of hyaluronic acid tended to be poorly correlated. This suggested that the Sperm Select® was affecting individual ejaculates differently, and the ANOVA results indicated that this was an animal-dependent effect. The results from section 3.5 had already indicated that the motility results collected were strongly affected by the animal from which the ejaculate originated, specifically ALH, and acrosome reaction data before and after addition of the solubilized zona pellucida (SZP; see below). Previously the motility parameters of bovine and canine sperm have been shown to be animal dependent (Budworth *et al.*, 1988; Ellington *et al.*, 1993 respectively).

LOW FERTILITY ANIMALS

Ultimately the results of this research have to be extrapolated for use by the commercial SDS operators. Collecting and analyzing data from SDS stud boars, rather than from an unselected population, greatly facilitates this translation of the research results into a practical tool. However, analysis of the motility results from SDS-rejected boars enabled a contrast to be made between low and high fertility animals.

Although the number of lower fertility animals was limited for this experiment, a trend towards lower mean VSL values was evident in the group of SDS rejected animals. In addition, ALH was found to be greater in the lower fertility animals after 1 and 1.5 hours incubation. This variable was positively correlated to conception rate within the logistic regression models described in section 3.4.

Animals suspected of being low fertility are quickly removed from the SDS centres for obvious economic reasons. The classification of these animal as 'low fertility' was based on the fertility results collected prior to removal of these animals from the stud. The number of inseminations performed using semen from these animals is relatively small and therefore the validity of these findings is uncertain and should be treated with caution.

SPONTANEOUS AND INDUCED ACROSOME REACTIONS

Several reports have indicated that the assessment of spontaneous and/or induced acrosome reactions is important in the clinical evaluation of human sperm samples (Spontaneous - Marshburn *et al.*, 1992; Takahashi *et al.*, 1992; Induced -

Cummins *et al.*, 1991; Marshburn *et al.*, 1991; Pilikian *et al.*, 1992 Pampiglione *et al.*, 1993; Both - Fénichel *et al.*, 1991). However, in the present study a significant relationship could not be established between spontaneous or induced boar sperm acrosome reactions and fertility results.

Both spontaneous and SZP-induced acrosome reaction results from trial 2 were found to be significantly animal-dependent. Interestingly, ANOVA of the acrosome reaction results collected after six hours incubation showed no effect of 'boar' on the proportion of exocytosed cells. This may indicate that the rate of spontaneous acrosome reactions varies for different animals and sampling specimens after 3.5 hours incubation may highlight this animal dependent rate. After six hours incubation, the frequency of exocytosis may converge across the samples and mask any variation between individuals. Unfortunately the data available did not allow further investigation of this observation.

PSA-FITC labelling proved to be a simple, convenient technique for the assessment of sperm acrosome reactions. Although the zona pellucida is a glycoconjugate and binds the PSA-FITC, we found that the lectin could be used to detect sperm acrosome reactions in the presence of solubilized zona pellucida glycoproteins (section 3.4 and chapter 4). Acrosome-intact sperm labelled in the presence or absence of SZP displayed intense fluorescence over the sperm head. PSA-FITC binds to the acrosomal contents (Cross *et al.*,1986); in samples containing SZP there will be additional labelling of this region due to the presence of the zona ligands (Yurewicz *et al.*, 1993). Disruption of the acrosomal contents and overlying membranes during exocytosis produced patchy, irregular labelling and reduced the fluorescence over the anterior head. At the magnifications used (x1000) this staining pattern could be visualized in both the presence and absence of SZP.

CLUSTER ANALYSIS

CASA systems have been developed to assess sperm movement objectively and to improve the accuracy and comparability of sample evaluations. In contrast, previous attempts to define hyperactivation criteria for routine CASA analysis have used subjective identifications of sperm movement (for a review see Katz *et al.*, 1993). The criteria defined in these studies do not reflect a 'gold standard' for hyperactivation, instead they are dependent on the CASA system used, the

preparation protocols employed and the subjective classification of a cell as hyperactivated. Application of criteria developed in one laboratory to samples assessed elsewhere may prevent the accurate assessment of hyperactivation (Young and Bodt., 1994; Zhu *et al.*, 1994).

The last section of this chapter investigated the use of a statistical technique to help discriminate hyperactivated sperm populations objectively. Subjectivity is involved in the initial division of the hierarchical trees, however, the actual pattern of sperm movement in each cluster is not known at this point (i.e. this division is made 'blind', without knowledge of the groups being created). The HST selection criteria for hyperactivated sperm can then be derived from these clusters without prior observation of the sperm movement biasing the choice of parameters.

To validate the application of cluster analysis, artificially generated sperm populations were used to assess whether the combination of CASA tracking and statistics could accurately cluster similar tracks together. The close association between the original and assigned groups for the majority of the track data confirmed this was possible. The original sections of tape were from genuine samples and as such contained some sperm that did not conform completely to the overall population characteristics. Hence some sperm with slow, linear trajectories were visible within each tape section. This explains why the derived groups are smaller than the original groups and accounts for the formation of a fourth group.

Dividing the artificial population using the variables VSL, ALH and LIN produced very different results from the original clustering using VAP, ALH and MAD. This suggests that the groups obtained are dependent on the variables used to differentiate the population, and care is needed when deciding which variables to choose.

When the technique was applied to real boar sperm samples, between 3 and 6 distinct groups were formed (the cluster analysis of human sperm produced a similar number of sub-populations (Davis *et al.*, 1991)). The 3-d scatterplots from the ejaculates were compared but no consistent positioning of the groups within the scatterplot could be discerned. However, by studying further motility measurements of these clusters, groups with the characteristics of hyperactivation were found to exist. Subsequently these sub-populations were identified on the original video-recordings of the sperm samples and found to display the characteristics of hyperactivation (Suarez *et al.*, 1992; Katz *et al.*, 1993;

Yanagimachi, 1994)

The two patterns of sperm movement identified in the cluster were similar to the star-spin and transitional motility previously described by Robertson and co-workers for human sperm (Robertson *et al.*, 1988a). It was possible to split the two populations by altering the HST 'Select' criteria slightly, and sperm with circular/star-spin trajectories were isolated in this manner (data not shown). However, the principle behind using cluster analysis was to identify hyperactivated sperm without simply changing criteria until the required sperm were isolated. Therefore this observation was not pursued.

Clinical studies suggest that hyperactivation may be an important indicator of fertility (Wang *et al.*, 1993; Zouari *et al.*, 1993; Mackenna *et al.*, 1993). These results indicate that hyperactivation can be identified by cluster analysis and that this statistical technique is a realistic method of dividing sperm populations based on their motion characteristics. Unfortunately, using the process proved to be very time-consuming and the methodology could not be applied to large numbers of ejaculates. Changes in computer software would increase the efficiency of the process and make the objective identification of hyperactivated sperm a realistic goal.

3.9 SUMMARY

Boar sperm motility during incubation in a capacitating medium was assessed using the Hobson Sperm Tracker. Results indicate that sperm motility, specifically velocity, over the first two hours of incubation is correlated to ejaculate fertility. These results were obtained for extended semen and although some characteristics are significantly correlated before and after storage it is not safe to assume that a similar relationship exists for fresh ejaculates. Increasing the viscosity of the media using Sperm Select® did not improve the correlation of sperm velocity to fertility. Animal-dependent variation was found in the response of ejaculates to incubation in media and media with the hyaluronic acid added.

Sperm populations were successfully split using the statistical technique 'Cluster Analysis'. Hyperactivated sperm were identified from the clusters produced, however, it was not possible to recognise other consistent groups within the sperm samples. This technique allows criteria for hyperactivation to be developed without the subjective classification of cell motility.

CHAPTER 4 - THE EFFECT OF ZONA PELLUCIDA GLYCOPROTEINS ON SPERM MOTILITY

This chapter describes experiments which were designed to investigate whether zona pellucida glycoproteins affect the movement patterns of spermatozoa. Methods to separate and solubilize the zona pellucida were established and the isolation of the glycoproteins verified. The binding sites of the glycoprotein on the sperm surface were identified using immunocytochemistry. The effect of the solubilized components of the zona pellucida on sperm motility was examined and subsequently the role of calcium in this system investigated.

4.0 INTRODUCTION

A spermatozoon may change motility pattern several times during its journey through the female tract (for a review see Katz *et al.*, 1989; Suarez *et al.*, 1990). Sperm movement alters when traversing the cervical mucus, the cumulus oophorus and the zona pellucida. The stimuli which cause these changes in motility are unclear. Simple physical factors such as the viscosity of the surrounding fluids may be responsible; alternatively specific signalling pathways could be involved.

Sperm penetration of the zona pellucida can be examined with light microscopy, and work from Drobnis' group in America indicates that hamster sperm exhibit qualitatively different motility as they penetrate this layer (Drobnis *et al.*, 1988a,b,c). Flagellar movement within the zona matrix was found to be bimodal; symmetrical, low amplitude, high frequency sinusoidal beats alternate with non-propagated, low frequency extreme principal bends[3]. The low frequency, high amplitude beats are asymmetric due to the lack of a complementary reverse bend and result in a 'lever' motion of the sperm against the zona pellucida. This is thought to increase the stresses against the zona material. Drobnis and co-workers estimated that this lever motion could result in forces of up to 2700 µdyn being exerted against the zona, far higher than previously suggested (Drobnis *et al.*, 1988b, Ishijima and Mohri, 1985). Interestingly extreme flagellar bends are observed during hamster sperm hyperaction but these are extreme reverse bends

[3] Hamster sperm possesses the falciform head shape common to all rodent sperm, easily observable with light microscopy. The hook-shaped nucleus confers an asymmetry to hamster sperm, enabling directionality to be assigned to the flagellar bending termed principal and reverse bending - a bend moving the flagellum towards the convex surface of the head is a principal bend (Ishijima and Mohri, 1985).

(Suarez *et al.*, 1984).

This chapter describes an attempt to quantify these motility changes using CASA. CASA machines have been developed to estimate small changes in sperm movement by analyzing displacement of the sperm head (see chapter 1). When the sperm head is anchored in the zona matrix, head movement is restricted and flagellar movement becomes the more informative parameter. In this chapter, the hypothesis was tested that zona pellucida glycoproteins in solution would elicit sperm motility changes that could be analyzed using the Hobson Sperm Tracker. Visual assessments of sperm motility have indicated that sperm movement is unaffected by the addition of solubilized zonae pellucidae (Pig - Berger *et al.*, 1989ab; Sacco *et al.*, 1989; Hamster - Gwatkin and Williams, 1977; Mouse - Bleil and Wassarman, 1980a).

Initial work was aimed at establishing methods for the collection of porcine oocytes and the solubilization of the zonae pellucidae (the phrase 'solubilized zonae pellucidae' is abbreviated to 'SZP' in the following text). A protocol was developed based on the methods used by Hedrick and Wardrip (1986) and the M.R.C. Reproductive Biology Unit in Edinburgh (M. Patterson, *pers comm*). The identities of the proteins isolated by this method were verified using SDS-PAGE, and gamete binding assays. The ability of different concentrations of the SZP preparation to induce the acrosome reaction was investigated and compared to results published previously. The effects of the SZP on sperm motility were then examined. The results from these experiments led to an investigation of the role of calcium within this system employing calcium-free media and the calcium-channel antagonist nifedipine. Subsequently antibodies raised against the porcine zona pellucida were used to identify the binding sites for the SZP on the sperm surface.

4.1 GENERAL MATERIALS AND METHODS

4.1.1 Collection of oocytes

Batches of frozen ovaries from gilts were obtained from the Dawkins International abattoir, Congerstone, Leicester. Blocks containing approximately 200 ovaries were stored frozen at -20°C and thawed overnight in a refrigerator before use. Extraneous tissue was trimmed, the ovaries placed in separation

media (Hedrick and Wardrip, 1986) and chopped using a razor-blade device constructed at the Institute of Zoology. This consisted of two rows of removable razor blades held securely in a plastic block which was attached to a handle. The device was used to slice the follicles finely, releasing the oocytes into the surrounding media. Separation media and the oocyte suspension were kept 'on ice' throughout the isolation procedure; approximately 7 litres of separation media were required for processing 200 ovaries. Plastic equipment was used throughout the protocol as ovarian tissue was found to adhere to the plastic whilst oocytes did not.

The oocyte suspension was rinsed through a series of nylon meshes of decreasing pore size (1500 µm, 200 µm and 80 µm: Lockertex, Warrington, U.K.) with separation media. The first two meshes removed pieces of tissue and large debris from the preparation but allowed passage of the oocytes. The 80µm mesh caught the oocytes but small items of debris were removed from the preparation. The oocytes and remaining debris were then washed from the final screen into a plastic measuring cylinder surrounded by ice and the suspension (a total volume of approximately 500 mL) allowed to stand for one hour. At this point all but the bottom 50 mL of liquid were carefully removed, checked for the presence of oocytes and then discarded.

The remaining suspension was removed from the cylinder and layered onto a Percoll gradient. This consisted of 5 mL each of 40%, 20% and 10% Percoll (made using PBS) layered into a 30 mL plastic universal tube (Nunc, Life Technologies Ltd, Paisley, U.K). Aliquots of the oocyte suspension (10 mL) were layered onto the gradient and the tubes centrifuged at 2000 x g for 30 minutes. The oocytes formed a band at the interface of the 10-20% layers and were collected using a fine plastic pipette. The oocytes were washed out of the Percoll solution by three cycles of centrifugation with PBS (5 minutes, 2000 x g) and resuspended in 5 mL of PBS. The number of oocytes collected from the Percoll layer was calculated by removing a 10 µL sample and counting the number of oocytes present, using a Wild M8 dissecting microscope (Wild, Heerbrugg, Switzerland) and x50 magnification. This figure was then used to estimate the total number of oocytes within the 5 mL volume. Approximately 30,000 oocytes were recovered from 100 ovaries.

4.1.2 Isolation and solubilization of the zonae pellucidae

Oocytes in PBS were gently homogenized using a 1.0 mL glass homogenizer (Uniform, Jencons, Leighton Buzzard, U.K.). The zonae were separated from ooplasm and nuclear material by three cycles of centrifugal washing (5 minutes, 500 x g) with 5mM ammonium bicarbonate, (pH 8.2), and finally resuspended in approximately 1.2 mL of 5mM ammonium bicarbonate in an Eppendorf tube. Heat solubilization was performed in a water bath at 70°C for 30 minutes. The solution was centrifuged for one hour at 20,000 x g at 4°C after which the supernatant was removed and frozen as 200 µL aliquots at -70°C. Subsequently these aliquots were lyophilized in an Edwards freeze-dryer (Jencons, Leighton Buzzard, U.K.) for a period of approximately three hours. Lyophilized aliquots were stored at -70°C until use.

Aliquots of the SZP prepared using this method were checked for the presence of debris. For this, lyophilized samples were dissolved in media to approximately 500 µg/mL (see below); 10 µL drops were placed on a slide and covered with a coverslip (diameter 13 mm). Slides were assessed at x 1000 magnification using a Nikon Diaphot-TMD microscope (Nikon, Tokyo, Japan) and phase-contrast optics.

4.1.3 Determination of protein concentration

The protein concentration was established using a modification of the Biuret assay, (Pierce, Illinois, U.S.A.). Briefly, Bovine Serum Albumin (BSA) standards of known protein concentration (0, 200, 400, 600, 800, 1000 and 1200 µg/mL) were constructed using the stock solutions provided. Aliquots (100 µL) of the BSA standards and the SZP sample were added to 2 mL of the copper sulphate reagent provided and incubated at 37°C for 30 minutes. Samples were allowed to cool to room temperature and then assayed using a spectrophotometer. Optical density was measured at 562 nM and the sample readings compared to the standard curve produced. The total protein yield obtained from 100 ovaries was approximately 300 µg.

4.1.4 One - dimensional sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed using the method described by Laemmli (1970;

see also Sambrook *et al.*, 1989). Prior to electrophoresis, SZP samples (A and B, B is the 1/10th dilution of sample A) and marker proteins of known molecular weight (Amersham International, Amersham) were incubated at 95°C for five minutes in the presence of mercaptoethanol to facilitate dissociation of the glycoproteins into their individual polypeptides. Gels were initially stained using Coomassie Brilliant Blue stain for protein (BDH, Poole, U.K.); to improve the resolution of the proteins, the gel was subsequently silver stained (Sambrook *et al.*, 1989).

4.1.5 Hemi-zona assay

Hemi-zona assays of boar sperm were performed in conjunction with the Department of Herd Health and Reproduction, University of Utrecht, Netherlands. Gilt ovaries were obtained from a slaughterhouse and the oocytes collected by puncturing antral follicles. Oocytes were resuspended in PBS and cumulus cells removed by vortexing the suspension for four minutes. The denuded oocytes were washed with PBS and microbisected using a CK2 inverted, phase-contrast microscope (x 200 magnification; Olympus, Tokyo, Japan) equipped with Narishige micromanipulators (Narishige, Tokyo, Japan). The oocyte contents were removed by vigorous pipetting and each hemi-zona placed in a 50 µL drop of SP-TALP medium (Parrish *et al.*, 1988) under mineral oil and stored overnight at 4°C. The hemi-zonae were pre-warmed to 39°C prior to insemination of the droplet with spermatozoa.

Sperm were washed three times with PBS (1000 x g, 10 minutes each), resuspended in pre-warmed SP-TALP and diluted to 2 x 10^6 sperm/mL. Sperm motility was assessed to check that all samples were >50% motile. Aliquots (50 µL) of the test sperm preparation (containing SZP - see section 4.2 for experimental design) were added to 50 µL incubation droplets containing a hemi-zona. The droplet containing the corresponding hemi-zona received 50 µL of the control sperm sample (no SZP).

Sperm and hemi-zonae were co-incubated for 4 hours at 39°C, 5% CO_2. The hemi-zona complexes were removed from the incubation droplets after the co-incubation period and washed through 5 cycles of PBS using a narrow-bore Pasteur pipette to remove loosely bound spermatozoa. Sperm/hemi-zona complexes were fixed with 2.5% glutaraldehyde in PBS for 10 minutes, washed

with PBS, and then stained with 1mg/mL Hoechst 33342 dye (Sigma Chemical Company, Poole, U.K.) in PBS for 10 minutes. After a final wash with PBS the stained complexes were mounted on slides and compressed with a coverslip.

The number of sperm bound to the inside of each hemi-zona and the number of sperm bound to the outside surface of each hemi-zona were counted using a SZ-1145 stereo zoom microscope (Olympus, Tokyo, Japan). The total number of sperm bound to each hemi-zona was then counted using a BH-2 microscope (Olympus, Tokyo, Japan) equipped with a DMU set of filters for the observation of Hoechst. The latter score was made independently of the first two as a method of checking the accuracy of counting.

4.1.6 Sperm viability assessment

5 µL sperm samples were stained with a combination of carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) (Sigma Chemical Company, Poole, U.K.) to establish the ratio of live/dead cells. The aliquots were mixed with 5 µL of the PI/CFDA solution (Harrison and Vickers, 1990) and incubated for 8 minutes at 30°C. Samples were mounted beneath a coverslip and viewed using an Axioskop microscope (Carl Zeiss, Oberkochen, Germany) equipped with 487909 and 487914 filters for the observation of FITC and rhodamine respectively.

4.1.7 Sperm motility assessment

5 µL aliquots were video-recorded at x 100 magnification using the method described in section 2.1. Quartered agar-coated coverslips (22mm x 22mm) were used as the volume of sample was small. The low numbers of progressively motile sperm meant that analysis by CASA was inappropriate (see discussion). Therefore instead of one continuous two minute recording, four fields were recorded for approximately 30 seconds each. The videotapes were carefully scrutinized and progressive motility and the proportion of H:H agglutination estimated from the recordings at a later time (assessments were made as a percentage of the population to the nearest 5%).

4.1.8 Nifedipine experiments

All experiments using the calcium channel antagonist nifedipine were performed under red lighting to minimise the deleterious effects of light on the

nifedipine. 500mM stock solutions of nifedipine in 100% ethanol were diluted with 0.9% NaCl to produce working solutions of various concentrations. These were determined such that 5 µL of working solution were added to 245 µL of sperm preparation to the required nifedipine concentration. Calcium concentrations were adjusted by the addition of 12mM calcium media (see appendix).

4.1.9 Immunocytochemistry

Sperm were fixed by the addition of an equal volume of 3.5% paraformaldehyde in PBS. Samples were washed in PBS by two cycles of centrifugation at 500 x g and the supernatant removed after the final centrifugation. Antisera (rabbit anti-porcine SZP and rabbit anti-ZP3 polyclonal antibodies) were diluted 1 + 9 with 10% heat-treated pig sera in saline and 100 µL of this solution used to re-suspend the sperm pellet. The sperm/antibody mixture was then incubated for 1 hour at 39°C.

After incubation, 400 µL of PBS were added to each sample, followed by two cycles of centrifugation (5 minutes at 500 x g) and re-suspension in saline. The sample was spun once more and the supernatant removed. FITC conjugated goat anti-rabbit IgG was diluted 1 + 49 with 10% pig sera in saline and 100 µL used to re-suspend the pellet. The sperm/anti-rabbit IgG mixture was then returned to the incubator for a further 30 minutes.

400 µL of PBS were then added to the sample, followed by two cycles of centrifugation (5 minutes at 500 x g) and re-suspension in PBS. The sample was spun once more and the supernatant removed. The pellet was then gently resuspended in 10 µL of deionized water and a 5µL drop gently smeared onto an agar-coated slide. This was allowed to dry, covered with a 2 µL drop of Citifluor and a coverslip added. The sample was then viewed on an Axioskop microscope (Carl Zeiss, Oberkochen, Germany) using a 487909 filter (x 1000 magnification).

4.1.10 Sperm preparation and addition of SZP

Extended boar semen from JSR Healthbred was washed using a Percoll gradient as described previously (See section 2.1). Sperm concentration was adjusted to 10 x 10^6 sperm/mL. Unless stated otherwise, sperm samples were incubated for 3.5 hours at 39°C in the presence of 5% CO_2, 95% air (100% humidity) prior to the addition of the SZP preparations.

Pre-warmed media was added to the thawed lyophilized SZP to make aliquots (50-100µL) with a protein concentration double that required in the experiments. An equal volume of the SZP in media was mixed with the sperm sample to produce a sperm concentration of 5 x 10^6 sperm/mL and the required SZP concentration.

4.1.11 Statistical analysis

All data described as percentages of sperm were subjected to arc sine transformation before statistical treatment. This includes the proportion of acrosome reacted sperm, the proportion of viable cells, the proportion of progressively motile sperm and the proportion of H:H agglutinated sperm. Different treatments were compared using analysis of variance (ANOVA). Where appropriate Repeated Measures ANOVA and Contrast Analysis were employed. Specific contrasts within the experimental design were examined by the use of orthogonal polynomial coefficients (Snedecor 1956).

4.2 CHARACTERIZATION OF THE PROTEINS OBTAINED BY ISOLATION AND SOLUBILIZATION OF THE ZONAE PELLUCIDAE.

The structure and properties of the porcine zona pellucida have been extensively characterized (for reviews see Dietl, 1989; Dunbar and O'Rand, 1991), and the molecular weights of the constituent protein families reported (Dunbar and Raynor, 1980; Hedrick and Wardrip, 1986; Yurewicz *et al.*, 1987). The following experiments were performed to identify the protein groups obtained using the separation method described in section 4.1.1-2. One-dimensional Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to compare the protein components of the SZP sample with marker proteins of known molecular weight.

Experiments were performed to confirm the ability of the SZP to block sperm-egg binding (Bleil and Wassarman, 1980a; Berger *et al.*, 1989b). Oocytes differ in their ability to bind sperm, therefore hemi-zonae were used to overcome intra-oocyte variation. Using this method, corresponding hemi-zonae can be used for test and control samples as these are known to bind equal numbers of sperm (Fazeli *et al.* 1992). Fluorescent labelling was used to detect accurately the

number of sperm bound to the zona in the presence and absence of the SZP.

4.2.1 Experimental design

SDS-PAGE - SZP preparations were analyzed using SDS-PAGE (see Materials and Methods) and compared to marker proteins of 14.3, 21.5, 30, 46, 69, 97.4 and 200 kD. Gels were originally stained with Coomassie Brilliant Blue; subsequently silver-staining was used to highlight protein groups.

Hemi-zona assay - Fresh ejaculates from three boars were prepared for the hemi-zona assay as described in section 4.1.5. 100 µL of each sperm sample was added to 100 µL of pre-warmed media containing either 200 µg/mL SZP (test) or media alone (control). Controls using BSA to create equivalent protein concentrations were considered, however the fraction of added BSA would have been very small relative to the 5mg/mL BSA content of the media, therefore controls only had media added.

Each sperm sample was then used for the two parts of the experiment; figure 20 shows the experimental design graphically. In both parts 1 and 2 of the experiment, three pairs of hemi-zonae were used for each of the three ejaculates

Part (1) - 20 µL of test or control sperm samples were immediately diluted with pre-warmed media to produce a sperm concentration of 1×10^5 sperm/mL and an SZP concentration of 5 µg/mL. These were then used in the hemi-zona assay as described in section 4.1.5.

Part (2) - Control and test sperm samples were incubated for 1 hour, after which the samples were diluted by the addition of pre-warmed media to a sperm concentration of 1×10^5 sperm/mL and an SZP concentration of 5 µg/mL. The diluted samples were then used in the hemi-zona assay as described above.

In both parts of this experiment, the eventual sperm:SZP:hemi-zona ratio in the incubation droplet was the same. However, in part 2 samples were pre-incubated for 1 hour at 100 µg/ml SZP.

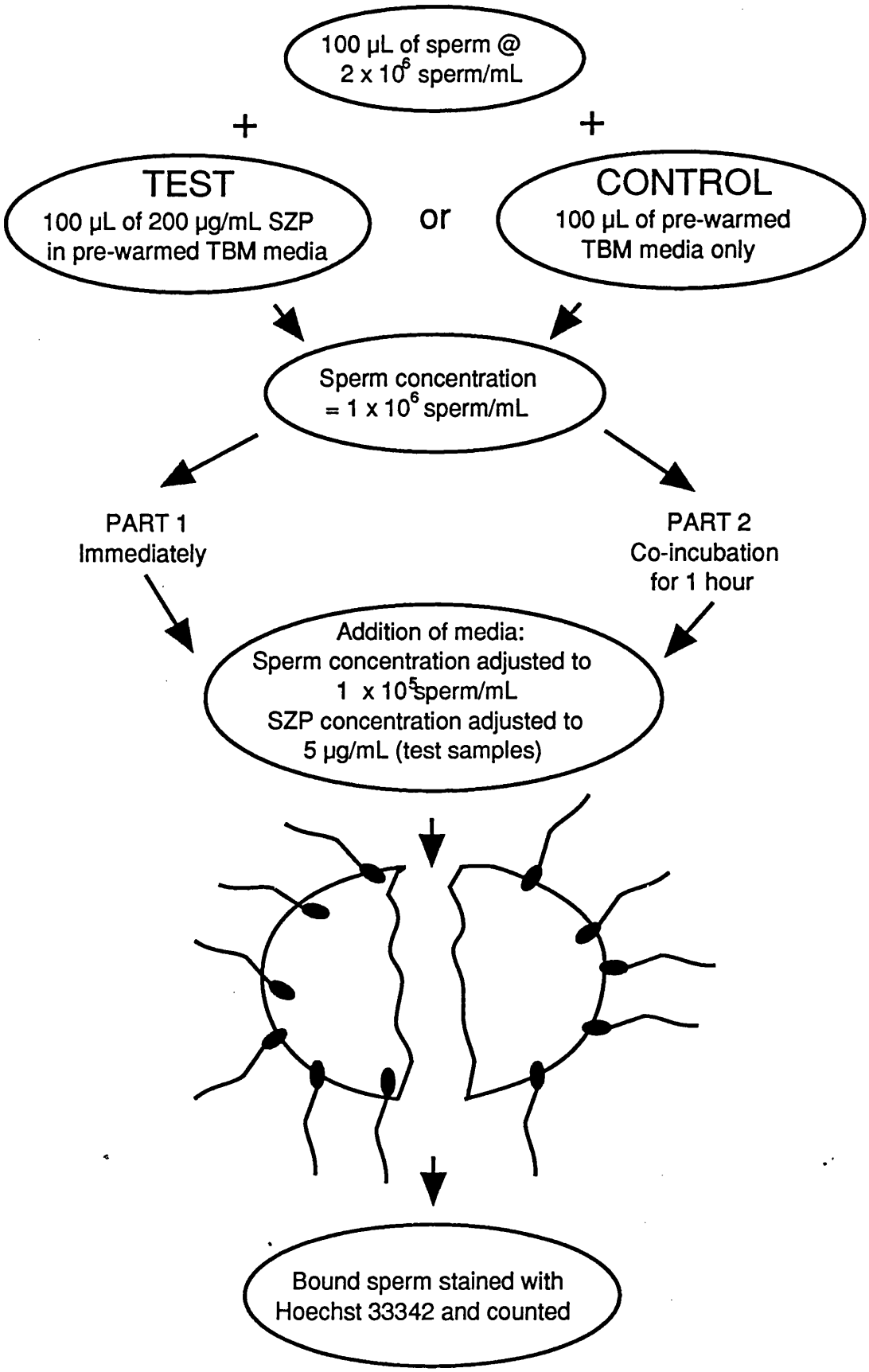


Figure 20. Schematic representation of the experimental protocol for section 4.2.

4.2.2 Results

Figure 21 shows the staining of the gel produced with the Coomassie Brilliant Blue and silver stain methods. Under the reducing conditions used, a diffuse band was visible depicting protein groups with an apparent molecular weight ranging from 45 kD-110 kD (both dilution A and B). Further smearing was evident in the region of the gel between the 21.5 and 30 kD bands. This staining pattern is characteristic of the electrophoretically heterogenous glycoprotein families of the porcine zona pellucida and indicates the presence of the 55 kD, 82-90 kD, 61-65 kD and 21-25 kD components. The family with a molecular weight of approximately 164 kD, previously reported by some groups (see Chapter 1) could not be discerned.

TABLE 20. The number of sperm bound to hemi-zona in the presence and absence of SZP.

NUMBER OF SPERM BOUND TO DIFFERENT REGIONS OF THE HEMI-ZONA†			
EXPERIMENT	OUTER SURFACE‡	INNER SURFACE‡	TOTAL*
PART 1 - TEST	16.7 ± 11.2	6.8 ± 5.0	23.1 ± 15.5
CONTROL	23.1 ± 13.7	8.0 ± 6.0	31.7 ± 20.7
PART 2 - TEST	8.7 ± 6.2	3.3 ± 3.2	10.6 ± 6.9
CONTROL	21.8 ± 7.8	12.3 ± 8.3	33.2 ± 6.1

Corresponding hemi-zonae were used for test (+SZP) and control (-SZP) sperm samples. In part 1., samples were immediately diluted and used in the hemi-zona assay; in part 2., samples were incubated for one hour prior to the hemi-zona assay.

†Values are means and standard deviations for the 9 hemi-zonae (3 ejaculates, 3 test and 3 control hemi-zonae per ejaculate).

‡ Evaluated using stereo-zoom microscopy.

*Evaluated using fluorescence microscopy.

Table 20 shows the mean number of sperm bound to the hemi-zonae in parts 1 and 2 of the experiment. The number of sperm bound to the inside and outside surfaces of the hemi-zonae differed significantly (dependent t-test $P<0.001$, pooled data from all treatments). The total number of sperm bound evaluated using fluorescence microscopy closely matched the sum of the values obtained using stereo-zoom microscopy.

In part 1, sperm and eggs were co-incubated in the presence of low

concentrations of SZP (5 µg/mL). The number of sperm bound to hemi-zonae was not significantly different for test or control (no SZP) samples (P=0.343). In part 2, sperm were pre-incubated with a high concentration of SZP (100µg/mL) prior to the hemi-zona assay. This resulted in a significant decrease in the number of sperm bound compared to controls (P<0.001).

TABLE 21. The mean number of sperm bound by individual boars in the hemi-zona assay.

THE TOTAL NUMBER OF SPERM BOUND IN THE HEMI-ZONA ASSAY				
	PART 1.		PART 2.	
	TEST	CONTROL	TEST	CONTROL
BOAR 1	39.0 ± 17.3	47.3 ± 21.7	17.7 ± 7.4	26.3 ± 3.1
BOAR 2	20.3 ± 2.5	32.7 ± 20.2	8.0 ± 1.7	37.3 ± 4.5
BOAR 3	10.0 ± 1.7	15.0 ± 7.0	6.0 ± 4.6	36.0 ± 3.5

The mean number of sperm bound to test (+SZP) and control (-SZP) hemi-zonae determined using Hoechst 33342 staining. Values are means and standard deviations for 3 hemi-zonae (3 test and 3 corresponding control hemi-zonae per ejaculate).

The results for Part 1 of this experiment indicated a significant effect of 'boar' on the number of sperm bound to the hemi-zonae (P=0.001; pooled test and control results, see table 21); however, this effect was not significant in Part 2 of the experiment (P=0.845). The SZP mediated decrease in sperm binding in Part 2 was also significantly animal-dependent (P<0.01).

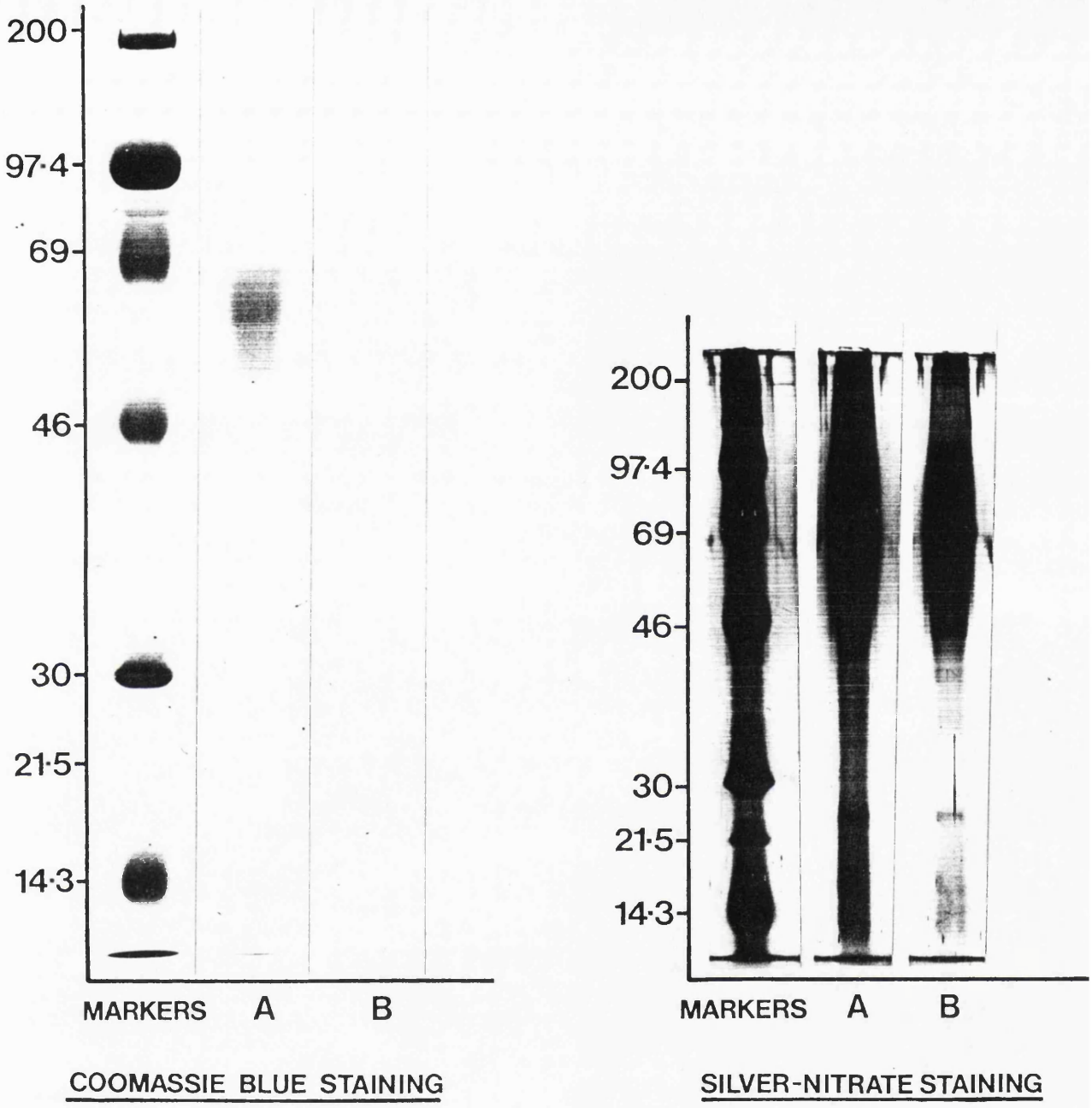


Figure 21. One-dimensional SDS-PAGE of glycosylated porcine SZP. This figure shows a single gel stained using the Coomassie Blue and Silver Nitrate methods. The molecular weights (kD) of known marker proteins are shown to the left of the gel. The smeared staining pattern indicates extreme electrophoretic heterogeneity and is characteristic of zona pellucida proteins.

4.2.3 Summary

The SDS-PAGE results from this section indicated that the SZP preparation contained electrophoretically heterogenous protein groups whose molecular weights resembled those of protein families previously identified in the porcine zona pellucida. Co-incubating sperm and eggs in the presence of low concentrations of SZP did not significantly block sperm-egg binding. However, pre-incubation of sperm with higher SZP concentrations subsequently reduced sperm-egg binding in the hemi-zona assay.

4.3 THE EFFECT OF SZP ON SPERM ACROSOME REACTIONS AND LOCALIZATION OF THE SZP BINDING SITES.

This experiment was performed to verify that the SZP preparation could induce the acrosome reaction (Berger *et al.*, 1989a). Varying concentrations of SZP preparation were used to induce exocytosis of sperm capacitated in TBM media. Subsequently this experiment was repeated with porcine IVF medium replacing the TBM media. This medium is based closely on SP-TALP media and was developed for pig IVF (Suzuki *et al* 1994). The media will sustain porcine IVF, and therefore must be capable of capacitating a proportion of the sperm population, but has a significantly lower calcium concentration (4.5mM) than TBM (40mM).

During fertilization the plasma membrane overlying the acrosome mediates initial sperm binding to the zona pellucida (for a review see Yanagimachi, 1994). Exposure of sperm to zona pellucida proteins in solution (rather than as a matrix) may enable receptor-ligand binding in novel areas of the sperm plasma membrane. To investigate this possibility, two polyclonal antibodies raised to porcine zona pellucida glycoproteins were used to localize SZP binding sites on the sperm surface. Polyclonal antibodies to undifferentiated porcine zona pellucida glycoproteins and to porcine 55 kD ZP3 were the kind gift of Dr R.J. Aitken, MRC Reproductive Biology Unit, Edinburgh. Specifically the ability of proteins within the SZP to bind to the sperm flagellum was investigated.

4.3.1 Experimental design

Induction of the acrosome reaction - Extended, stored, semen samples from SDS

boars were prepared as described previously (see section 2.1) using TBM media (n=5) or porcine IVF medium (n=8). After 3.5 hours incubation, SZP in the relevant media were mixed with sperm samples to produce aliquots with a sperm concentration of 5 x 10^6 sperm/mL and a range of SZP concentrations of 50, 75, 125 or 250 µg/mL. Controls were produced by adding only warmed media to the sperm samples.

Aliquots (10 µL) were removed from the samples immediately and after 30, 60, 90 and 120 minutes for preparation of smears for acrosomal assessment using PSA-FITC (see section 3.1.4). ANOVA was used to evaluate the effects of 'time' and 'SZP concentration' on the proportion of exocytosed sperm.

Immunocytochemistry - Three extended semen samples (each from a different boar) were pooled and subsequently prepared as described previously using porcine IVF media. After the 3.5 hour incubation, SZP in pre-warmed media were added to the 'test' sperm sample to produce an SZP concentration of 250 µg/mL and a sperm concentration of 5 x 10^6 sperm/mL. Control samples received media only. All samples were then co-incubated for 1 hour at 39°C, 5%CO_2, 100% humidity. At this point the samples were fixed and stained using the process described in section 4.1.9. Half the test/control samples were stained using antisera raised against undifferentiated zona pellucida glycoproteins and half were stained using antisera raised against the 55 kD porcine ZP3 family.

4.3.2 Results

Tables 22 (a) and (b) show the proportion of acrosome reacted sperm for each SZP concentration at each sampling time. Figure 22 shows this data for each of the protein concentrations after 1 hour co-incubation of the sperm samples in both TBM and porcine IVF media.

TBM media - The results from this section indicated that the proportion of exocytosed sperm increased significantly with duration of incubation ($P<0.001$) and was dependent on the concentration of SZP in the media($P<0.001$). The interaction of the two factors was not significant ($P=0.896$).

Applying a L.S.D. post-Hoc test to the data indicated that for samples containing 250 µg/mL of SZP there was a significant increase in the proportion of

acrosome reacted sperm after 30, 60 and 90 minutes (P=0.033, P=0.0007 and P=0.009 respectively). Similarly, 125 µg/mL of SZP produced a significant effect after 60 minutes (P=0.008). At later sampling times the proportion of acrosome reacted sperm in the test samples stabilized whilst spontaneous acrosome reactions in control samples continued to increase. This caused the differences between test and control samples to become non-significant.

IVF media - The results for this section were similar to those described above. Both increased SZP concentrations and longer co-incubation periods resulted in a greater proportion of acrosome reacted sperm (P=0.024 and P=0.001 respectively). Again the interaction between these factors was not significant (P=0.968). The L.S.D. post-Hoc test indicated that the increase in the proportion of acrosome reacted sperm was significant for 250 µg/mL SZP after 60 minutes (P=0.012) and 120 minutes (P=0.019).

Immunocytochemistry - Spermatozoa co-incubated with SZP and exposed to anti-SZP anti-sera were labelled over the apical ridge, acrosome and mid-piece (figure 23, a). No fluorescence was visible in the equatorial segment, post acrosomal region or the principal- or end- piece of the flagella. Labelling of test samples using anti-ZP3 antisera produced identical staining patterns (figure 23, b). Using either anti-sera, a small proportion of cells (approximately 25%) showed labelling over the mid-piece only (figure 23, c). These two staining patterns could reflect differences in acrosomal status of sperm, however, no attempt was made to differentiate acrosome intact and acrosome reacted sperm.

Control samples (no SZP) exposed to either antisera were faintly labelled over the whole sperm (Figure 23, d + e). On some sperm brighter, but patchy, fluorescence could be observed over the sperm head.

4.3.3 Summary

Addition of the SZP increased the proportion of acrosome reacted sperm. The minimum concentration of protein and the co-incubation period required to induce a significant increase in exocytosed sperm were dependent on the media used. Fluorescent staining of the sperm indicated that components of the zona pellucida were binding to the sperm mid-piece as well as the area overlying the

acrosome. Labelling of the sperm mid-piece occurred when sperm incubated with SZP were exposed to either antisera. Therefore the SZP component binding to the sperm mid-piece is recognized by both anti-SZP and anti-ZP3 antibodies.

Table 22. The effect of SZP concentration on the proportion of acrosome reacted spermatozoa.

(a) - TBM media

SZP-INDUCTION OF THE ACROSOME REACTION - TBM MEDIA					
CO-INCUBATION TIME (MINUTES)	SZP CONCENTRATION (µg/ml)				
	0	50	75	125	250
0	44.6±4.8	44.1±2.5	46.5±3.3	49.0±3.2	46.0±2.2
30	60.3±2.6	59.7±2.5	61.8±3.6	64.4±4.4	68.5±3.7
60	56.2±1.7	56.9±2.5	60.3±2.3	66.6±1.5	69.6±1.6
90	64.2±3.7	66.5±1.3	64.6±3.2	70.0±0.7	74.4±2.6
120	67.3±3.6	66.7±2.6	70.1±2.2	72.6±1.5	73.5±2.7

The proportion of acrosome reacted sperm was determined for samples co-incubated with varying concentrations of SZP. Samples were pre-incubated for 3.5 hours in TBM media, SZP added and aliquots removed after 0, 30, 60, 90, 120 minutes for assessment with PSA-FITC. Values are means and s.e.m for five ejaculates.

(b) - IVF media

SZP-INDUCTION OF THE ACROSOME REACTION - IVF MEDIA					
CO-INCUBATION TIME (MINUTES)	SZP CONCENTRATION (µg/ml)				
	0	50	75	125	250
0	41.2±3.3	45.5±2.6	43.5±2.3	43.6±2.6	43.0±2.4
30	44.5±2.5	47.6±2.5	45.1±2.7	46.8±3.1	49.5±2.4
60	44.8±1.6	49.0±2.0	49.0±1.8	50.2±1.9	54.2±2.2
120	44.0±2.6	51.5±2.2	49.3±2.6	50.2±1.9	52.8±2.3

The proportion of acrosome reacted sperm within samples co-incubated with varying concentrations of SZP. Samples were pre-incubated for 3.5 hours in porcine IVF medium, SZP added and aliquots removed after 0, 30, 60, 120 minutes for assessment with PSA-FITC. Values are means and s.e.m. for eight ejaculates.

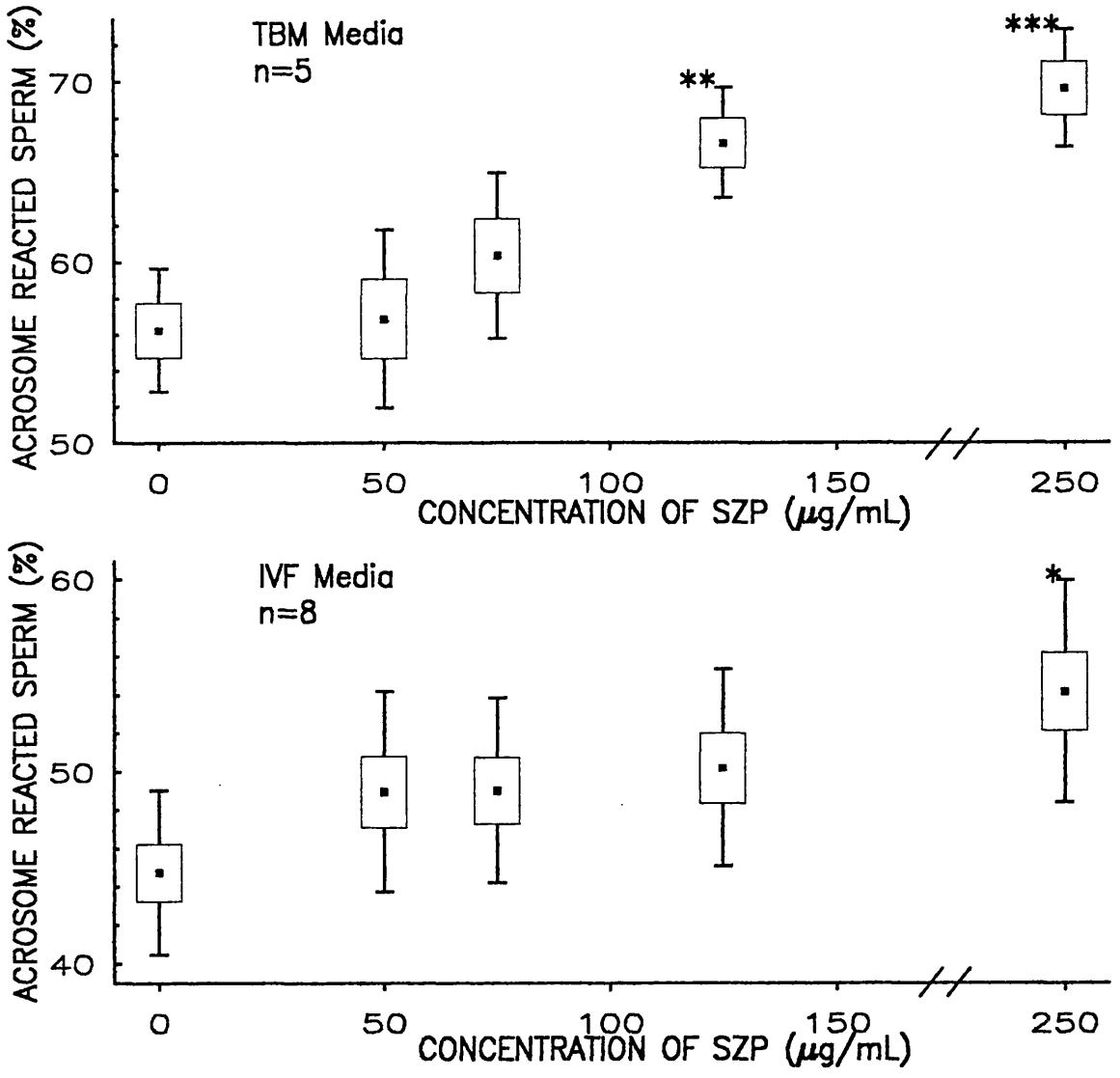


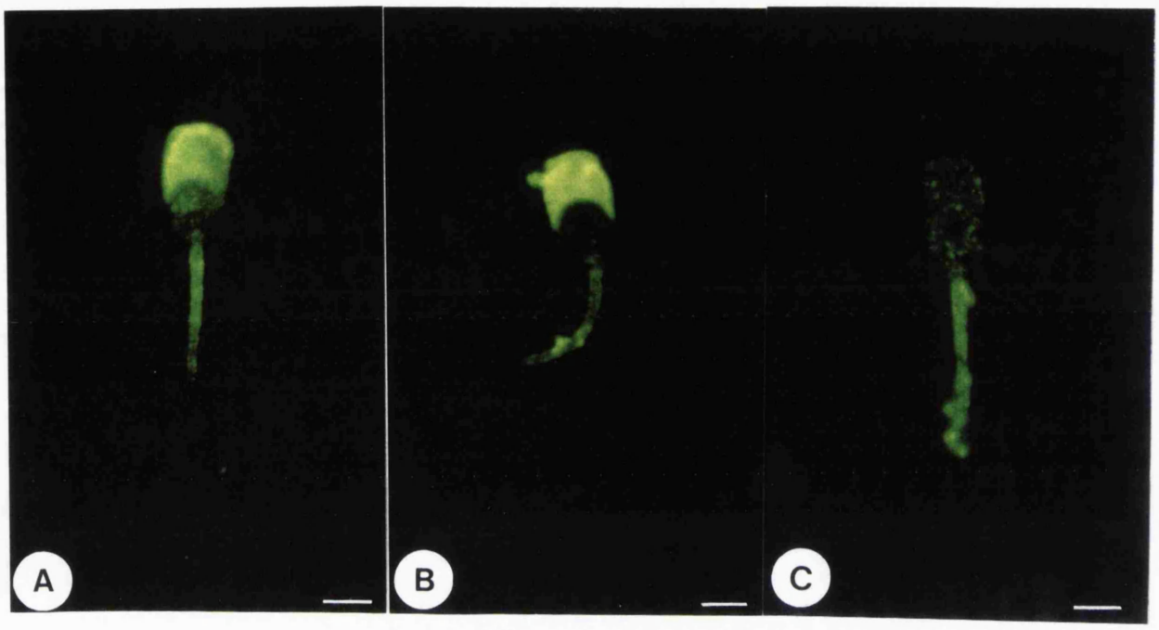
Figure 22. The effect of SZP concentration on the proportion of acrosome reacted sperm. Washed, extended sperm samples were incubated for 3.5 hours and then exposed to varying concentrations of SZP. The proportion of sperm that have undergone the acrosome reaction after 1 hour is shown for sperm incubated in TBM and IVF media (box-whisker plots illustrate the mean, s.e.m. and s.d. of the samples).

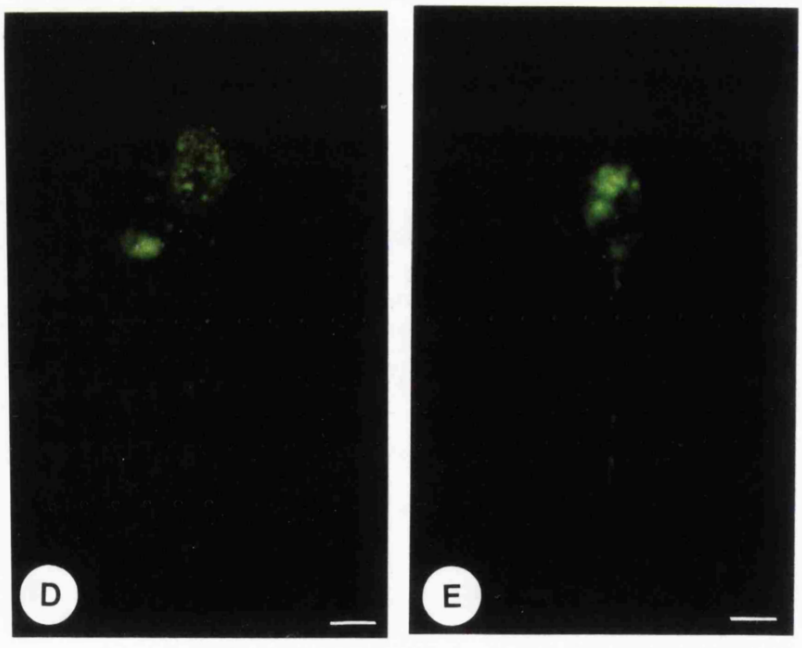
*indicates significant difference from 0 µg/mL SZP value. *=P<0.05; **=P<0.01; ***=P<0.001.

Figure 23. Localization of the zona pellucida binding sites of sperm. Boar sperm were exposed to the SZP preparation and then stained using antibodies raised against either undifferentiated ZP proteins (SZP) or against porcine ZP3.

(A and C)	Test samples (co-incubated with SZP) labelled using anti-SZP antibody.
(B)	Test samples labelled using anti-ZP3 antibody.
(D)	Control samples (no SZP) labelled using anti-SZP antibody.
(E)	Control samples labelled using anti-ZP3 antibody.

Test samples (labelled with either antisera) were stained over the acrosomal and mid-piece regions (A,B) or over the mid-piece region alone (C). In contrast, control samples labelled with either antisera were faintly stained over the whole sperm (D,E). Bars represent 3 μm.


A
B
C


D
E

4.4 THE EFFECT OF SZP ON SPERM MOTILITY

Previously spermatozoa penetrating the zona pellucida have been observed to exhibit 'hatchet-like' movements (Drobnis *et al.*, 1988b; Katz *et al.*, 1989). These experiments were designed to establish whether the addition of SZP in solution to sperm preparations produced similar patterns of sperm movement. This would facilitate CASA assessment of this type of motion.

Spermatozoa were incubated in TBM medium to enable comparison of the results with the motility data from the preceding chapter. An SZP concentration of 125 µg/mL and a co-incubation period of up to 60 minutes were chosen as these conditions were known to promote the acrosome reaction. The effects of the SZP on sperm viability and motility were also examined.

4.4.1 Experimental design

Extended, stored ejaculates (n=8) from different boars, were prepared as described previously and incubated for 3.5 hours. At this point sperm samples were split into 'test' and 'control' aliquots. SZP preparation in pre-warmed media was added to the test sample to a final SZP concentration of 125 µg/mL, control populations received only pre-warmed media. Sub-samples were removed after 5, 10, 15, 30 and 60 minutes continuous incubation.

At each time point, the aliquots removed were used for the following purposes;

1) Smears were used to assess the occurrence of the acrosome reaction (PSA-FITC fluorescent stain; see section 3.1.4 for method).

2) 5 µL samples were stained with a combination of carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) to establish the ratio of live/dead cells.

3) 5 µL aliquots were video-recorded and the proportion of progressively motile sperm and the proportion of sperm in H:H agglutinations assessed.

4.4.2 Results

Figure 24 summarises the results from this experiment. The main outcome was the decline in numbers of motile sperm within the first 15 minutes of co-incubation with the SZP (figure 24, a and b). Progressive motility was significantly reduced in test samples compared to controls ($P<0.0001$); the mean score for control samples remained >60% throughout the experiment, but fell to below 20%

in test samples. This was partly due to the increased H:H agglutination in the test samples compared to the controls ($P<0.005$). These aggregations were greater than the groups of four or five sperm observed previously and whilst the agglutinated sperm displayed high frequency, quivering flagellar movement, there was no overall displacement. The sperm that remained single or in small agglutinations also showed flagellar beating with a loss of progressive motility. Control samples retained progressive motility throughout the experiment and only a few small agglutinations (<10 sperm), were observed.

Assessing the CFDA/PI staining of agglutinated sperm proved to be time-consuming - judging the staining patterns of sperm from a single sample took approximately ten minutes. To evaluate each of the different treatment groups would have required suspension of the cells in the CFDA/PI preparation for extended periods of time (>15 minutes). This would have produced an inaccurate estimation of cell viability as both CF and PI will breach the plasma membrane. Therefore it proved necessary to estimate cell viability to ensure each sample was analyzed within a few minutes of entering the fix. A minimum of ten microscopic fields were scrutinized for each slide and the proportion of viable cells estimated to the nearest 5 %.

The CFDA/PI data suggested that the SZP caused destabilization of the sperm plasma membrane thereby reducing the number of CFDA positive (membrane intact) cells ($P<0.0001$; figure 24, c). Although this figure is highly significant, the inability to estimate precisely the status of sperm in the large groups reduces the reliability of the result.

As observed previously, the number of acrosome reacted sperm was significantly increased by exposure to the SZP preparation ($P<0.01$; figure 24, d).

4.4.3 Summary

Co-incubation of the sperm with the zona pellucida proteins reduced cell motility and increased cell aggregation.

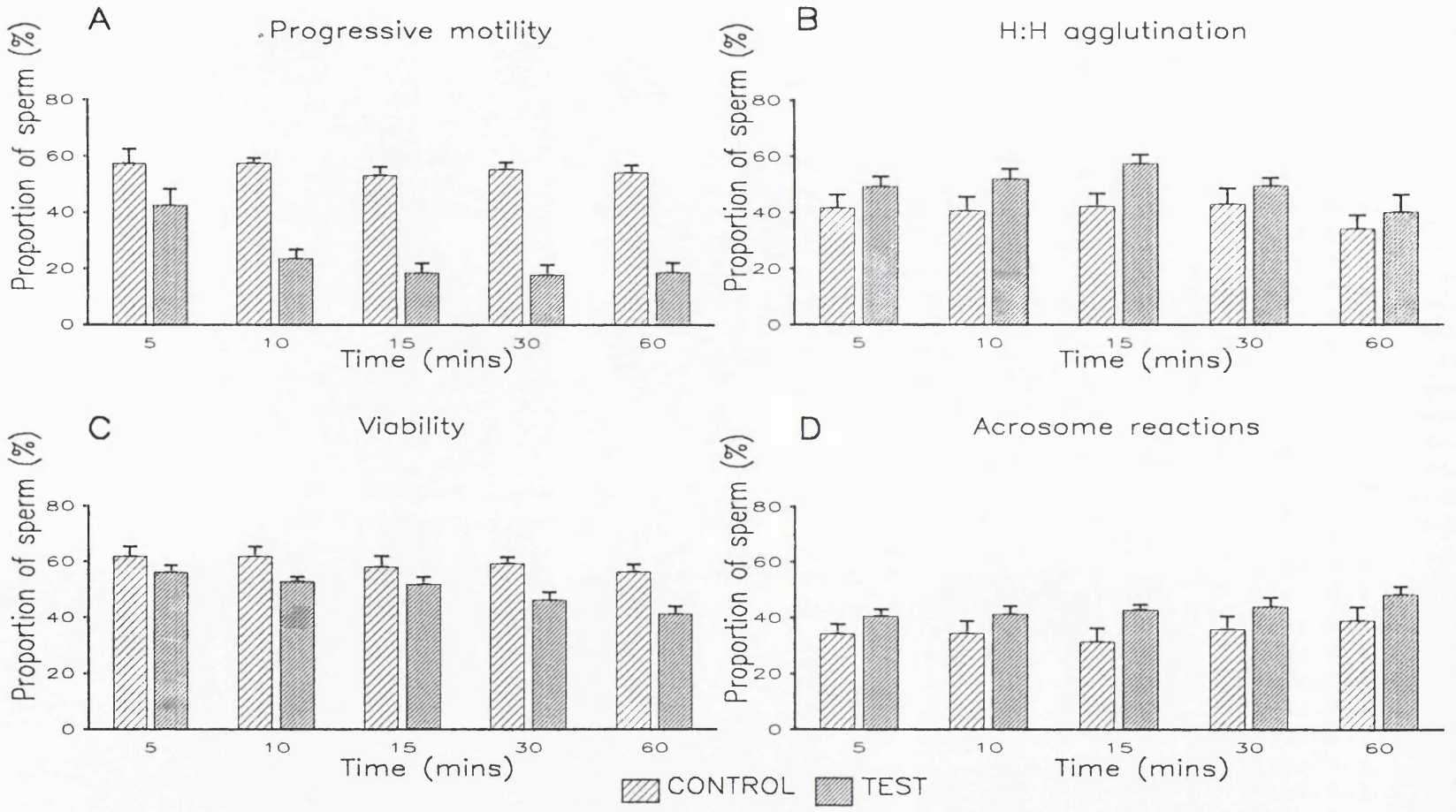


Figure 24. The effect of SZP on sperm motility, viability and acrosome reactions. Washed sperm samples were incubated in TBM medium (3.5 hours), then exposed to 125 µg/mL SZP (test aliquots) or only pre-warmed media (control populations). Sub samples were removed after 5, 10, 15, 30 and 60 minutes incubation. Histograms show the mean and s.e.m. for eight samples.

4.5 CALCIUM IONS AND THE ACTION OF THE SZP

The following two experiments were designed to investigate the role of calcium in the SZP-induced loss of sperm motility observed previously. The first experiment compared the action of the SZP at three calcium concentrations corresponding to

(a) the previously used concentration (40mM Ca^{2+}),

(b) a concentration comparable to normal sperm incubation media (2mM Ca^{2+}) and

(c) trace calcium media (Ca^{2+}-free).

The second experiment used the calcium channel antagonist nifedipine to investigate whether the SZP-induced loss of sperm motility required a rise in intracellular calcium.

If spermatozoa are incubated in media from which all the calcium has been removed (e.g. using the chelator EGTA), they become immotile and lose the ability to undergo the acrosome reaction (Feng *et al.*, 1988). This effect is irreversible after approximately 2 hours. The 'Ca^{2+}-free' medium used in the following experiments was prepared without calcium or chelating agents, but contained trace amounts of the Ca^{2+} ion from the other components of the media. Spermatozoa in this media retain motility. The addition of 50µM EGTA removed all free calcium from the media as evinced by the abolition of both sperm motility and spontaneous acrosome reactions (data not shown). Therefore the calcium concentration of the 'calcium-free' media can be estimated at <50µM.

4.5.1 Experiment 1 - The effect of calcium concentration

The aim of this experiment was to establish whether the effects of the SZP preparation on sperm motility reflected the high calcium concentration of the TBM media. The majority of studies examining SZP:sperm interactions have employed media with lower calcium concentrations, therefore sperm motility was examined in the presence of 2mM Ca^{2+}. The Ca^{2+}-free media was used to establish if the SZP-induced loss of progressive motility and H:H agglutination required the presence of the Ca^{2+} ion.

4.5.2 Experimental design

The effects of calcium were examined in a factorial experiment with 3 treatments (media) and 4 replicates (ejaculates). Extended, stored ejaculates were each split into three aliquots for use with each of the treatments: 'Ca^{2+}-free', 2mM Ca^{2+} or 40mM Ca^{2+} media. The compositions of the media used in this experiment are shown in the appendix. Samples were prepared as described previously (Percoll gradients were also constructed using each of the three media).

After incubation of spermatozoa for 3.5 hours, SZP in the relevant media were added to the sperm samples to a final protein concentration of 125µg/mL. Two 5µL aliquots were removed from the samples at the following times:

(a) just after addition of the SZP,

(b) after 30 minutes and

(c) after 60 minutes co-incubation.

One aliquot was processed for acrosomal assessment using PSA-FITC as described previously, the second was used to assess progressive motility using the method described in section 4.1.7.

4.5.3 Results

Figure 25 contains a summary of the results from this section. Repeated measures ANOVA of the acrosome reaction data (see table 23.) indicated that the proportion of acrosome reacted sperm increased significantly as the Ca^{2+} concentration was raised ($P<0.0001$). Significantly fewer sperm were acrosome reacted in Ca^{2+}-free media compared to the calcium-containing media (Ca^{2+}-free treatments vs 2mM Ca^{2+} and 40mM Ca^{2+} treatments $P<0.0001$) and the presence of 40mM Ca^{2+} increased the frequency of exocytosis compared to 2mM Ca^{2+} (2mM Ca^{2+} treatments vs 40mM Ca^{2+} treatments $P=0.002$).

Previously a SZP concentration of 125 µg/mL induced a significant increase in exocytosis within 60 minutes; in this experiment this effect was not significant within the 60 minute incubation period ($P=0.0607$). This may indicate batch variations in the activity of the protein from different separations.

All the sperm samples showed good motility after the initial 3.5 hours of incubation. Control samples retained this level of motility throughout the experiment. Samples incubated in 'Ca^{2+}-free' media contained very few agglutinated sperm compared to those incubated in 2mM or 40mM Ca^{2+} media.

Samples in 2mM Ca^{2+} media were less agglutinated than samples in 40mM Ca^{2+} media.

The 'Ca^{2+}-free' test (+SZP) medium did not cause agglutination and retained sperm motility at control levels throughout the incubation period. Test samples incubated with either 2mM or 40mM Ca^{2+} media exhibited the loss of progressive motility seen in the previous experiment. As previously, a few large agglutinations were formed and the majority of sperm retained high frequency flagellar movement without progressively motility. The loss of progressive motility in both the 2mM Ca^{2+} and 40mM Ca^{2+} media appeared to be similar.

One ejaculate responded differently to the other three. Sperm in this ejaculate retained good motility for all test samples, regardless of calcium concentration; however the acrosome reaction results obtained for this boar were similar to those obtained for the other 3 animals.

4.5.4 Summary

Incubation of sperm in Ca^{2+}-free media reduced the incidence of SZP-induced acrosome reactions compared to calcium-containing media. The deleterious effects of the SZP on sperm motility were not observed for samples incubated in 'Ca^{2+}-free' media. Increased sperm agglutination and loss of progressive movement were observed in three out of four samples containing 2mM or 40mM Ca^{2+}.

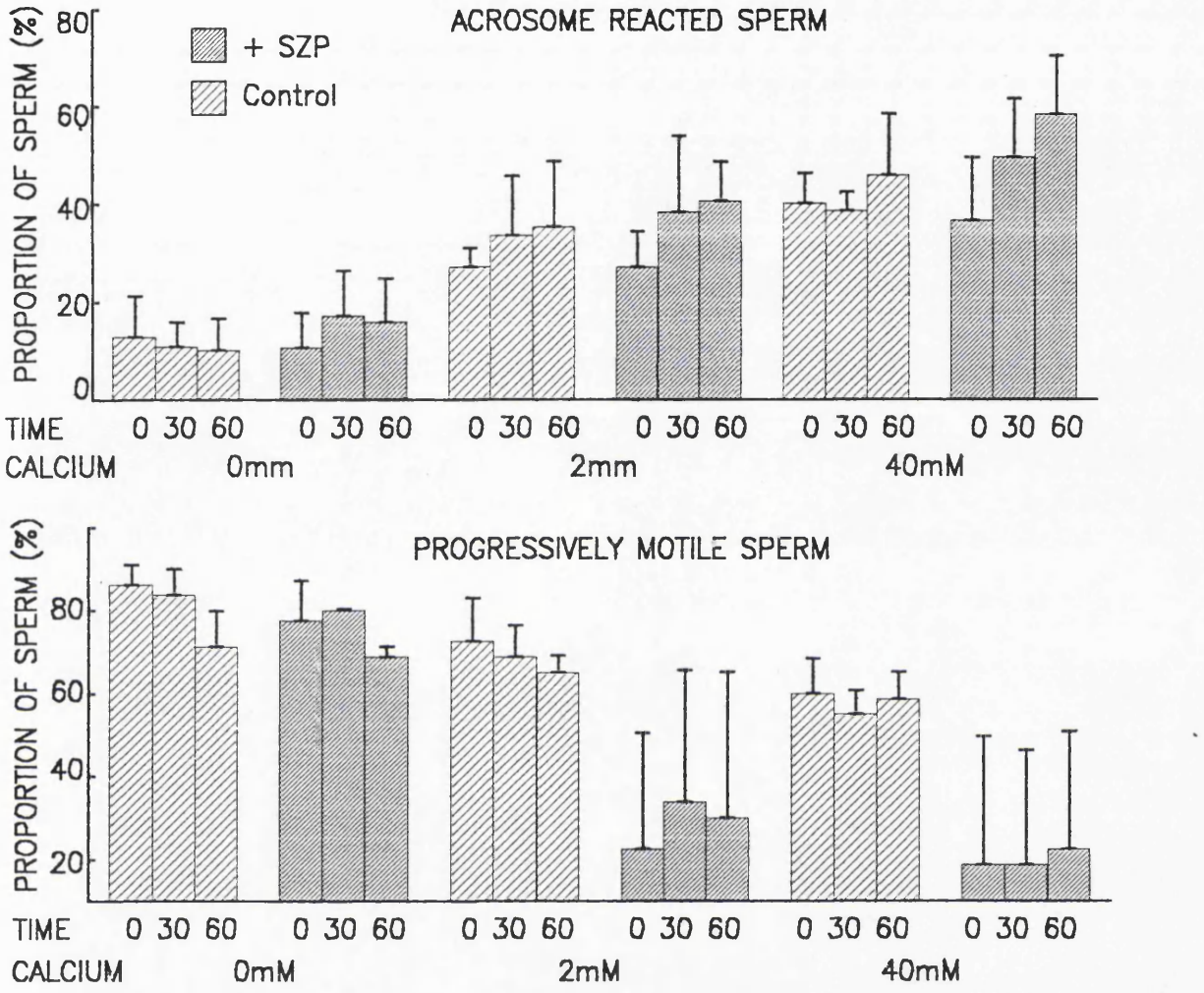


Figure 25. The effect of SZP on sperm incubated in 'Ca^{2+}-free', 2mM Ca^{2+} or 40mM Ca^{2+} media. Washed, extended ejaculates were incubated for 3.5 hours in each of the three media and then SZP added to a final concentration of 125µg/mL. Samples were removed after 0, 30 and 60 minutes and the proportion of acrosome reacted sperm and the proportion of progressively motile sperm assessed. Histograms show the mean and s.e.m for four ejaculates.

Table 23. The effect of calcium concentration on SZP-induced acrosome reactions - Repeated measures ANOVA of the effects of time, calcium concentration and presence/absence of SZP.

REPEATED MEASURES ANOVA			
SOURCE OF VARIATION	D.F.	F. VALUE	SIGNIFICANCE
SZP	1	5.310	P=0.0607
TIME *	2	2.143	P=0.1601
CALCIUM CONC.	2	141.610	P<0.0001
SZP x TIME	2	0.911	P=0.4282
SZP x CALCIUM CONC.	2	0.244	P=0.7872
TIME x CALCIUM CONC.	4	0.736	P=0.5764
SZP x TIME x CALCIUM CONC.	4	0.156	P=0.9586

Sperm samples (n=4) were incubated in media containing trace, 2mM or 40mM Ca^{2+} for 3.5 hours after which media containing SZP (test) or media only (controls) was added to the samples. Assessments of the proportion of acrosome reacted sperm were made after 0, 30 and 60 minutes.
*'Time' indicates the sampling time-point, i.e. 0, 30 or 60 minutes after addition of the SZP

4.5.5 Experiment 2 - The effect of nifedipine, a calcium channel antagonist, on the SZP-induced motility loss.

To undergo the acrosome reaction, sperm require an increase in intracellular [Ca^{2+}] and therefore exocytosis is retarded in low calcium media (Fraser 1987). The results from the previous experiment demonstrated this effect; significantly fewer sperm underwent exocytosis (either spontaneous or SZP-induced) after incubation in 'trace-calcium' media than after incubation in 2mM Ca^{2+} or 40mM Ca^{2+} media. Similarly, the inclusion of SZP proteins did not affect the movement of sperm incubated in the absence of calcium whilst sperm incubated in calcium-containing media became poorly motile. Therefore this experiment was performed to investigate whether the SZP-induced motility loss required an increase in intracellular [Ca^{2+}].

Several studies have demonstrated that mammalian sperm possess voltage-dependent calcium channels (VDCC) within the plasma membrane (Babcock and

Pfeiffer, 1987; Cox and Peterson, 1989). Activation of the VDCC initiates the influx of Ca^{2+} responsible for acrosomal exocytosis and is triggered by the binding of zona agonists to the plasma membrane (Fraser, 1993; Florman *et al.*, 1992). Recent evidence that ZP-activation of the VDCC elevates internal calcium in both the sperm head and tail indicates the presence of these channels on the sperm flagellum (Florman, 1994).

In the present study, nifedipine was used to block the entry of calcium into the cell whilst external calcium concentrations were elevated. This compound is a specific antagonist of VDCC and does not interact with other cationic channels (Triggle 1991). Previous experiments have demonstrated the ability of this compound to inhibit calcium-stimulated acrosome reactions and to impair fertilization in vitro without affecting sperm motility (Fraser and McIntyre, 1989).

4.5.6 Experimental design

Pooled, extended, sperm samples (n=4) were prepared in Ca^{2+}-free media using the method described previously (see section 2.1). After incubation for 3.5 hours, aliquots of each sperm sample were treated in the following manner (summarized in table 24):

1) Nifedipine was added to three aliquots to achieve final concentrations of 1, 10 or 100 nM nifedipine (treatments B-D respectively).

2) Two 'control' aliquots received only the nifedipine solvent PBS (treatments A and E). Control A evaluated the effect of elevating calcium concentrations and adding SZP in the absence of nifedipine; control E evaluated the effects of raising external calcium concentration in the absence of SZP or nifedipine.

All samples were incubated for 7 minutes, and then external calcium concentration raised to 2mM Ca^{2+}. After incubation for a further 5 minutes. SZP in 2mM calcium media were then added to achieve a final SZP concentrations of 125 µg/mL (treatments A-D); control treatment E received 2mM Ca^{2+} media without SZP. Sperm motility and exocytosis were assessed in test and control samples after incubation in the Ca^{2+}-free media (prior to the addition of any substances) and 10, 30 and 60 minutes after SZP addition.

Table 24. Summary of the experimental treatments used in section 4.5.6.

TREATMENT	NIFEDIPINE	EXTERNAL CALCIUM CONCENTRATION	SZP CONCENTRATION
A	0 nM	2 mM	125 µg/mL
B	1 nM	2mM	125 µg/mL
C	10 nM	2mM	125 µg/mL
D	100 nM	2mM	125 µg/mL
E	0 nM	2mM	0 µg/mL

Sperm samples incubated with varying concentration of nifedipine (0, 1, 10 and 100 nM) prior to elevation of the calcium concentration. The effects of SZP addition (125 µg/mL) on sperm motility and acrosome reactions were evaluated.

TABLE 25. The effect of nifedipine on SZP-induced acrosome reactions - the coefficients used for between treatment contrasts in the analysis of variance.

CONTRAST	TREATMENT				
	A	B	C	D	E
A v B,C,D,	-3	1	1	1	0
E v B,C,D,	0	1	1	1	-3
A v D	1	0	0	0	-1

4.5.7 Results

Sperm motility - After incubation in the Ca^{2+}-free media all samples contained >60% progressively motile sperm. Sperm in control samples (treatment E) remained motile throughout the subsequent 1 hour sampling period (mean progressive motility score at the end of the experiment = 40%). Samples in treatment group A displayed loss of progressive motility and the increased agglutination observed in previous experiments. These effects were observed at 10 minutes and persisted throughout the subsequent sampling period. Addition of nifedipine (treatments B-D) followed by addition of calcium and SZP also resulted in loss of progressive motility and the increased agglutination observed in previous

experiments.

Acrosome reactions - The effect of the different treatments on the incidence of acrosome reactions was examined using ANOVA and specific contrast evaluated by the use of orthogonal polynomial coefficients (table 25 shows the matrix of coefficients for the contrasts made at each time point). A summary of the results obtained is shown in table 26 and figure 26.

TABLE 26. The effect of nifedipine on SZP-induced acrosome reactions - ANOVA results.

ANALYSIS OF VARIANCE				
SOURCE OF VARIATION		D.F.*	F. VALUE	SIGNIFICANCE
TIME		2	55.965	P<0.0001
TREATMENT		4	21.295	P<0.0001
TIME x TREATMENT		8	3.625	P<0.005
CONTRAST ANALYSIS				
TIME	CONTRAST	D.F.*	F. VALUE	SIGNIFICANCE
10 MINUTES	A vs B-D	1	4.749	P<0.05
	B-D vs E	1	0.371	N.S.
	A vs E	1	5.184	P<0.05
30 MINUTES	A vs B-D	1	10.679	P<0.01
	B-D vs E	1	14.368	P<0.001
	A vs E	1	33.213	P<0.0001
60 MINUTES	A vs B-D	1	11.149	P<0.01
	B-D vs E	1	23.176	P<0.001
	A vs E	1	45.531	P<0.0001

D.F. indicates 'degrees of freedom'

Effects of time and treatment obtained in the analysis of variance were further investigated by contrasting the treatment effects at each of the three sampling times (see text).

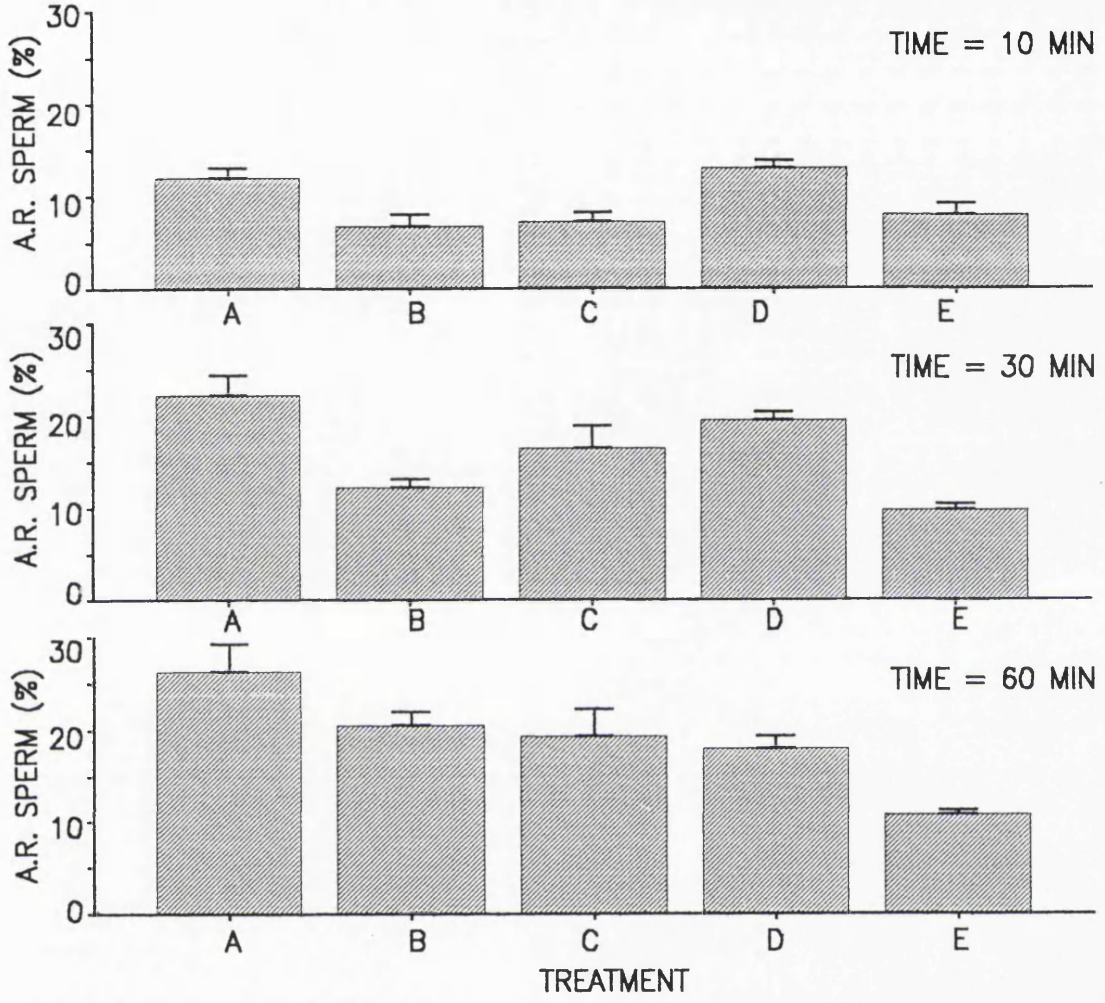


Figure 26. The effect of nifedipine on SZP-induced acrosome reactions. Pooled, extended, semen samples were washed and incubated in Ca^{2+}-free media for 3.5 hours. Each sample was then divided into 5 aliquots, and one aliquot used for each of the treatments A-D (summarised in table 24). Sperm motility and acrosome reactions were assessed in test and control samples after incubation in the Ca^{2+}-free media (data not shown, see text) and 10, 30 and 60 minutes after SZP addition. Histograms show the mean and s.e.m. for four samples.

Incubation in the Ca^{2+}-free media resulted in a low rate of spontaneous acrosome reactions (mean proportion of acrosome reacted sperm prior to addition of any chemicals was 5.50% ± 0.53 s.e.m.). For all treatment groups the incidence of exocytosis increased over time. Addition of SZP resulted in a significant increase in acrosome reacted sperm (treatment A vs E). This effect was blocked by the presence of nifedipine (treatments B-D vs A). After 10 minutes incubation, exocytosis in samples with nifedipine and SZP was similar to samples containing no nifedipine and no SZP (treatments B-D vs E). Subsequently acrosome reactions in the presence of nifedipine increased compared to control E, indicating a reduction in the ability of the nifedipine to prevent SZP-induced acrosome reactions, but values remained below those for control A (added SZP but no nifedipine).

4.5.8 Summary

Addition of the nifedipine reduced SZP-induced exocytosis but did not prevent loss of progressive motility or increased sperm agglutination. These results indicate that the deleterious effects of SZP on sperm motility do not require calcium to be internalized.

4.6 SPERM MOTILITY WITHIN A SZP GRADIENT

The aim of this experiment was to establish whether the impairment of sperm movement by the SZP was a concentration-dependent effect, as has been shown for SZP-induction of the acrosome reaction (Berger *et al.*, 1989a and see section 4.3). Specifically, the hypothesis that lower concentrations of SZP have less damaging effects on sperm motility was evaluated.

SZP concentration gradients were used to study the effect of a continuum of protein concentrations on sperm motility. A similar approach has been used previously to study sperm chemotaxis (Ralt *et al.*, 1994). SZP gradients were constructed by drawing sperm samples into microslides and then using capillary action to introduce SZP. Preliminary experiments were performed using FITC-labelled mouse IgG to verify that a concentration gradient could be established in this manner. Subsequent experiments examined sperm motility at points along the SZP gradient.

4.6.1 Materials and methods

Establishing and validation of gradient method

Porcine IVF media (39°C) was drawn 50 mm into pre-warmed 1 mm width, 100µm depth microslides (Camlab, Cambridge, U.K.) by capillary action. Mouse IgG-FITC (500 µg/mL, also at 39°C) was then drawn up the microslide a distance of 10 mm, also by capillary action. Both ends of the microslide were sealed using Critoseal (Fisher Scientific, Edmonton, Canada) and the capillary was placed on a glass slide pre-warmed to 39°C. Observations of the dye progression were made at four sites (A-D; see figure 27.) after 0, 2, 4 and 6 minutes using four microslides in a latin squares design (see box 1). The four sites were the point of protein introduction (close to the sealed end of the tube) and 15, 30 and 45 mm along the microslide, (A-D respectively).

Sperm motility within a SZP gradient

Subsequently pooled sperm samples (n=3), each derived from bottled semen from three boars, were used in SZP gradients. The samples were prepared using the porcine IVF media as described previously (see section 2.1) and samples incubated for 3.5 hours prior to use. The concentration gradients were produced using the same method as for the preliminary experiment. The capillaries were filled with the diluted sperm samples and the protein used was either 500µg/mL SZP (test) or 500µg/mL B.S.A. (control). Microslides were placed on a pre-warmed (39°C) glass slide and viewed on an Olympus BH-2 microscope (x100 magnification) equipped with a heated stage. Video-recordings were made of the spermatozoa at sites B-D immediately after introduction of the protein and after 2 and 4 minutes. Assessment of the sperm motility was made from these video-recordings at a later time; the proportion of agglutinated and progressively motile sperm were estimated. As before a latin squares design was employed, using three microslides for each pooled semen sample (see box 1).

4.6.2 Results

The fluorescence of the IgG protein spread along the microslide creating a smooth gradient. The movement of the protein was slow and no colour was discernible at D by the end of the six minute observation period, although low concentrations of protein may have been present.

Box 1.
Explanation of the latin squares design employed in section 4.6

LATIN SQUARES DESIGN USED FOR CONCENTRATION GRADIENT EXPERIMENTS				
TIME (MINUTES)	SITE A	SITE B	SITE C	SITE D
0	M1	M4	M3	M2
2	M2	M1	M4	M3
4	M3	M2	M1	M4
6	M4	M3	M2	M1

The table shows how four microslides were used in section 4.6 to examine protein diffusion at four sites and four time points using a 4 x 4 latin square.
M1 = microslide 1, M2 = microslide 2, etc.
In this section a similar experimental design was used to examine sperm motility at sites B-D after 0, 2 and 4 minutes incubation in the SZP gradient, (but a 3 x 3 latin square was used. Video-recordings of the sperm movement were made at the three sites at each of the three time-points to facilitate assessment.

Figure 27 illustrates the results obtained using the microslides. In both control and test chambers the area into which the protein had been drawn (site A.) contained few sperm as the protein had displaced the sperm sample. Further up the capillaries at site B, adjacent to the protein interface, sperm in the test samples displayed high frequency, quivering, flagellar movement but no progressive motility. This effect was obvious immediately and less than 5% of sperm were progressively motile after 4 minutes. This was the only site at which sperm motility appeared to be affected. Elsewhere, test and control samples retained progressive motility and the proportion of agglutinated sperm remained similar to the levels recorded previously.

4.6.3 Summary

The motility of spermatozoa close to the area of SZP introduction was reduced, elsewhere motility was unaffected. Visual observations indicated that the effect on sperm motility was an 'all or nothing effect'. Motility was either abolished (site B, test slides) or unaffected (sites C+D all slides and site B, control slides) by the preparation.

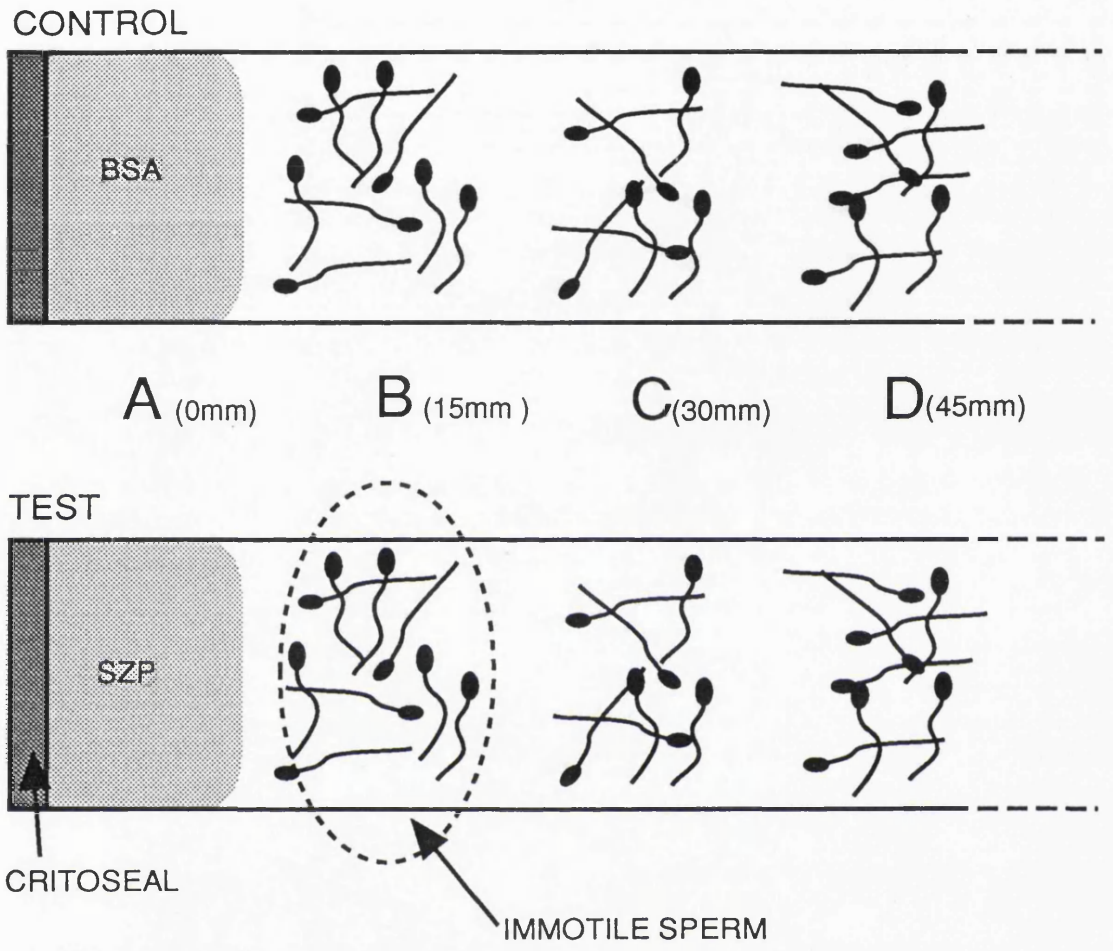


Figure 27. Sperm motility in a SZP gradient. Microslides were filled with sperm samples and then 500 µg/mL SZP (test) or 500 µg/mL BSA (controls) introduced at one end of the capillary. Sperm motility was assessed at the positions B-D after 0, 2, and 4 minutes. Sperm at site B of the test samples showed the adverse effects of SZP on sperm motility.

4.7 DISCUSSION

The work described in this section was intended to complement the previous chapters by providing CASA information about sperm movement during fertilization. Achieving this proved to be problematic and ultimately it was not possible to use the Hobson Sperm Tracker to analyze sperm exposed to solubilized zona pellucida glycoproteins. The experimental work underlines the importance of calcium in the action of the SZP and suggests that systems using solubilized SZP may not accurately mimic sperm:oocyte interactions.

PREPARATION OF THE SZP

The isolation procedure proved to be time-consuming and severely limited the range of experiments possible. The number of oocytes recovered from the ovaries was calculated to be in the order of 3×10^4 from 100 ovaries. This compares with typical yields from other investigators of between $0.5\text{-}6 \times 10^4$ zonae per 100 ovaries (Nakano, 1989). The most inefficient part of the procedure was the initial chopping of the ovaries as it was not possible to establish if the majority of oocytes had been released from the tissue. Subsequent steps in the protocol were briefly examined to evaluate oocyte wastage (e.g. the number of oocytes retained on the 1500µm and 200µm sieves) but relatively few oocytes were lost in this manner (<1000). Slicing the ovaries more finely would release additional oocytes into the isolation media, however, this approach increased the amount of debris within the oocyte preparation. This impeded sieving of the isolation media and hampered the separation of the oocytes on the Percoll gradient.

The total protein yield obtained from 100 ovaries was approximately 300µg. If each zonae contains approximately 33ng of protein (Nakano, 1989) this reflects a loss of 2/3rds of the protein theoretically available. However, both mature and immature oocytes were collected from the ovaries using this separation technique. The amount of glycoprotein within the zona pellucida is known to increase during oocyte maturation (Bleil and Wassarman, 1980b), therefore the mean amount of protein per oocyte collected would be less than the 33ng quoted above. In addition, it is probable that some protein is retained within the pellet after centrifugation of the solubilized zona pellucidae.

The large smears are typical of zona pellucida proteins separated under

reducing conditions (Dunbar and Raynor, 1980; Hedrick and Wardrip, 1986; O'Rand and Fisher, 1987) and are caused by the heterogeneity of the polypeptide glycosylation within each glycoprotein family. The smeared protein represents reduced and non-reduced components of the 55 kDα, 55 kDβ and 82-90 kD groups. Berger and co-workers (1989b) reported that the stoichiometric ratio of these three glycoprotein families is 1:1:1. The presence of only a relatively small band of the 21-25kD component therefore suggests that reduction of the 82-90kD group may not have been complete.

INHIBITION OF SPERM:ZONA BINDING

After establishing a standard isolation protocol it was important to verify that this procedure did not destroy the activity of the SZP. The results from the hemi-zona assay and acrosome reaction induction experiments appeared to confirm that the separation methods were suitable. The porcine hemi-zona assay was used to assess sperm:zona binding as this technique compensates for inter-oocyte variation using relatively few oocytes (Burkman *et al.*, 1988; Fazeli *et al.*, 1992). Sperm:hemi-zona binding was significantly reduced after co-incubation of the sperm with the SZP preparation, and this was interpreted as evidence for the binding of SZP-ligands to receptors on the sperm surface. Similar experiments have demonstrated the capacity of zona proteins from several species to block binding of homologous gametes (pig - Sacco *et al.*, 1984 and 1989; Berger *et al.*, 1989b; mouse - Bleil and Wassarman, 1980a; hamster - Gwatkin and Williams, 1977).

The observation that sperm from different boars vary in their ability to bind to homologous hemi-zona has been the basis for the development of the hemi-zona assay as a test of fertility (Fazeli *et al.*, 1992). The results for Part 1 of this experiment supported this observation; there was a significant effect of 'boar' on the number of sperm bound to the hemi-zonae. Interestingly, the results for Part 2 indicated that the SZP mediated decrease in sperm binding was also animal-dependent. Similarly, previous studies have examined the inhibition of sperm-oocyte binding by various concentrations of SZP and found that the resulting dose response curves differ significantly between boars (Berger *et al.*, 1989b). These observations imply that different individuals produce diverse responses to the SZP. The ability to quantify these inter-animal variations may aid the assessment of

sperm fertility. Berger and co-workers also reported variation in the response of different ejaculates to induction of the acrosome reaction by SZP (Berger *et al.*, 1989a). Approximately one third of ejaculates examined displayed no significant stimulation of the acrosome reaction by SZP, and individual boars were found to produce both 'non-responsive' and 'responsive' ejaculates. Further research is required to clarify the relationship between these animal-dependent and ejaculate-dependent SZP responses.

The results from subsequent experiments suggest that sperm used in the hemi-zona assay may have experienced negative effects on their motility, which could have caused the observed decrease in sperm:hemi-zona binding. Sperm motility was assessed prior to addition of the SZP and again before addition of the sperm to the hemi-zona. At these times the proportion of progressively motile sperm was still relatively high (>50%). Therefore the motility of these samples was less affected by the SZP than in subsequent experiments (and was almost certainly not the cause of the decrease in sperm:hemi-zona binding). The retention of sperm motility in these experiments could reflect the method of sperm preparation used (see later) or the dilution of the SZP concentration to 5 µg/mL prior to the hemi-zona assay. This latter hypothesis would suggest that the deleterious effects on sperm motility are reversible and therefore distinct from the processes creating the non-reversible block to sperm:hemi-zona binding.

In both parts of the hemi-zona assay experiment, the eventual sperm:protein:hemi-zona ratio in the incubation droplet was the same. Part 1 indicated that the inhibition of sperm-zona binding was not due to the presence of SZP in the media during co-incubation. However, pre-incubation of sperm with a higher concentration of SZP (part 2) followed by reduction of SZP concentration prior to insemination did block sperm-hemi-zona binding. This indicates a specific interaction of the sperm and glycoproteins that occurred during the initial co-incubation period when the SZP concentration was 100µg/mL.

INDUCTION OF THE ACROSOME REACTION

The addition of SZP preparations to sperm populations significantly increases the proportion of acrosome reacted sperm (pig - Berger *et al.*, 1989a; rabbit - O'Rand and Fisher, 1987; mouse - Bleil and Wassarman, 1983; human - Cross *et al.*, 1988; hamster - Cherr *et al.*, 1986). In the present study, co-

incubation of boar sperm in TBM media containing 125 µg/mL SZP or porcine IVF media containing 250 µg/mL SZP significantly increased exocytosis after 30 and 60 minutes respectively. In a previous report of the use of porcine SZP to stimulate the acrosome reaction, Berger and co-workers pre-incubated sperm for 6 hours in Earles M199 medium and then co-incubated for 30 minutes with various concentration of SZP. (Berger *et al.*, 1989a). Using this protocol they found that SZP concentrations of 125µg/mL and 250µg/mL caused a significant increase in the number of acrosome reacted sperm. Although protein concentrations are comparable, the proportions of sperm defined as exocytosed by Berger and co-workers were lower than found in the present study. Mean values for acrosome reacted sperm after 30 minutes were <20% (Berger and co-workers) compared to approximately 60% after 30 minutes in section 4.3. This is partly a reflection of the choice of media (the TBM media is used to promote the proportion of acrosome reacted sperm) and partly a result of the different subjective definitions of an acrosome reacted spermatozoon. There may also be a difference between fresh and extended sperm as discussed later.

LOCALIZATION OF ZONA BINDING SITES ON THE SPERM SURFACE

The immunocytochemistry performed using antibodies to the porcine SZP indicated that binding occurred over the acrosomal region and the flagellum. Exposure of the sperm mid-piece to the zona pellucida glycoproteins would not be expected in the early events of fertilization, where initial contact between gametes is via the sperm head (Katz *et al.*, 1989; Yanagimachi, 1994). However, pig zonae are approximately 16µm thick (Dunbar *et al.*, 1991) whilst the length of the boar sperm head is approximately 8µm (Bonet and Briz, 1991). Sperm penetrate the oocyte obliquely and the sperm mid-piece would enter the zona during fertilization. A debilitating effect of the zona glycoproteins on sperm motility at this time would be disadvantageous and is therefore unlikely. This suggests that the binding of the SZP to the sperm mid-piece may not be physiologically relevant.

Previous reports of the binding of zona components to the sperm mid-piece are contradictory. Both hamster and rabbit sperm co-incubated with undifferentiated SZP demonstrated increased labelling of the mid-piece compared to controls (Gwatkin 1978; O'Rand and Fisher 1987). The methods of detecting binding were different in the two studies: Gwatkin employed fluorescein-labelled

antisera to the hamster zona whilst O'Rand and Fisher labelled whole rabbit zonae with fluorescein, heat-solubilized the zonae and applied the labelled solution to live sperm. In contrast, studies using mouse ZP3 glycoproteins, porcine SZP and ZP3 glycoproteins found that receptor activity was confined to the anterior head region (mouse - Bleil and Wassarman 1986; pig - Gwatkin and Klein, 1985; Yurewicz *et al.*, 1993). These studies used ^{125}Iodine radiolabelling, fluorescein-labelled anti-zona serum and biotinylation, respectively, to localise the zona glycoprotein receptor sites on the sperm surface.

The presence of zona binding sites on the sperm mid-piece therefore remains controversial. The results from the present study suggest that porcine ZP3 ligands will bind to the sperm mid-piece contrary to the evidence from Gwatkin and Klein (1985) and Yurewicz and co-workers (1993). This result is interesting in view of the effect of the SZP preparation on sperm motility.

SZP INDUCED MOTILITY LOSS

The experiment described in this chapter revealed that co-incubation of sperm with SZP impaired sperm movement. The observed loss of progressive motility was unexpected. The majority of studies examining the action of SZP on viable sperm have concentrated on the sperm acrosome, but a few make reference to sperm motility in the in vitro systems used (pig - Berger *et al.*, 1989ab; hamster - Gwatkin and Williams 1977; mouse - Bleil and Wassarman, 1980a; human - H. Moore *pers comm*). These studies have not revealed deleterious effects of zona pellucida glycoproteins on sperm movement. It is possible therefore that the SZP-induced effects on sperm motility observed in the present study are artefactual. An examination of the SZP product and the preparative methods used does not support this hypothesis.

Protein solubilization with ammonium bicarbonate is a common method of protein isolation and was chosen because the ammonium bicarbonate is removed during lyophilization of the protein. In addition, both the method of preparing the solubilized SZP and the concentrations used have been employed previously by Berger and co-workers who reported normal sperm motility throughout the experiments (Berger *et al.*, 1989a). It would seem unlikely therefore that the method of protein preparation subsequently affected sperm motility.

A further possibility was that unidentified components were co-purified with

the zona pellucida proteins during the processes of zona isolation and heat-solubilization. However, sperm motility is unaffected by the addition of the SZP preparation in the absence of Ca^{2+} ions. This would not be expected if the sperm motility loss was caused by non-specific detrimental component(s) within the SZP. Alternatively the SZP preparations could have contained debris, such as small pieces of zona, which acted as loci for the aggregation of sperm. This possibility was checked by microscopic examination of SZP samples. However the preparations appeared clear and no debris could be seen (data not shown).

Alternatively, the presence of SZP may have promoted non-specific binding, increasing the number of sperm bound to debris within the sperm preparation. Solubilized porcine SZP are known to promote the binding of human sperm to human hemi-zonae (Windt *et al.*, 1992). If this was the cause of the extensive agglutination observed, a decrease in the proportion of bound sperm might be expected as the number of acrosome reacted sperm increased. This effect was observed in the test samples after 15 minutes. Although this hypothesis may explain the increase in sperm agglutination it cannot account for the observation that samples contained single (i.e. unagglutinated) sperm which were non-progressive and that spermatozoa displayed high frequency vibrations of the flagella after co-incubation with the SZP (see section 4.4).

The protocol routinely employed for these experiments used extended semen, which may have had a significant effect on sperm:SZP interaction. Where normal sperm motility has been reported in the presence of SZP preparations (see above for references) freshly ejaculated or epididymal sperm have been used. Liquid storage of semen is known to cause disruption of the peri-acrosomal region of sperm (apical ridge) and to impair sperm motility (Clarke and Johnson, 1987, Gadella *et al.*, 1991). Further studies have shown that incubation of sperm in the presence of seminal plasma increases the resistance of these cells to cold-shock, possibly as a result of changes to the plasma membrane (Tamuli and Watson, 1992; Robertson *et al.*, 1988b). Most significantly, ZP binding-glycoproteins are present within boar seminal plasma and these are known to adhere to the surface of spermatozoa on ejaculation (Hanqing *et al.*, 1991; Parry *et al.*, 1992; Sanz *et al.*, 1992). The role of these glycoproteins in fertilization is unclear; evidence exists that they may act as primary ligands involved in the initial binding of sperm to the zona pellucida (Hanqing *et al.*, 1991; Jonáková *et al.*, 1991; Sanz *et al.*, 1992).

Extended storage in the presence of these glycoproteins could increase binding of the SZP to the sperm surface, potentiating the actions of the ligand.

It seems plausible, therefore, that extended liquid-storage of sperm in the presence of seminal plasma may have made these cells more susceptible to the effects of the SZP, causing the observed disruption of sperm motility. Addition of the SZP preparation to fresh sperm samples would enable clarification of this hypothesis but was not performed for the present study. However, the hemi-zona assay experiments, described in section 4.2, used fresh ejaculates, and estimates of sperm motility were higher than in subsequent experiments.

THE INTERACTION OF SZP WITH CALCIUM

The experiments described here were performed with the high calcium TBM media (to facilitate comparisons with sperm motility data obtained in the preceding chapter). The same medium has been used previously in similar experiments (Berger *et al.*, 1989a) involving low concentrations of SZP (25 to 50 µg/mL) with no report of SZP-induced motility loss.

Zona induced acrosome reactions are known to be calcium mediated (for a review see Fraser, 1992). The interaction of the SZP with receptors on the sperm plasma membrane results in a calcium influx; the actual mechanism is unclear but available evidence suggests that voltage dependent calcium channels are involved (Fraser and McIntyre, 1989; Florman *et al.*, 1992; Okamura *et al.*, 1993). It was hypothesized that the SZP-induced loss of motility, observed here, may be a reflection of a high intracellular [Ca^{2+}] environment and experiments were performed to investigate the involvement of this ion.

Incubating spermatozoa in a calcium-free environment prevented the deleterious action of the SZP on motility and the SZP-induced increase in exocytosis[4]. Co-incubation of the sperm and SZP preparation in the presence of

[4] Within this experiment, one ejaculate responded differently to the other three; sperm in this ejaculate retained good motility for all test samples regardless of calcium concentration. Berger and co-workers also reported that some ejaculates do not undergo an SZP-induced increase in acrosome reacted sperm (Berger *et al.*, 1989a). The 'unresponsive' ejaculates came from several boars, most of which also produced 'responsive' ejaculates. Although the ejaculate used in section 4.5 produced anomalous motility results, the acrosome reaction results were similar to those obtained for the other ejaculates in this experiment.

higher concentrations of calcium (2mM or 40mM) reproduced the deleterious effects on sperm motility and induced acrosome reactions. These results indicated that the loss of motility was calcium-dependent.

The immunocyochemistry performed in section 4.3 demonstrated that components within the SZP (possibly ZP3) were binding to the sperm flagellum as well as the sperm head. Binding of the zona ligands to receptors on the sperm head results in an increase in intracellular calcium concentration (see above); a parallel receptor pathway in the sperm mid-piece could produce similar increases in flagellar [Ca^{2+}]. Sperm movement is highly calcium-dependent and increases in intracellular calcium are known to have deleterious effects on sperm motility (for a review see Tash and Means, 1983). Recent research has confirmed that co-incubating sperm with zona agonists increases internal [Ca^{2+}] within the sperm head *and flagellum* via VDCC (Florman, 1994). However, the inability of the calcium channel antagonist nifedipine to prevent the detrimental effects on motility suggests that either (a) the effects on motility do not require the internalization of calcium or (b) that mechanisms other that the VDCC channels cause the putative increase in flagellar [Ca^{2+}].

These results indicate that the action of the SZP on motility is a specific, calcium-dependent event. To elucidate the actual mechanism by which this process occurs is beyond the scope of this thesis. A putative mechanism could involve calcium-mediated binding of SZP components to the plasma membrane over the acrosome and mid-piece, simultaneously causing agglutination of the sperm and impairment of flagellar activity through raised internal calcium levels.

SZP CONCENTRATION

The possibility that the SZP-induced effects on sperm motility were concentration dependent was investigated using SZP gradients. These experiments indicated that the change in sperm motility was a threshold response. Exposure to more than the threshold concentration of SZP resulted in sperm agglutination and immotility. Spermatozoa observed swimming into the area characterized by immotile sperm rapidly became non-progressive.

Sperm motility at concentrations below this threshold level appeared unaffected. A gradual loss of sperm motility at sites further down the protein concentration gradient was not observed in the time course of these experiments

(6 minutes). It is possible, however, that the SZP did not form a true concentration gradient in the manner of the mouse IgG, and that protein diffusion did not occur (thereby restricting the SZP-induced motility loss to the sperm sample:SZP interface).

SPERM AGGLUTINATION *IN VIVO*

All boar semen samples prepared using the protocols outlined in section 2.1 contained a proportion of agglutinated spermatozoa (see chapter 2 and 3). Addition of the SZP preparation greatly increased the size and frequency of the sperm agglutinations. Lindahl (1978) suggested that hormones present in the female reproductive tract, the cumulus cells and the oocyte can induce agglutination of bovine sperm (adrenaline, noradrenaline, estradiol-17β and corticosterone) This hormone-induced H:H agglutination was regarded as distinct from the H:H agglutination that occurs after washing of ejaculated sperm (Lindahl and Sjöblom 1981). Sjöblom later observed that the extent of estradiol-17β induced sperm:sperm binding in vitro was closely correlated to sperm:egg binding and IVF success in mice (Sjöblom, 1986; Sjöblom and Lindahl, 1986). He concluded that increased 'induced' agglutination could be physiologically relevant for fertilization resulting from a general promotion of sperm binding by hormones in the female tract. (Evidence that estradiol promotes the binding of boar sperm to mucosal explants of the oviduct supports this hypothesis (Suarez *et al.*, 1991b)). This is similar to the concept of 'progesterone-priming' of spermatozoa in the female tract whereby binding of progesterone to sperm surface receptors (Tesarik and Mendoza, 1993) facilitates, but does not cause, exocytosis.

In contrast, maximal egg binding ability of epididymal sperm occurs when the spermatozoa originate from regions of the epididymis where the incidence of agglutination is low (cauda, Dacheux *et al.*, 1983). The sperm agglutination observed by Dacheux and co-workers probably represents the association of cells described in chapter 2 and 3 of this thesis. This suggests that the relevance of sperm agglutination to fertilization in vivo depends on the type of agglutination.

Further experimental evidence would be needed to assess whether the SZP-induced agglutinations are related to sperm function. In section 4.2, the presence of the SZP was shown to reduce sperm:zona binding. In this respect, the SZP-induced binding is at variance with the hormonally induced agglutination

demonstrated by Lindahl (Lindahl 1978). A further difference is that the agglutination induced using cAMP, adrenaline, noradrenaline, estradiol-17β and corticosterone is unaffected by the absence of calcium from the media (agglutination of bovine sperm occurred at 2-3 x 10^{-6} M calcium; Lindahl and Sjöblom, 1981). This was clearly not the case for SZP-induced agglutination, although this could be a reflection of species-different requirements for calcium.

4.8 SUMMARY

Methods to isolate and solubilize the porcine zona pellucida were adapted from previous studies and the resulting solubilized zona pellucida preparation was found to contain the glycoprotein families commonly identified in the porcine zona pellucida. Addition of this preparation to sperm samples resulted in increased sperm agglutination and the loss of progressive motility. Subsequent experiments indicated that this was a calcium-dependent event which does not require internalization of this ion. The cause of the SZP-induced motility loss was not elucidated but may arise through binding of components within the SZP preparation to the sperm mid-piece in conjunction with a non-specific promotion of sperm binding.

CHAPTER 5 - DISCUSSION

Artificial insemination is a valuable technique for both pig breeding companies and commercial farmers, and its use is now widespread within the U.K. pig industry (Reed, 1990b). Cost-benefit analyses indicate that by increasing the usage of AI by only 5%, the U.K. pig industry could save nearly £1 million pounds *per annum* (G.Boon, *pers comm*). Awareness of the economic rewards of AI has resulted in a rapid increase in the demand for bottled semen over the last decade (Reed,1990a).

Semen delivery services within the U.K. are distributed throughout the country. The ability to transport bottled semen through the postal system means that SDS companies are in competition both with each other and with similar services operating within Europe, and must always seek to increase efficiency in order to remain viable. The ability to predict ejaculate fertility would facilitate the efficient use of semen by these companies and enable the rapid removal of low fertility boars from the SDS stud. The methods of semen assessment currently in use can only discern gross infertility (e.g. asthenozoospermia, oligozoospermia) and not probable fertility (for a review see Woelders, 1990, Reed, 1988). Several clinical studies of human semen have shown that sperm motility is a useful 'indicator' of fertility (for references see chapters 1 and 3). The aim of the present study was to provide a rapid and objective method of assessing boar sperm movement and to examine the value of motility measurements for the prediction of fertility.

The HST is a novel system for assessing sperm motility and has been developed through collaboration with engineers from Sense and Vision Electronics Systems Limited (Sheffield, U.K.). Refining the HST software whilst simultaneously using the system at the Institute of Zoology proved to be a productive approach. It was possible to define the facilities required for sperm assessment, and to discuss the implications of engineering theory on CASA analysis (Boyers *et al.*, 1989; Davis *et al.*, 1992a; Owen and Katz, 1993). The resulting system allowed flexible and comprehensive analysis of specimens, and is now commercially available.

HST set-up parameters were optimized for a user-defined grey-threshold and a sperm concentration of 20×10^6 sperm/mL (used throughout this research). Several studies have shown that grey-threshold values significantly affect motility results (Knuth *et al.*, 1987; Mack *et al.*, 1988, Toth *et al.*, 1989; Davis *et al.*,

1992b), but at present no objective method exists to establish optimal grey-threshold levels. Although standard microscope settings were maintained throughout all of the experiments in this study, the light intensity of video-recordings fluctuated slightly between samples (the factor that most significantly affects grey thresholds). An effective way of equilibrating the brightness of the sample image would greatly facilitate the standardization of CASA systems (Davis and Katz, 1993a).

The sperm concentration employed in these studies was low compared with the range of concentrations commonly used in CASA (Davis and Katz, 1993). Tracking cells for extended periods of time increases the probability of sperm colliding or crossing paths (Robertson and Middleton, 1992), which are factors known to distort CASA results (Vantman *et al.*, 1988; Davis and Katz, 1993). HST analysis therefore requires samples to be more dilute than for CASA analysis using frame-store systems. Accordingly, the HST would be the preferable CASA system when sperm concentrations are low - for valuable, poor quality, material such as asthenozoospermic samples or for the analysis of single spermatozoa (Ishijima and Mohri, 1985; Ishijima *et al.*, 1992).

Although sperm concentration was adjusted to 20 x 10^6 sperm/mL, incubation of the washed samples resulted in the formation of sperm agglutinations which reduced the concentration of single, activated, spermatozoa. Davis and Katz (1992) have stated that samples containing more than 10% agglutinated sperm are not suitable for CASA analysis, although they give no reason for this assertion. In the future it may be possible to prevent sperm agglutination prior to CASA by the addition of antagglutinin compounds identified in the epididymal and seminal plasma of the boar (Dacheux *et al.*, 1983; Harayama *et al.*, 1994). However, in the present study, the most significant correlations between sperm motility and fertility were obtained after two hours incubation of the sample, at which point sperm agglutination was extensive. Therefore the occurrence of agglutination may be relevant to subsequent fertility (demonstrated for hormonally induced agglutinations of mouse sperm by Sjöblom, 1986), and their motility an important measure of sperm samples.

The process of system validation, described in chapter 2, demonstrated the sensitivity of HST results to the user-defined set-up parameters, in common with other CASA systems (Mack *et al.*, 1988; Mortimer *et al.*, 1988a,b; Toth *et al.*, 1989;

Olds-Clarke *et al.*, 1990; Owen and Katz., 1993; Slott *et al.*, 1993; Davis and Katz, 1993). HST result were found to depend on the search radius and minimum frame number chosen. Parallel studies using marmoset sperm indicated that set-up parameters are species-dependent, probably as a result of variations in size, morphology and motility patterns.

The accuracy and precision of the HST were evaluated and the results found to be comparable to similar examinations of other CASA systems (Mack *et al.*, 1988; Davis *et al.*, 1992b, Slott *et al.*, 1993). Accuracy was evaluated using a standard videotape of moving bright spots, rather than actual boar sperm. The HST set-up parameters had been optimized for boar sperm and may have been inappropriate for assessment of the moving spots. Therefore the evaluations made using the calibration tape probably underestimated the accuracy of the HST. Further error arises in the data from all CASA systems because measurement calculations are made using an approximation to the sperm track (see figure 4., chapter 1.). In the HST this produces an error of <5% (G.Hobson, *pers comm*) which would not be detectable when measuring normal biological systems. In view of these sources of error, the accuracy obtained by the system was good.

The calibration tape used in these experiments was a gift from Sense and Vision Electronic Systems Ltd. Using computer graphics it is possible to generate artificial sperm images which move with a known velocity. However these analogues do not exactly reproduce the erratic, phasic motion of spermatozoa. (Additionally, assessment of computer generated motion at inappropriate framing rates will be inaccurate owing to the process of aliasing described in chapter 2). A standardized tape of real sperm would ensure that representative movement characteristics were analyzed. However, this requires the initial accurate evaluation of the sperm movement for which no method currently exists. The circularity of this problem has prevented the commercial production of a calibration tape. Access to such a tape would greatly facilitate both clinical research using CASA systems and evaluation of the systems themselves. In a clinical setting, standardization would improve the comparability of results from different locations (Davis *et al.*, 1992b) and could be vital for ensuring quality control within centres requiring any form of licensing (Davis and Katz., 1993a and b). Further improvements to CASA analysis have already been identified through the use of artificially generated sperm tracks (Davis *et al.*, 1992a; Owen and Katz, 1993) and

similar research would be promoted by wider access to analogues of sperm movement.

The HST is unique amongst the CASA systems as it performs continual tracking over periods of minutes rather than seconds. It is unclear whether tracking many sperm for a short period of time is preferable to tracking fewer sperm for a longer period[5], and may depend on the type of research being performed. In the context of establishing a fertility predictor, evaluation of the most suitable approach would require comparative studies using the HST and a frame-store system, in conjunction with fertility trials. Alternatively, analysis of the video-recordings made in the course of this research would provide a useful insight into this question. However, the sperm concentration used (20 x 10^6 sperm/mL) is comparatively low for the frame-store systems (see above).

Analyzing each spermatozoon for extended periods of time may be advantageous - increasing the duration for which a sperm trajectory is measured should compensate for limitations in the number of sampling points per beat cycle (Davis and Katz., 1993a). Increasing the number of sampling points describing a trajectory improves the accuracy of the CASA measurements. The frame-store systems, limited to approximately 30 sampling points, obtain most accurate data by sampling a few oscillations of the trajectory at high frequencies, 60 Hz or more (Owen and Katz, 1993). The HST attains similar levels of accuracy by sampling many oscillations of the trajectory using lower framing rates, i.e. longer sampling interval vs higher sampling rate (Davis and Katz, 1993a). The production of an HST with a framing rate of 50 Hz should further improve the accuracy of trajectory measurements.

The extended periods of analysis which are possible using the HST do have associated disadvantages. The multiple tracking of sperm cells moving in a circular pattern (which pass repeatedly through the analysis window of the HST) can distort motility results if the proportion of circular swimming cells is relatively large. In addition, a single measurement is produced for each sperm track which may mask changes in motility that occur within the trajectory. Sperm movement

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[5] Ergodic theory would suggest that the two strategies are equivalent, i.e. from a random population as much information can be obtained from many sperm tracked over a short time as from fewer sperm tracked over a longer time.

is phasic (e.g. hyperactivation, Katz and Yanagimachi, 1980) and reporting a measurement as a mean value may reduce the merit of the information from the trajectory. A prototype HST system capable of evaluating the movement change along each track has been developed, and future versions of software could include modal, maximum and minimum CASA measurements. This will enable the regularity of sperm trajectories to be investigated, and facilitate quantification of the episodic movement of spermatozoa.

The issue of fertility calibration in terms of sperm motility analysis necessarily has to be approached differently with different species groups. For domestic animals comprehensive fertility data can be derived through the multiple insemination of split ejaculates from many individuals, and experiments can be designed to accommodate considerations such as sperm dose and female fertility (discussed in section 3.4 and reviewed in Den Daas, 1992). In a human clinical setting the number of inseminations performed using semen from a particular donor is restricted, for ethical reasons. Similarly, insemination procedures are designed to maximize the probability of a conception. These factors prevent accurate quantification of the *in vivo* fertility of human sperm samples and dictate the type of research that can be performed. (*In vitro* fertility is more easily assessed and several papers have correlated semen characteristics with this parameter (Holt *et al.,* 1985; Liu *et al.*, 1988, 1991; Liu and Baker, 1992; Oehninger *et al.,* 1992). However, *in vitro* fertility is not analogous to *in vivo* fertility, hence the use of IVF for infertile couples). Clinical studies often partition males into categories (e.g. 'infertile', 'poorly fertile' and 'fertile') on the basis of insemination outcome, and semen characteristics are compared across these groups (Holt *et al.*, 1989; Davis *et al.*, 1991; Marshburn *et al.*, 1992; Mackenna *et al.*, 1993). Analyses of human spermatozoa therefore concentrate on the differences between fertile and infertile individuals whilst studies of domestic species tend to use fertile individuals to examine the correlates of fertility.

Two fertility trials were performed in the present study and each indicated that velocity data gathered over a two hour incubation period were significantly related to fertility measures (both conception rate and litter size). In trial 2 the change in sperm velocity over the first two hours was found to be the most significant factor - VSL decrease (VSL_0 - VSL_2) was positively correlated with fertility whilst VAP change (VAP_0 - VAP_2) was negatively correlated with fertility.

This indicates that spermatozoa in more fertile ejaculates maintain their velocity (maintenance of kinetic activity) whilst producing less progressive trajectories (smaller VSL). Rapid sperm motility with little displacement is characteristic of hyperactivation (Katz *et al.*, 1993), and clinical studies have found the frequency of hyperactivated human sperm to be higher in semen from donors than in semen from patients (Burkman *et al.*, 1984; Pilikian *et al.*, 1991; Mackenna *et al.*, 1993). Therefore the correlations described above may reflect the incidence of this movement pattern in boar sperm samples.

This hypothesis is supported by the strong, though non-significant, correlation of ALH after 2 hours to conception rate in trial 1. However, the velocity results from trial 1 are in direct contradiction - VSL after two hours was positively correlated with average litter size and the corresponding VAP measurement was negatively correlated to average litter size. These results suggest that after 2 hours, more fertile samples contain sperm swimming slower with straighter trajectories (low VAP but high VSL). It has not proved possible either to reconcile these two findings or to identify the reason for the disparity. The fact that both trials resulted in significant correlations of HST measurements with fertility is extremely encouraging. However, as the results from trial 1 and trial 2 differ, it is unclear whether these results are reproducible.

The aspects of sperm function *in vivo* represented by these measures are discussed in chapter 3. In brief, the maintenance of sperm velocity probably indicates the capacity of sperm to survive within the female tract, whilst the positive correlation of ALH to fertility may indicate the ability of sperm to pass through the female tract[6] (for references see chapter 3). Both litter size and conception rate were significantly correlated with similar motility variables. This would seem logical as properties of a sperm population that increase conception rate would presumably increase the number of conceptions within one female (i.e. litter size).

In the present study, the measurements correlated with fertility were mean values for sperm samples. However, the number of inseminated spermatozoa that eventually fertilize an oocyte (0-30 assuming 30% embryonic loss (Hunter, 1991))

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[6] Analysis of ejaculates from low fertility boars contradicted this hypothesis. Sperm from the SDS-rejected boars were found to have greater mean ALH than the SDS-stud boars after 60 and 90 minutes incubation (see section 3.6). Additionally, the reduced STR and LIN values suggest that the movement of sperm from the SDS-rejected boars was more erratic and less progressive.

eventually fertilize an oocyte (0-30 assuming 30% embryonic loss (Hunter, 1991)) is small compared to the number inseminated (2 doses of 1.5 billion sperm). Analyzing sperm sub-populations may therefore prove to be more informative than considering samples as a single population (Amann, 1989; Amann and Hammerstedt, 1993; Davis *et al.*, 1991). To investigate this hypothesis, data on boar sperm populations generated by the HST were sub-divided using the statistical technique of cluster analysis (Afifi and Clark, 1990; Culasso *et al.*, 1993). Video-recordings of sperm displaying extreme movement characteristics were used to verify this methodology (Holt *et al.*, 1992). Artificial analogues of these sperm movements would have greatly facilitated this process and enabled clearer assessment of the accuracy of the technique; this further demonstrates the need for calibration tapes (discussed above).

The use of cluster-analysis enabled sub-populations containing hyperactivated sperm to be identified. Hyperactivated spermatozoa have known characteristics (high VCL, low VSL, high ALH and low LIN; Robertson *et al.*, 1988a; Burkman, 1991; Katz *et al.*, 1993) and these sub-populations are therefore easily recognizable. Further analyses of sperm samples using this technique may reveal equally important (though less visually distinctive) sperm movement patterns; however, careful examination of the sub-populations obtained using the variables VAP, MAD and ALH did not reveal any consistent groupings besides the hyperactivated cells. More extensive research using cluster analysis and alternative combinations of motility variables may prove instructive.

Previous studies have described the modification of movement patterns that accompanies sperm penetration of the cumulus oophorus (Drobnis *et al.*, 1988a) and factors within this cellular mass are known to modulate sperm motion (Fetterolf *et al.*, 1994). Further movement changes occur when sperm encounter the zona pellucida (Drobnis *et al.*, 1988b; Katz *et al.*, 1989), but at present there is no evidence that these are caused by stimuli from the zona. Visual assessments suggest that sperm motility is unaffected by exposure to SZP (Berger *et al.*, 1989ab; Sacco *et al.*, 1989; Gwatkin and Williams, 1977; Bleil and Wassarman, 1980a), however, subtle motility variations would not be detectable in this manner.

Having developed methods for the CASA analysis of boar sperm, it was proposed that these techniques could be used to detect SZP-induced motility changes. Quantifying these movement patterns would enable identification of a

'responsive' population of sperm within ejaculates, and could aid fertility assessment. Assays evaluating the reaction of motile sperm to the SZP would record whether sperm produced the appropriate response to physiological stimuli, and may be more sensitive than evaluations of sperm acrosomes or motility alone (see Amann and Hammerstedt, 1993).

Unfortunately, addition of the SZP to spermatozoa in TBM medium produced unexpected deleterious effects on motility which meant that analysis using the HST was inappropriate. Possible reasons for the disruption of motility are discussed in chapter 4. The effects of SZP on sperm motility were found to be calcium-dependent - motility was preserved in the absence of this ion. Several studies have shown that the zona agonist causes an influx of calcium into the spermatozoa (Florman *et al.*, 1992: Clark *et al.*, 1993) in both the head and tail regions (Florman, 1994). Elevating internal calcium concentration above a critical threshold disrupts protein phosphorylation resulting in the inhibition of sperm motility (for a review of these mechanisms see Tash and Means, 1983). It seems probable therefore that binding of the SZP to the sperm mid-piece (identified in this study using anti-SZP and anti-ZP3 antibodies) increased flagellar calcium levels causing the observed loss of progressive motility.

Sperm motility is modulated in the female tract by factors from the follicular fluid (Revelli *et al.*, 1992; Lee *et al.*, 1992) and the cumulus cells (Fetterolf *et al.*, 1994). Hypothetically, binding of the zona pellucida glycoproteins to the sperm mid-piece during fertilization could effect further movement changes. However, a debilitating effect on sperm motility during penetration of the oocyte would presumably be disadvantageous, and is unlikely. Therefore the physiological relevance of the SZP-induced disruption of motility remains uncertain.

Further investigation of SZP concentration effects and the cause of the SZP-induced motility loss were not possible owing to time restrictions. Future research could determine whether motility loss is prevented by the addition of anti-SZP antibodies prior to the introduction of the SZP, and thereby establish whether the motility effects are artefactual. In addition it would be interesting to determine whether the effects of the SZP on acrosome reactions (observed in chapter 3) are accompanied by similarly animal-dependent motility changes.

The presence of an animal-dependent effect on acrosome reactions in trial 2 but not trial 1 may reflect the incubation periods used in the two experiments.

In trial 1, sperm were not challenged with ionophore until after 6 hours incubation. By this time the rate of spontaneous acrosome reactions was high and did not differ significantly between the boars. In contrast, spontaneous and SZP-induced acrosome reactions (after 3.5 hours incubation and one hour co-incubation respectively) were strongly animal-dependent. Shortening the incubation period prior to analysis of the ionophore-induced acrosome reactions could result in an assay more relevant to fertility, and in agreement with previous studies using human sperm (Cummins *et al.*, 1991; Pilikian *et al.*, 1992; Pampiglione *et al.*, 1993). However, in a clinical setting ionophore-challenge assays have been shown to partition fertile (donor) or sub-fertile (patient) samples - such assays may be less discriminatory when applied to highly fertile samples only.

In common with other assays of the male gamete (e.g. evaluation of spontaneous and induced acrosome reactions, labelling of cells with antibodies and lectins, morphological assessments), CASA analysis is simple to perform. In contrast, assays of sperm:oocyte interaction are technically complex and involve extended execution and assessment periods (e.g. hemi-zona assay, hamster egg penetration assay, binding assays). Additionally, obtaining the oocytes required for such assays may be difficult (e.g. the majority of rare or exotic species). Therefore although the discriminating power of such assays appears good (hemi-zona: Oehninger *et al.*, 1992; Franked *et al.*, 1993; hamster egg penetration assay: Berger and Parker, 1989; Prasad, 1984; binding assays: Liu and Baker, 1992; Fazeli *et al.*, 1993), if controversial (Talbert *et al.*, 1987; Marshburn *et al.*, 1992; Liu and Baker, 1992), their practical application is limited.

A further advantage of CASA is that the process is automated, and does not require the labour-intensive analysis of individual sperm. Although flow cytometry can aid the assessment of fluorescently labelled cells, the cost and complexity of this technology is prohibitive. Automated morphologizers are available (Wang *et al.*, 1991b,c; Kruger *et al.*, 1993), however subjective morphological assessments performed routinely at the Thorpe Willoughby Centre have demonstrated a low correlation of this variable with fertility (Reed, 1988).

The present research indicates that analysis of sperm samples using the HST would provide a useful method of assessing fertility, either alone or in conjunction with traditional assessment methods. (Reed, 1988). For maximum efficiency, the SDS companies require a rapid, simple (or automated) test that can

be performed shortly after ejaculate collection. The protocol developed in this study requires a 2 hour incubation of washed, 24-hour liquid stored sperm - therefore future work must adapt the present methodology to a more convenient form, without incurring a loss of test sensitivity.

Converting the current procedures to a more simplified or automated form would be uncomplicated. An automated microscope stage has been interfaced with HST software and analysis of multiple samples contained within multiwell plates has been performed (H.D.M. Moore *pers comm*). The incorporation of a 2 hour period between ejaculate collection and semen bottling may prove more problematic. However, the present study indicates that motility results collected without this period of incubation do not provide informative data. Therefore between 1.5 and 2.5 hours is envisaged for processing and analysis of samples. Effective application of CASA technology may require SDS companies to alter slightly their production methods, perhaps through the earlier collection of ejaculates or the later dispatch of bottled semen.

Evidence from this research suggests that the motility of fresh and stored sperm samples is highly correlated initially, but that the significance of these correlations is reduced on incubation. The assumption that valuable motility data will be obtained by using fresh sperm in the established protocols is therefore unsafe. Further trials will be required to evaluate the effectiveness of HST analyses of fresh samples. Although less efficient, it may prove necessary to analyze samples after a period of liquid storage. Ultimately, the desire for convenience should not impair the effectiveness of an assay. A single rapid test performed on the morning of semen collection may be more convenient, but less useful, than a more elaborate test performed later.

The present research has demonstrated the value of motility characteristics in the assessment of boar sperm fertility, and the results concur with previous studies of human sperm (see chapter 3 for references). It would be valuable to ascertain whether these findings have a more universal application - to establish whether the general principles apply across a wide variety of species. Whilst comparative studies using domestic species would be possible (providing financial resources and fertility information were available) the evaluation of this technique for rare or exotic species is more problematic. Background motility data do not exist for the majority of species, which prevents sensible interpretation of any

CASA measurements made. The accumulation of such data at every possible opportunity would greatly aid these and similar comparative studies.

We have demonstrated that CASA using the HST is an effective method of sperm assessment and appropriate conditions for the analysis of boar sperm have been derived. Future research should aim to automate these processes and so facilitate the use of this system by the SDS companies. Primarily, correlations between the motility of fresh sperm and the fertility of liquid stored sperm should be evaluated, concentrating on motility data collected over a two hour incubation period.

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ADDENUM

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APPENDIX

Composition of media

CHEMICAL	MEDIA				
	IVF	TBM	12mM	2mM	Calcium-free
$CaCl_2$ (mM)	4.5	40	12	2	0
TRIS (mM)		20	20	20	20
GLUCOSE (mM)	5	11	11	11	11
Na Pyruvate (mM)	1	5	5	5	5
KCl (mM)	3.1	3	3	3	3
NaCl (mM) ‡	90-120	~84	~126	~141	~144
$NaHCO_3$ (mM)	15				
NaH_2PO_4 (mM)	0.3				
HEPES (mM)	20				
$MgSO_4$ (mM)	0.4				
Lactate (mM)	16.7				
Kanamycin (µg/mL)	100				
Gentamycin (µg/mL)	50				
Phenol Red	+	+	+	+	+
BSA (mg/mL)	5	5	5	5	5
pH †	7.4	7.85	7.85	7.85	7.85
Osmolarity (mOsm)‡	300	335	335	335	335

IVF media was gassed with carbon dioxide prior to addition of the pyruvate and BSA.

‡ The osmolarities of all media were adjusted by the addition of NaCl.

† pH adjusted using NaOH