Sperm structure and quantitative sperm motility analysis of three wild antelope species: *Aepyceros melampus, Damaliscus dorcas phillipsi and Connochaetes taurinus*

By

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Declaration

I, the undersigned, hereby declare that: "***Sperm structure and quantitative sperm motility analysis of three wild antelope species: Aepyceros melampus, Damaliscus dorcas phillipsi and Connochaetus taurinus*** ", is my own work and has not previously in its entirety, or in part, been submitted at any university for a degree. All the sources I have used or quoted have been indicated and acknowledged by means of complete references.

29-03-2001

J. M. Brinders　　　　　　　　Date

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With love and appreciation,

To my wife, Jacqueline and sons, Kyle and Matthew

Thank you

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"Then God commanded, "Let the earth produce all kinds of animal life: domestic and wild, large and small" – and it was done. So God made them all and He was pleased with what He saw" Gen. 1: 24,25

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Order: Artiodactyla
Family: Bovidae
Subfamily: Aepycerotinae

Order: Artiodactyla
Family: Bovidae
Subfamily: Alcelaphinae

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Family: Bovidae
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ABSTRACT

The aim of this study was to compare sperm morphology, motility and the effects of cold treatment on sperm of three wild antelope species; *Aepyceros melampus* (impala), *Connochaetes taurinus* (blue wildebeest) and *Damaliscus dorcas phillipsi* (blesbok). Animals were obtained from nearby hunting farms during the annual hunting season. For seasonal studies, special arrangements were made with game farmers to also collect material during the non-hunting (summer) season. Samples were taken for the respective time-dependent experiments to assess morphology by means of (a) light microscopy, (b) scanning and transmission electron microscopy, (c) computer aided sperm motion analysis, (d) sperm precooling to 4°C in Triladyl cryodiluent and (e) freezing of blue wildebeest sperm. For the sperm motility studies, cauda epididymal sperm from the three species were incubated at various time intervals i.e. 15 minutes, 1, 3 and 6 hours.

The sperm head of impala, blesbok and blue wildebeest have a distinctive dorso-ventrally flattened/spatulate structure. Scanning electron microscopy (SEM) studies revealed possible species specific differences in head length:head width ratios; impala (0.63), blesbok (0.71), blue wildebeest (0.67). Various sperm abnormalities were identified with forms of the Dag defect being the most prominent.

The results showed very small changes in the percentage of viable sperm over a period of six hours. Blue wildebeest had the highest percentage of live sperm at 1 hour (81.67%) followed by blesbok (76.5%) and then impala (74.13%). The highest percentage of dead acrosome reacted sperm was found at 6 hours in blesbok (19.67%)

Sperm motility studies indicated species-specific differences. Sperm aspirated from the epididymis were incubated in Ham's F10 culture medium. Motility was assessed at 15 minutes (blue wildebeest and impala), 1, 3 and 6 hours after incubation. The following sperm motility parameters were selected: Curvilinear velocity (VCL), Straightline velocity (VSL), Linearity (LIN), Average Path velocity (VAP), Beat Cross Frequency (BCF), Dancemean (DNCmn), mean Amplitude of lateral head displacement (mnALH), maximum Amplitude of Lateral head displacement (mxALH) and Mean Angular Deviation (MAD). At 15 minutes, statistically significant ($p<0.05$) differences were found for VCL, VSL, VAP, mnALH, mxALH, LIN, BCF and DNCmn, respectively among species. At 1 hour, VCL, VSL, VAP, MAD, LIN, BCF and DNCmn were respectively statistically significantly different for all three species. Mean amplitude of lateral head displacement differed statistically significantly ($p<0.0001$) when comparing Blue Wildebeest to Blesbok. After 3 hours, VCL, VSL, VAP, mnALH, mxALH and MAD were the only parameters that were respectively statistically significantly different for the three species. After 6 hours, VCL, VAP, mnALH, mxALH, LIN, BCF and DNCmn differed statistically significantly for all three species. Hyperactivation was not observed in two of the three species, but indications

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were evident in Blesbok sperm. This was marked by increased VCL ($p<0.0001$), DNCmn ($p<0.0001$), mnALH and mxALH ($p<0.0001$), decreased VSL ($p<0.0001$), VAP ($p<0.0001$) and LIN ($p<0.0001$).

Data collected for impala during the rainfall period and dry season showed that there was a seasonal effect on sperm motility parameters. The results were divided into winter period (group1) and rainy season (group2). Data compared for group 1 *vs* group 2 showed differences for VCL (253.25 ±5.44 vs 251.0 ±7.97, $p > 0.05$), VSL (146.35 ±3.46 vs 62.44 ±2.8, $p<0.05$), LIN (60.28 ±0.95 vs 29.77 ±1.54, $p<0.05$), BCF (53.70 ±1.38 vs 29.02 ±1.54, $p<0.05$), VAP (170.91 ±3.22 vs 116.39 ±3.53, $p<0.05$) and mnALH (4.33 ±0.11 vs 6.51 ±0.22, $p<0.05$). From the data obtained, it appears that season has an effect on sperm motility parameters, except for VCL.

Precooling was performed on impala and blue wildebeest sperm. Impala cauda epididymal sperm VCL after precooling showed no statistically significant differences from 2 to 4 hours after rewarming. Precooled cauda epididymal sperm VSL, LIN, VAP, BCF and MAD showed a decline from 2 to 6 hours where DNCmn increased within the same time interval. Blue wildebeest precool data on the other hand showed an initial decrease from 1 to 2 hours with an increase from 2 to 3 hours for VCL, VSL mnALH, mxALH, BCF, VAP DNCmn and MAD. The difference between fresh and precooled sperm was determined for impala and blue wildebeest. Differences between treatments were dependent on time and the parameter measured. From the results obtained, it appears that both impala and blue wildebeest need only to be precooled for 2 hours.

Blue wildebeest cauda epididymal sperm frozen at -196°C were analysed by means of transmission electron microscopy. Sperm for motion analysis were recovered by means of a swim-up technique. Results showed that only 35% of sperm recovered were live. TEM studies showed damage to the sperm acrosome and plasma membrane. Live sperm were detected by means of an acidophilic fluorescent dye (SYBR-14 Live/Dead kit) dual stained with Propidium Iodide (PI) staining the nuclei of dead sperm red. Sperm motility analysis showed a recovery of post thaw sperm motility similar to that found in fresh sperm. Data of precooled sperm were in most instances (VCL, VSL and BCF) the same as motility immediately after thawing. This was probably due to the presence of glycerol in the medium. Post thaw sperm were washed in glycerol-free medium and allowed to swim-up. The swim-up technique is widely used to isolate motile, progressively swimming sperm from a freeze/thaw or semen sample. For the swim-up sample, in this study, only a few were progressively motile with individual sperm displaying high progressive motility.

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CHAPTER 1

General Introduction

The purpose of this study was firstly, to describe sperm structure and quantitative sperm motility of cauda epididymal sperm collected post-mortem in three antelope species, namely, impala (*Aepyceros melampus)*, blesbok (*Damaliscus dorcas phillipsi)* and blue wildebeest (*Connochaetes taurinus).* Secondly, the effects of cooling on sperm motility in these species as well as the effects of liquid nitrogen freezing on sperm morphology, sperm motility and acrosome status of blue wildebeest were investigated.

Gamete and embryo cryopreservation is of particular importance in preserving bio- and genetic diversity in animals and in particular those, which could become extinct (Wildt, 1992). Wildt (1992) stated that by preserving genetic vigor and enhancing captive propagation, cryopreserved gene banks could provide a high level of insurance against further losses of diversity or entire species. The process of freezing gametes is straightforward, but the real success of this method resides in producing live young from thawed material. It is here that a good understanding of cryobiology of living material and the physiology of living cells converge. Besides the conservation of the genes, cell metabolism and metabolic processes need to remain intact both before and after freezing. In addition, membrane components necessary during sperm capacitation (Chang,

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1984) and sperm-egg fusion (Harayama *et al.*, 1993) need to remain unchanged. It is therefore assumed when cryopreserving sperm that a sound knowledge base exists of basic sperm characteristics such as sperm morphology and sperm motility. These facets will subsequently be discussed.

Sperm Morphology

One of the most important criteria in evaluating bovine seminal quality is the percentage normal sperm present (Garner, 1997). According to Soley *et al.* (1985), a strong association exists between reduced fertility and the occurrence of certain sperm defects in ejaculates of mammals. Defects in sperm morphology can be a consequence of various metabolic, hormonal or environmental influences. Cryopreservation of sperm requires that a large percentage of sperm be free of abnormalities. Abnormal sperm may fertilize ova, but result in early embryonic failure (Saacke, 1982).

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The acrosome is directly involved in the fertilization process, and acrosomal integrity is an important feature in sperm morphology assessment. Prior to fertilization, sperm undergo capacitation, which is a crucial step towards successful fertilization, *in vivo*. The acrosome reaction can not take place unless capacitation has taken place (Bedford and Yanagimachi, 1991).

Despite the wealth of information on sperm morphology in domestic bovid species (Anzar and Graham, 1996; Lapointe *et al.*, 1998, Martinus and Molan,

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1991; Soley *et al.*, 1985) relatively few studies report on sperm morphology and semen parameters in wild bovid species e.g.; african buffalo (*Syncerus caffer)* (Brown *et al.* (1991), and impala (Ackerman, 1995); *Gazelle dama mhorr, G. dorcas neglecta,* and *G. cuvieri* (Cassinello *et al.*, 1998). The lack of information on sperm morphology assessment in antelope species necessitates further investigation and elucidation.

Sperm motility

Sperm motility is essential for fertility (Hafez, 1987). However, sperm may lose their fertilizing ability before they lose their motility (Hafez, 1987). Even more important is the type of motility exhibited by sperm in their transit through the female reproductive tract. Sperm undergo capacitation which is accompanied by progressive changes in their swimming patterns. Once sperm reach the oviducts, they become hyperactivated (Fraser, 1977; Johnson *et al.,* 1981; Suarez and Osman, 1987, Suarez, 1988). Several investigators showed that there is a correlation between the percentage hyperactivation of sperm and fertilization outcome (Burkman, 1991; Olds-Clarke, 1986). Hyperactivation of sperm is therefore a prerequisite for fertilization (Burkman, 1991). Computer aided sperm motility analysis (CASA) greatly assists in defining sperm motility quantitatively including hyperactivation in capacitation media (discussed in detail in Chapter 3). This latter approach has been adopted in this investigation to describe sperm motility in wild antelope species. No research has been

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performed on this important facet in wild antelope species and serves as an important motivation for its inclusion in this study.

Cauda epididymal sperm *versus* ejaculate sperm

The above discussion predominantly referred to sperm characteristics of the ejaculate. However, sperm is usually obtained from the cauda epididymis of culled animals since an electro-ejaculate cannot be obtained from animals post-mortem. It is therefore important to establish the validity of using sperm from the cauda epididymis for cryopreservation purposes. Van der Horst *et al.* (1999), demonstrated that the percentage sperm motility of cauda epididymal sperm is significantly higher than that of ejaculate sperm in the vervet monkey, *Cercopithecus aethiops*. Cryopreserved cauda epididymal sperm has a significantly higher percentage sperm motility than cryopreserved ejaculate sperm in *C. aethiops* (Van der Horst and Seier, 2000).

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It furthermore appears that epididymal sperm were superior to ejaculate sperm in IVF (*in vitro* fertilization) trials (Goto *et al.*, 1989). More important, these authors showed that there was less intraspecies variation in the fertilising ability of epididymal sperm, compared to ejaculate sperm from different bulls. Seminal plasma may have a deleterious effect on sperm (Martinus and Molan, 1991). Katska *et al.*(1994), have demonstrated that removal of seminal plasma from bull ejaculates immediately after collection and prior to freezing, significantly increased blastocyst yield. The above discussion therefore indicates that cauda

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epididymal sperm is of sufficient quality to be used for artificial propagation of mammalian species in general and validates its use in this investigation. In further support of the use of epididymal sperm, no research appears to be published on this aspect of applied reproductive technology (ART) in wild antelope species.

Environment, seasonality and condition

Environmental factors like day length and season play an important role in the reproductive cycles of most animals. Most ram breeds in the UK show marked seasonal fluctuations in reproductive performance (Martin *et al*., 1999). These fluctuations can be seen in decreased testicular size, *libido* and semen quality during the non-breeding seasons.

The exact mechanisms whereby animals anticipate seasons and integrate environmental information are not completely known. It is well established in animals living in temperate conditions, that the pineal gland is implicated in the integration of photoperiodic information via the daily pattern of melatonin synthesis (Vivien-Roels *et al*., 1999). Brown *et al*. (1991), found in african buffalo that there was no seasonal variation in the total number of sperm per ejaculate. However, during the breeding season bulls produced ejaculates with a greater volume and lower sperm concentration than bulls during the non-breeding season. They also found that the percentage of motile and morphologically normal sperm were lower in ejaculates collected during the non-breeding season.

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The most prevalent sperm abnormalities seen in the ejaculate of the african buffalo during the non-breeding season were the presence of cytoplasmic droplets and deformities of the midpiece and flagellum (Brown *et al.*, 1991). Similar results were found by Walkden-Brown *et al.* (1994) working on Australian cashmere goats. They found that both diet and month of the year influenced testicular mass.

Impala have a restricted mating season in autumn with territorial males focusing on females in estrus, whilst body condition declines during this period. Males reach a nadir in spring (Skinner and Smithers, 1990). There are no reports in the literature on a comparison of semen parameters in the blue wildebeest between reproductively active and reproductively inactive bulls. Blesbok are seasonal breeders, mating in autumn, and further oestrus synchronization is induced by the presence of a male (Skinner and Smithers, 1990). It is therefore important to relate results to sampling period.

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Species specificity

The species selected for this study all belong to the same Order (Artiodactyla) and family (Bovidae). Blue wildebeest and blesbok belong to the same sub-family (Alcelaphinae) but impala belong to the sub-family Aepycerotinae (Skinner and Smithers, 1990). Therefore, comparisons among more closely related and less closely related species may assist in establishing whether species specific differences exist.

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The species selected for this study were furthermore based on availability. Commonly occurring species that are frequently hunted were selected. In addition, the advantage of comparing three commonly occurring antelope species is that basic research on the gametes can be performed. Knowledge obtained from such a study could be used to investigate the preservation of genetic material of endangered species.

Specific aims of the study

From the knowledge currently available, it is clear that basic standards for spermatological parameters such as sperm morphology and sperm motility still need to be determined for many of the wild antelope species, particularly in view of the need to cryopreserve their sperm. The aims of this study were the following:

1. To provide a description of sperm morphology for the three antelope species by means of light microscopy and scanning electron microscopy. Transmission electron microscopy was only used to validate certain aspects of normal and abnormal sperm morphology (Chapter 2).
2. To quantitatively assess sperm motility by means of computer aided sperm motion analysis (CASA). The author attempted to elucidate possible species specific differences in the three antelope species based on selected CASA parameters (Chapter 3).
3. To establish the effect of cooling on quantitative sperm motility in the three antelopes species under consideration (Chapter 3), since pre-cooling of

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sperm before cryopreservation is probably the most important facet of the entire cryopreservation procedure.

4. To investigate the effect of cryopreservation on sperm motility, sperm structure and acrosome status in one of the three antelope species, the blue wildebeest (Chapter 4).

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Chapter 2

Post-mortem cauda epididymal sperm morphology and viability of sperm of three antelope species.

2.1 Introduction

Normal sperm morphology has been suggested to be one of the most reliable indicators of male fertility and infertility (Garner, 1997; Canale *et al.,* 1994; Seier *et al.*, 1996). Ejaculates of highly fertile bulls contain 10 - 25% abnormally shaped sperm (Garner, 1997). However, it is the number of normal sperm that forms one of the most important criteria in assessing potential male fertility.

Much research has been done in the field of bovine spermatology (Blom, 1950; Phillips and Kalay, 1984; Soley *et al.,* 1985). Some information is available on sperm structure (Dott and Skinner, 1989), sperm physiology (Dhindsea *et al.*, 1995) and semen parameters of wild antelope species (Howard *et al.*, 1986). This chapter attempts to add to the existing body of knowledge relating to sperm structure of wild antelope species.

During spermiogenesis, there is a marked reduction in nuclear volume of the sperm head. During this development, histones are removed and replaced by

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transitional proteins, which in turn are replaced by arginine-rich, basic protamines of low molecular mass (Breed, 1997). Van der Horst *et al.*, (1991), state that certain argentophillic proteins are involved in stabilizing sperm head morphology during the maturation process by means of disulfide bond formation. The perinuclear material accumulates at the apex of the head and is referred to as the perforatorium. The perforatorium is not present in monotremes or marsupials and presents an erect tapering profile, which is also stabilised by disulfide bonds (Calvin and Bedford, 1971). The percentage of normal sperm within a semen sample gives an indication of the intactness of all the abovementioned processes which ultimately translates into the sperm's ability to fertilize an oocyte by properly binding, penetrating and decondensing during conception. Wildt *et al.* (1995), found that higher cleavage rates were obtained in normospermic domestic cats and tigers than the teratospermic sperm of cheetah and puma, during their *in vitro* fertilization trials.

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Scanning and transmission electron microscopy do not provide any information relating to sperm vitality at the time of fixation. Information is purely morphological with reference to detailed surface structure (scanning electron microscopy) and the sperm's internal structure (transmission electron microscopy). SEM and TEM can provide insight into sperm capacitation at the ultrastructural level. Sperm capacitation is a process that sperm undergoes prior to fertilization. This process results in both sperm biochemical and structural

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changes. Suzuki and Foote (1995) showed, with the aid of SEM, vesiculation of the bovine sperm surface after capacitation.

The main aim of this section was to identify and describe normal sperm morphology of cauda epididymal sperm of impala, blesbok and blue wildebeest by means of light and scanning electron microscopy and to establish the percentage of viable sperm using light microscopy. Transmission electron microscopy was only used to confirm certain normal and abnormal features of sperm in the three antelope species.

2.2 Materials and Methods

2.2.1. Collection Procedure

2.2.1.1 Animals used and transport of material

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Three free-ranging antelope species were selected for this study *viz* impala (*Aepyceros melampus*), blue wildebeest (*Connochaetes taurinus*) and blesbok (*Damaliscus dorcas phillipsi*). Three blue wildebeest specimens were collected in Ellisras in the Northern Province (23°45'S, 27°42'E). The other three blue wildebeest and six blesbok were collected on the game farm, Rhino Game Lodge, about 100km from Delmas (26°10'S, 28°41E') in the Mpumalanga province.

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Impala were collected in the Thabazimbi area on a game reserve, Ferroland Ground Trust (24°41'S, 27°21'E). Initially, four adult impala specimens were collected during May and another four during December in this area. This formed the basis for the seasonality experiment (Chapter 3, section 3.3.3.). In order to increase the number of specimens to n=6, two additional impala were killed the following December. Therefore, in total, this study comprised of impala (n=8), blue wildebeest (n=6) and blesbok (n=6).

All animals were killed with a hunting rifle. All adult animals collected were in good physical condition.

2.2.1.2 Transport of material

Almost immediately after the animals were killed, the scrotal sacs containing the testes with attached epididymi were removed from the animal and placed in a plastic bag and then in a cooler box with crushed ice or ice packs. This was done to cool the testes, slow down all metabolic processes in the epididymis, and preserve sperm longevity by decreasing the rate of oxidative and peroxidative processes. Material usually arrived back at the laboratory within three hours after the kill.

2.2.1.3 The Epididymis

The scrotal sac containing the gonads was removed from the icebox and allowed to warm to room temperature. The epididymis was separated from the testis and

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cleaned with the aid of a dissection microscope. All visible connective tissue and blood vessels were carefully removed. The epididymis was rinsed in clean Ham's F10 culture medium. An incision was made in the cauda region of the epididymis and dense clouds of sperm flowed into the surrounding medium. Of the freshly aspirated sperm, 40µl was collected with a Finnpipette and placed in 2ml of prewarmed Ham's F10 supplemented with 10% fetal calf serum (culture medium) for motility studies (See chapter 3 section 3.2.1.4).

For electron microscopy studies, freshly aspirated sperm was gently mixed with 2.5% phosphate-buffered Glutaraldehyde (PBGA).

2.2.2 Microscopy

2.2.2.1 Scanning Electron Microscopy (SEM)

Sperm samples were processed for SEM according to a modified method described by van der Horst *et al.* (1989). Twenty microliters of epididymal sperm were aspirated into 4ml Ham's F10 and 10µL aliquots placed into 500µl 2.5% PBGA.

The cell suspension was centrifuged at 300x*g* for 10 minutes and the supernatant removed. The resulting pellet was resuspended in a 2.5% PBGA solution. The cell suspension was placed onto small pieces of mica for about 20 minutes to adsorb sperm. The mica sheets were then dehydrated in 50, 70, 80,

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90, 95% ethanol and twice in 100% ethanol allowing 10 minutes for each step. Once dehydrated, the samples were dried by the critical point method and mounted on aluminium stubs. The samples were sputter-coated with carbon and gold and viewed with a Leica Stereoscan 420 (LEO Electron Microscopy Ltd, Cambridge, England) microscope.

2.2.2.2 Transmission Electron Microscopy

Undiluted sperm samples were fixed in 2.5% PBGA as explained for SEM. The sperm/GA suspension was centrifuged at 300x*g* and the pellets which formed, were washed in distilled water and buffer for ten minutes each, respectively. The pellets were then post-fixed in 1% osmium tetroxide for two hours, after which they were again washed in buffer and distilled water. The samples were then dehydrated in a graded series of ethanol (70%, 80%, 90%, 95% and 100%) allowing ten minutes for each step. The pellets were infiltrated with 100% propylene oxide for 10 minutes and then placed in a 1:1 resin: propylene oxide mixture overnight at room temperature. The mixture was replaced with 100 % resin (EM bed812) and placed in a vacuum evaporator for two hours. Specimens were resin embedded and polymerised for 24 hours at 70°C. Semi-thin sections (0.1µm) were stained on glass slides with toluidine blue and viewed with an Olympus, BH microscope. Ultra-thin sections (70nm) were double stained with lead citrate and uranyl acetate and viewed with a Jeol, JEM 1200EX-transmission electron microscope.

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2.2.2.3 Light Microscopy

2.2.2.3.1 Nigrosin/Eosin -fast green (FCF) stain

The FCF staining method was performed on sperm incubated for 15 minutes, 1, 3 and 6 hours (see Chapter 3 for incubation methods). The nigrosin-fast green (FCF) staining was performed according to Way *et al.* (1994). The vital dye was made up by dissolving 2% (w/v) of fast green and 0.4% (w/v) of eosin B in phosphate buffer (320mOsm). Fresh, unfixed sperm was diluted in Ham's F10 to $5X10^8$ sperm/ml. Aliquots of 50µl were added to 100µl of vital dye and mixed rapidly. Smears were prepared on glass slides and placed on a 37°C warm plate to dry. Slides were mounted with DPX and covered with coverslips for evaluation at a later stage. For each slide, at least 200 sperm were evaluated for live (non-eosinophilic), dead (eosinophilic) and pre-and post acrosomal staining. Classification of sperm abnormalities was done according to Curry *et al.* (1989), Pinart et *al.* (1998), Hafez (1987) and Mortimer (1994).

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Sperm images were captured with the aid of a video capture card (Tekram 205, Tekram Technology Co., Ltd). The capture card was connected to a Panasonic CP220 color CCD camera mounted onto an Olympus CH-2 microscope. Images were viewed by using a 100X Phase (A100PI, 1.30 oil) oil immersion objective.

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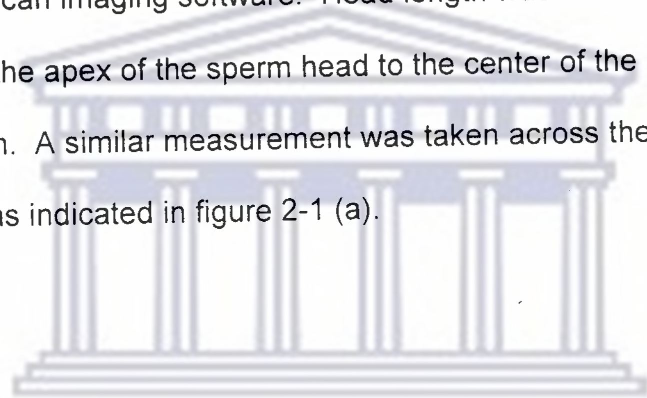
2.2.2.3.2 **Sperm head measurements**

A Leica Stereoscan 420 (LEO, Electron Microscopy LTD, Cambridge, England) was used to quantify sperm head measurements. Scanning electron micrographs were used to measure head length and head width of 20 sperm from each animal for blesbok, blue wildebeest and impala.

Measurements were taken as shown in figure 2-1 (a) for head length and width using the Stereoscan imaging software. Head length was calculated from the furthest point on the apex of the sperm head to the center of the head base of normoaxial sperm. A similar measurement was taken across the widest point of the sperm head as indicated in figure 2-1 (a).



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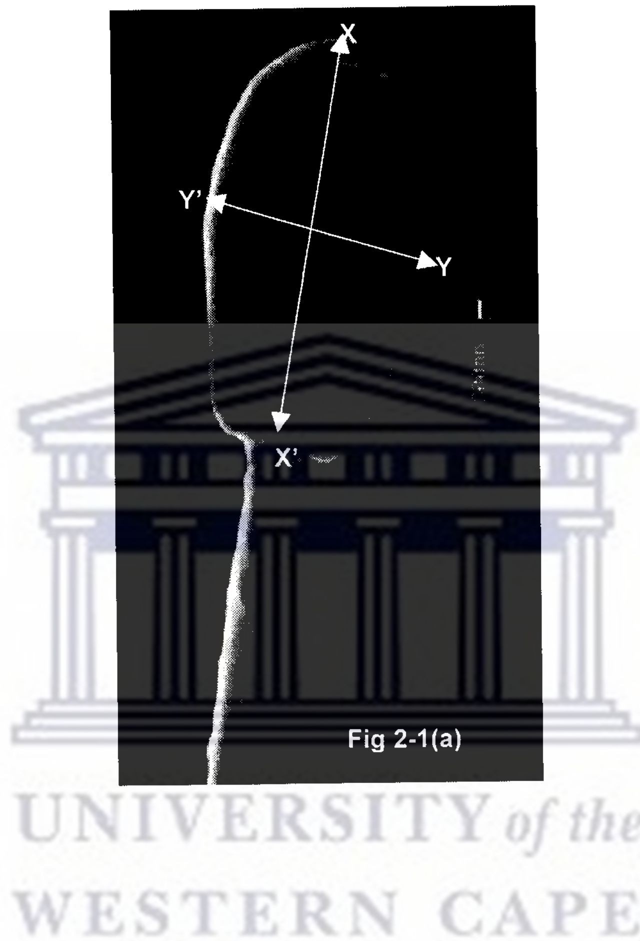

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Fig. 2-1 (a) Shows a normal cauda epididymal sperm of impala. The arrows indicate the points used for head length (X-X') and head width (Y-Y') measurements

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2.3 Results

2.3.1. Scanning Electron and Light Microscopy of Cauda Epididymal Sperm

Results of impala, blue wildebeest and blesbok cauda epididymal sperm head length ±SD and width ±SD as determined by scanning electron microscopy are shown in table 2-1. For impala, blesbok and blue wildebeest the ratios of sperm head width to sperm head length were respectively 0.62, 0.71 and 0.67. Immature sperm were recognized by the presence of proximally situated cytoplasmic droplets or droplets found halfway between the neck and the annulus (Oko *et al.*, 1993). Normal sperm were selected for measurement in each species on the basis of the descriptions presented below.

2.3.1.1. Impala

2.3.1.1.1 Normal Sperm (Scanning Electron Microscopy)

For this study, normal sperm were assessed using both sperm head as well as tail characteristics. The position on the neck of the cytoplasmic droplet was an important indicator of normal sperm structure (Figure 2-1). Here, sperm with a cytoplasmic droplet at the end of the annulus were considered normal and mature cauda epididymal sperm. The normal sperm of the impala is typically spatulate in shape and dorso-laterally flattened (Bedford and Nicander, 1971). The anterior part of the head up to the postacrosomal border is wider than the postacrosomal area of the sperm head. For sperm head normality, it is important to note that the contours are even and bilaterally equal. The anterior part of the

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Table 2-1: The relative head length, width and head width/length ratio for each species were measured and calculated with the aid of the scanning electron microscope. The $\bar{x}$ ± SD are presented for each individual from n=20 to n=25 sperm.

	Head Length µm (±SD)	**Head Width** µm (±SD)	**Ratio** ($\frac{Width}{Length}$)	**Average Ratio** $\bar{x}$ ± SD
Impala 1	6.64(0.37)	4.14(0.27)	0.62	0.62 (0.00)
Impala 2	6.64(0.27)	4.09(0.18)	0.62	
Blesbok 1	5.43(0.27)	3.74(0.19)	0.69	0.71(0.03)
Blesbok 2	5.67(0.29)	3.98(0.18)	0.70	
Blesbok 3	5.67(0.33)	4.18(0.22)	0.74	
Blue wildebeest 1	6.00(0.30)	4.04(0.13)	0.67	0.67(0.02)
Blue wildebeest 2	6.25(0.17)	4.04(0.16)	0.65	
Blue wildebeest 3	6.08(0.19)	4.26(0.10)	0.70	

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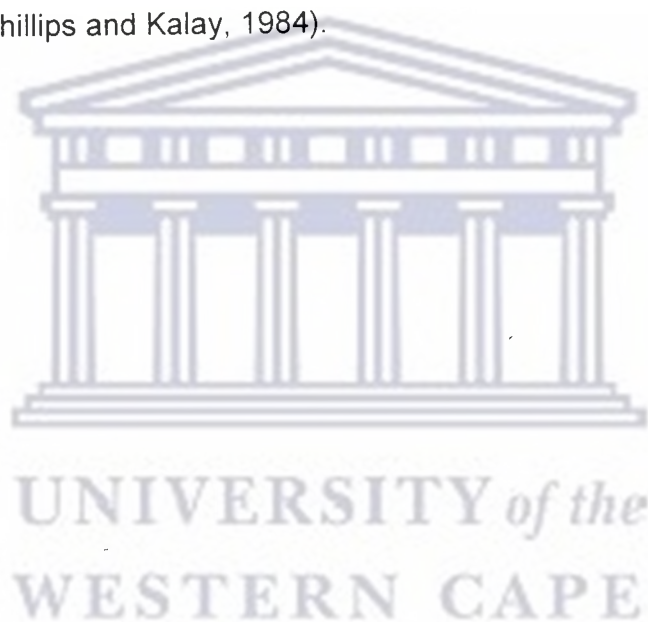
acrosome has a distinct ridge called the acrosomal ridge (Figure 2-2) and the equator separates the acrosome from the dense postacrosomal lamina (Ackerman, 1995).

The tail can be subdivided into a middle piece, principal piece and end piece, all of which are well demarcated. While the linear dimensions of these components have not been measured, they appear to be typically bovid (Bedford and Nicander, 1971; Phillips and Kalay, 1984).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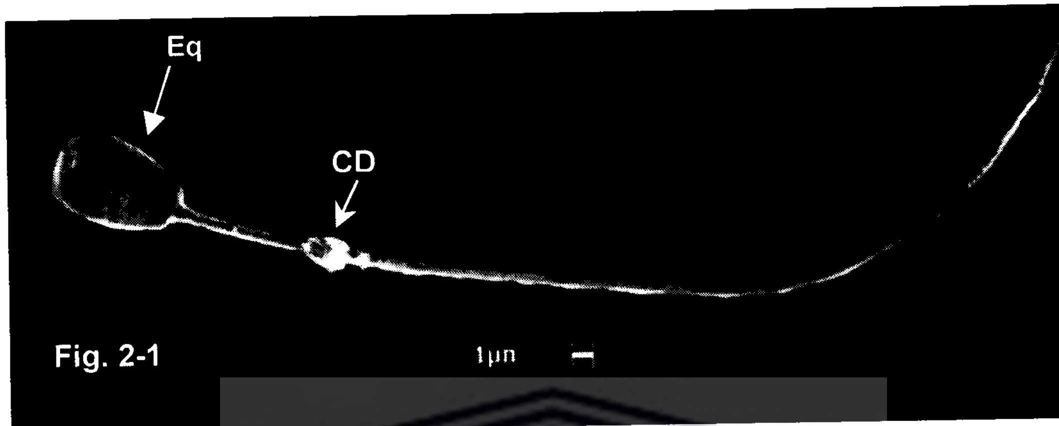

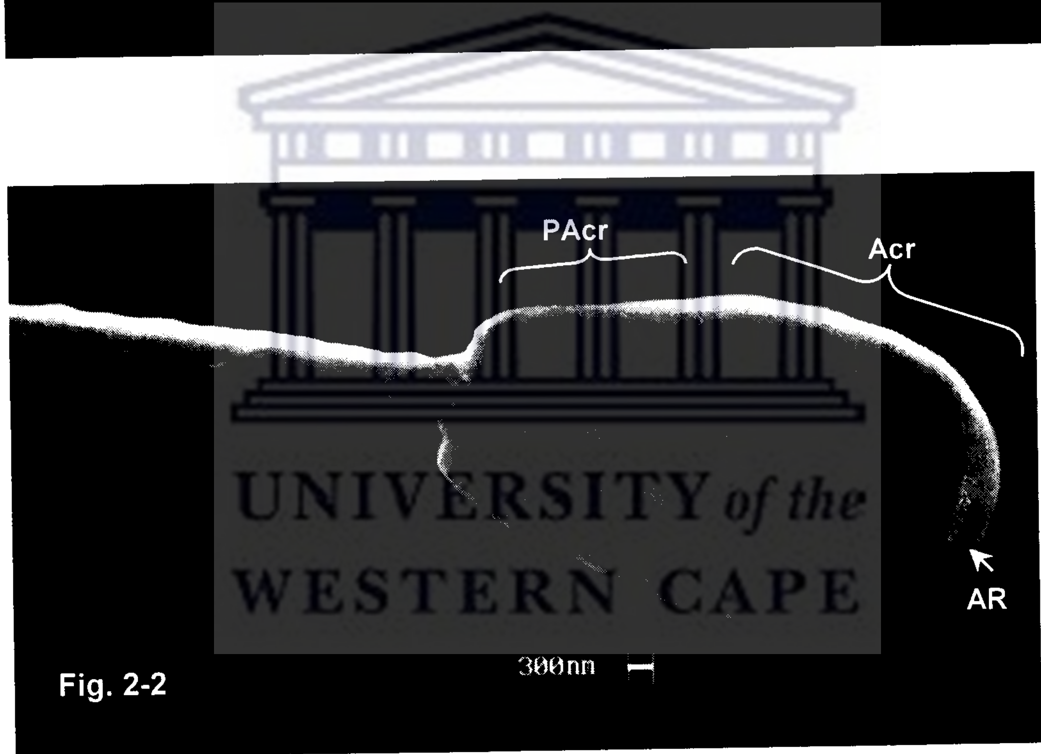

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Fig. 2-1: Shows a normal cauda epididymal sperm of impala. Sperm head shape appears normal. CD (Cytoplasmic droplet). Eq = equatorial region **Fig. 2-2**: Shows a cauda epididymal sperm head. The acrosomal ridge (AR) is clearly defined. The acrosomal region (Acr) covers more than 50% of the sperm head as compared to the post acrosomal region (PAcr).

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2.3.1.1.2 Abnormal Sperm (Scanning Electron Microscopy)

Figures 2-3 to 2-9 show abnormal sperm morphology found in impala. Figures 2-3 and 2-4 show the proximal placement of the cytoplasmic droplet. This is indicative of sperm immaturity and possibly of epididymal malfunction. Figure 2-5 shows a sperm with a broken or non-inserted neck (Mortimer, 1994).

Figures 2-6 show at least four types of abnormalities in impala sperm. They are the absence of the acrosome, a proximally placed cytoplasmic droplet, abaxial insertion of tail to the head, and a biflagellate tail. Figure 2-7 shows a biflagellate sperm with a coiled tail and head which appears asymmetrical. This abnormality has been documented by Hafez (1987) for bull ejaculates.

Figures 2-8 and 2-9 are examples of folded tails. In Figure 2-8 the tail fold starts at the border of the midpiece and principal piece (annulus) and in Figure 2-9 bending occurs along the principal piece. Both forms of tail folding are associated with cytoplasmic droplets.

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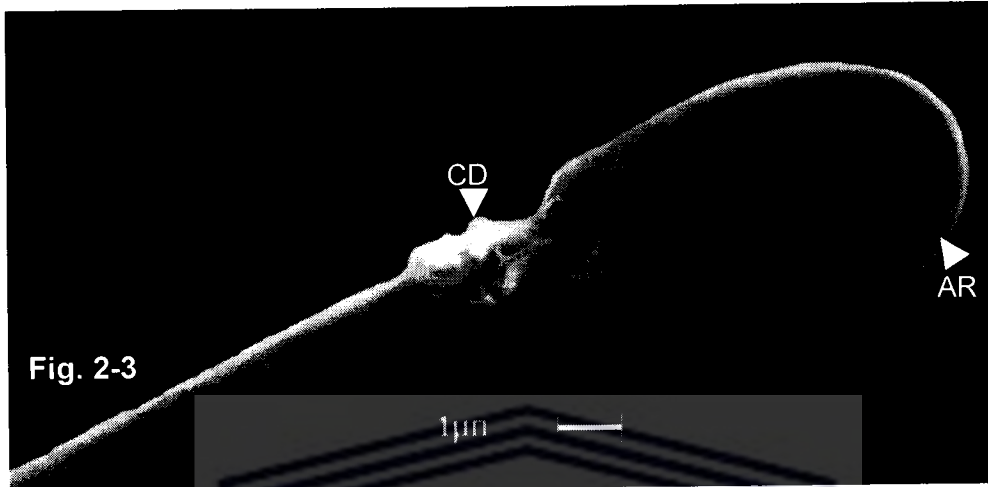

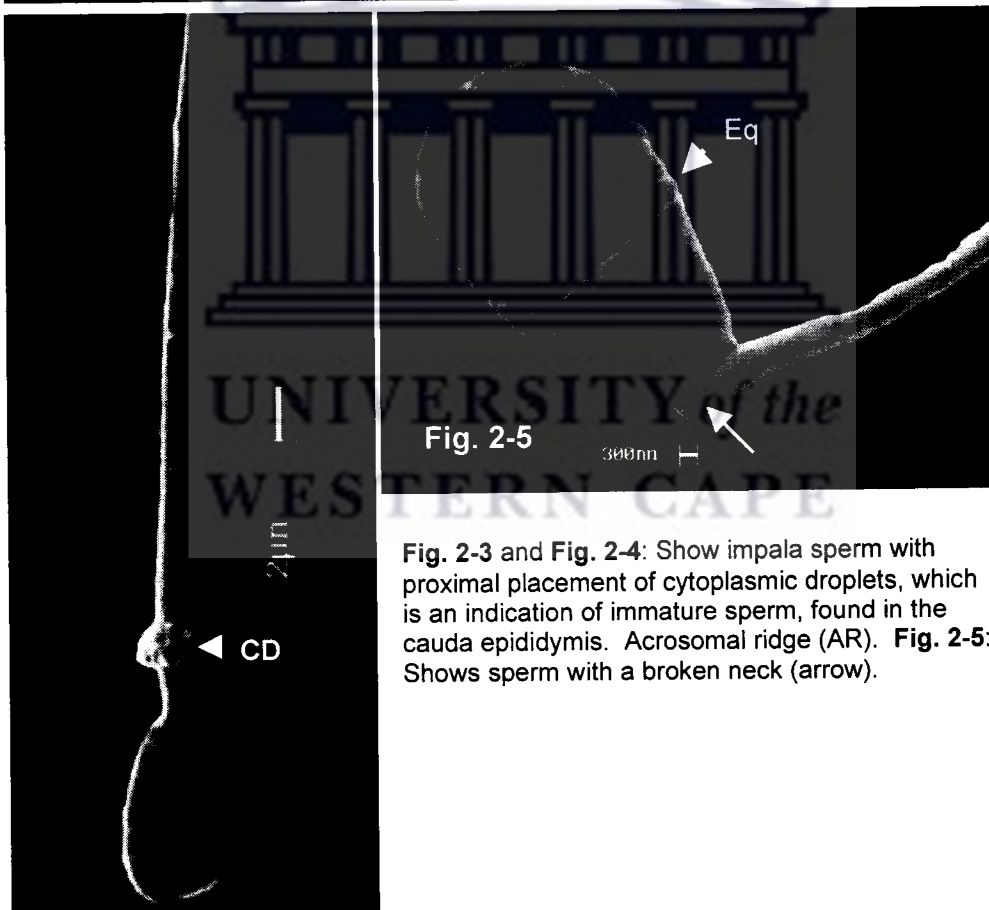

Fig. 2-4

Fig. 2-3 and **Fig. 2-4**: Show impala sperm with proximal placement of cytoplasmic droplets, which is an indication of immature sperm, found in the cauda epididymis. Acrosomal ridge (AR). **Fig. 2-5**: Shows sperm with a broken neck (arrow).

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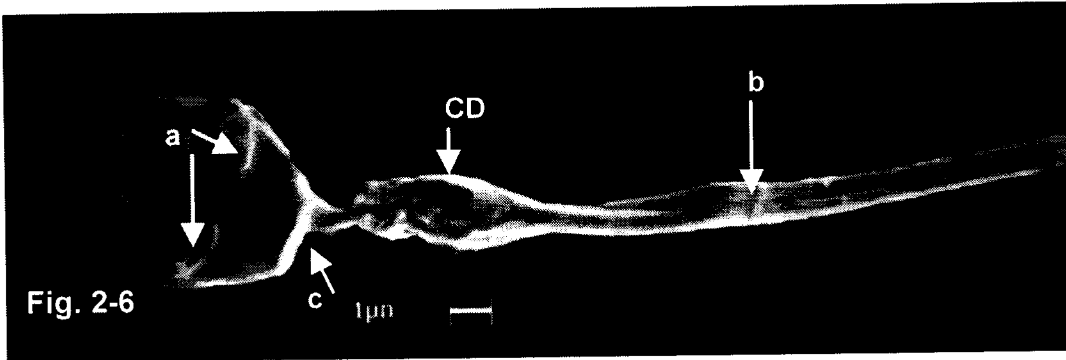

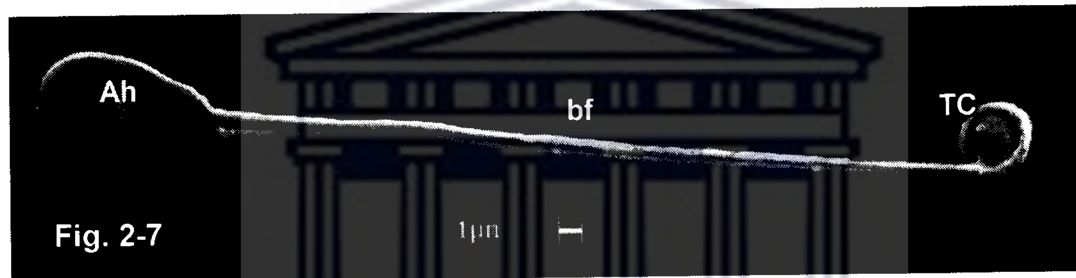

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Fig. 2-6: Abnormal cauda epididymal sperm of impala where (a) indicates the damaged acrosome of an abnormally shaped sperm head, (b) biflagellate tail abaxially attached to the sperm head (c), (CD) proximally attached cytoplasmic droplet. **Fig. 2-7:** Abnormal cauda epididymal sperm of impala with an irregularly shaped head (Ah), biflagellate tail (bf), coiled at the tip (TC).

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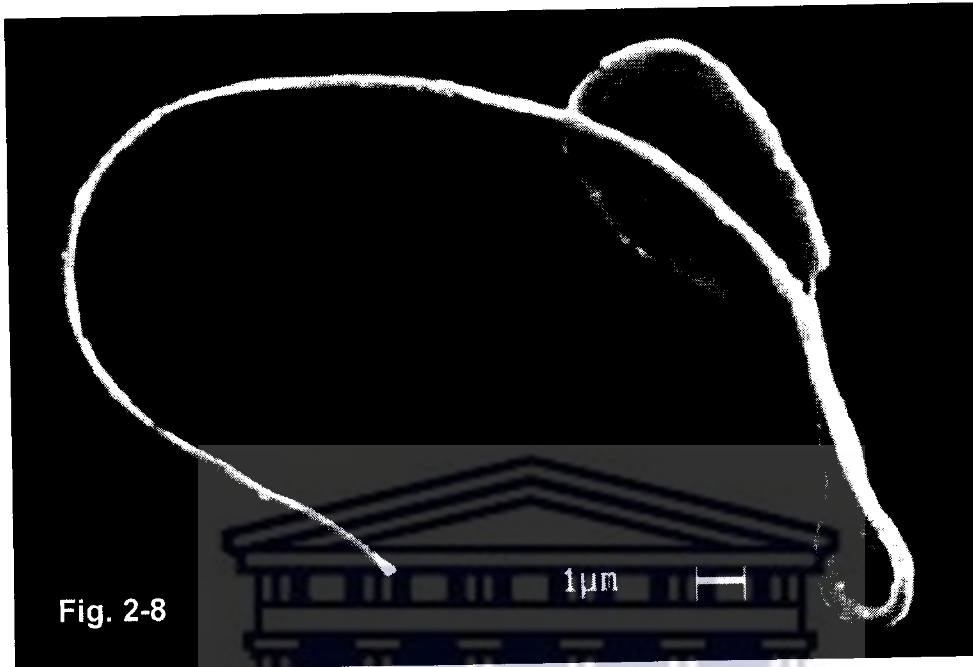

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Fig. 2-8: Impala cauda epididymal sperm folded at the annulus, an abnormality found in all three species studied. **Fig. 2-9**: Shows sperm tail folding with cytoplasmic droplet (CD) still attached.

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2.3.1.1.3 Light Microscopy of normal and abnormal impala sperm

Light microscopic evaluation of impala sperm was mainly used to assess sperm viability. However, light microscopy was also used to establish whether most of the structural features of sperm observed by means of SEM could be detected. Normal sperm could be identified by the shape of the head and the presence or absence of any tail defects. The head of impala sperm were relatively large (when compared to the other two species), with a wider acrosomal region compared to a narrower postacrosomal region (Figures 2-10 (a) and (b)). The acrosome covered about two thirds of the sperm head. The presence of a distally situated, i.e. on or near the annulus, cytoplasmic droplet (Figure 2-10 (b)) was one of the markers of normal sperm collected from the cauda epididymis. Decapitated sperm heads (Figure 2-10 (d)) were frequently observed in impala. Tail defects i.e. tail bends, coiling, Dag defect and broken necks were commonly found (Figures 2-10 (c), (e) and (f)) in impala sperm samples.

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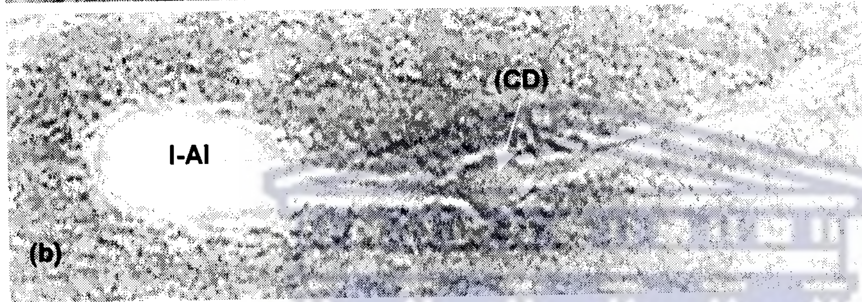

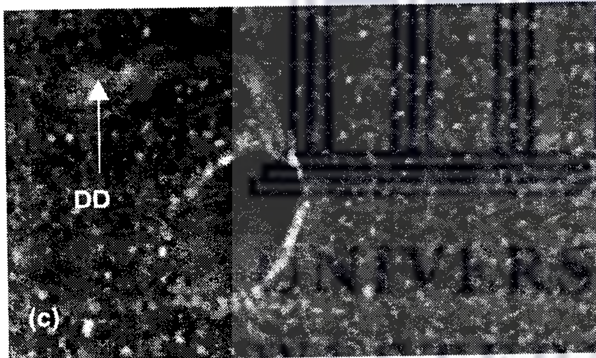

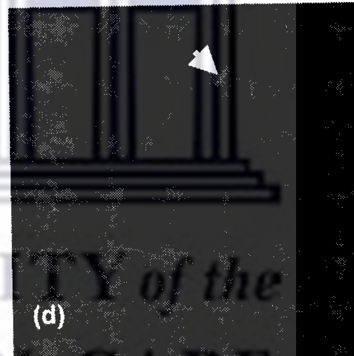

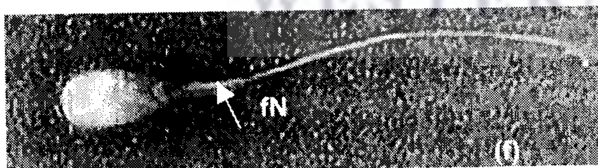

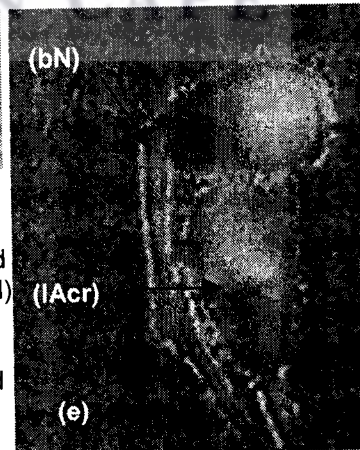

Fig. 2-10 (a) and (b) show normal cauda epididymal sperm of impala where (a) is an example of a dead, acrosome reacted or damaged sperm (D-AR) and (b), a live acrosome intact (L-AI) sperm, **(c)** example of Dag defect (DD) seen in impala, **(d)** decapitated sperm head (arrow), **(e)** show two sperm, one with a broken neck (bN) and one with a loose acrosomal cap (lAcr) and **(f)**. impala sperm with a folded midpiece

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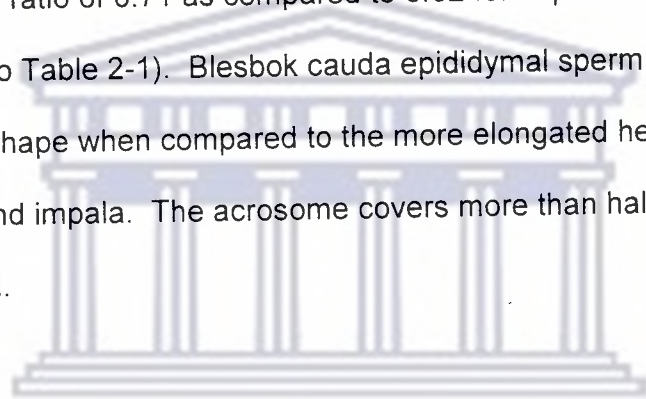
2.3.1.2 Blesbok

2.3.1.2.1 Normal Sperm (Scanning Electron Microscopy)

The morphological criteria for 'normal' blesbok sperm were essentially the same as for impala. Figure 2-11 shows a normal cauda epididymal sperm of blesbok. The head is dorso-ventrally flattened and oval. The head furthermore tapers sharply posteriorly from the postacrosomal area to the base of the head. Blesbok sperm differs from impala and blue wildebeest in that blesbok has a larger width/length ratio of 0.71 as compared to 0.62 for impala and 0,67 for blue wildebeest (refer to Table 2-1). Blesbok cauda epididymal sperm also appear more rounded in shape when compared to the more elongated head shapes of blue wildebeest and impala. The acrosome covers more than half of the surface of the sperm head.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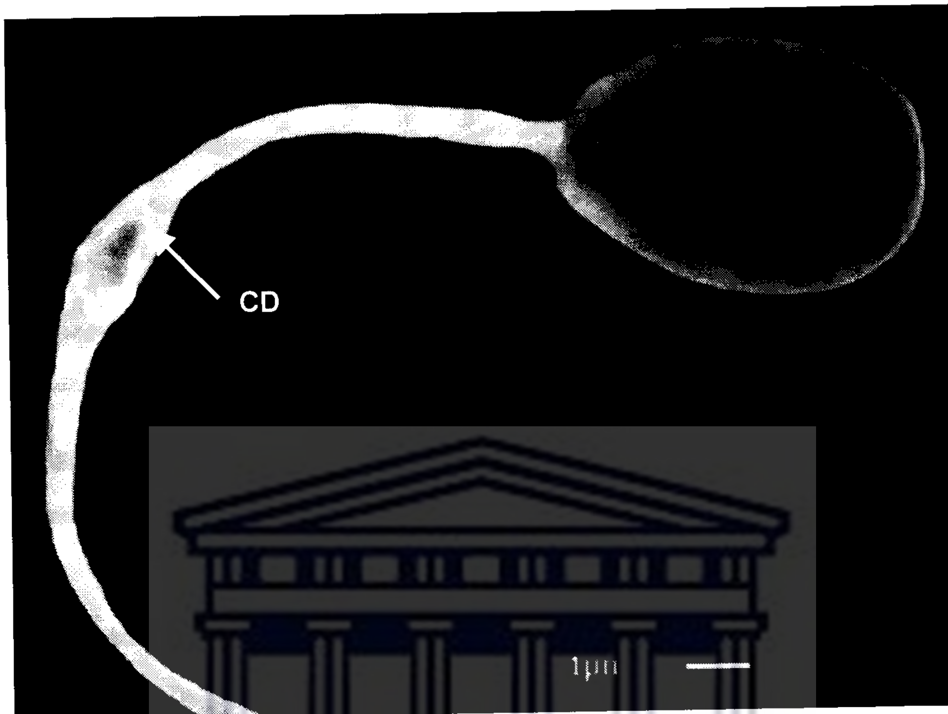

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Fig. 2-11

Fig. 2-11 is a representation of normal blesbok sperm. The acrosome occupies about 60% of the sperm head and the cytoplasmic droplet (CD) has migrated toward the annulus, typical of normal sperm from the cauda epididymis.

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2.3.1.2.2 Abnormal Sperm (Scanning Electron Microscopy)

The abnormalities seen in blesbok cauda epididymal sperm were similar to those observed in impala. In some instances, sperm heads were amorphous (elongated or tapered) while the rest of the sperm appeared to be normal. Tail defects appeared to be the most common abnormality. The three commonly observed regions of tail folding in the blesbok were in front of the annulus i.e. midpiece folding (Figure 2-12), at the end of the midpiece (Figure 2-15) and along the principal piece (Figure 2-13).

Various degrees of tail coiling were observed in blesbok cauda epididymal sperm. Tail coiling/folding is shown in Figure 2-13. It appears that the tail is broken and lies parallel to the midpiece and folds over and around the head. Figure 2-14 shows a blesbok sperm tail forming regular loops with possible cytoplasmic debris trapped by the coils. Figure 2-16 shows two sperm with tails which appear to be encapsulated by a membranous sheath. Loose fibrils and a broken neck are also apparent in this figure.

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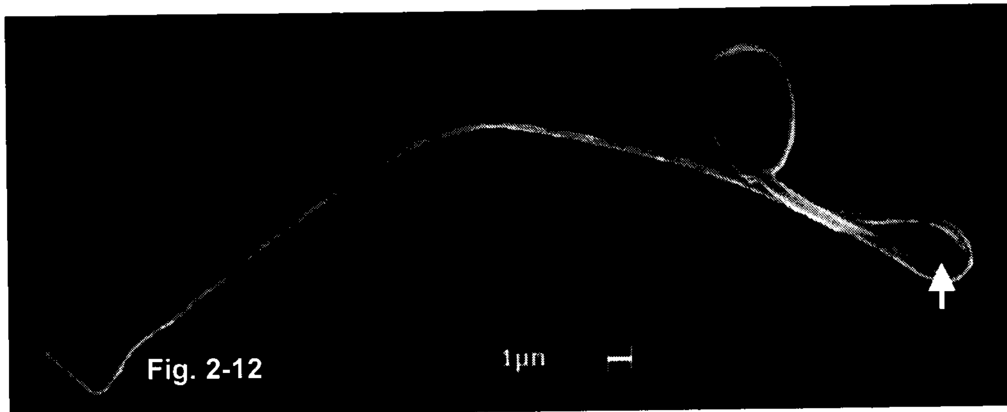

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Figures 2-12 and **2-13**: Represent various types of tail defects found in blesbok cauda epididymal sperm. **Fig. 2-12** is a folding of the midpiece (arrow) and **Fig. 2-13** showing a type of Dag defect (arrow), looping back onto itself and over the sperm head (arrow head)

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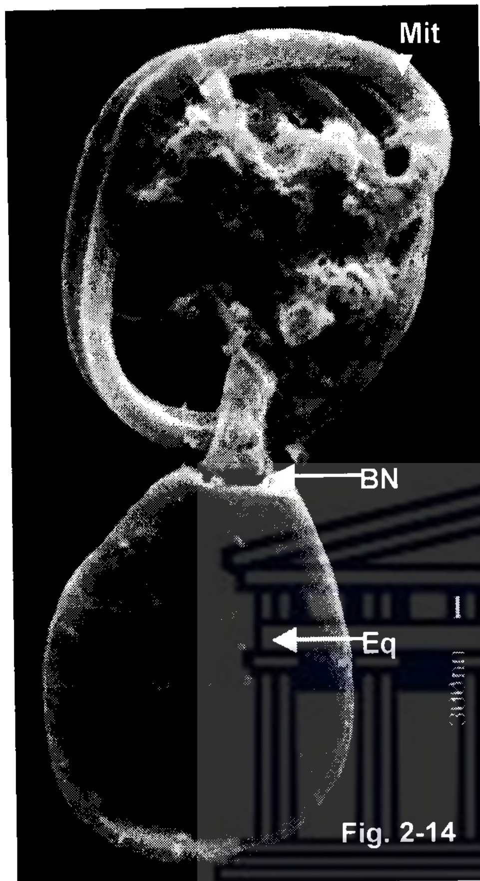

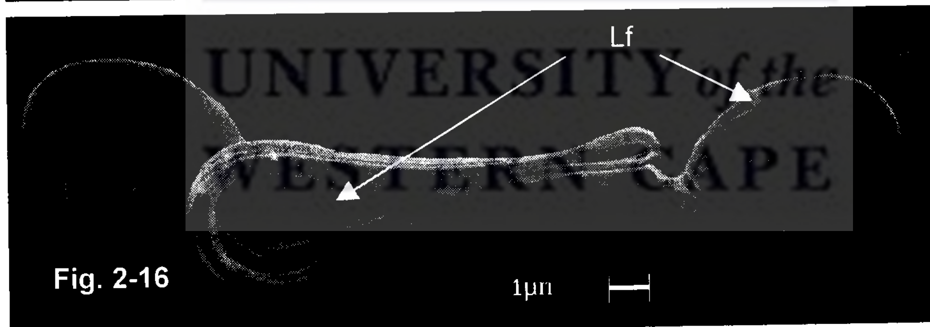

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Fig. 2-14: This micrograph shows a blesbok cauda epididymal sperm with a completely coiled tail. The tail forms circular loops with what appears to be cytoplasmic debris trapped in the center. Mitochondria (Mit) can clearly be seen in the sperm midpiece. The tail is broken at the head (arrow, BN). A clear demarcation of the equatorial region (Eq) can be identified separating the acrosomal from the postacrosomal region. The acrosomal region also appears smoother in surface texture that the postacrosomal region. **Fig. 2-15** is an example of sperm tail bending at the annulus (An). This sperm has an amorphous head with no clear indication of any of the surface structures such as the acrosome **Fig. 2-16** shows two blesbok cauda epididymal sperm with excessive folding of the tails. The tails appear to be encapsulated by a membranous sheath. Loose fibrils (Lf) and a broken neck are apparent.

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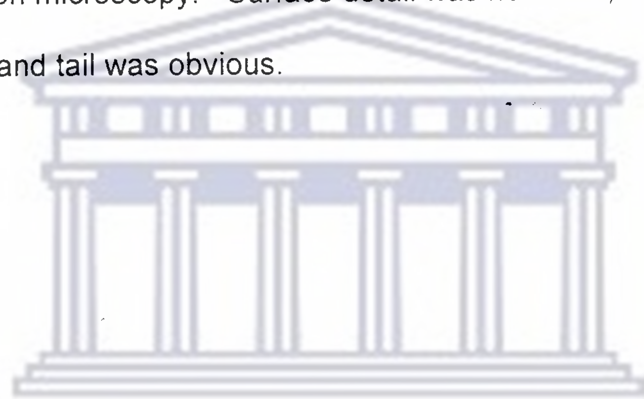
2.3.1.2.3 Normal and Abnormal sperm (Light Microscopy)

Figure 2-17 shows normal blesbok cauda epididymal sperm as seen with the aid of the light microscope. The sperm head of blesbok appear less elongated compared to the other two species. The acrosome spans slightly more than 50% of the blesbok sperm head (Figure 2-17(a)). Dag defects and other tail defects were common. Figure 17(c) shows an example of a sperm head with a torn or damaged acrosomal cap. However, this defect was not seen in the samples prepared for electron microscopy. Surface detail was not clear, but the normal shape of the head and tail was obvious.



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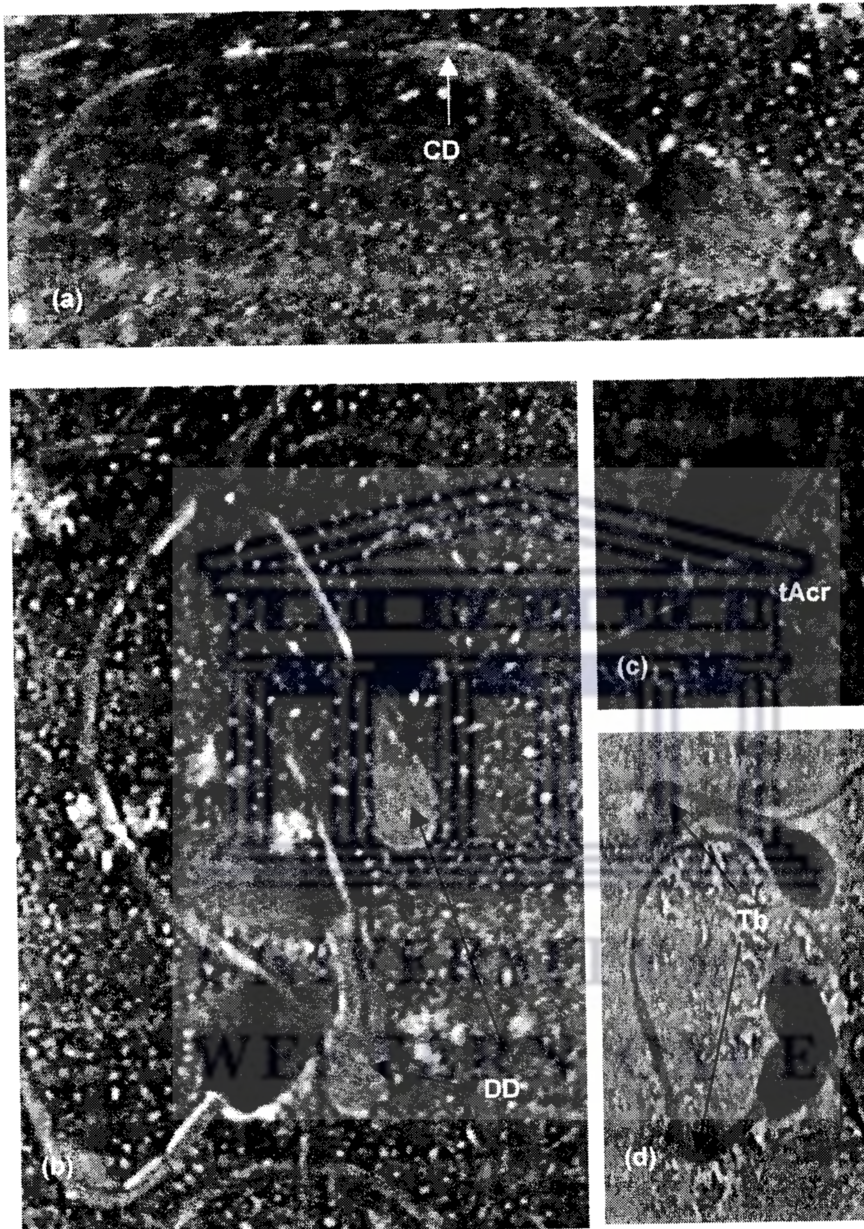

Fig 2-17: (a) Normal cauda epididymal sperm of blesbok with a distally placed cytoplasmic droplet (CD). **(b)** shows different types of tail (Tb) bending and Dag defect (DD), similar to that seen in **(c)** tACR = damaged acrosome; **(d)** shows sperm tails encapsulated by a membranous sheath, the plasmalemma.

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2.3.1.3 Blue wildebeest

2.3.1.3.1 Normal sperm (Scanning Electron Microscopy)

Normal sperm were identified on the same basis as for impala and blesbok. Normal sperm displayed a spatulate head and a single tail with or without a distally placed cytoplasmic droplet. The anterior part of the sperm head exhibited a prominent acrosomal ridge (Figure 2-19). The acrosome occupied about two thirds of the entire head (Figure 2-18). The equatorial segment was also much more prominent in blue wildebeest than in either impala or blesbok. The tail of the sperm was inserted centrally into the base of the head.

2.3.1.3.2 Abnormal sperm (Scanning Electron Microscopy)

One of the important criteria for identifying normal cauda epididymal sperm is the placement of the cytoplasmic droplet. Figures 2-20 and 2-21 are examples of a proximally and a centrally i.e. in the center of the midpiece, placed cytoplasmic droplet. The sperm head in Figure 2-21 appears to be normal with even contours and is bilaterally symmetrical.

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Fig. 2-18

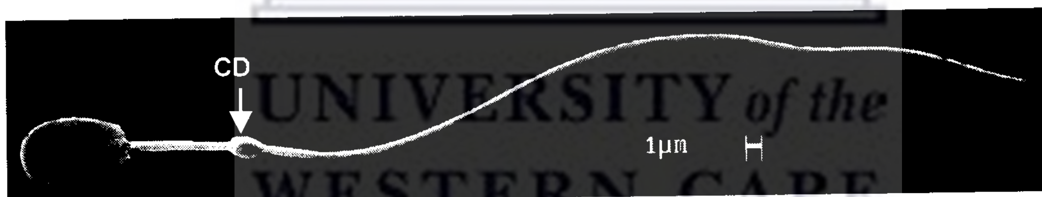

Fig. 2-19

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Figures 2-18 and **2-19**: Show normal cauda epididymal sperm of blue wildebeest. **Fig 2-18** is a normal cauda epididymal sperm without a cytoplasmic droplet. A distal cytoplasmic droplet (CD) can be seen in **Fig 2-19**.

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Fig. 2-20 Abnormal blue wildebeest cauda epididymal sperm with a proximally placed cytoplasmic droplet (CD). The boundary between the acrosome and the postacrosomal region is not as well defined as in **Fig 2-21.** which indicates a prominent equatorial segment.

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Figures 2-22 and 2-23 are examples of the various degrees of tail bending observed. Many of the tail folds occur at the site where the cytoplasmic droplet is situated (Figures 2-22 and 2-23). This type of tail defect has been observed in other species. Figure 2-22 shows that the sperm head is bilaterally symmetrical with a well-defined equatorial segment. The acrosomal ridge appears irregular.

There is a similarity between the tail fold seen in Figure 2-23 and that seen in Figure 2-22. However, the part folded back lies tightly against the midpiece with the cytoplasmic droplet trapped in the loop. This type of folding has been described as one form of the Dag defect.

Figure 2-24 shows blue wildebeest cauda epididymal sperm with extensive coiling of the principal piece. Dense outer fibers seen along the tail create the appearance of a multiflagellated sperm tail. Remnants of the cytoplasmic droplet are trapped within the coils. The head of the sperm appears regular in shape except for a slight indentation in the postacrosomal region. There is a clear distinction between the acrosomal and postacrosomal regions. The acrosomal region is smooth in texture as opposed to the coarse texture of the postacrosomal region. Figure 2-25 shows extensive coiling of the mid –and principal piece, and the mitochondrial sheath is prominent. The sperm head is completely amorphous and has an acrosomal lip. Figure 2-26 shows a coiled sperm tail covering the entire surface of the sperm head.

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Figures 2-27 to 2-28 show what appear to be multiple tailpieces attached to the same sperm, but could also be another form of Dag defect with extensive folding of the sperm tail.

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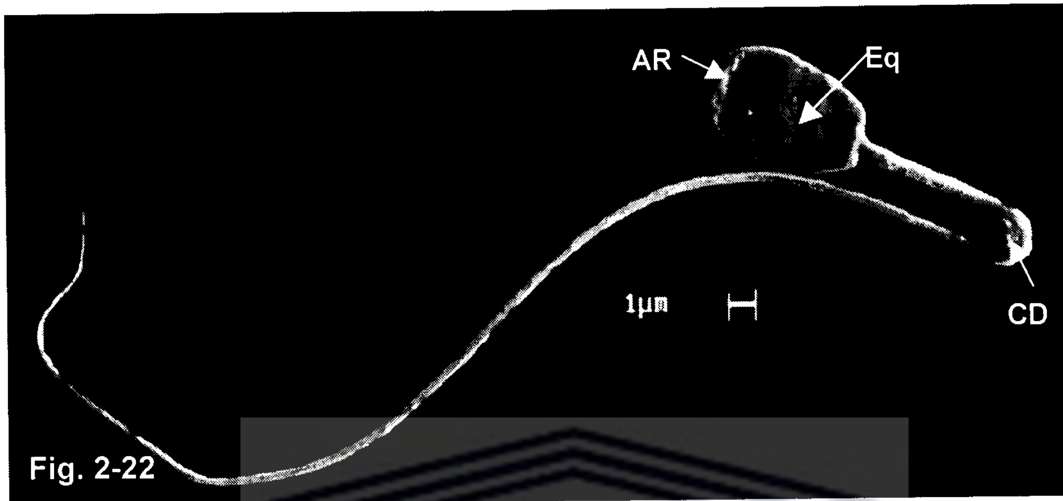

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Fig. 2-22 Blue wildebeest cauda epididymal sperm with a bent tail and an abnormal acrosomal ridge. The equatorial region (Eq) is well-defined. **Fig. 2-23** cauda epididymal sperm with an abnormal tail and head. The head is slightly elongated, tapering towards the tip of the head. The contours are uneven and the equatorial region is not well defined as compared to Fig. 2-22

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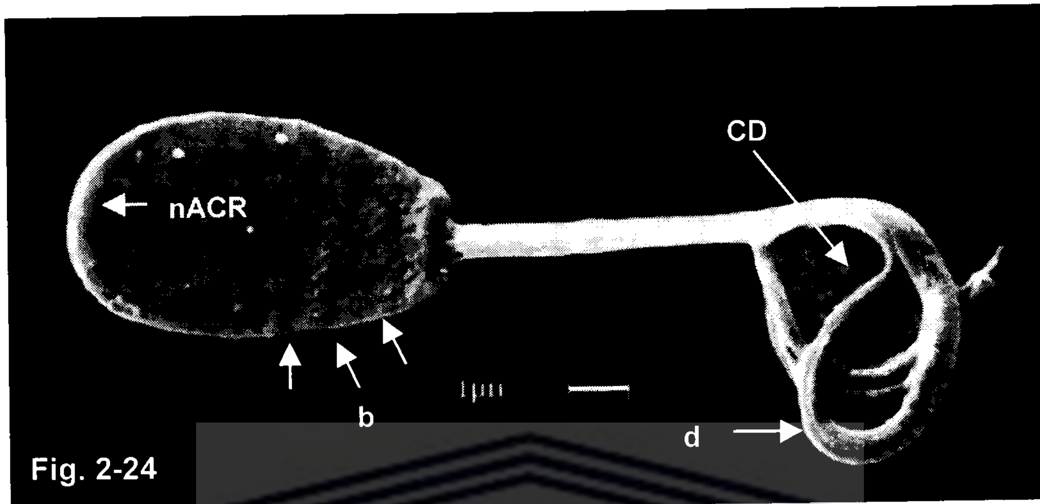

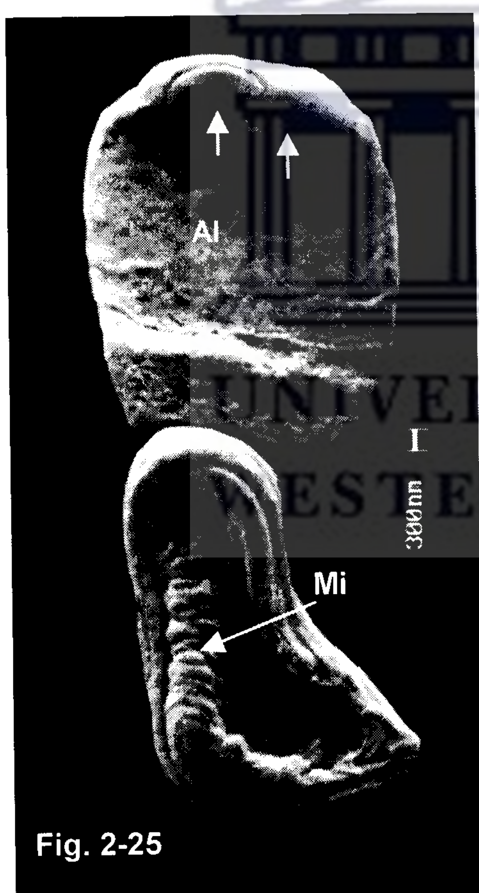

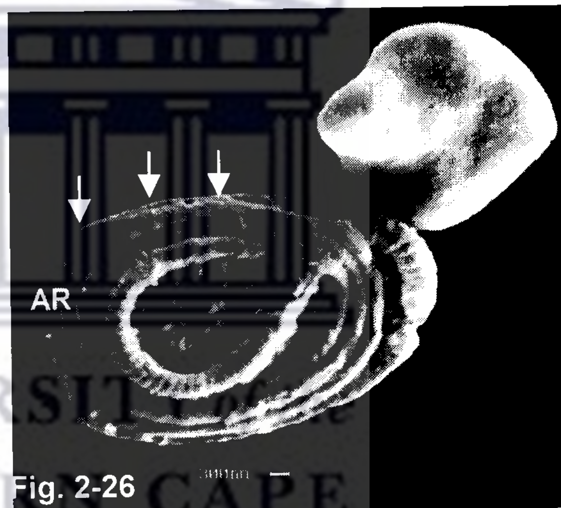

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Fig. 2-24: Blue wildebeest cauda epididymal sperm showing several abnormalities including an abnormal acrosomal ridge (nACR); uneven contours of the head in the postacrosomal region (b), (d) shows what appears to be dense outer fibers. **Fig. 2-25** shows sperm with severe head and tail deformities. The sperm head is irregular with an acrosomal lip (Al). The tail is coiled and covered by a membranous sheath or plasmalemma. Mitochondrial (Mi) sheath is also very prominent. **Fig. 2-26** This is an example of a coiled tail, covering the entire surface of the sperm head (arrows). In this figure, the acrosomal ridge (AR) can be seen.

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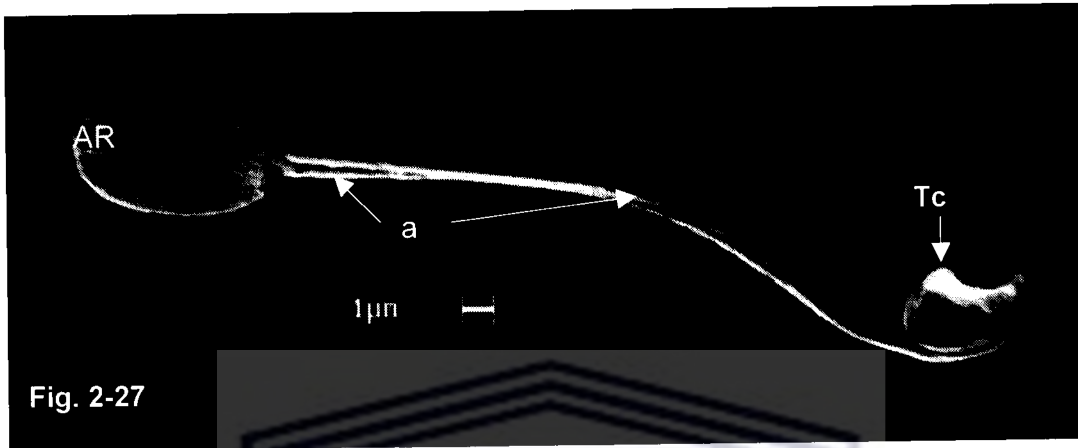

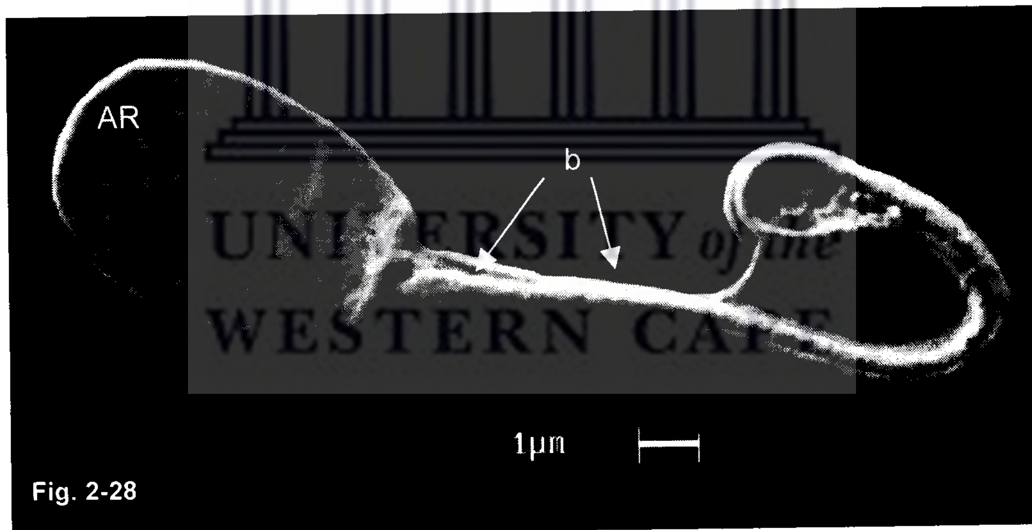

Fig. 2-27. Blue wildebeest cauda epididymal sperm with (a) abnormal biflagellate tail which is coiled at the tip. The head shape is abnormal and appears oval. The acrosomal ridge (AR) is not present or is poorly developed. **Fig. 2-28** is an example of a possible Dag defect with tail coils creating the appearance of a multiple flagellated sperm tail. The flagellae are encapsulated by a membranous sheath. The head appears to be normal in shape and about two thirds of the head is covered by the acrosome.

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2.3.1.3.3 Normal and abnormal sperm (Light microscopy)

Examples of normal cauda epididymal sperm in the blue wildebeest can be seen in Figures 2-29 (a) and (d). Blue wildebeest sperm are more elongated than impala sperm with a wider acrosomal region. The acrosome covers about two thirds of the head surface. Tail defects varied from tail bending to tail coiling (Figure 2-29(c)).

2.3.2 Transmission Electron Microscopy: *a validation of some normal and abnormal features found in impala, blue wildebeest and the blesbok*

Figure 2-30 depicts a normal sperm head and the condensed nuclear material appears to be homogenously distributed. The cell membrane appears regular and fits tightly around the sperm head. The equatorial region is clearly demarcated separating the sperm head into an acrosomal and postacrosomal region.

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Figure 2-31 shows a sagittal section of the sperm cytoplasmic droplet. From the micrograph, it is clear that several membranous structures (Golgi structures) are present (Oko *et al.*, 1993). Sperm ultrastructural abnormalities and specifically those that occur internally can only be seen with the aid of the transmission electron microscope. Figure 2-32 is an example of such an abnormality and shows a vacuole situated at the base of the neck (Ackerman, 1995).

Figure 2-33 is a cross section through a blesbok sperm with the Dag defect. This defect has been described in other species (Hafez, 1987; Pinart *et al.*, 1998), and was also seen in impala and the blue wildebeest. In this instance, the sperm

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head is completely deformed with vacuoles found in the nucleus. The axoneme appears unaffected

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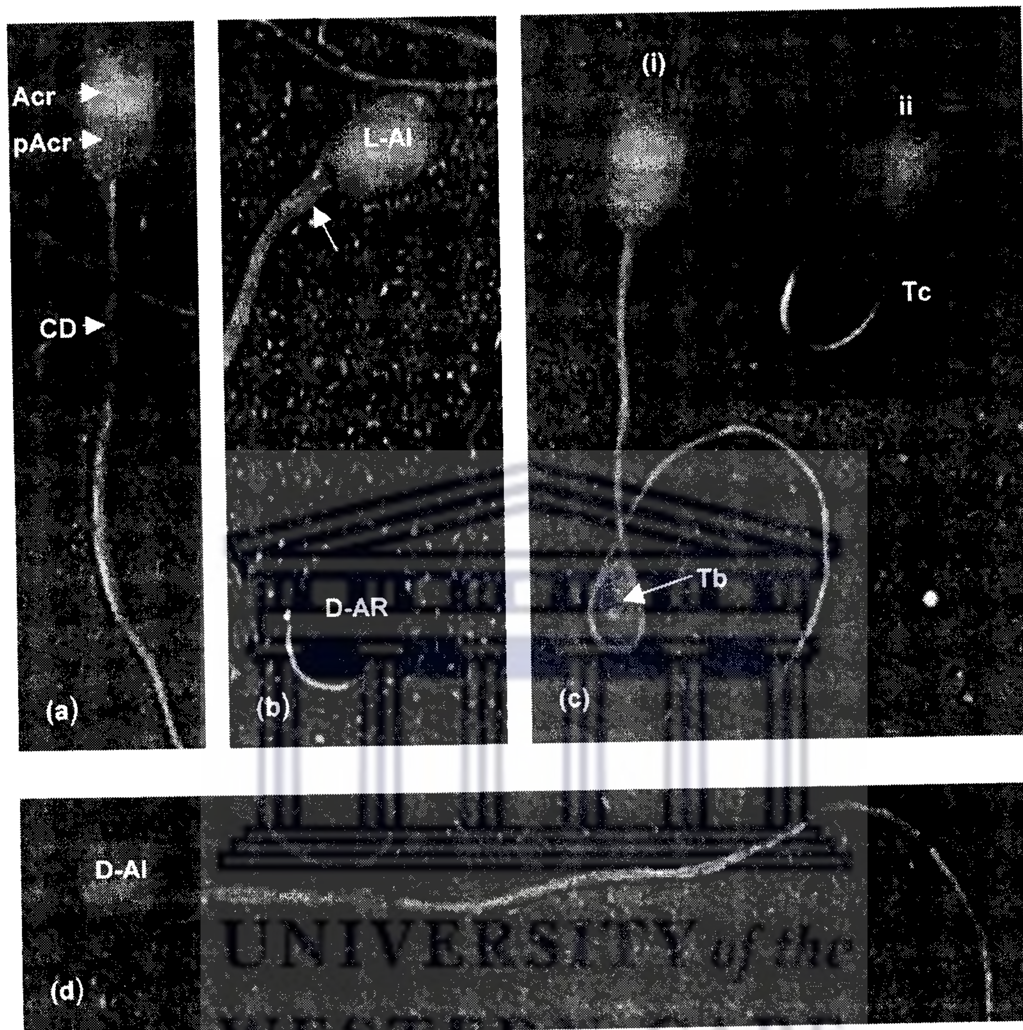

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Fig. 2-29 (a) Normal cauda epididymal sperm of blue wildebeest. The sperm head is regular in shape with a faint acrosomal (Acr) and postacrosomal (pAcr) region. The acrosome covers about two thirds of the head. The cytoplasmic droplet is distally placed, typical of normal cauda epididymal sperm. **(b)** two cauda epididymal sperm of which one is dead with either a damaged or reacted acrosome (D-AR), the other shows a live, acrosome intact sperm head (L-AI). The neck of this cell appears abnormal (arrow) due to thickening of this region. **(c)** Shows two sperm (i and ii) where (i) is an example of sperm tail bending (Tb) and (ii) tail coiling (Tc). **(d)** Normal cauda epididymal sperm. Staining pattern suggests that the sperm was dead, as indicated by the dark postacrosomal region, but that the acrosome was intact (D-AI), as indicated by the lightly stained acrosome.

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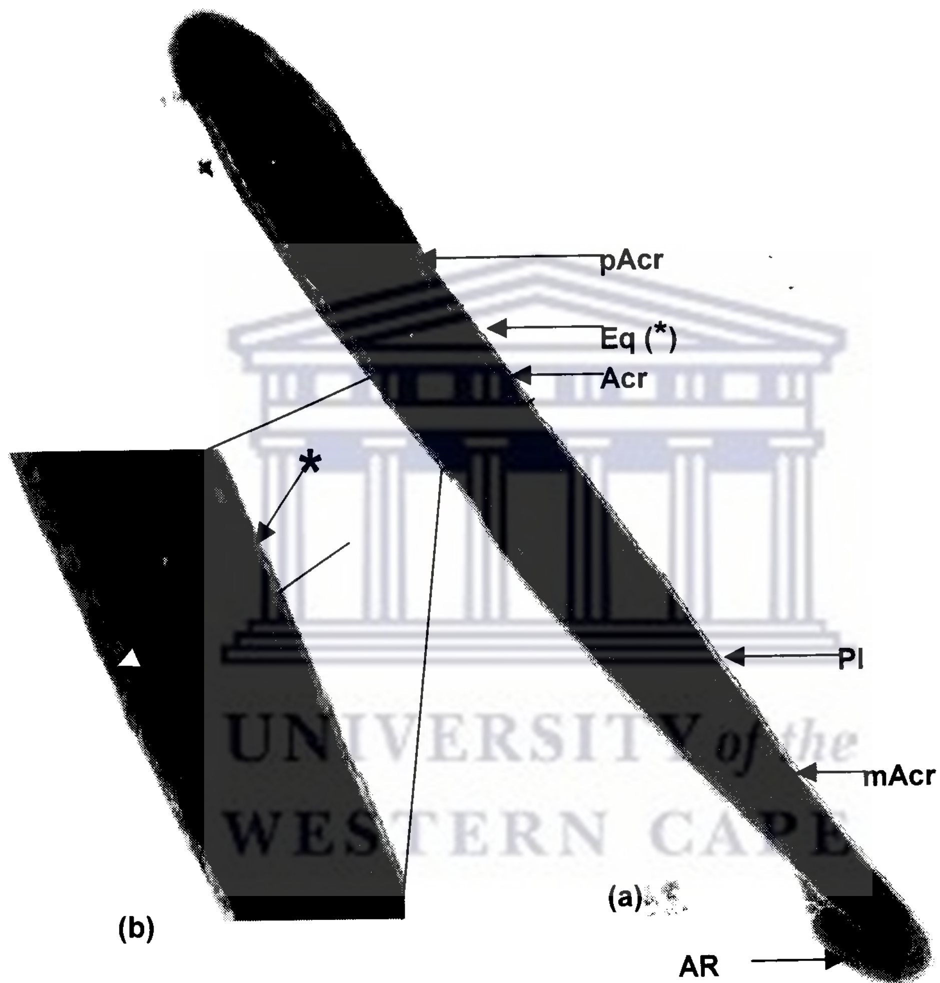

Fig. 2-30 (a) Oblique TEM section of a normal cauda epididymal sperm head of blesbok. The sperm head has an intact plasma membrane (Pl) and acrosomal membrane (mAcr). The acrosomal ridge (AR) can be clearly identified. The sperm head is further subdivided into an acrosomal (Acr) and postacrosomal region (pAcr) separated by, in this micrograph, the end the equatorial segment (Eq). Due to the oblique nature of this section, the beginning of the equatorial segment is not visible **Fig. 2-30 (b)** is an enlargement of the equatorial segment. Asterisk (*) shows the end of the equatorial segment.

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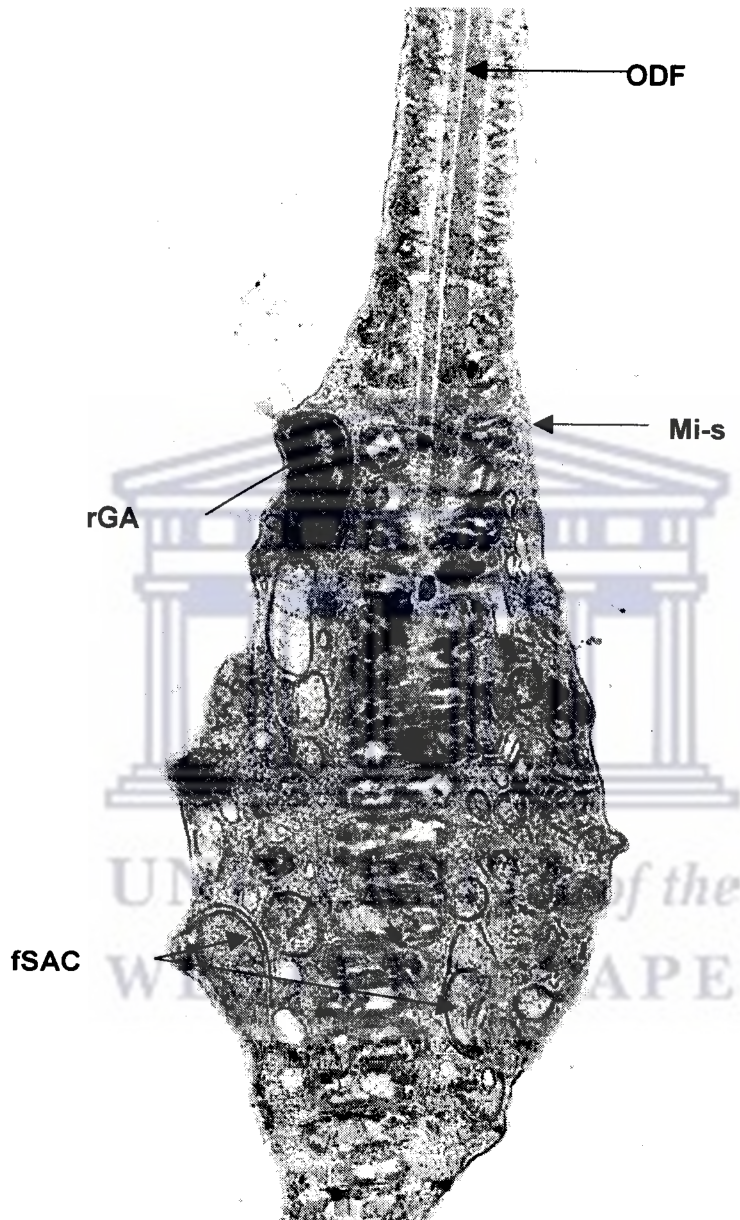

Fig. 2-31: Shows a sagittal section through a cytoplasmic droplet of an impala cauda epididymal sperm. The cytoplasmic droplet contains many remnants of the Golgi apparatus (rGA) and other flattened saccular structures (fSAC) (Oko, *et al.*, 1993). The outer dense fibre (ODF) and the mitochondrial sheath (Mi-s) can also be seen in this figure.

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Fig. 2-32: shows a TEM sagittal section of the neck and midpiece of an impala sperm. Note the presence of a neck vacuole (arrow) at the base of the neck near the proximal centriole (C).

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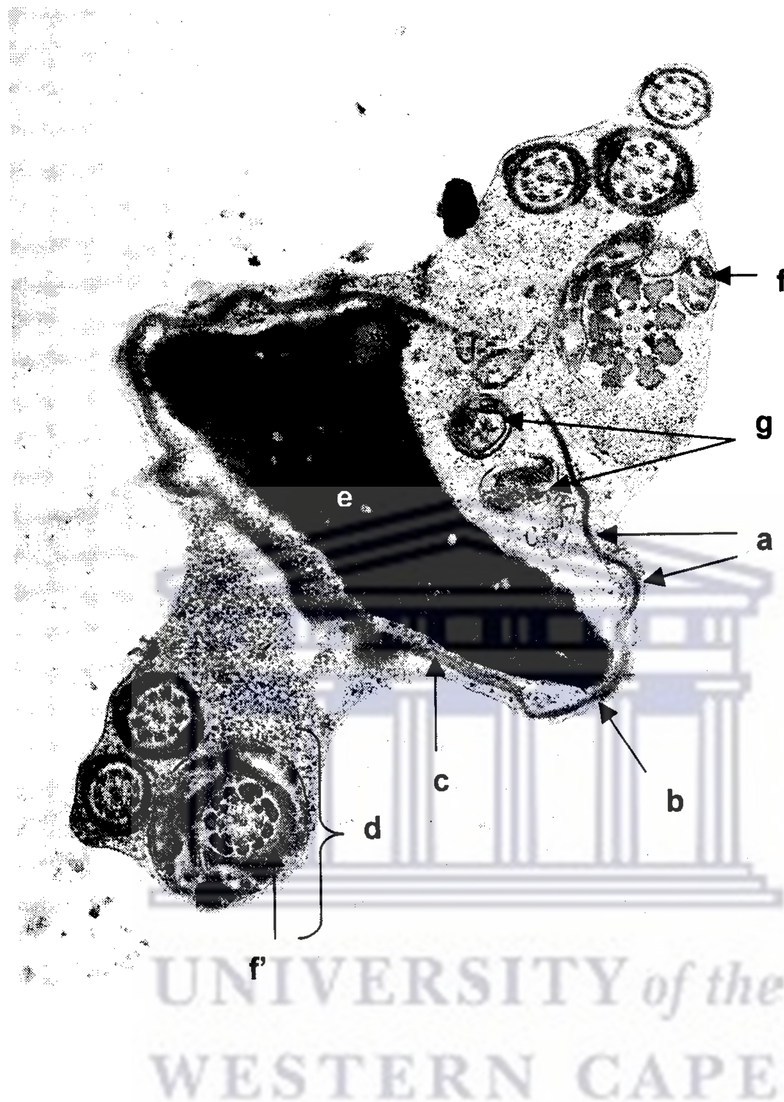

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Fig. 2-33: Shows a section through the head and tail of a coiled blesbok cauda epididymal sperm encapsulated by the sperm membrane (Dag defect).
Labels: **(a)** equatorial region; **(b)** thinning of the equatorial region; **(c)** acrosome; **(d)** section through coiled tail encapsulated by plasma membrane; **(e)** amorphous nuclear material in sperm head with vacuoles; **(f) and (f')** indicate a section through the midpiece of the sperm tail; **(g)** saccular structures as normally found in the cytoplasmic droplet.

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2.3.3 Light Microscopic evaluation of Live/Dead status

Sperm live/dead staining patterns were assessed by means of light microscopy. FCF-fast green staining patterns were determined from images such as those seen in Figures 2-10 (a) and (b); and Figure 2-17. Figures 2-34 to 2-37 show the percentage of live versus dead sperm observed after staining with Nigrosin-Fast Green (FCF) and Eosin B after 15 minutes, 1 hour, 3 hours and 6 hours incubation respectively. Data (mean ± SEM) are represented in the figures.

Live sperm appear white with no stain taken up by the sperm. The sperm head can be divided into the acrosomal and postacrosomal regions. From Figures 2-10 (a) and (b) it is clear that sperm staining patterns differed according to which region of the head was stained. If the whole head was unstained (Figure 2-10(b)) the sperm was considered live. Dead sperm were further subdivided according to the presence or the absence of the acrosomal cap. Dead sperm without the acrosome (AR) (Figure 2-29(b)) stained completely dark as opposed to dead sperm with an intact acrosome which did not stain and appeared white (AI) (Figure 2-29(d)). The results show very little change in the live/dead status amongst the three species over a six hour incubation period in Ham's F10. The results obtained also showed no live, acrosome reacted sperm for any of the three species.

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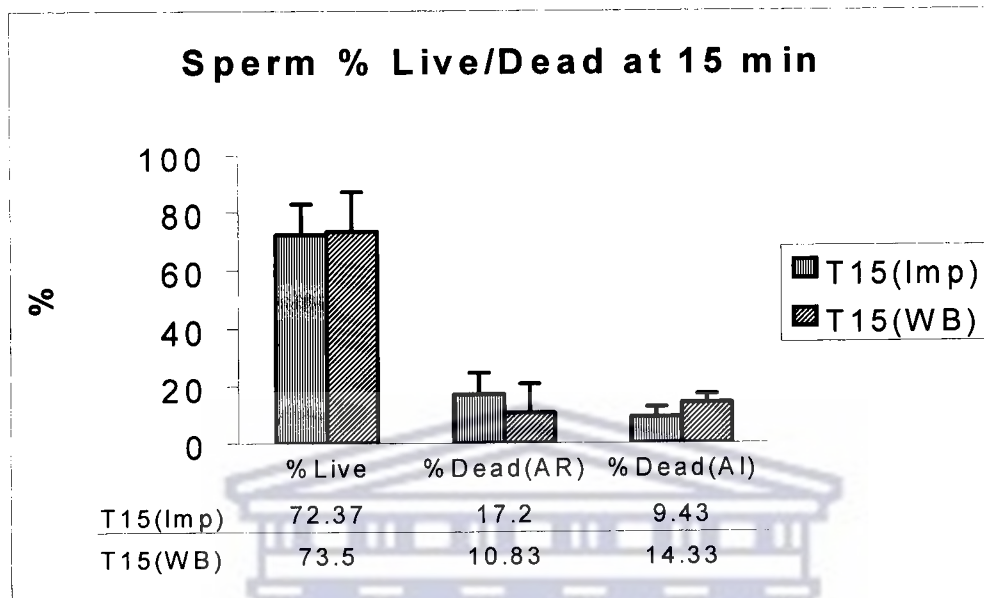
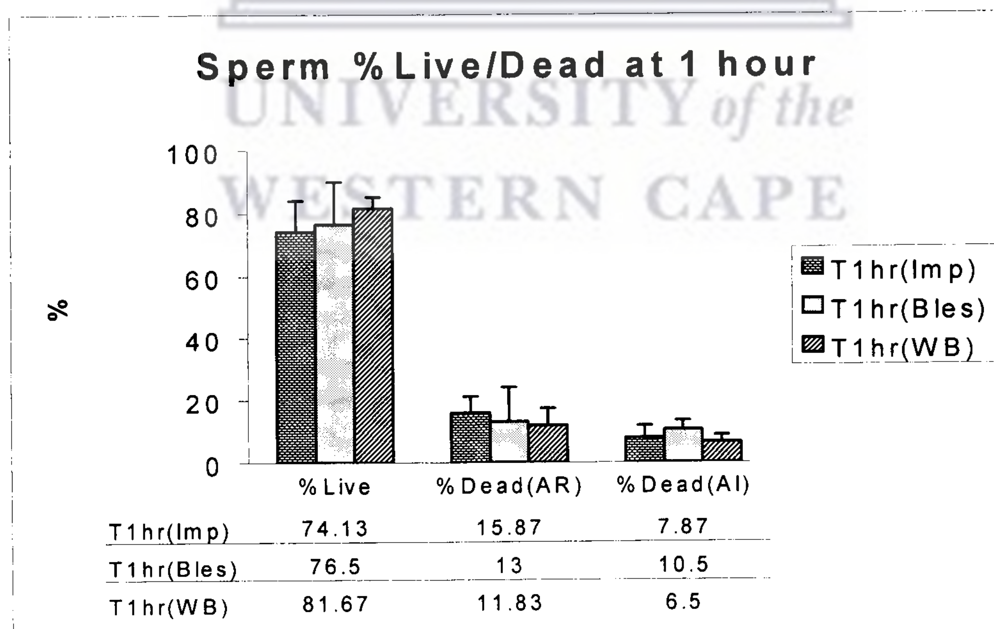


	% Live	% Dead(AR)	% Dead(AI)
T15(Imp)	72.37	17.2	9.43
T15(WB)	73.5	10.83	14.33

Fig. 2-34: Histogram (mean±SEM) and results (%) of a live/dead count for impala and blue wildebeest after 15 minutes incubation in Ham's F10. For definitions of AR and AI, refer to Figure (2-29).



	% Live	% Dead(AR)	% Dead(AI)
T1hr(Imp)	74.13	15.87	7.87
T1hr(Bles)	76.5	13	10.5
T1hr(WB)	81.67	11.83	6.5

Fig. 2-35: Histogram (mean±SEM) and results (%) of a live/dead count for impala, blesbok and blue wildebeest after 1 hour incubation in Ham's F10. For definitions of AR and AI, refer to Figure (2-29).

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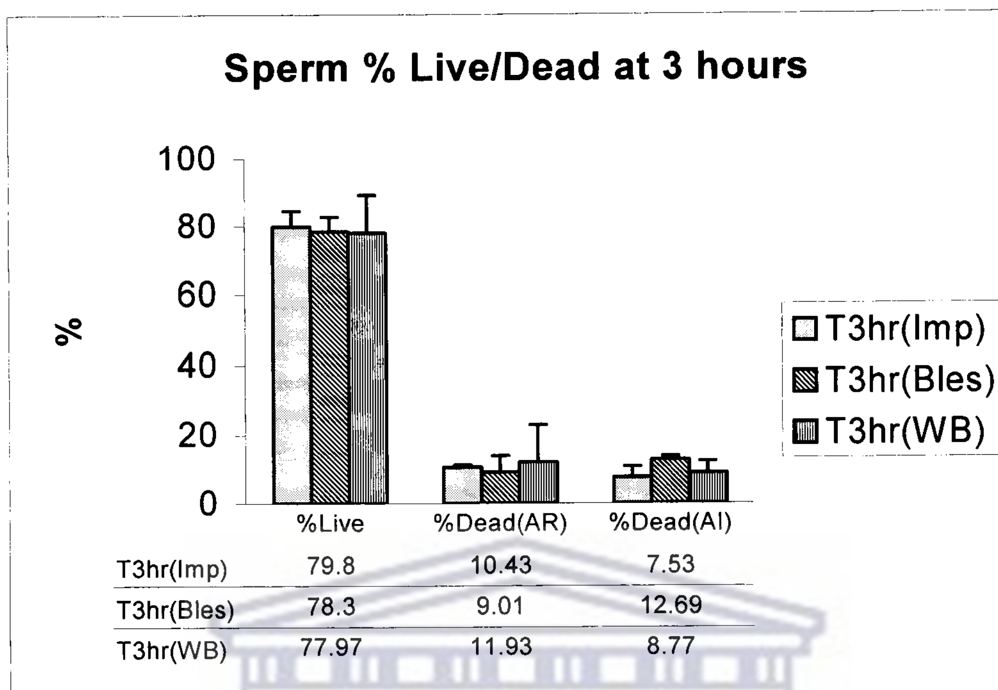
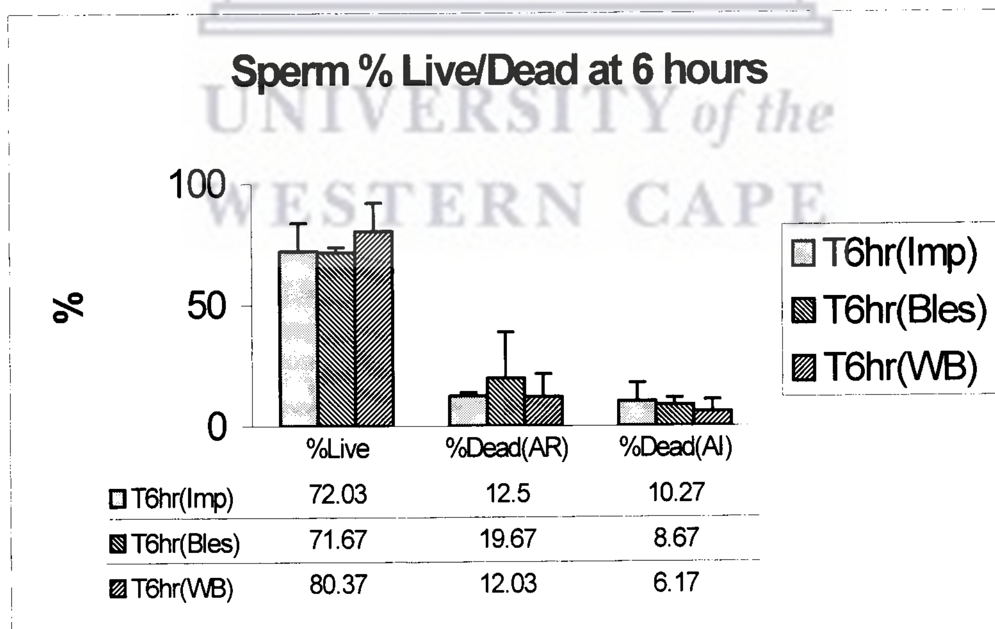


	%Live	%Dead(AR)	%Dead(AI)
T3hr(Imp)	79.8	10.43	7.53
T3hr(Bles)	78.3	9.01	12.69
T3hr(WB)	77.97	11.93	8.77

Fig. 2-36: Histogram (mean±SEM) and results (%) of a live/dead count for impala, blesbok and blue wildebeest after 3 hours incubation in Ham's F10. For definitions of AR and AI, refer to Figure (2-29).



	%Live	%Dead(AR)	%Dead(AI)
T6hr(Imp)	72.03	12.5	10.27
T6hr(Bles)	71.67	19.67	8.67
T6hr(WB)	80.37	12.03	6.17

Fig. 2-37: Histogram (mean±SEM) and results (%) of a live/dead count for Impala, Blesbok and Blue Wildebeest after 6 hours incubation in Ham's F10 For definitions of AR and AI, refer to Figure (2-29).

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2.4 Discussion

The sperm of impala, blesbok and blue wildebeest are like most other bovid species dorso-ventrally flattened and spatulate in structure (Ackerman, 1995). However, sperm of each antelope species display distinctive characteristics as revealed by scanning electron microscopy and light microscopy.

The sperm head of impala has a smooth and regular appearance and is slightly elongated. There is a prominent acrosomal ridge and the acrosomal region is wider than the postacrosomal region. These two regions are separated by the equatorial segment which could clearly be recognized in the impala. The acrosomal region occupies about two thirds of the sperm head surface.

In the cauda epididymis, the cytoplasmic droplet is present on or near the annulus. It has been shown, in the rat, that the cytoplasmic droplet migrates along the midpiece of the tail from the neck region of the sperm to the annulus and eventually detaches from the sperm in the corpus and cauda epididymis to be endocytosed by the epithelial layer (Hermo *et al.*, 1988; Robaire and Hermo, 1988).

Abnormalities observed in impala sperm were irregular head shapes and tail defects. Some impala sperm were observed with cytoplasmic droplets along the midpiece and close to the head and were considered to be immature cells(Pinart

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et al., 1998). Light microscopic evaluation confirmed some of the abnormalities seen with the aid of the scanning electron microscope.

Normal sperm in the blesbok are dorsoventrally flattened and oval. Blesbok have a more rounded sperm head when compared to impala and blue wildebeest. Measurements for sperm head length and head width confirmed that impala and blue wildebeest differed from blesbok (see later, Table 2-1). The most commonly observed abnormalities for blesbok sperm were abnormally shaped heads with uneven symmetries. Tail deformities ranged from folded to coiled tails. In some instances loose tail fibrils and broken necks were observed. Broken necks or detached heads are associated with testicular degeneration according to Barth and Oko (1989). This defect develops during the first stages of spermiogenesis and manifests most frequently in the epididymis when sperm acquire motility (Zamboni, 1991, 1992).

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It was difficult to evaluate detailed head morphological differences in blesbok sperm at the light microscopic level. The most obvious abnormalities were tail deformities and abnormally shaped heads. At higher magnifications, loose acrosomal caps were identified.

Normal sperm in the blue wildebeest are similar in shape compared to impala. Blue wildebeest sperm heads are characterised by an elongated acrosomal region and a narrower postacrosomal region. The equatorial segment is

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particularly well defined in blue wildebeest sperm and could be better resolved than in impala and blesbok sperm at the scanning electron microscope level. This is suggestive of differences in the surface glycoproteins among the three antelope species. Normal sperm from the cauda epididymis were either with or without a cytoplasmic droplet. The acrosomal region has a smooth texture compared to the coarse appearance of the postacrosomal region in blue wildebeest; the acrosomal ridge is particularly well defined in blue wildebeest.

Abnormally shaped blue wildebeest sperm relate to both head and tail abnormalities. The cytoplasmic droplet was a marker for sperm maturity. Immature sperm typically revealed proximally placed cytoplasmic droplets. Tail folding usually occurred at a point that coincided with the location of the cytoplasmic droplet close to the annulus. Briz *et al.* (1996) stated that tail folding develops in mature spermatids due to disturbances of tail organisation in the folded piece.

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Impala and blue wildebeest sperm often have multiple tails. Tail abnormalities develop in the early stages of spermiogenesis (Kojima and Kinoshita, 1975; Barth and Oko, 1989) and are the expression of anomalies in remodeling processes of the flagellum during the late stages of spermiogenesis (Ross and Christie, 1973; Barthelemy *et al.*, 1990).

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Sperm head length and width measurements were used in an attempt to highlight species specificity. Results obtained may indicate that species specific differences exist. Several investigators have determined sperm head measurements in impala and blesbok. Table 2-2 lists results obtained by various investigators over the past three decades for head length, head width and width/length ratios of these two antelope species.

Table 2-2: Sperm head measurements of impala and blesbok obtained by different investigators.

Impala

	Ackerman(1995)	Dott and Skinner (1989)	Morgenthal (1967)	*This study
HL	7.59±0.61	9.75	6.52±0.02	6.64
HW	4.81±0.49	6.05	4.13±0.02	4.12
Ratio	0.63	0.62	0.63	0.63

Blesbok

	Dott and Skinner (1989)	This study
HL	7.53	5.59
HW	4.78	3.97
Ratio (HW/HL)	0.64	0.71

Measurements are in µm, *For SEM values-refer to table 2-1 (Results)

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Similar results were obtained in this study when compared to the results obtained by Morgenthal, (1967). In contrast, there was a decline in sperm measurements of about 30% in relation to FCF-stained sperm measurements obtained by Dott and Skinner (1989). Van der Horst *et al.* (1991), demonstrated that a shrinkage of about 20% may occur after processing sperm for SEM. The shrinkage was attributed to the critical point drying process during the preparation of samples for SEM viewing.

The results obtained for impala in this study showed a 31.8% reduction for head length and 31.9% for head width when compared to the results obtained by Dott and Skinner (1989). For blesbok sperm there was a reduction of 25% for head length and 17% for head width when compared to measurements for FCF stained sperm as reported by Dott and Skinner (1989). The results obtained by Ackerman (1995), showed a 22% reduction in length compared to the results of Dott and Skinner (1989)(sperm FCF stained values) and a 20.5% reduction in width for impala sperm. This is in agreement with the 20% reduction suggested by van der Horst *et al.* (1991).

No sperm head measurements could be found for blue wildebeest in the literature. Sperm obtained from BW had the same morphological features, as the other two species except that sperm surface details of BW were more prominent when viewed with the scanning electron microscope. The pre- and post acrosomal regions could clearly be distinguished. The acrosomal region covered

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almost two thirds of the sperm head surface which is similar to that of impala and blesbok sperm.

Light microscopy was used to confirm features of normal and abnormal sperm. It was clear that surface details could not be as clearly observed by means of LM when compared to scanning electron microscopy. However, gross sperm abnormalities were evident. These included features such as head shape and tail abnormalities.

The use of a water soluble dye such as the Nigrosin and Eosin B-fast green (FCF), was sufficient for the assessment of the percentage live and dead sperm in a sample. According to the results obtained, sperm live/dead status remained relatively unchanged throughout the six-hour incubation period for all three species. It may have been expected that the number of dead sperm would increase over six hours. From the data, however, it appears that blue wildebeest sperm had the highest percentage of live sperm at six hours after incubation. The percentage dead sperm never increased to more than 25% for any of the species for the duration of the incubation period. It was not possible to identify live acrosome reacted sperm from any of the samples. A possible reason is that the stain could not detect the acrosome reaction or that very little or no acrosome reaction occurred. The results furthermore indicate that the live/dead ratio remained relatively constant during incubation and accordingly also during the six hour incubation period. In practice, this proves to be advantageous that more

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than 70% of the sperm may remain viable and may not undergo capacitation. Accordingly no acrosome reacted sperm would be present prior to exposure to the oocyte during an ART (assisted reproductive technology) procedure. Early capacitation of sperm could lead to the loss of the sperm's fertilising ability, *in vitro* or *in vivo*.

In this study, the transmission electron microscope was only used to validate some of the ultrastructural features of normal and abnormal sperm in impala, blue wildebeest and blesbok. Sections through normal sperm showed the typical structure of the various sperm membranes with a clear demarcation of the acrosomal and postacrosomal regions being observed. The nucleus appears homogenous without vacuoles in normal sperm. Abnormalities in head size and shape develop in the round spermatids because of alterations in the pattern of chromatin condensation (Fawcett *et al.*, 1971; Barth and Oko, 1989; Schill, 1991). In addition, transmission electron microscopy proves useful to study abnormalities since many of these could not be detected using light or scanning electron microscopy. An example of such an abnormality was the neck vacuole found in the impala sperm. Dag defects (Ackerman, 1995) were common in all three species studied. A section through a sperm with a Dag defect in blesbok indicated malformation of the sperm head. The sperm head was completely deformed with vacuolisation. This malformation is primary (Pinart *et al.*, 1998) and originated in the testes during spermiogenesis due to alterations in the pattern of chromatin condensation.

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In conclusion, normal and abnormal sperm have been described for the three species by means of scanning and light microscopy. Some of the aspects of normal and abnormal sperm structure were validated with the aid of the transmission electron microscope. From the data obtained, it appears that species differences exist based on both qualitative differences as well as on the basis of sperm head dimensions.

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Chapter 3

Wild antelope cauda epididymal sperm: kinematic analysis following incubation at 37°C

3.1 Introduction

Routine semen analysis in both human andrology laboratories and livestock/animal production systems comprise a series of tests aimed at investigating the physical properties of the semen. This is done in order to establish whether the sample obtained is viable. Sperm viability is in turn an indication of the patient or animal's fertility status. The fertilising ability of sperm is the most important quality and various methods and criteria have been devised to assess fertilising potential. Both semen and sperm physical characteristics play an important role. Of the seminal criteria, semen volume, pH, colour etc (Mortimer, 1994; Hafez, 1987) are compared with documented normal data.

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Factors that influence semen and sperm quality assessment include methods of sampling and handling of samples, hierarchical status of the animal (i.e. reproductively dominant or suppressed), environmental factors like pollutants (estrogenic, etc.) and the constantly changing new technologies (Garner, 1997). For humans, the World Health Organization and Tygerberg strict criteria are accepted standards in human andrology evaluation, (WHO Manual, 1992; Kruger *et al.,* 1986; Mortimer, 1994) but not many

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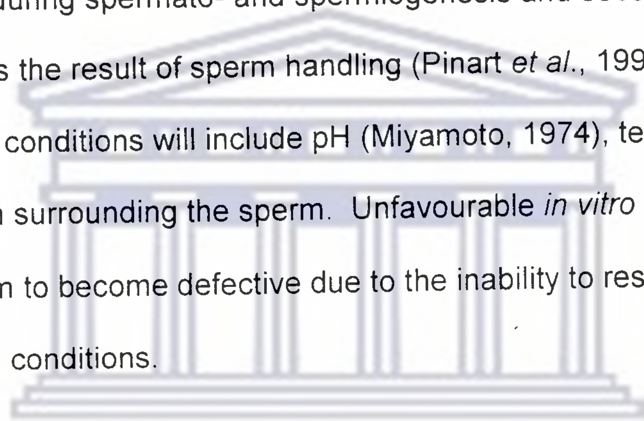
standards exist for other non-human and non-primate species (Salisbury and Vandemark, 1961).

In vitro handling of sperm is important as the conditions to which the sperm are exposed could influence the quality and functionality of the sperm. Sperm defects, for example, can be either of a primary type or a secondary type, where primary type abnormalities stem from the testes during spermato- and spermiogenesis and secondary types from the epididymis, and as the result of sperm handling (Pinart *et al.*, 1998). Examples of hostile environmental conditions will include pH (Miyamoto, 1974), temperature and tonicity of the medium surrounding the sperm. Unfavourable *in vitro* conditions could therefore cause sperm to become defective due to the inability to resist these "micro-environmental" stress conditions.



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Media and sperm extenders are designed to minimize secondary sperm defects and "environmental" stress on sperm function. Medium composition has several effects on sperm function including stimulation of the sperm to fertilise the oocyte. Many of the media supplements can alter sperm function. For example, sodium acts as a hydrogen exchanger in order to increase sperm intracellular pH and thereby activating motility by raising the intracellular pH (Hyne *et al.*, 1984). On the other hand, some elements, although crucial in the living animal and for maintaining important functions in the animal body, could be detrimental for sperm survival (Lapointe *et al.*, 1998). Lapointe *et al.* (1998), showed that certain amino acids of the aromatic type could influence sperm

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motility. Their study has shown that the addition of amino acids like phenylalanine can reduce the average path velocity of sperm from 120 µm/s in the control to about 50 µm/s. They suggested that dehydrogenation and deamination of aromatic amino acids form hydrogen peroxide and ammonia could adversely affect sperm motility. These detrimental processes occur because of enzyme release (amino acid oxidase) from dead sperm in the system.

Carbohydrates play an important role in the initiation of sperm motility (Tso *et al.*, 1987). Tso *et al.* (1987) suggested that sugar structural specificity play a role in the initiation of motility. Monosaccharides containing more than four carbons were all good motility initiators. Tso *et al.*, (1987) concluded that a potential motility initiator should possess a carbon structure of no less than five carbons irrespective of stereospecificity. Fraser and Quinn (1981) obtained similar results whilst studying the effects of combinations of pyruvate, lactate and glucose on mouse fertilization, *in vitro*. They showed that preincubating sperm with media lacking glucose led to loss of fertilising ability, but regardless of the composition of the initial medium, the addition of glucose immediately induced fertilising ability. Fraser and Quinn (1981), furthermore, showed that if fructose, rather than glucose, was added as a sole energy source, no fertilization was obtained.

Studies on Murciano-Granadina goats (Roca *et al.,* 1992), showed that the poorest quality semen was collected during winter and spring. Similar results were obtained for impala (Brown *et al.,* 1991). Although there may be an abundance of post-mortem

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material (testes with epididymis) during the normal hunting season, sperm quality could be inferior due to seasonality. Most hunting activity takes place during winter, i.e. May to July.

Sperm motility is an indicator of sperm viability and a function of the physiology of sperm. The differences in semen quality including motility seen during different seasons have been ascribed to seasonal fluctuations in the release of gonadotropins (Brown *et al.*, 1991).

3.1.1 Sperm motion analysis and capacitation

Computer aided sperm motility analysis (CASA) provides information such as sperm velocity, progression and amplitude of lateral head movement, which cannot be obtained by routine semen analysis. Data obtained from CASA can be used to predict fertilising potential (Burkman *et al.*, 1984), sperm maturation in the epididymis (Van der Horst *et al.*, 1999), and is used in toxicological studies (Katz, 1991). Identifying subpopulations within the sample, based on kinematic parameters, can be related to the fertilising potential of the animal or provide information relating to the physiology or electro-mechano-chemical function of sperm (Katz, 1991). Computer aided sperm motion analysis is useful in identifying sperm hyperactivation, which has been seen in most mammals studied (Gwatkin and Anderson, 1969; Yanagimachi, 1970; Burkman, 1990 and 1991). The onset of hyperactivation is species specific and is a useful biological biomarker as it is related to capacitation. Capacitation is a necessary

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physiological and biochemical process which sperm undergo to enable them to fertilise the oocyte. Hyperactivation in sperm is identified by complex, jerky paths of the sperm (Katz, 1991).

The time taken to induce capacitation is species specific (Fouquet and Kann, 1992). During hyperactivation, the swimming pattern of the sperm changes from a straight, regular swimming pattern to a vigorous irregular pattern (Brinders, 1994; Suarez, 1988). Hyperactivation is part of the final maturation process and may be a possible indicator of sperm capacitation.

3.1.2 Possible sources for error during analysis

For most species, sperm motion represents a three dimensional swimming pattern and recording free-swimming sperm on videotape introduces at least three sources of error in determining or comparing the properties of sperm swimming behaviour (Tessler and Olds-Clarke, 1985):

a). Only two-dimensional (planar) movements are captured, therefore, movement in the third dimension is not recorded or represented.

b). Movement is not recorded as a continuous process, but consists of discrete positions of the sperm head in time and space, with the time interval between positions being determined by the flash exposure rate.

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c). Total time over which sperm movement can be observed is limited by both the microscope field of view and the actual swimming speed of the spermatozoan.

Suarez *et al.*, (1983) showed that the error imposed by a two- dimensional bias on velocity estimates for helical rolling rabbit sperm is less than 10% and considered statistically unimportant. Mortimer (1997) showed that head movement which is used to track sperm by means of CASA is representative of flagellar movement of the sperm. Owen and Katz (1993) showed that sampling above 50HZ for 0.5 seconds reduced errors compared to one second or longer at sampling rates below 30Hz. Suarez (1996) therefore assumed that the three-dimensional swimming patterns generate two-dimensional tracks containing enough information for detecting differences in sperm movement parameters.

For larger sperm, such as rodent sperm, a 3-5mm (depth) chamber is necessary for recording sperm motion (e.g. Wistar rat). The usual glass-slide and coverslip method is adequate for species with smaller sperm e.g. ungulates, primates.

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The influence of frame rate on the estimation of movement parameters such as, curvilinear velocity (VCL) and progressiveness, straightline velocity (VSL) and linearity (LIN) can be dramatic (Sherins, 1991) (for definitions, see table 3-1). There is an increased loss of information and accuracy as the frame rate used for analysis decreases, due to a "corner cutting" effect (Tessler and Olds-Clarke, 1985). Brinders (1994), showed that significant changes in results can occur from 50Hz to 6.25Hz analysis for VCL (353.5µm/s to 180.1µm/s), VSL (96µm/s to 79.1µm/s), LIN (27.3% to

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44.2%) and VAP (164.2µm/s to 89.8µm/s) in Wistar rats. Many studies on sperm motility convincingly showed that a quantitative approach (CASA) is required to be able to differentiate differences in swimming patterns and in particular sperm kinetics.

3.1.3 Effect of season on sperm

All three of the selected species are seasonal breeders. Seasonality impacts on circulating gonadotropins and therefore influences sperm production and maturation. Sperm quality can become affected by changes in season.

Bahga and Khokar (1991) studied the effect of different seasons on sperm quality and the freezability of buffalo sperm and found that after thawing, sperm motility was significantly affected by season. Sagdeo *et al.* (1991) also found that postthaw motility in buffalo was affected by season. Brown *et al.* (1991) found that ejaculate sperm of impala during the non-breeding period was significantly poorer than during the breeding period. It was therefore necessary to confirm this phenomenon for epididymal sperm in the current investigation.

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3.1.4 Specific aims for Chapter 3

The main aim of this investigation was to quantitatively characterise sperm motion of cauda epididymal sperm in a capacitating medium (Ham's F10) by means of computer aided sperm motion analysis (CASA) in impala, blesbok and blue wildebeest. Since the

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time of onset of hyperactivation varies both in individuals of the same species as well as in different species, a time-based study was performed.

The effect of season on quantitative sperm motility of cauda epididymal sperm was determined in impala in an effort to establish a relationship between this work and earlier reports that season affected impala ejaculate sperm quality (Brown *et al.*, 1991).

3.2 Materials and Methods

3.2.1 Collection of material

3.2.1.1. Animals

Animals were collected in areas as described in Chapter 2 (refer to section 2.2.2.1). For this part of the study, six animals belonging to each species were analysed. For the motility studies, 90 to 110 motile sperm were analysed for each animal.

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3.2.1.2. Testis with intact epididymis

After the animals were killed, the testes with intact epididymides were removed in the field and placed in a plastic bag. The bags were then placed in a cooler box with ice, padded with tissue paper, so that the temperature of the specimens could decrease and stabilise at 4°C. Specimens reached the laboratory within three to four hours. The epididymis of impala was processed within three hours after the animal was killed. The

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three hours limit was established through observation and the time that expired from field sampling to the laboratory processing.

3.2.1.3 Preparation of the epididymis before sperm collection

The epididymis was carefully cleaned of all connective tissue, adipose tissue and blood vessels. Blood vessels surrounding the epididymal tubules are extremely thin and a fine iridectomy scissors and forceps were used for removal of capillaries surrounding the epididymis. Blood vessels were removed because blood contains a number of factors, such as antibodies, enzymes and clotting factors that could be harmful to sperm Damianova *et al.*, 1999; Attia *et al.*, 2000).

3.2.1.4 Sperm collection and aspiration

After the epididymis was cleaned, a small incision was made in a tubule of the cauda epididymis. By applying slight pressure behind the incision, condensed epididymal sperm flowed into the surrounding Ham's F10 medium. The sperm was then collected with a pipette and transferred to a petri dish containing culture medium, prewarmed to 35°C, in order to activate sperm.

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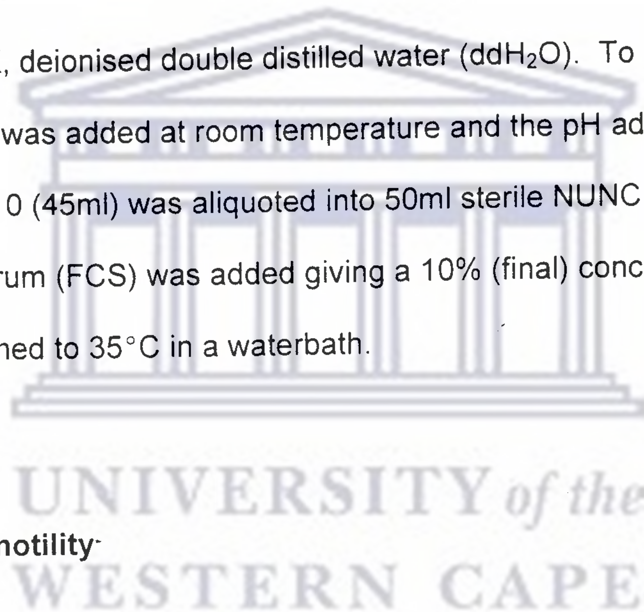
3.2.2 Culture medium

3.2.2.1 Preparation of Ham's F10 Culture medium

This medium was prepared according to the manufacturer's specifications (SIGMA Cat No 6635). In order to prevent wastage, the powdered medium was initially made up with deionised double distilled water to a 10 times concentration. This solution contained no sodium bicarbonate or fetal calf serum. The 10X stock solution was stored for not longer than one week at 4°C. On the day of use, 10ml was diluted by adding 90ml of SABAX, deionised double distilled water (ddH_2O). To this, 0.12g (1.2g/l) of sodium bicarbonate was added at room temperature and the pH adjusted to 7.4 (1N NaOH). The Ham's F10 (45ml) was aliquoted into 50ml sterile NUNC culture bottles to which 5ml fetal calf serum (FCS) was added giving a 10% (final) concentration of FCS. The medium was warmed to 35°C in a waterbath.



3.2.3 Sperm motility

3.2.3.1 Sperm videomicrography

After 15 minutes incubation in Ham's F10, a sample was collected with a 10µl micropipette. The drop was placed onto a 36°C prewarmed glass slide and covered with a prewarmed coverslip. An Olympus-CH30 light microscope, fitted with a 20 times negative phase objective lens and warming stage was used to observe motility. A Panasonic high-resolution black and white CCD videocamera, a videocasette recorder and a digital temperature regulator (set at 35°C, range 34.8°C to 35.2°C) were

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connected to the microscope for sperm motion recording on tape. Sperm motility was recorded on videotape at random for 10 fields at five seconds per field.

3.2.3.2 Computer Aided Sperm Motion Analysis

Twenty microlitres of concentrated aspirated epididymal sperm were placed in 4ml of prewarmed Ham's F10 supplemented with 10% fetal calf serum (FCS). Cauda epididymal sperm were incubated at 35°C in Ham's F10 for periods between one to six hours. Sperm motility was recorded at 15 minutes, 1, 3 and 6 hours. Sperm motility was quantified by means of computer aided sperm motion analysis (CASA). This was done using the Sperm Motility Quantifier (Wirsam Scientific). The S.M.Q calculates twelve motility parameters for each sperm (See table 3-1). For this study, VCL (Curvilinear velocity), VSL (Straight-line velocity), LIN (Linearity), ALH (Amplitude of lateral head displacement) and DNC (Dance) were selected. Although LIN and DNC are derived from other parameters (DNC = VCL X mean ALH, LIN = VCL/VSL), they have a close relationship to the actual motility pattern. VCL, VSL and ALH are independent factors and they have a close relationship to the swimming pattern of sperm. Figure 3-1 graphically represents a hypothetical head movement of a sperm, indicating the sperm motion parameters. All motile sperm in a sample was used for analysis and some of these may be morphologically abnormal but it is not possible to ascertain this. It must therefore be assumed that the motility analysis is representative of the motile sperm population of a particular animal, which include normal and morphologically abnormal sperm. At least 100 motile sperm per sample were analysed.

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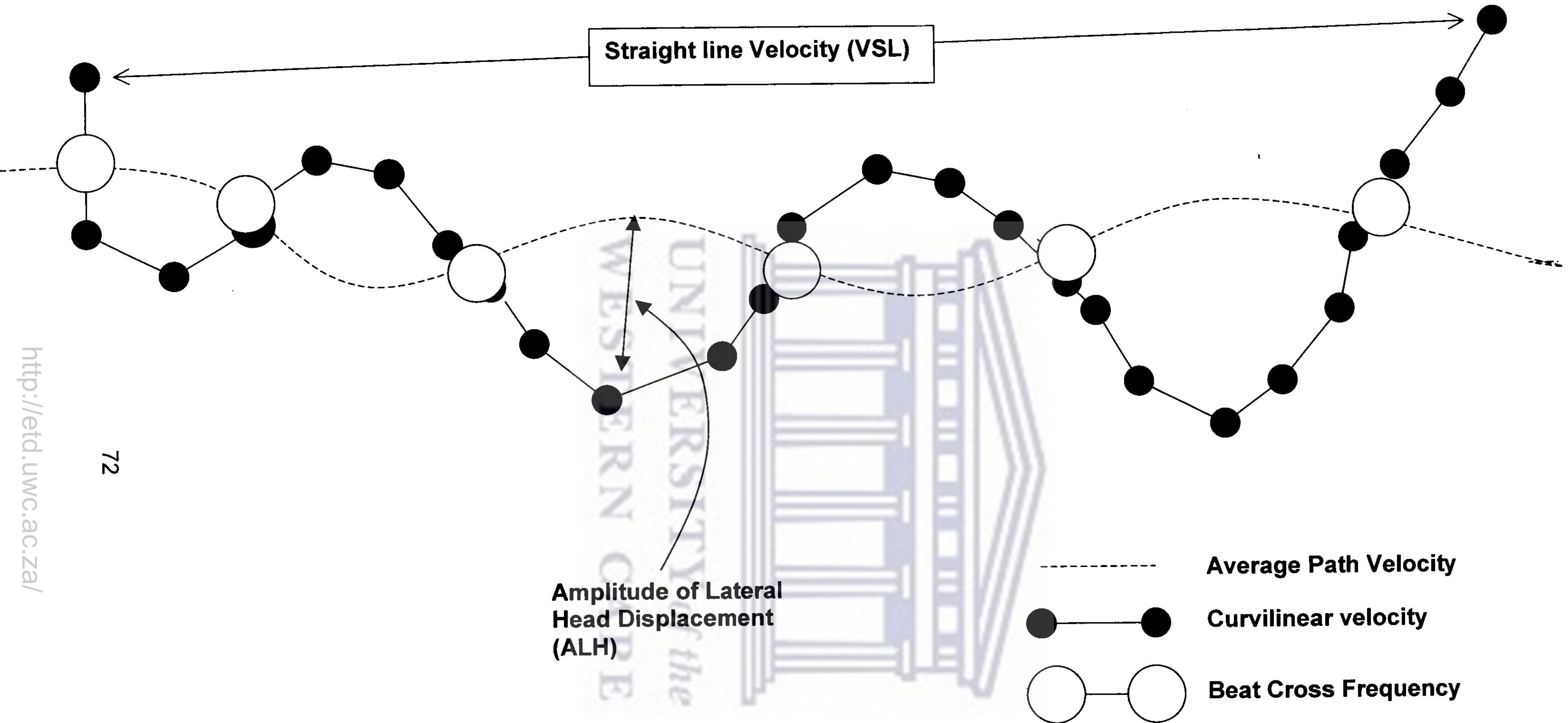

Fig. 3-1: Idealized sperm track indicating some of the motion parameters calculated by the Sperm Motility Quantifier (SMQ) (van der Horst,1995,1999)

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Table 3-1: Definitions, abbreviations and units for all CASA parameters assessed by the SMQ system (Van der Horst *et al.*, 1999).

Parameter	Definition	Abbreviation	Units
Curvilinear velocity	Time-averaged velocity of sperm head along its actual path	VCL	μm/s
Straightline velocity	Time-averaged velocity of sperm head projected along straightline between its first and last detected position	VSL	μm/s
Linearity	Ratio of projected length to to total length of curvilinear trajectory (LIN=VSL/VCL)	LIN	%
Amplitude of lateral head displacement	Maximum amplitude of lateral distances of the sperm head trajectory about its spatial average path	ALH	μm
Dance	Describes sperm motion as space occupied by sperm head path during one second (DNC=VCL X ALH)	DNC	$\mu m^2/s$
Wobble	Expression of the degree of oscillation of the curvilinear path about its spatial average path (WOB=VAP/VCL)	WOB	%
Straightness	Expression of the straightness of average path (STR=VAP/VSL)	STR	%
Average path Velocity	Time-averaged velocity of sperm head projected along its spatial average trajectory	VAP	μm/s
Mean Angular Displacement	Gives information on average angle at which spermatozoa turn when motile and enables detection of circling spermatozoa.	MAD	Radians
Curvature	Reflects progressiveness of movement and ranges between 0 and 1, the smaller the CURV, the straighter the sperm path and higher its progressiveness; also gives information on the mode of movement and a value >0.5 indicates that spermatozoa swim in a circular fashion (CURV=1 – (VSL path/VCL path)	CURV	none

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3.2.3.3 The possible effects of season on sperm motion parameters

Initial studies were performed by the author to compare the sperm motion parameters of animals that were culled during the winter period as opposed to those culled during summer. This initial information suggested that seasonal differences might be evident in sperm motion characteristics.

Blesbok and blue wildebeest material was obtained during the respective breeding seasons. However, impala samples were collected at two different times during 1997. The first (n=4) was collected during the hunting season, which coincides with the winter period, and the second were collected during December 1997 (n=4) (Ferroland Groundtrust). The December period coincided with the rainfall period when males become reproductively active. Males of two years and older were selected for this study. Sperm motility was accordingly compared on a seasonal basis in impala as indicated below.

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3.2.3.4 Time-based study in a defined medium

Sperm become hyperactivated after a variable period in culture medium. The time taken to induce sperm capacitation and hyperactivation is species specific and is dependent on the medium used to induce capacitation. CASA of impala and blue wildebeest cauda epididymal sperm were accordingly studied at 15 minutes, 1,3 and 6 hours and

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blesbok at 1, 3 and 6 hours as outlined under section 3.2.3.2. At least 100 motile sperm were analysed per sample.

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3.3 Results

3.3.1 Sperm Motion Analysis

Table 3-2 shows the motility data of impala, blue wildebeest and blesbok sperm after 15 minutes, one hour, three hours and six hours respectively. Figures 3-2 (a-c), 3-3(a-c), 3-4 (a-c) and 3-5 (a-c) represent data graphically depicting VCL, VSL, VAP, mnALH, mxALH, MAD, LIN, BCF and DNCmn. The above data were statistically analysed by means of Wilcoxon-Mann-Whitney non-parametric analysis, comparing the different parameters by species and time.

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Table 3-2: Motility data for blue wildebeest (BW), impala (IMP) and blesbok (BL) at 15 minutes, 1, 3 and 6 hours incubation at 37°C.

15 Minutes													
	VCL	VSL	LIN	mnALH	MxALH	BCF	DNC	DNCmn	VAP	WOB	STR	MAD	CURV
IMP	239.18	142.02	62.25	4.15	8.90	52.13	2317.05	40.10	163.86	0.72	0.85	1.85	0.54
±SEM	22.03	13.72	4.67	0.52	0.98	4.59	447.57	10.10	12.61	0.04	0.03	0.16	0.03
BW	304.84	156.96	52.97	5.88	12.48	53.31	3888.49	89.18	187.49	0.63	0.81	1.94	0.50
±SEM	19.13	17.34	4.48	0.42	0.92	4.99	426.30	26.88	13.30	0.02	0.05	0.07	0.03
1 Hour													
	VCL	VSL	LIN	mnALH	MxALH	BCF	DNC	DNCmn	VAP	WOB	STR	MAD	CURV
BW	318.71	169.60	55.28	5.83	12.42	57.22	4014.05	72.49	201.17	0.65	0.83	1.90	0.50
±SEM	13.65	15.42	4.40	0.34	0.73	3.79	321.55	17.13	11.11	0.03	0.04	0.08	0.02
IM	226.52	152.54	69.01	3.46	7.43	55.59	1823.64	26.58	172.64	0.78	0.87	1.79	0.50
±SEM	24.06	14.95	3.34	0.40	0.82	3.21	355.32	6.10	15.55	0.02	0.02	0.12	0.03
BL	282.91	257.46	90.62	3.14	6.67	60.18	1823.57	14.47	265.27	0.94	0.97	0.86	0.29
±SEM	4.08	3.14	0.72	0.10	0.20	1.85	80.15	0.71	3.76	0.01	0.00	0.06	0.01
3 hour													
	VCL	VSL	LIN	mnALH	MxALH	BCF	DNC	DNCmn	VAP	WOB	STR	MAD	CURV
IMP	174.46	121.50	71.82	2.79	6.28	41.83	1214.87	29.22	135.76	0.81	0.88	1.73	0.45
±SEM	16.91	10.69	2.50	0.37	0.73	3.79	234.01	11.13	10.57	0.03	0.02	0.12	0.02
BW	303.56	165.08	58.28	5.53	11.84	54.66	3707.96	69.13	200.28	0.69	0.82	1.84	0.51
±SEM	19.40	13.39	5.99	0.57	1.04	2.55	543.91	15.27	8.43	0.05	0.04	0.17	0.04
BL	265.70	214.66	82.44	3.70	7.73	53.90	2202.02	43.09	229.07	0.87	0.93	1.14	0.33
±SEM	14.81	14.20	2.71	0.32	0.54	2.02	298.71	19.44	13.14	0.02	0.02	0.10	0.02
6 hour													
	VCL	VSL	LIN	mnALH	MxALH	BCF	DNC	DNCmn	VAP	WOB	STR	MAD	CURV
IM	149.32	103.09	71.04	2.46	5.73	37.98	934.65	23.19	113.85	0.79	0.89	1.76	0.44
±SEM	19.14	14.06	2.97	0.38	0.62	5.64	202.41	9.32	14.17	0.02	0.02	0.11	0.03
BW	251.46	150.42	64.30	4.49	9.72	47.49	2696.00	59.44	181.79	0.76	0.82	1.68	0.48
±SEM	27.97	18.83	7.64	0.86	1.79	4.17	654.08	24.77	17.52	0.06	0.05	0.11	0.04
BL	294.67	167.51	62.67	5.75	11.30	48.59	3843.72	106.79	203.49	0.73	0.80	1.72	0.48
±SEM	29.30	18.39	11.13	1.28	2.14	1.18	1251.56	59.66	4.64	0.07	0.09	0.19	0.01

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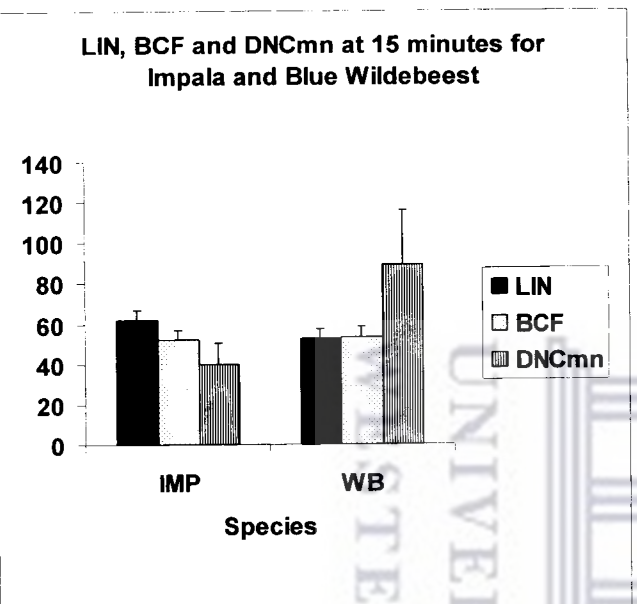

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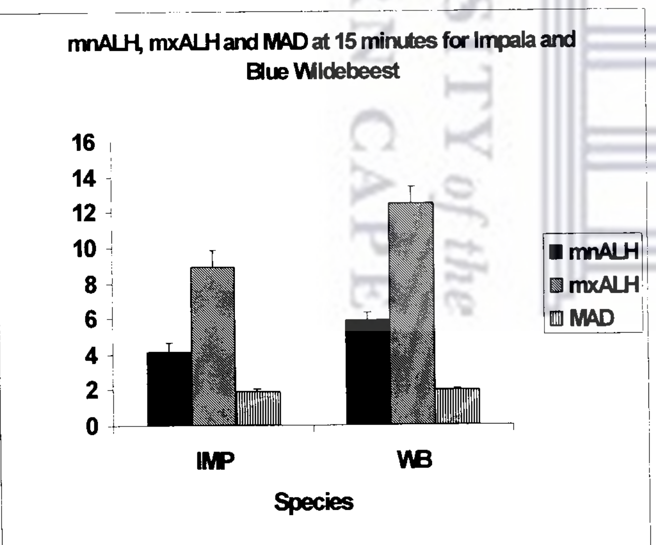

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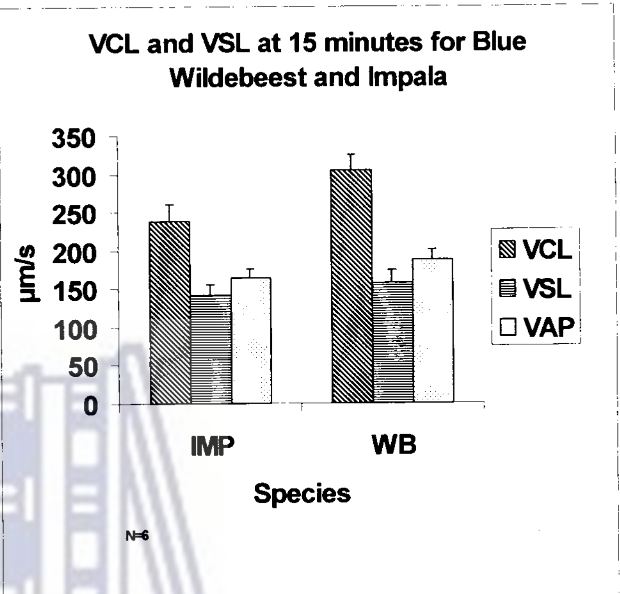

(c)

Fig. 3-2 (a): Histograms comparing blue wildebeest and impala at 15 minutes for LIN, BCF, DNCmn. Significant differences ($p<0.05$) were evident for LIN between the two species, for BCF between the two species and DNCmn between the two species.
Fig. 3-2 (b) Histograms comparing blue wildebeest and impala at 15 minutes for mnALH, mxALH and MAD. Significant differences ($p<0.05$) were evident for mnALH between the two species, for mxALH between the two species and MAD between the two species.
Fig. 3-2 (c) Histograms comparing blue wildebeest and impala at 15 minutes for VCL, VSL and VAP. Significant differences ($p<0.05$) were evident for VCL between the two species, for VSL between the two species and for VAP between the two species.

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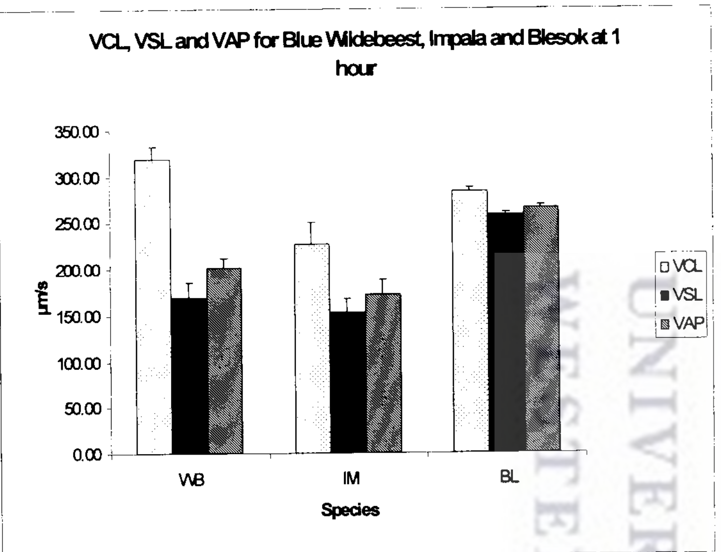

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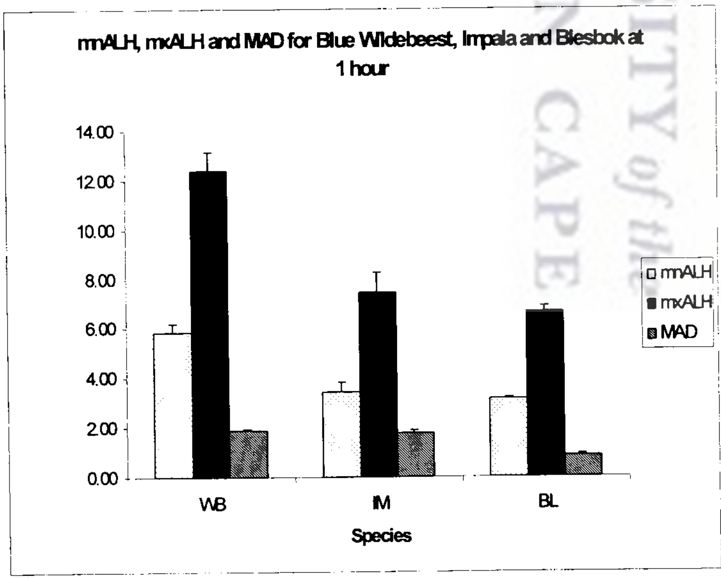

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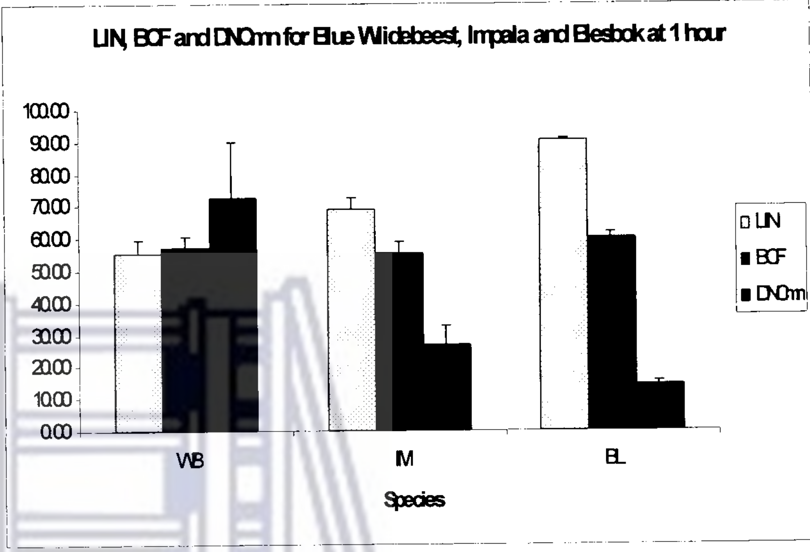

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Fig. 3-3 (a): Histograms comparing blue wildebeest, impala and blesbok at 1 hour for VCL, VSL, and VAP. Significant differences ($p<0.05$) were evident for VCL among the three species, VSL among the three species and VAP among the three species.
Fig. 3-3 (b): Histograms comparing blue Wildebeest, impala and blesbok at 1 hour for mnALH, mxALH and RAD. mxALH for Im vs Bl ($p=0.136$), mnALH (WB vs Bl, $p<0.001$), mnALH (WB vs IM, $p=0.123$). MAD was significantly different among the three species.
Fig. 3-3 (c) Histograms comparing blue wildebeest, impala and blesbok at 1 hour for LIN, BCF and DNCmn. Significant differences ($p<0.05$) were evident for LIN among the three species, BCF among the three species and DNCmn among the three species.

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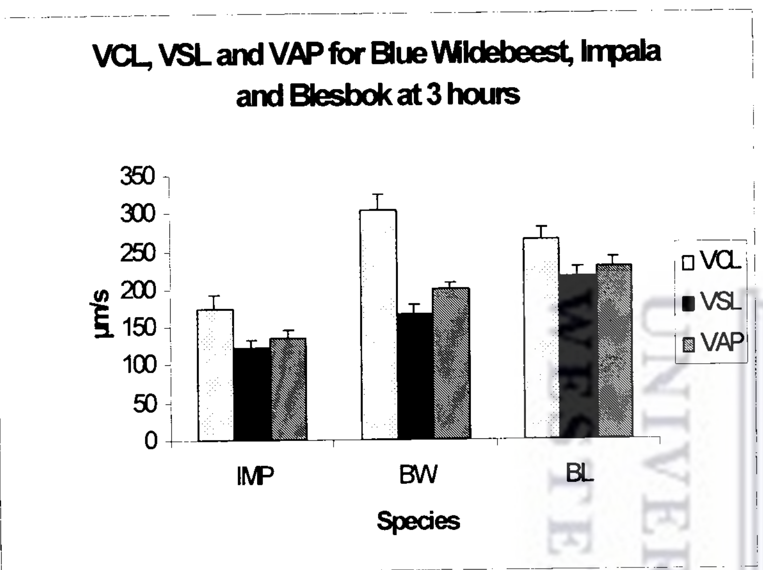

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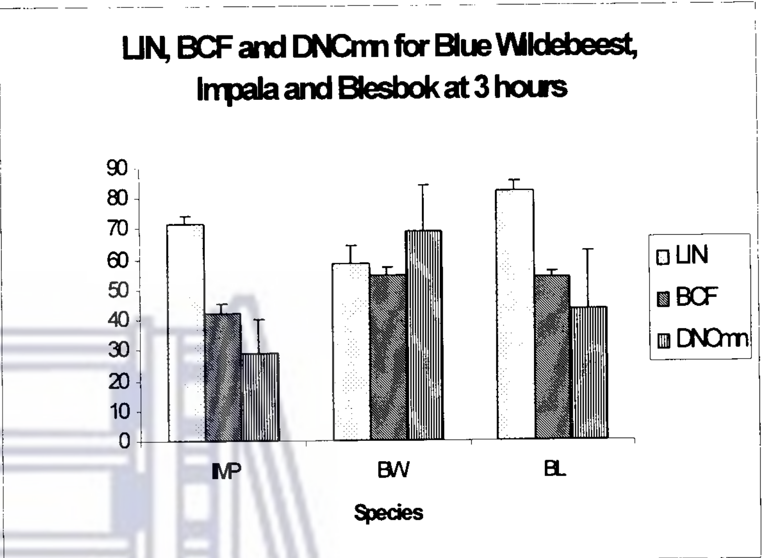

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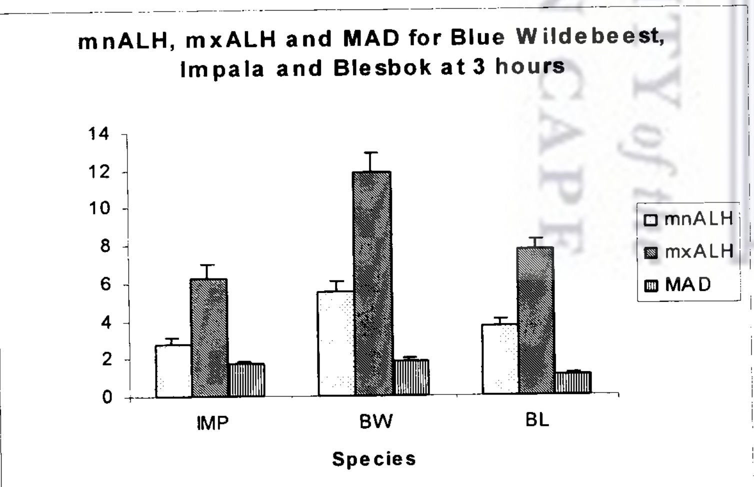

(b)

Fig. 3-4 (a): Histograms comparing blue wildebeest (BW), impala (IM) and blesbok (BL) at three hours for VCL, VSL and VAP. Significant differences ($p<0.05$) were evident for VCL among the three species, for VSL among the three species and for VAP among the three species.
Fig. 3-4 (b): Histograms comparing blue wildebeest, impala and blesbok at three hours for mnALH, mxALH and MAD. Significant differences ($p<0.05$) were evident for mnALH among the three species and for mxALH among the three species.
Fig. 3-4 (c): Histograms comparing blue wildebeest, impala and blesbok at three hours for LIN, BCF and DNCmn. Significant differences ($p<0.05$) were evident for LIN among the three species and for DNCmn among the three species. BCF for BL was not significantly different from BW ($p=0.7637$).

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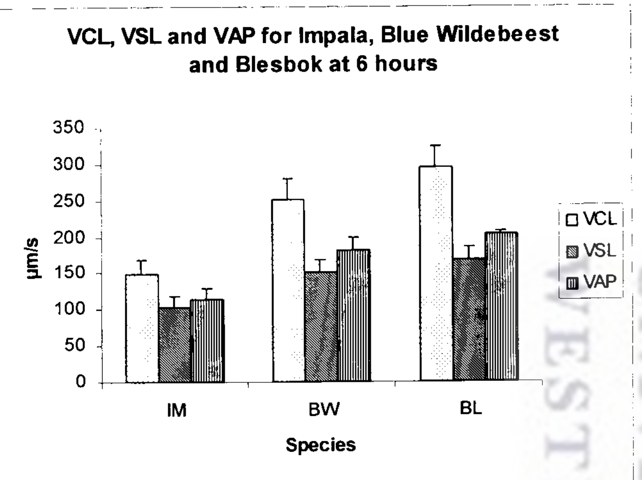

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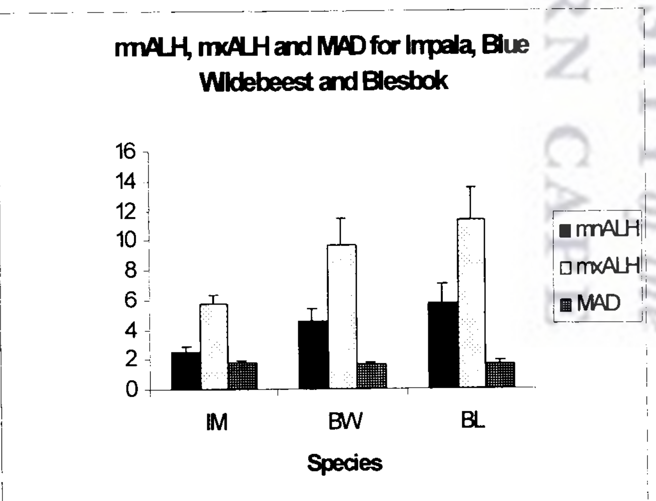

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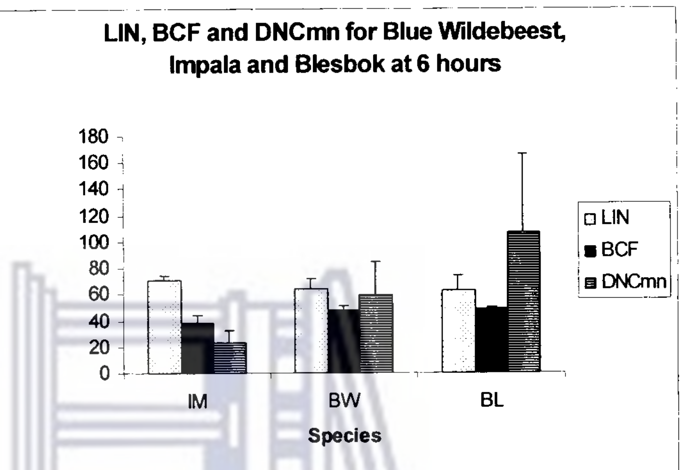

(c)

Fig. 3-5 (a): Histograms comparing blue wildebeest (BW), impala (IM) and blesbok (BL) at six hours for VCL, VSL and VAP. Significant differences ($p<0.05$) were evident for VCL among the three species and for VAP among the three species. VSL of IMP differed significantly from BW and BL ($p<0.05$) but VSL of BW and BL did not differ significantly ($p=0.1959$).
Fig. 3-5 (b): Histograms comparing Blue Wildebeest, Impala and Blesbok at six hours for mnALH, mxALH and MAD. Significant differences ($p<0.05$) were evident for mnALH among the three species and for mxALH among the three species.
MAD was not significantly different ($p>0.05$) among the three species.
Fig.3-5 (c): Histograms comparing blue wildebeest, impala and blesbok at six hours for LIN, BCF and DNCmn.
DNCmn was significantly different among the three species. BCF was not significantly ($p>0.05$) different among the three species and LIN was significantly different for IM vs BW, $p<0.05$; IM vs BL, $p<0.05$ but not BW vs BL, $p>0.05$.

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3.3.2 Time-based motility analysis

Only blue wildebeest and impala sperm motility were recorded at 15 minutes. Each kinematic parameter respectively differed significantly ($p < 0.05$) at 15 minutes when blue wildebeest was compared to impala. Figures 3-6, 3-7 and 3-8 are line graphs (legend on page 84) for the motility data obtained in blue wildebeest, blesbok and impala respectively. Data were compared by means of ANOVA (F-Test) and non-parametric Wilcoxon-Mann-Whitney analysis. Significance was determined at a 95% confidence level.

3.3.3. Impala – Seasonal data

Motility data presented in Table 3-3 was taken after 15 minutes. Data were statistically analysed by means of analysis of variance (F-Test) and compared by means of Wilcoxon-Mann-Whitney non-parametric methods.

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Figures 3-9 (a), (b), (c) and (d) show histograms for VCL, VSL, LIN, BCF and mnALH. Except for VCL, all other motion parameters differed significantly and respectively based on season ($p<0.05$). During the rainy season VSL, LIN, BCF, and VAP were significantly higher than during the winter months, but mnALH was significantly lower during the rainy season.

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Blue Wildebeest

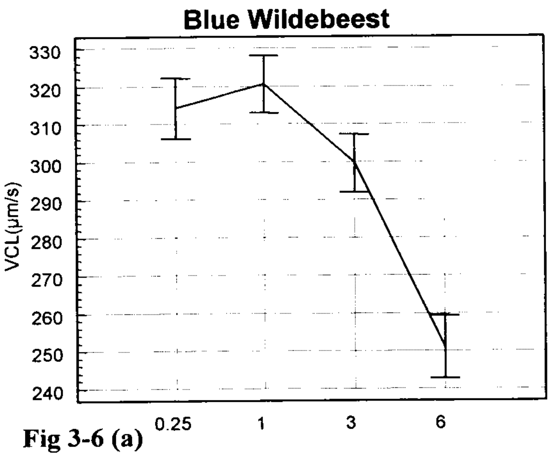
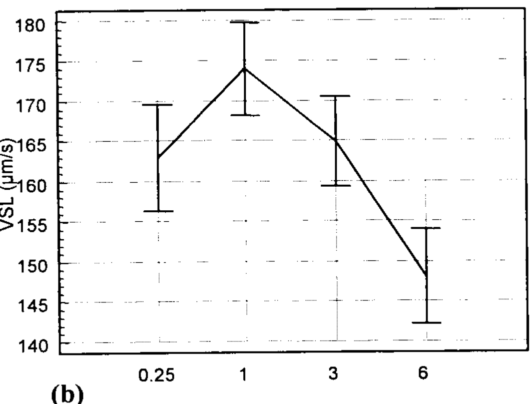


Fig 3-6 (a)



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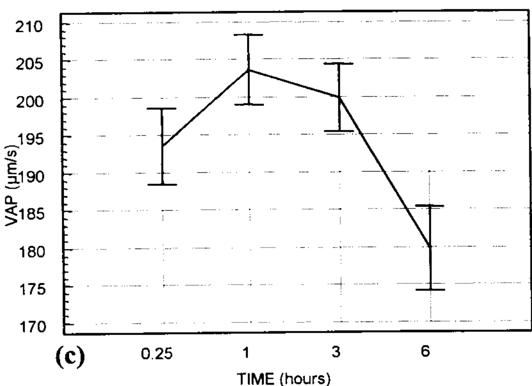


(c)

Blesbok

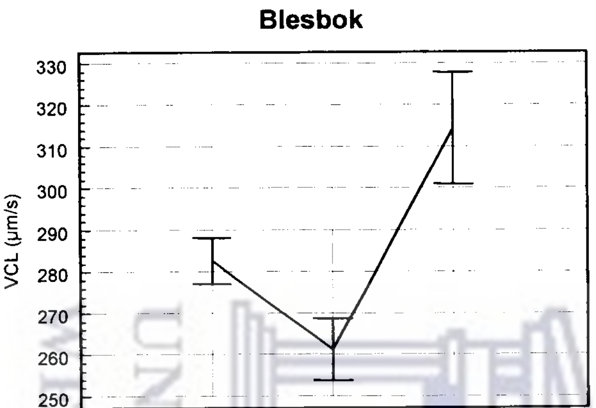
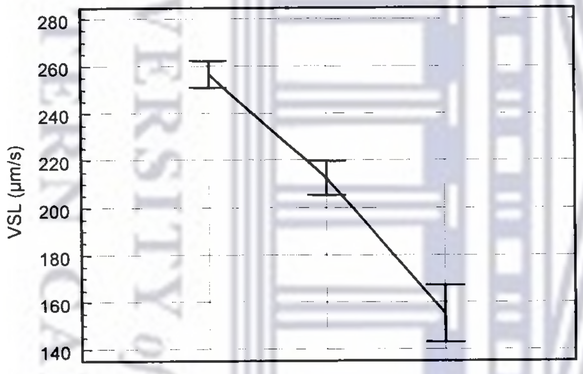


Fig 3-7 (a)



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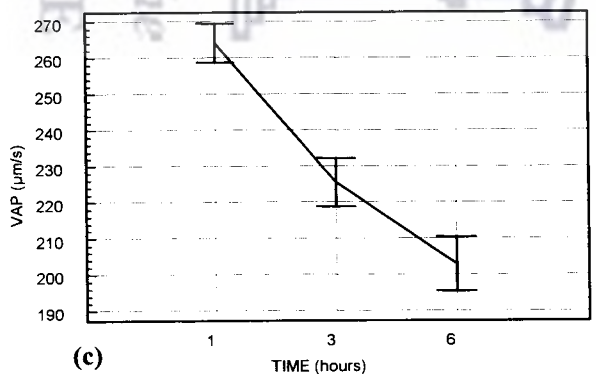
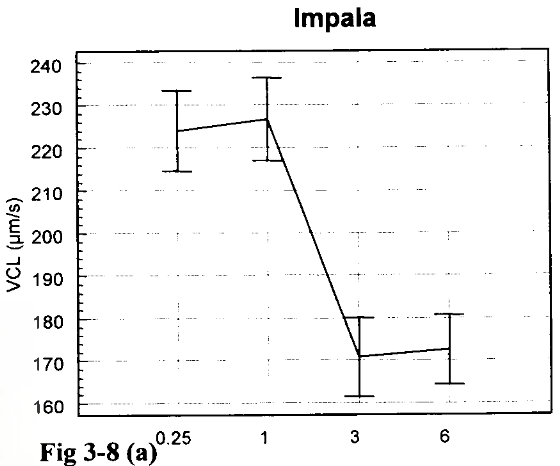


(c)

Impala



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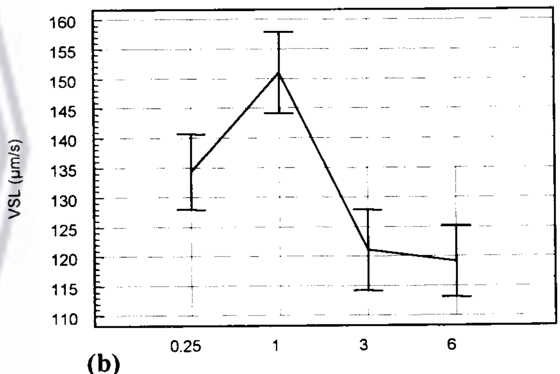

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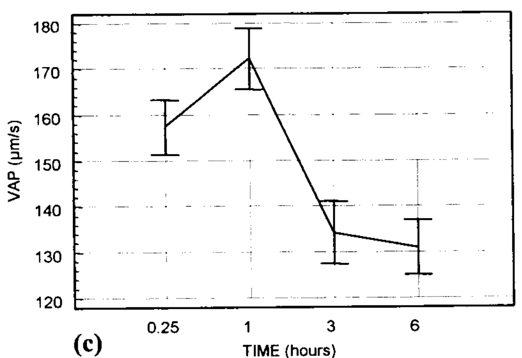

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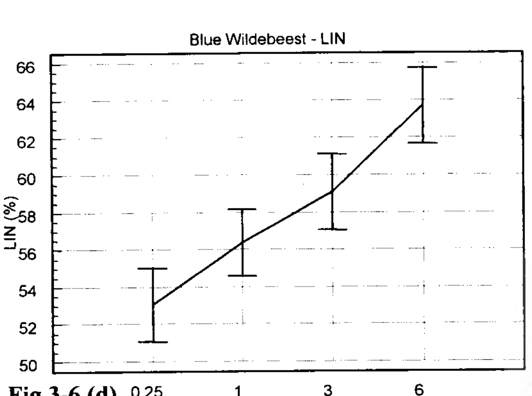

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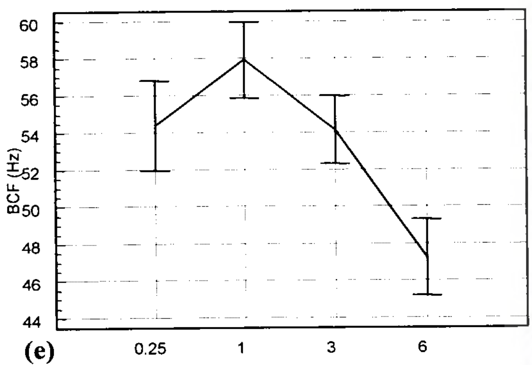

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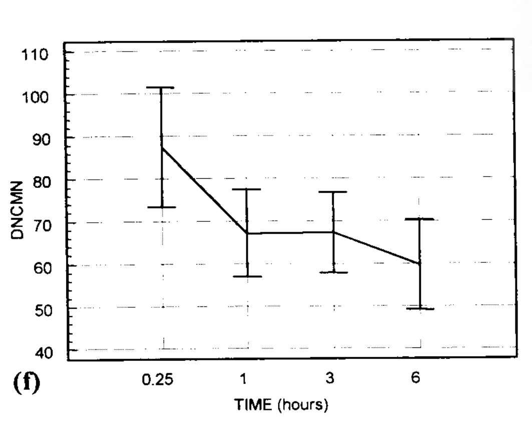

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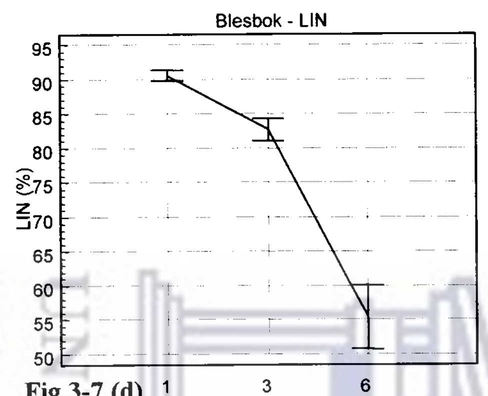

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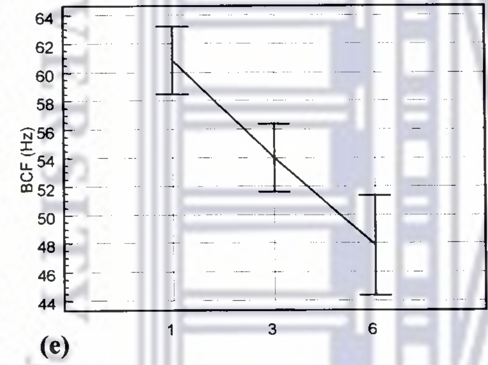

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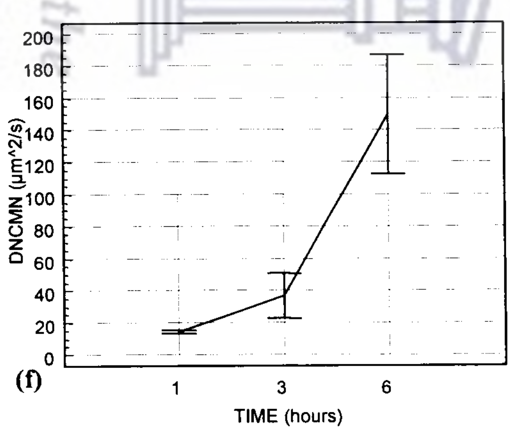

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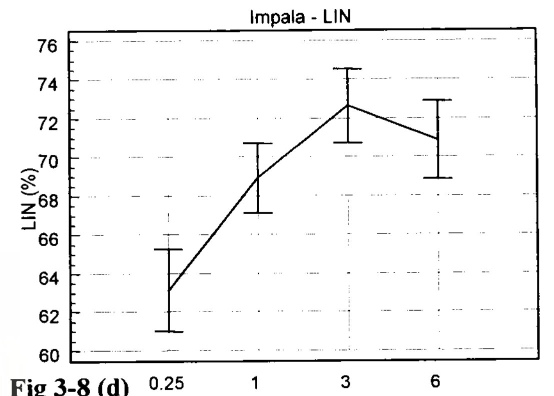

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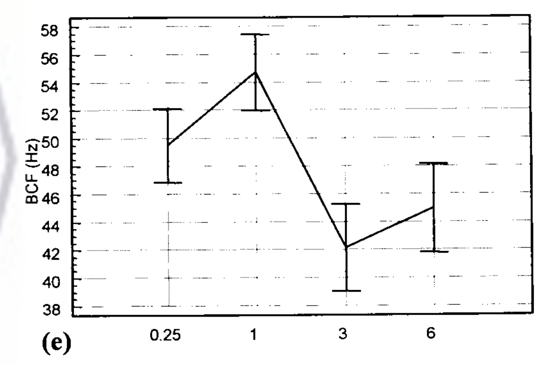

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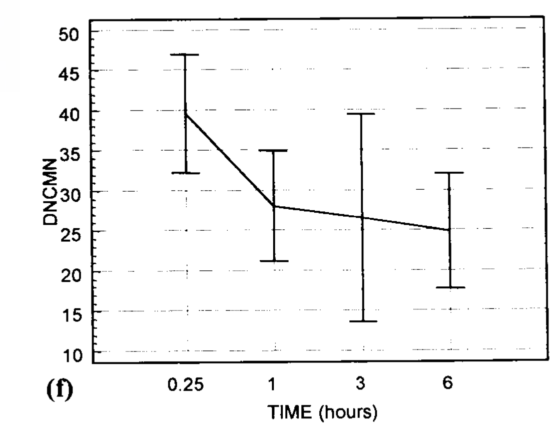

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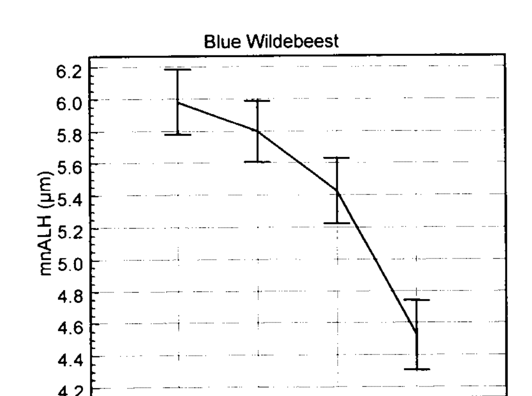

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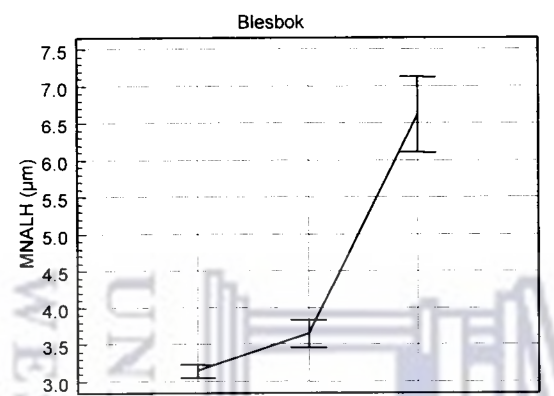
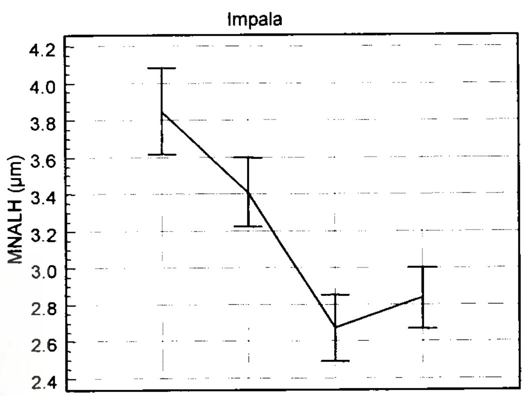


Fig 3-7 (g)



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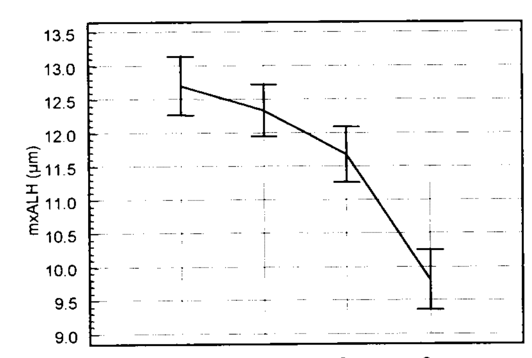

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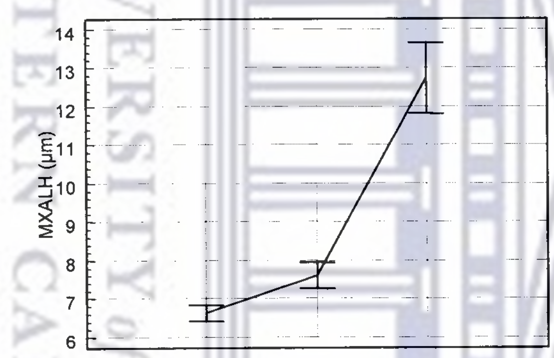

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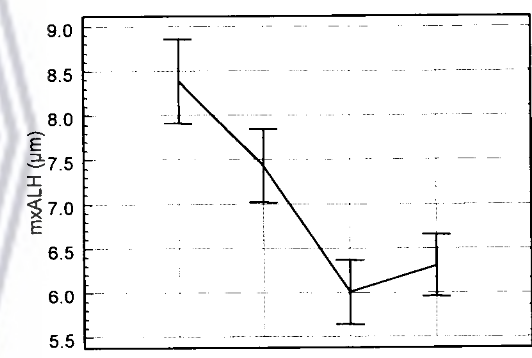

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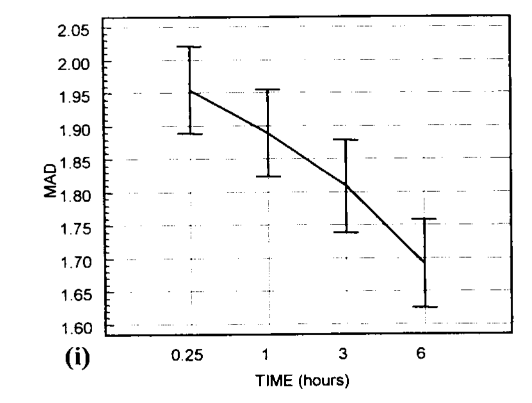
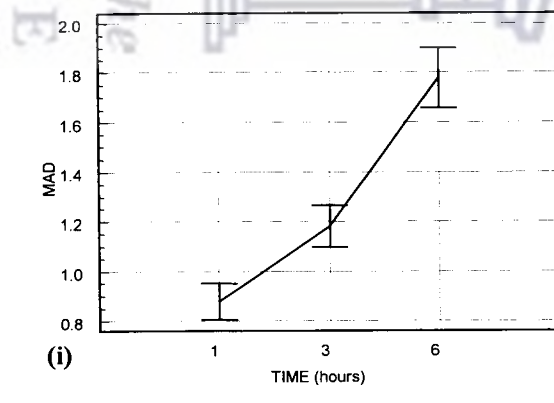


(i)



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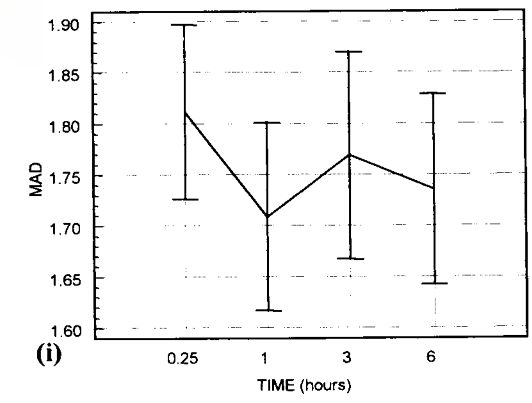

(i)

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Legend (Figures 3-6, 3-7 and 3-8)
Figures 3-6 (a-i), 3-7 (a-i) and 3-8 (a-i) depict line graphs of selected sperm motility parameters after a six hour incubation period in Ham's F10 supplemented with 10% fetal calf serum for blue wildebeest, blesbok and impala. Each point represents the mean ± 2SEM for 90 to 110 sperm of each species (n=6). Sperm were manually analysed with the aid of the Sperm Motility Quantifier (SMQ) to show species differences in sperm swimming patterns.

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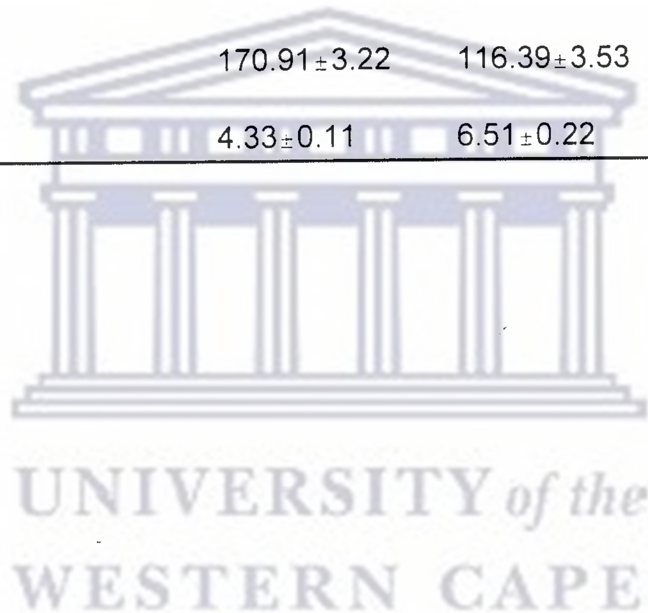
Table 3-3: Shows data (mean ± SEM) of selected sperm motion parameters of impala that were killed during the winter period (Group 2) and the rainy season (Group 1).

Calculated Motion Parameter (CASA)	Group 1 (n=4)	Group 2 (n=4)	Significance (p value)
VCL(µm/s)	253.25 ±5.44	251.0±7.97	> 0.05
VSL(µm/s)	146.35±3.46	62.44±2.8	< 0.05
LIN(%)	60.28±0.95	29.77±1.2	< 0.05
BCF(Hz)	53.70±1.38	29.02±1.54	< 0.05
VAP(µm/s)	170.91±3.22	116.39±3.53	< 0.05
mnALH(µm)	4.33±0.11	6.51±0.22	< 0.05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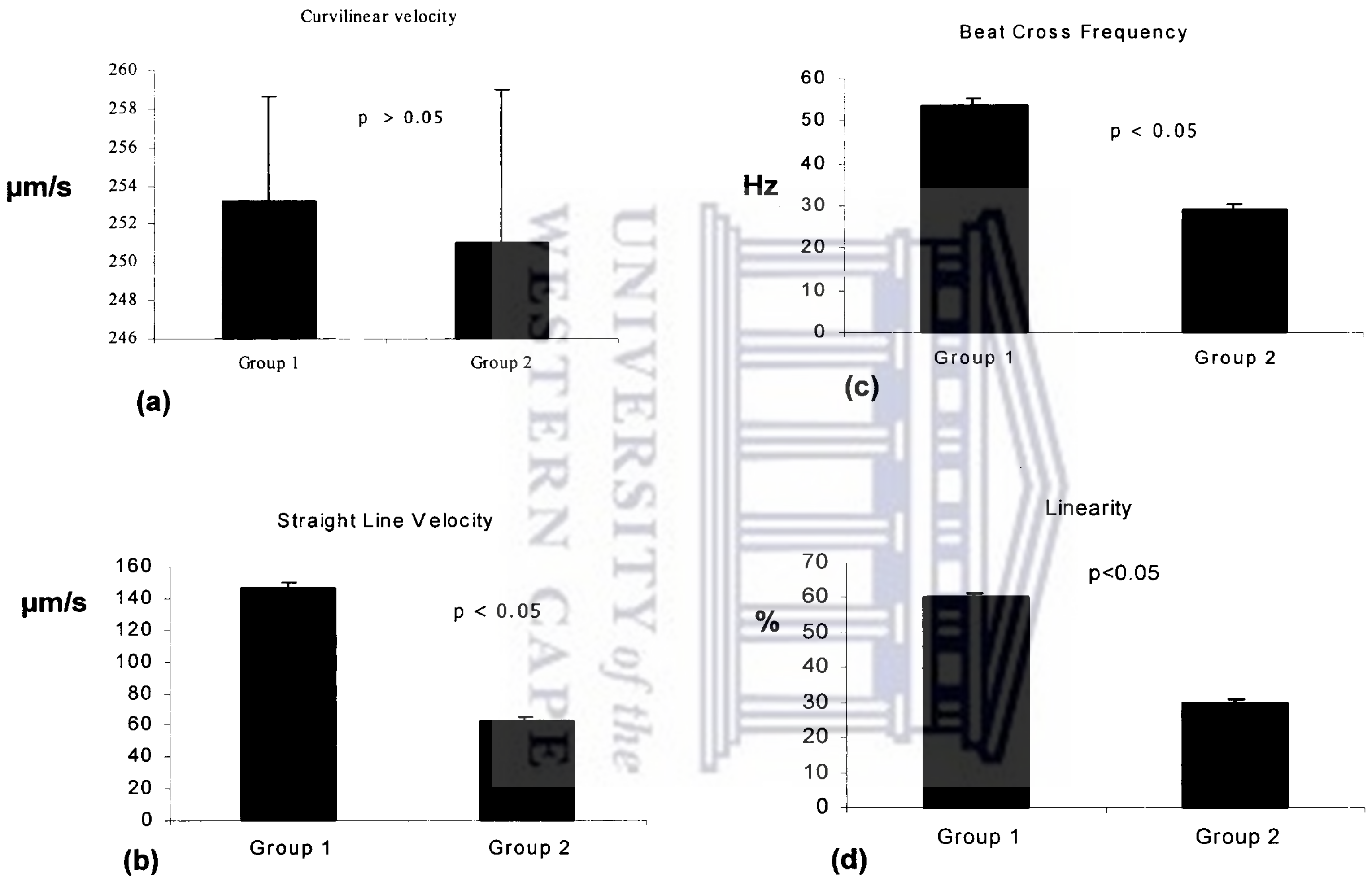

Fig. 3-9: Shows motility data of impala cauda epididymal sperm analyzed during the summer rainfall (group 1) and the winter, dry season (group 2). (a) Shows VCL for the two groups but with no significant differences between the groups. However, (b), (c) and (d) show that VSL, BCF and LIN respectively differed significantly between group1 and group2 ($p<0.05$).

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3.4 Discussion

3.4.1 Sperm Motility – Differences among species

Species specific differences were evident when sperm motility of the three species of antelope were compared at all time intervals. Clear visual differences in swimming patterns were observed. Blue wildebeest had a greater percentage of motile sperm upon dilution after it had been kept on ice for longer than three hours when compared to the other two species.

The most prevalent finding was that all motion parameters for blue wildebeest and impala follow the same trend over time. There was a decrease in the values of all the motion parameters for blue wildebeest and impala over time except for linearity (LIN) which increased from approximately 53% to over 63%. However, in blesbok, VCL, LIN, DNCMN, ALH and MAD show exactly the opposite trend (e.g. when VCL declined over time in blue wildebeest and impala it increased in blesbok).

After 15 minutes, blue wildebeest sperm displayed a significantly higher VCL, VSL, mnALH, mxALH, DNCmn and VAP than impala sperm. However, LIN was higher ($p<0.05$) in impala. At one hour of incubation in culture medium, the sperm of blue wildebeest had the highest ($p<0.05$) mnALH, mxALH, DNCmn and MAD whereas blesbok sperm had the highest VSL, LIN and therefore the lowest MAD of all the animals in the one hour incubation group.

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One of the most prominent kinematic features observed was the high linearity seen in blesbok [90.62±0.72% *vs* 55.28±4.40%(impala), 69.01±3.34% (blue wildebeest)]. A possible contributing factor to the high linearity observed could have been due to the smaller head dimension of the sperm of blesbok (5.59µmX3.9µm) compared to the sperm of impala (6.64µmX4.12µm) and blue wildebeest (6.11µmX4.11µm).

3.4.2 Time-based differences in sperm motion parameters

From Figures 3-6(a-i), 3-7(a-i) and 3-8 (a-i), sperm motility changes were shown to be species specific. The sperm of blue wildebeest showed a decline in VCL, VSL, BCF and DNCmn with a linear increase in LIN. VCL, VSL, BCF and VAP, showed a turning point at one hour indicating that maximum velocity was reached after one hour. For blesbok, data was only recorded at 1, 3 and 6 hours and motility behaviour was significantly different from that seen in blue wildebeest and impala sperm. For blesbok sperm motion parameters, there was an initial drop in VCL (282µm/s to 265µm/s, p=0.0001) from 1 hour to 3 hours with an increase in VCL from 265µm/s to 294µm/s (p=0.0001) after 6 hours. In addition, there was a decrease in VSL, VAP, LIN, and BCF with an increase in mnALH, mxALH and MAD over 6 hours. The sperm from impala had a similar profile to that observed in blue wildebeest with a peak VCL, VSL, VAP and BCF reached after 1 hour in incubation medium. When comparing Figures 3-6, 3-7 and 3-8, it appears that blue wildebeest and impala sperm display a similar type of behaviour over six hours of incubation for all parameters.

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There is an initial progressive type of motility when cauda epididymal sperm motility is initiated (Suarez, 1988, Suarez, 1996; Suarez *et al.*, 1991; Suarez & Osman 1987). Sperm swimming pattern is then transformed from a linear, progressive type of swimming pattern into a more non-linear pattern associated with an increased lateral amplitude of head displacement and increased curvilinear velocity or VCL. This type of motility pattern has been described as transitional before changing over into a very erratic, vigorous and non-linear type of motion referred to as hyperactivation. From the data presented, it appears that blesbok sperm show signs of hyperactivation, evidenced by an increase in VCL, DNCmn and a decrease in VSL, VAP, LIN and BCF. It is difficult to explain the reverse to have occurred in the sperm of impala and blue wildebeest. There could have been structural and biochemical damage in the latter two species causing a decrease in sperm velocity and some of the components in Ham's F10 are known to prevent hyperactivation. There may accordingly be a differential effect exerted by Ham's F10 medium on the sperm of the species under investigation.

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Generation of reactive oxygen species (ROS) and peroxidation of the sperm membrane can adversely affect sperm motility, and sperm – oocyte fusion and cause sperm midpiece abnormalities. According to Kim & Parthasarathy (1998), ROS also triggers sperm hyperactivation and supports sperm capacitation. Impala sperm showed increased sperm agglutination, which might indicate sperm membrane damage/false acrosome reaction and manifests as a pseudo-capacitated state.

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Hyperactivation, in species e.g. the hamster (Cooper, 1984) and human (Aitken *et al.*, 1986) can be induced by stimulants like caffeine and 2-deoxyadenosine. Ham's F10 contains anti-hyperactivation components i.e. glucose and aromatic amino acids, tryptophan (0.0006g/L), tyrosine (0.00261g/L) and phenylalanine (0.00496g/L). Phenylalanine has been implicated in free radical formation, causing damage to sperm and cell cultures (Lapointe *et al.,* 1998).

3.4.3. Seasonal variation in Impala

The results for impala indicate significant differences between the two groups (ie winter and summer – Table 3-3) for parameters VSL, LIN, BCF, VAP and mnALH, but not for VCL. Sperm collected during the rainy season had a straight progressive swimming pattern compared to the winter period when sperm exhibited larger head oscillations and accordingly a lower linearity or a poorer progressive motility.

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Based on number of semen collections for each month, Gross (1991) found that season and time of collection could affect the semen quality in gaur (*Bos gaurus*). Their results indicated a decline in the number of semen collections that met the minimum pre-freeze criteria of >50% motility and >3.0 progressive status. Brown *et al.* (1991), studied the seasonal variation in pituitary-gonadal function in free ranging impala. Anaesthetized animals were treated, intravenously, with saline, gonadotropin-releasing hormone (GnRH) and human chorionic gonadotropin (hCG) in order to determine testosterone production from animals collected during the breeding and non-breeding season. They

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found that testosterone production and secretion, stimulated by GnRH and hCG, were approximately nine times higher during the rut than during the non-breeding season. The seasonal increase in testosterone production was associated with a doubling in testicular volume and concentrations of Luteinizing hormone (LH) receptors.

In their study Brown *et al.* (1991), demonstrated that the seminal characteristics of the impala presented differences between male impala during the breeding and the non-breeding season. Sperm concentration and the percentage sperm motility were significantly reduced and the proportions of sperm abnormalities were significantly greater in ejaculates from impala in the non-breeding season compared to impala in the breeding season (Brown *et al.*, 1991). Furthermore, Brown *et al.* (1991) made similar observations for ejaculate sperm of breeding and non breeding buffalo, and Nunes *et al.* (1982) reported similar findings in the goat. Despite the small sample size used in the current investigation, the results obtained are therefore supported by those found by Brown *et al.* (1991) in the impala and in other species as indicated above.

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3.5 Conclusion

The evaluation of sperm motility by means of CASA, was a sensitive method to discriminate among the three antelope species, and between time based incubation of sperm as well as sperm collected during different seasons (impala).

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Species specificity and time based studies

Various parameters that relate to sperm head movement have been shown to highlight differences and similarities among sperm from the different species. As stated earlier, sperm motion parameters change over time as the sperm are allowed to go through different physiological and biophysical changes. These changes could clearly be seen for the different parameters over time for the three species. The data also suggest that important changes like hyperactivation might be present in species like the blesbok as indicated by the increasing curvilinear velocity or VCL and the decrease in linearity (LIN). An increase in the mean amplitude of lateral head displacement (mxALH) and an increase in dancemean (DNCmn) further support the occurrence of hyperactivation in blesbok. In comparison to blesbok, the other two species, blue wildebeest and impala, showed the reverse in terms of most of these motion parameters.

Seasonality

Although sample size was small, clear differences in sperm motion parameters were observed between the two groups collected during different seasons (impala). The finding of this study is supported by research performed on impala during 1991 in the Kruger National Park (Brown *et al.*, 1991). Sperm curvilinear velocity was not significantly affected, but VSL, LIN, BCF, VAP and mnALH elucidated seasonal differences quantitatively in impala. This study is the first CASA investigation on African wild antelope species and should be considered in future applications such as sperm cryopreservation studies.

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Chapter 4

The effects of cauda epididymal sperm cold treatment on sperm motion parameters in two antelope species and the effects of cryopreservation on blue wildebeest sperm.

4.1 Introduction

Cryoprotectants are essential for the survival of cells during precooling (equilibrating) and cryopreservation (Pontbriand *et al.*, 1989; Check *et al.*, 1991; Dhami *et al.*, 1992). The most commonly used cryoprotectant is glycerol. The exact mechanism by which glycerol protects cells from freeze-thaw damage is not completely understood, but it has been shown to modify the crystal formation in the medium by preventing the electrolyte concentration from rising above a harmful level (Kumar *et al.*, 1992; Watson, 1995)

4.1.1 Plasma membranes

Plasma membranes are most affected during freezing and are complex structures when considering recent concepts of lipid distribution, lipid asymmetry, lipid to lipid and lipid to protein interaction (Aloia, 1988). These complex

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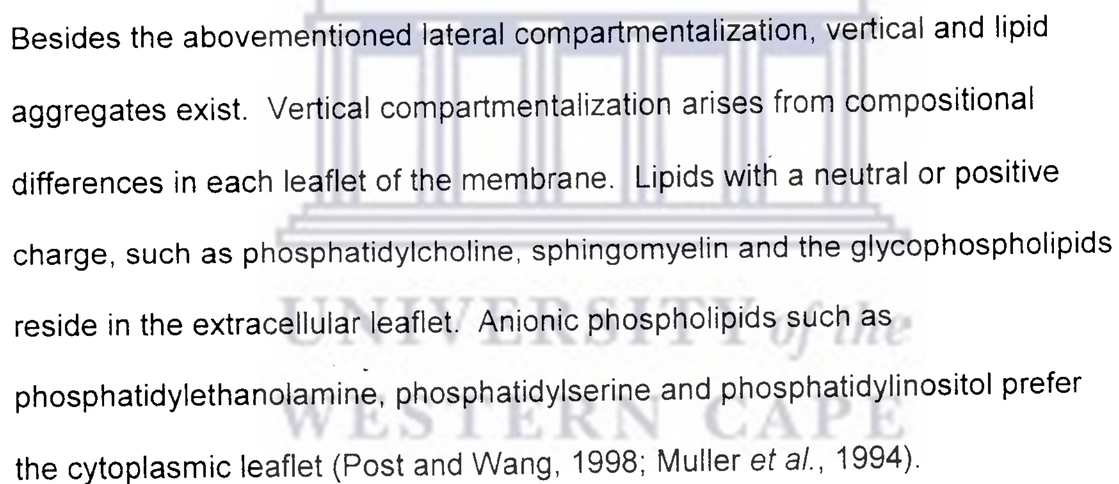
interactions are the basis for ordered domains with the plane of the plasma membrane, resulting in compartmentalization of the plasma membrane (Jain and Arora, 1988). Such compartmentalization is observed in the plasma membrane e.g. with the differences in the distribution of both lipids and proteins between the periacrosomal region of the sperm head, the midpiece and the principle piece of the flagellum. Maintenance of these domains in the sperm cell is critical to overall sperm function as each plays a very specific role during fertilization (Jain and Arora, 1988).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Besides the abovementioned lateral compartmentalization, vertical and lipid aggregates exist. Vertical compartmentalization arises from compositional differences in each leaflet of the membrane. Lipids with a neutral or positive charge, such as phosphatidylcholine, sphingomyelin and the glycophospholipids reside in the extracellular leaflet. Anionic phospholipids such as phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol prefer the cytoplasmic leaflet (Post and Wang, 1998; Muller *et al.*, 1994).

Lipid aggregates exist due to various phases in which lipid species find themselves (Parks and Graham, 1992). The bilayer form dominates biological membranes, but a hexagonal phase may occur. Most lipids, which prefer the hexagonal arrangement, are termed non-bilayer lipids. These non-bilayer lipids are involved in forming an annular ring around integral membrane proteins, creating a tight seal at the protein-lipid transition areas in the membrane.

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Lipids generally exhibit freedom for lateral motion as long as the temperature is above a critical value (Parks and Graham, 1992). Reorganization of the bilayer is possible when the membrane is cooled. Cooling of the membrane causes it to change from a liquid crystal to a gel phase. During the transition of liquid-crystalline to gel phase, non-bilayer lipids exhibit a higher phase transition than bilayer lipids. The result is that, during cooling of sperm membranes, non-bilayer lipids could first undergo phase transition and aggregate into gel microdomains leaving bilayer lipids in a liquid crystalline phase with proteins. This arrangement, according to Parks and Graham (1992), is not likely to affect the membrane during cooling, but upon rewarming the non-bilayer lipid may no longer be returned to the bilayer configuration. The resulting regions of hexagonal aggregates could therefore destabilize the membrane. In addition, altered lipid-protein interaction could lead to altered protein function.

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4.1.2 Sperm Freezing

Mortimer (1994) described cryopreservation as a branch of cryobiology concerned with the suspension of life during storage in the frozen state at ultra low temperatures. Much work regarding semen and sperm cryopreservation has been done in various mammalian species such as bovine (Thomas *et al.*, 1998; Beorlegui *et al.*, 1995); the ram (Fiser and Fairfull 1989; Fiser *et al.*, 1987); human (Mahadevan and Trounson, 1983), and boar (Osinowo and Salamon, 1976a, b). Cryopreservation methods have been well established in the past because of the need to store sperm of genetically superior sires especially in

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terms of animal breeding programs. From sperm morphological data, it appears that the species under investigation have sperm that typically resemble that seen in bovine in terms of general morphology (Chapter 2) and it was assumed that bovine sperm freezing protocols could be employed in this investigation.

In this study, cauda epididymal sperm was used. The advantage of using epididymal sperm is that sperm handling is minimized as opposed to the ejaculate and that epididymal sperm is not subjected to the harmful agents in seminal plasma (Cerolini *et al.*, 1999). Epididymal sperm capacitation can be controlled by controlling medium pH (Ijaz and Hunter, 1989). Sperm is stored within the epididymis at a pH of 6.0 or lower in the bull (Salisbury and VandeMark, 1961). Upon exposure to seminal plasma or saline solution (pH 7.2), epididymal sperm becomes activated. Ijaz and Hunter(1989), found that increasing pH above 7.2 did not affect sperm motility significantly, but that the acrosome reaction was enhanced with increasing pH. Physiological and biochemical processes could therefore be controlled when using epididymal sperm as opposed to ejaculate sperm that are exposed to seminal plasma.

4.1.2.1 Epididymal sperm and the effects of cooling

Most acrosomal damage occurs during the dilution and precooling (equilibration) period from 35°C to 4°C. According to Niemann (1990), ice begins to form in the extracellular medium between -5°C and -10°C and while ice crystals grow in the

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extracellular medium, intracellular solute concentration increases. Cooling rate is the major factor determining what further happens to the intracellular water. With slow cooling, the cells become increasingly dehydrated and with more rapid cooling, cells freeze intracellularly. Intracellular freezing can be detrimental to the cells, depending on the amount of ice and the size of ice crystals. Very rapid cooling leads to very small intracellular ice crystals, which might not be as harmful to cells. Larger ice crystals tend to form during slow thawing procedures. It is therefore necessary to thaw cells rapidly that were rapidly frozen for maximum survivability. Likewise, cells frozen slowly should be thawed slowly to avoid damage from too rapid entry of water. The optimal freezing rate is, therefore, slow enough to avoid extensive intracellular ice formation (Niemann, 1990).

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Most of the existing protocols include glycerol in combination with egg yolk as a cryoprotectant in the freezing medium. It has been shown previously in goats that an enzyme Phospholipase A secreted from the bulbourethral gland degrades the lecithin on contact with egg yolk. This contact with the egg yolk produces toxic substances that affect spermatozoa and simultaneously induce medium coagulation (Ronkko *et al.*, 1991; Ronkko, 1995). The production of these toxic agents therefore would not be expected for epididymal sperm samples with the absence of accessory gland secretions.

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The specific aims of this chapter were to:

a) compare quantitative sperm motility after treatment of sperm with a cryoprotectant commonly used in domestic cattle (Triladyl) and during cooling in blue wildebeest and impala.

b) determine the effects of freezing on cauda epididymal sperm in blue wildebeest. Sperm motility and sperm morphology (including acrosome status) of frozen-thawed sperm were studied in blue wildebeest only.

(3) Sperm viability (live/dead status) of post-thaw sperm was assessed in blue wildebeest to assist in determining whether viable sperm were present after cryopreservation.

4.2 Materials and Methods

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4.2.1 Sperm Collection and Precooling

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Sperm were aspirated from the cauda epididymis of impala and blue wildebeest collected as indicated in Chapter 3 (3.2.4). The epididymis was processed as described earlier in Chapter 3 under section 3.2.3. To prevent excessive fluctuations in temperature, sperm processing for cryopreservation was performed at room temperature. For this study, a time limit of three to six hours (maximum for blue wildebeest only) was allowed after killing, before sperm processing for cryopreservation.

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The epididymis was cleaned (refer to section 2.2.3) and sperm were gently aspirated into a petri dish containing Ham's F10 supplemented with 10% fetal calf serum. This was done at room temperature (22-24°C). Of the sperm/Ham's F10 suspension, 500µl samples were aliquoted into 1500µl minicentrifuge vials. Cryodiluent was added to the sperm suspension at a rate of approximately one drop per second. It is crucial that each drop is well dispersed in the medium so that high concentrations of glycerol do not accumulate around the sperm, which could be cytotoxic (Holt, 2000). This creates a high osmotic gradient in the immediate vicinity of the sperm and thus could damage the sperm membrane even before equilibration is reached. Equilibration or precooling refers to the time it takes to cool the sperm to 4°C and remove sufficient intracellular water from the sperm prior to freezing. After the glycerol was added to the sample, the vials were placed in a water bath at 4°C for periods of 2, 4 and 6 hours (impala) and 1, 2 and 3 hours (blue wildebeest).

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4.2.2 Media and cryodiluents

For this experiment, a standard bovine protocol was used to cryopreserve the sperm (Chen *et al.*, 1993; Hinsch *et al.*, 1997). Bovine sperm morphology shows many similarities to that of wild antelope species used in this study.

The standard bovine protocol will subsequently be described. The cryodiluent used was Triladyl locally purchased from Taurus (Irene, South Africa). It is a

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TES/TRIS based medium containing 14% glycerol and no egg yolk. Medium preparation was done according to the manufacturer's instructions. Triladyl was aliquoted into 15ml sterile centrifuge tubes to which 40%(v/v) egg yolk (commercially available chicken eggs) was added to the undiluted cryoprotectant medium. If not used, the freshly prepared cryodiluent was stored at -20°C for up to six months. On the day of the sperm freezing experiment, the Triladyl was thawed and kept at room temperature. The sperm/Hams F10 suspension was diluted 1:1 with the cryodiluent to yield a 20% egg yolk and 7% glycerol final (v/v) concentration. Cryopreservation studies were performed in 1500µl conical cryovials. These vials had a relatively large surface area to volume ratio required during the precool/equilibration stage of the experiment. The cryodiluent (500µl) was added at one drop per second to the sperm sample and mixed thoroughly after each drop to minimize the effects of glycerol on the sperm.

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4.2.3 Cauda epididymal sperm motility at different time intervals during precooling at 4°C

Once the cryoprotectant medium was added to the sperm suspension, the vials were placed in a waterbath at 4°C and allowed to cool and equilibrate. At intervals of 2, 4 and 6 hours, a 10µl sample was taken for motility assessment in impala. Blue wildebeest samples were assessed at 1, 3 and 6 hours. Both impala and blue wildebeest experiments were performed simultaneously. In order to synchronise the experiment, both started at the same time with an hour difference between the first blue wildebeest motility recording and the first impala

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motility recording. Samples were placed on a prewarmed (36°C) slide and motility was recorded using computer aided sperm motility analyses as previously described in Chapter 3, section 3.2.4.2.

4.2.4 The effects of freezing Blue Wildebeest cauda epididymal sperm at (-196°) centigrade

4.2.4.1 Sperm freezing in liquid nitrogen

Figure 4-1 is a schematic representation of the freezing method used. After sperm precooling had taken place (2-6 hours), the sperm suspension was aspirated into 250µL French straws and sealed with sealant (Taurus, Irene, South Africa). The straws were then placed 4cm above liquid nitrogen (-90°C) inside a styrofoam container for 10 minutes before being plunged into the liquid nitrogen.

[1]4.2.4.2 Post thaw evaluation

Straws were rapidly removed from the liquid nitrogen and thawed at 36°C for 60 seconds. The straws were cut at both ends and the contents were decanted into 1500µl vials. One of the vials was placed into a larger plastic tube to fit into the centrifuge. Samples were centrifuged at 300X*g* for 10 minutes. The supernatant was discarded and the pellet was overlaid with Ham's F10 supplemented with

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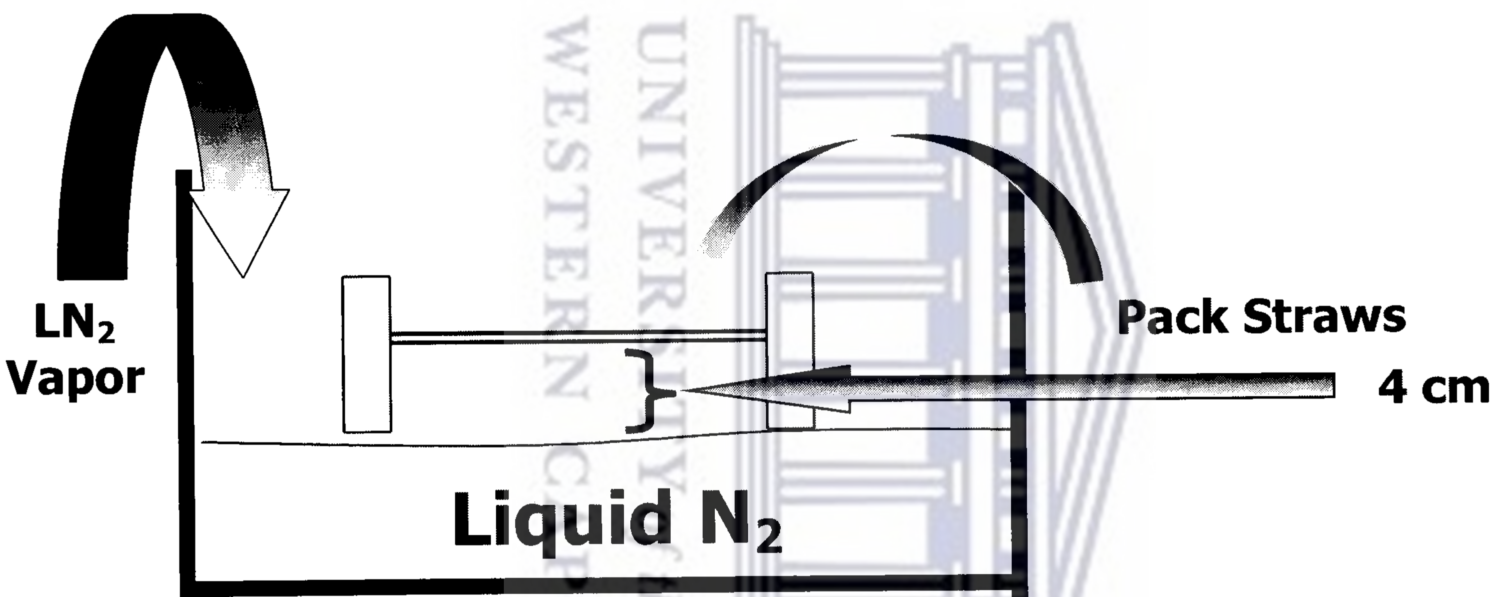

Fig. 4-1: Shows a schematic representation of the sperm freezing method, first, liquid nitrogen vapour (-90°C) before being plunged into the liquid phase at –196°C.

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10% fetal calf serum. The tubes were placed in the incubator for one hour at 37°C. After one hour, the supernatant was carefully removed and transferred to a clean sterile microcentrifuge tube. The sample, containing the swim-up sperm was centrifuged at 300X*g*[1] for 10 minutes to concentrate the sperm. This step was necessary for all samples, as the recovery of motile sperm was low in all instances.

4.2.4.3 Sperm motility

Sperm motility was assessed as described previously. Briefly, 10μL samples were taken and placed on a prewarmed glass slide. Motility was observed by means of a light microscope (negative phase contrast) fitted with a digital black and white camera (Panasonic WV-BP310). Images were recorded onto VHS videotape. Video images were then later analysed with the aid of the Sperm Motility Quantifier or SMQ as described under 3.2.4.2. Between 90 to 110 motile sperm were manually analysed for each of the individual samples.

4.2.4.4 Sperm morphology

4.2.4.4.1 Transmission Electron Microscopy

After thawing, the straw content was decanted into 1500μL vials and centrifuged at 300X*g*. The supernatant was discarded and the pellet was resuspended in

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[1] $g = 1118 \times 10^{-8} \times R \times N^2$ where g = centrifugal force, R = distance in cm from center of the rotor, N= revolutions per minute

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fresh Ham's F10. This process was repeated two to three times in order to remove most egg yolk in solution. The last centrifugation was to condense the sperm for analysis. About 10µL of the pellet was fixed in 2.5% glutaraldehyde and then subsequently processed for transmission electron microscopy as indicated in Chapter 2, section 2.2.2.2.

4.2.4.4.2 Sperm lectin binding for postthaw acrosome detection

A lectin binding technique was applied to detect the acrosome. This was done as described by Gabriel *et al.* (1995). FITC-conjugated lectin was prepared and made up to a concentration of 1mg/ml. Of this, 5µl was added to 20 X 10^{6} sperm and incubated at 36°C for 15 minutes.

4.2.4.4.3 SYBR-14/Propidium Iodide Live/Dead assay in Blue Wildebeest

The LIVE/DEAD Sperm Viability Kit (L-7011) was obtained from Molecular Probes, Leiden, The Netherlands. The solutions were prepared according to the manufacturer's recommendations and modified in some instances as according to Garner (1997). The kit consists of SYBR-14 dye dissolved in dimethylsulphoxide (DMSO) and a Propidium Iodide solution. Prior to assaying sperm for live/dead status, a Sperm-TALP (Tyrodes, albumin, lactate, pyruvate) solution was prepared (10mM HEPES, 0.85% NaCl, 10mM BSA at pH 7.4). The SYBR-14 was diluted ten times with Sperm-TALP. Of the diluted SYBR-14, 5µl was added to 5ml diluted sperm or 1µl to1ml (333nM). This mixture was

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incubated at 36°C for 10 minutes after which, 5µl of Propidium Iodide (PI) was added to 1ml (93nM concentration, final) of the sperm-SYBR-14 preparation. Sperm preparations were viewed with an Olympus BX-40 epifluorescence microscope.

4.3 Results

4.3.1 Species specific sperm motility changes after precooling

The sperm motility changes after cooling will first be described in impala (4.3.1.1) and then in blue wildebeest (4.3.1.2)

4.3.1.1 Impala

From Figures 4-2 (legend on page 108), impala cauda epididymal sperm curvilinear velocity (VCL) after precooling showed no statistically significant differences ($p>0.05$) from 2 to 4 hours after rewarming. There was a significant decrease in VCL from 4 to 6 hours ($p<0.0001$), which also differed significantly ($p=0.0094$) from 2 to 6 hours. VCL decline was accompanied by significant decreases in VSL (Fig 4-2 b) from 2 to 4 hours ($p=0.0475$) and 2 to 6 hours ($p<0.0001$). Linearity (LIN) decreased significantly from 2 to 4 ($p<0.0001$) and 4 to 6 hours ($p=0.0165$) as well as average path velocity (VAP), and beat cross frequency (BCF) decreased ($p<0.05$) almost linearly from 2 hours to 6 hours as seen in figures 4-2 (c) to (e), respectively. There was a tendency for DNCmn

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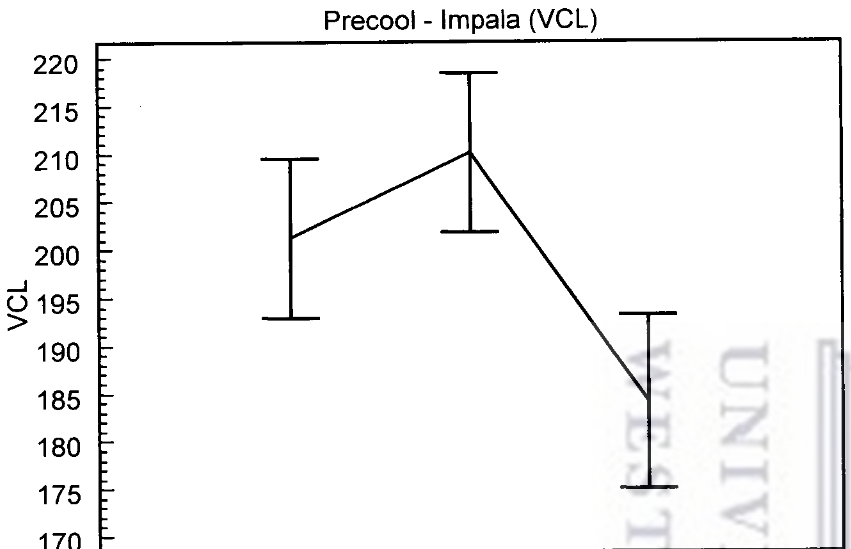

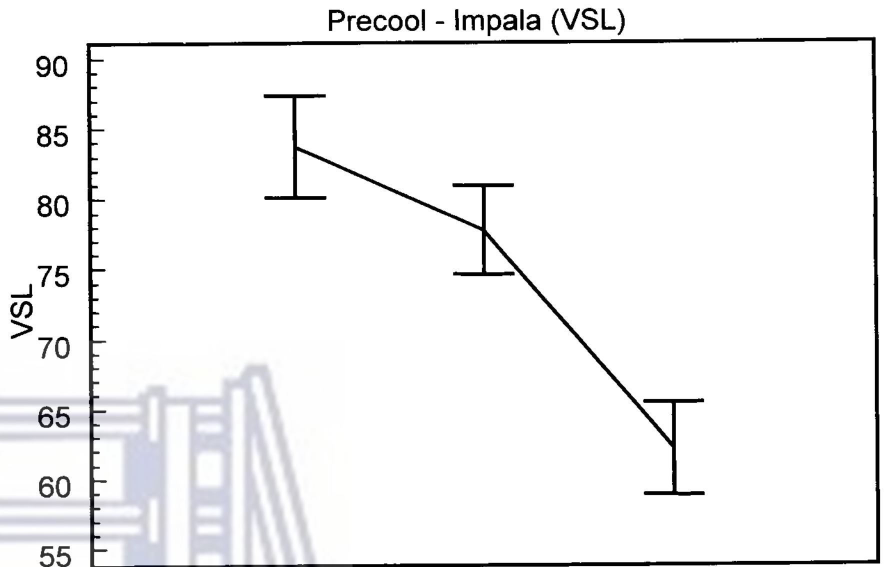

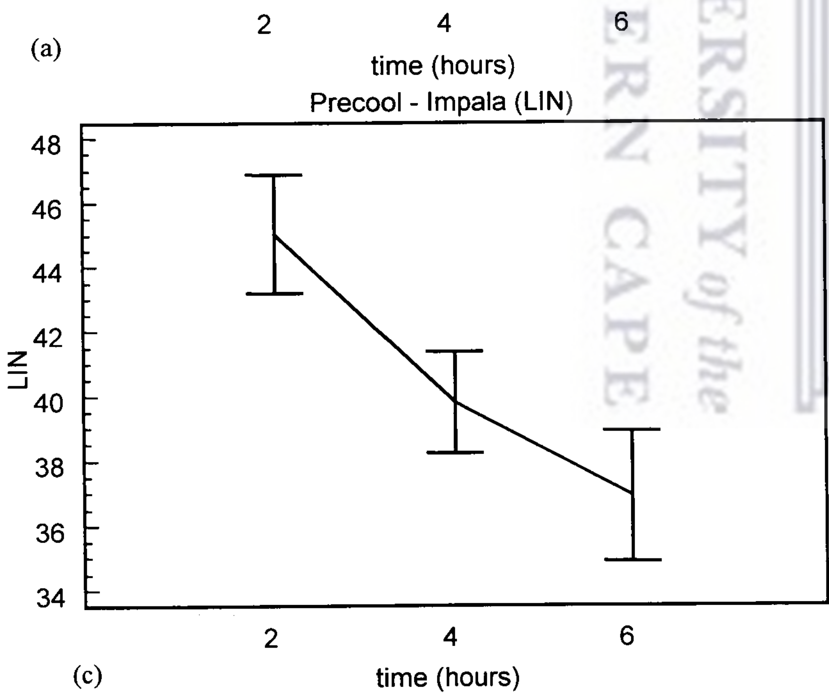

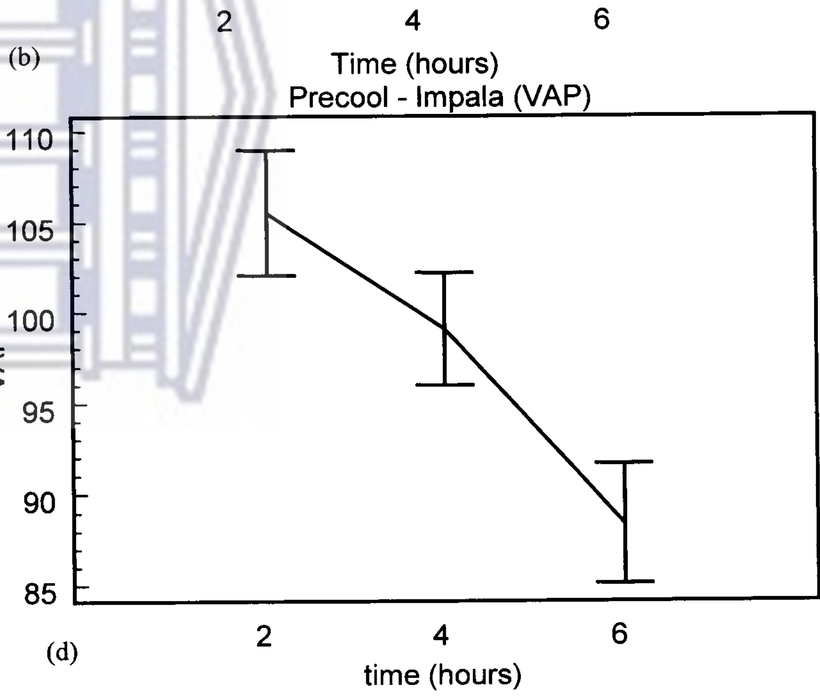

Fig 4-2

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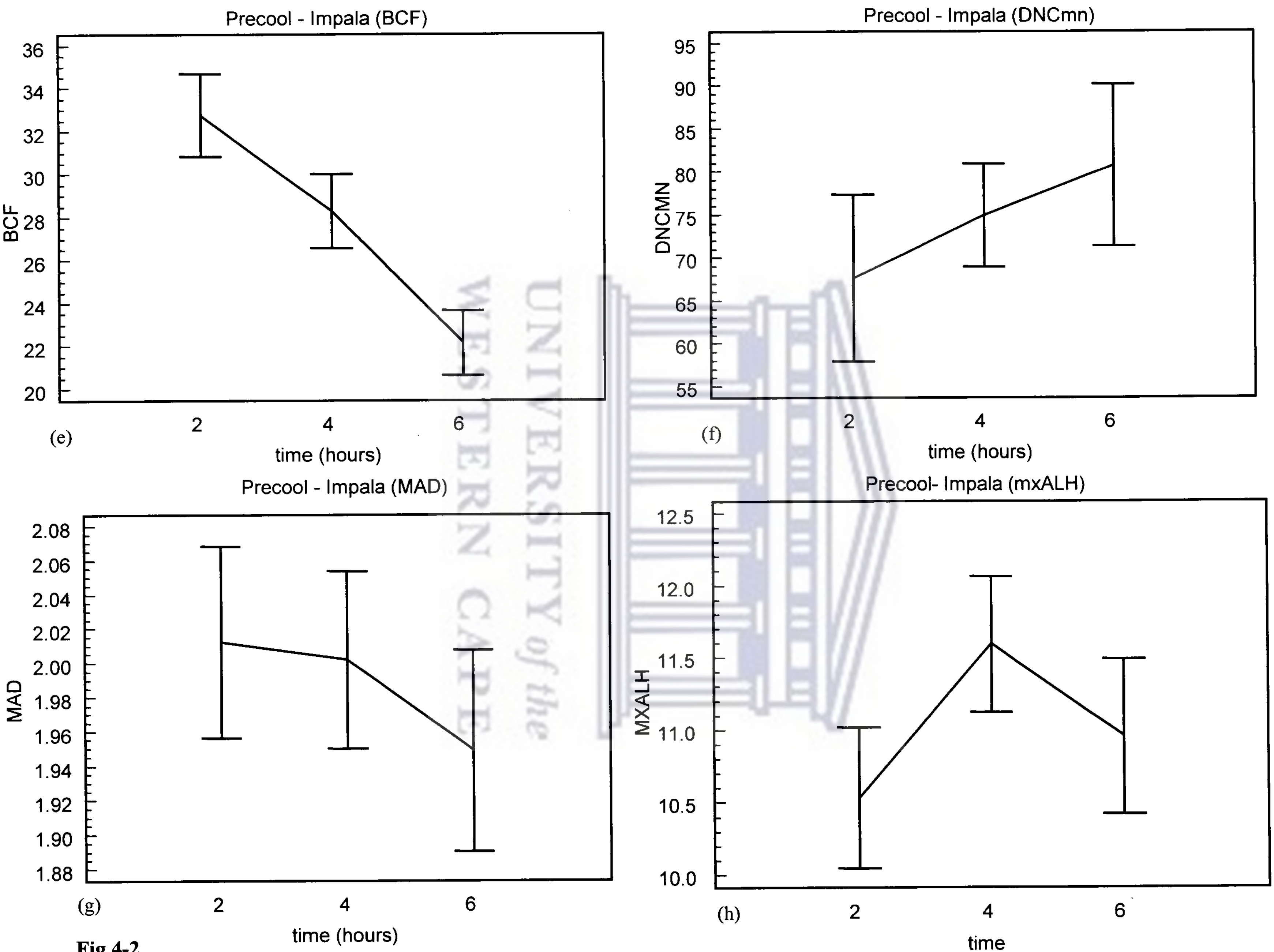

Fig 4-2

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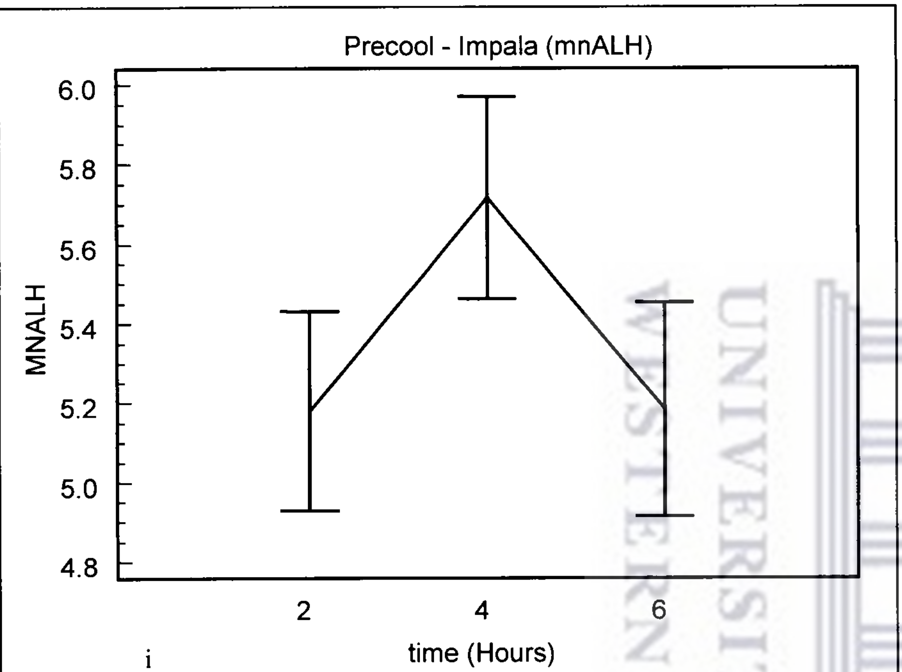

Figures 4-2 (a to i): Depicts various cauda epididymal sperm motion parameters, after precooling (2, 4 and 6 hours). **Fig. 4-2 (a)** shows an initial increase in sperm VCL within first 2 hours followed by a decline in swimming velocity. **Fig. 4-2 (b)** Shows an almost constant decrease in VSL irrespective of an initial VCL increase. Data points are represented as the mean ±2SEM (equivalent to 95% confidence interval).

Fig. 4-2 (c): Shows decrease in impala precooled sperm linearity (LIN) after 2, 4 and 6 hours incubation. Both a decrease in sperm VSL (Fig. 4-2 b) and linearity indicate a loss of progressive forward motility. **Fig. 4-2(d)** indicates a decrease in precooled impala sperm average path velocity. Data points are represented as the mean ±2SEM (equivalent to 95% confidence interval).

Fig. 4-2 (e): The decrease in beat cross frequency is an indication of a decrease in the sperm head crossing the average path which requires an increase in amplitude of lateral head displacement (**Fig. 4-2 (h), (i)**) as well as an increase in space occupied by the sperm head path (refer to Table 3-1) and indicated by dancemean (DNCmn), shown in **Fig. 4-2 (f).** Data points are represented as the mean ±2SEM (equivalent to 95% confidence interval).

Fig 4-2

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(Figure 4-2 f) to increase and this was statistically significantly different from 2 to 4 hours ($p<0.0001$) but not from 4 to 6 hours ($p=0.9313$). Both mxALH and mnALH surged at two hours with statistically significant increases from 2 to 6 hours for mnALH but not mxALH ($p=0.1419$). Figure 4-2 (g) shows the tendency for MAD to decrease but were not significant at all stages.

4.3.1.2 Blue Wildebeest

Blue wildebeest cauda epididymal sperm motility readings were taken at intervals of 1, 2 and 3 hours (Figures 4-3 a-i; legend on page 112). The incubation period for impala sperm differed from that of blue wildebeest, and sperm motility patterns over this period could not be compared. Figure 4-3 (a) shows that sperm VCL showed an initial sharp decline ($p<0.0001$) from 1 to 2 hours. Thereafter, VCL increased between 2 to 3 hours ($p<0.0001$). There was no statistical significant difference between 1 hour and 3 hours for VCL ($p=0.6717$). The same phenomenon was observed for VSL, being significantly different after two hours, increasing after three hours and with no statistically significant difference between 1 and 3 hours. This pattern was found for all other parameters (mnALH, mxALH, BCF, VAP, DNCmn and MAD), except LIN, which showed an increase after 2 hours, as can be seen in Figures 4-3 (d-i).

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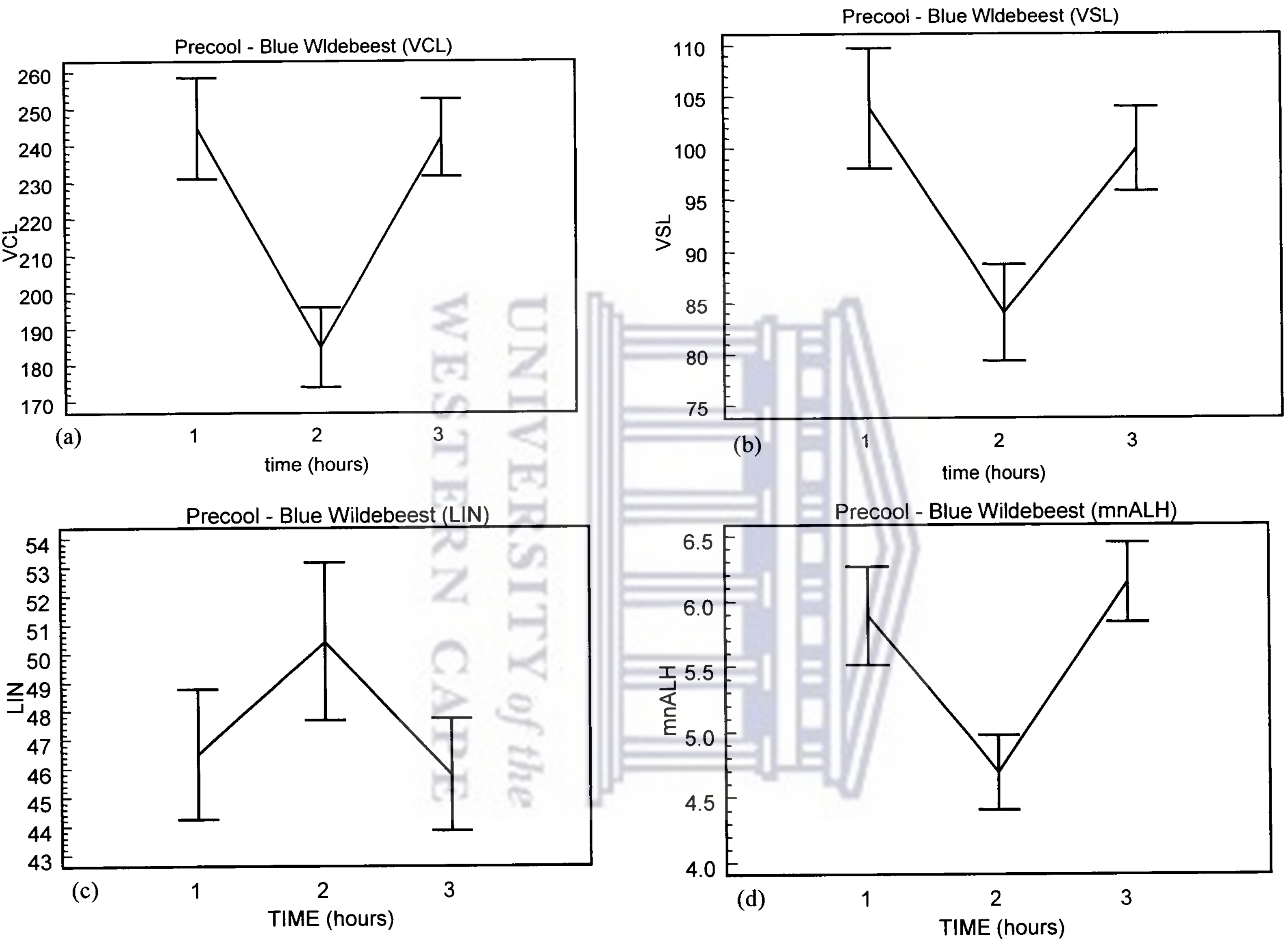

Fig 4-3

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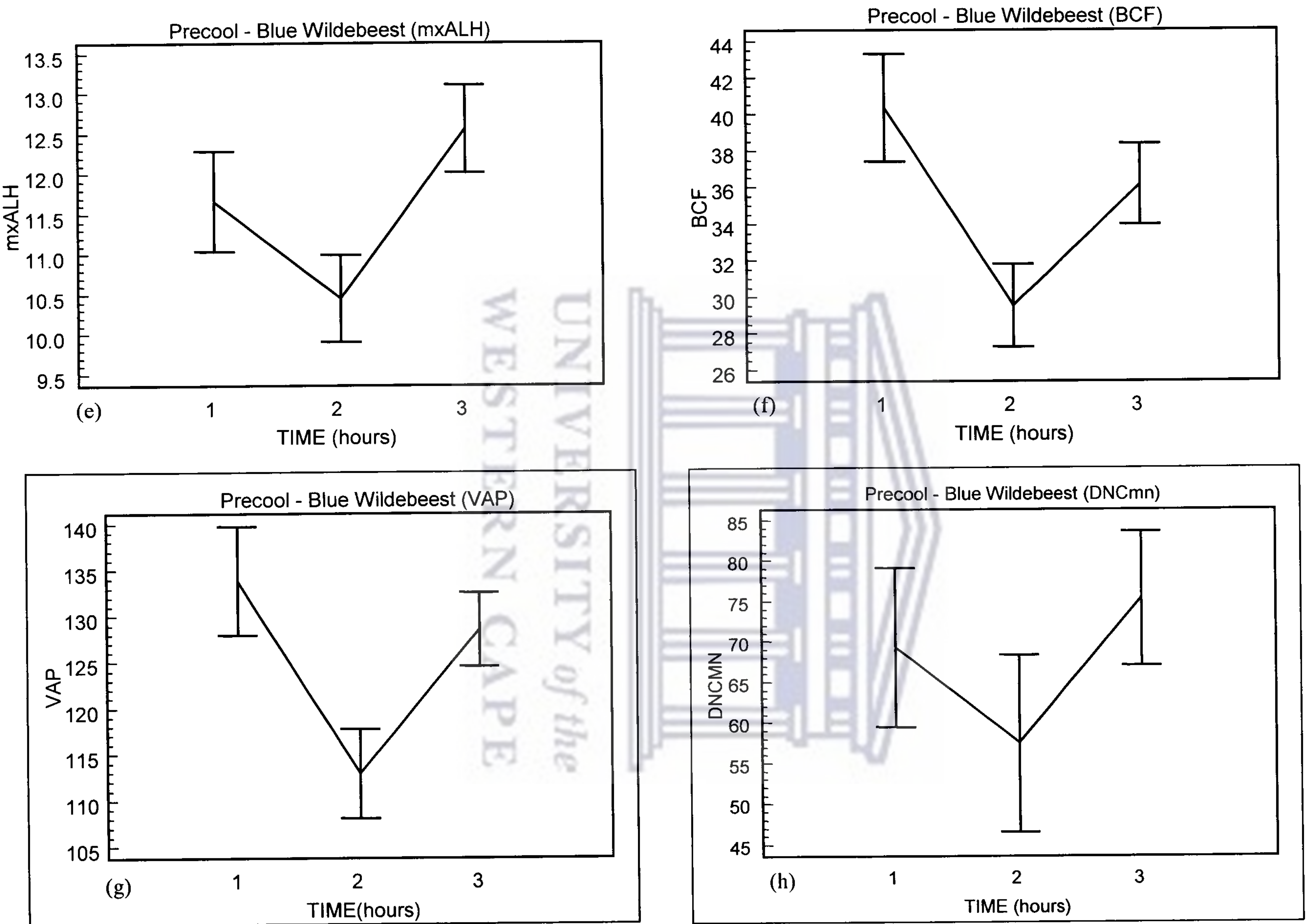

Fig 4-3

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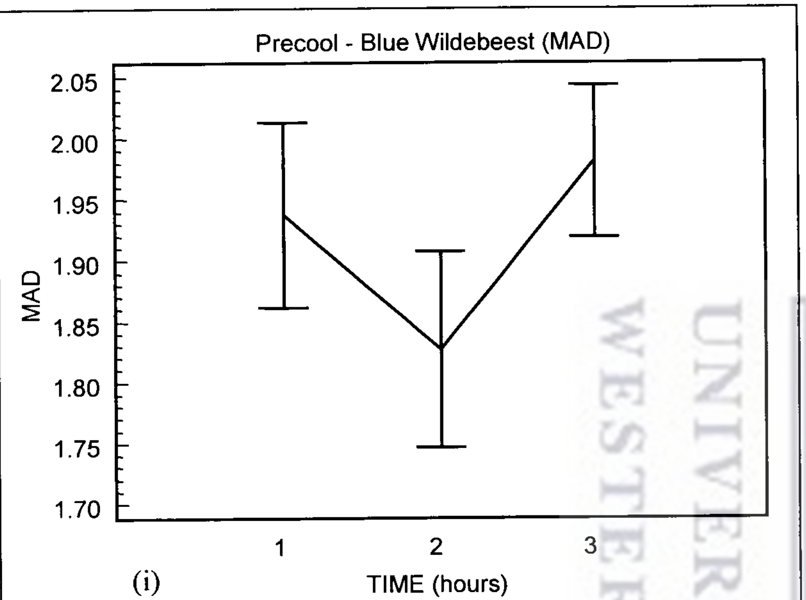

Fig. 4-3 (a): Depicts precooled blue wildebeest cauda epididymal sperm curvilinear velocity or VCL (mean ± 2 SEM). Sperm motility was taken at 1, 2 and 3 hours after precooling. After an initial decrease in VCL, sperm velocity partly recovered ($p<0.0001$). The same trend can be seen for straightline velocity (VSL), **Fig. 4-3(b)**

Fig. 4-3 (c): Shows an increase in linearity from 1 to 2 hours. This coincides with the decrease in the mean amplitude of lateral head displacement (mnALH), **Fig. 4-3(d)**. From 2 hours to three hours, LIN decreases and mnALH and mxALH **(Fig. 4-3 (e)**) increase indicating the decrease in progressive motility.

Fig. 4-3 (f): shows a similar pattern to the other parameters, where values decrease after two hours incubation. The decrease in beat cross frequency (BCF) also coincides with the initial decrease in VAP and DNCmn **(Fig. 4-3 (g)** and **Fig. 4-3 (h)**). At two hours, VCL, VAP, BCF, DNCmn and MAD decrease (**Fig. 4-3(i)**) and coincide with an increase in sperm linearity (LIN). The increased linearity can be explained by a decrease in mean angle of displacement (MAD) and the decrease in space occupied by the sperm head as indicated by DNCmn.

Fig 4-3

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4.3.2. Sperm motility of fresh versus precooled sperm

In this section sperm motility of fresh versus precooled sperm will be described for impala (4.3.2.1) and subsequently for blue wildebeest (4.3.2.2).

4.3.2.1 Impala

Figures 4-2 (a-i) show impala sperm that were precooled (PC) for 2, 4 and 6 hours. Fresh non-precooled (NPC) sperm parameters were measured at intervals of 1, 3 and 6 hours. The different time-based comparisons were performed because material for impala and some blue wildebeest arrived at the same time. Synchronization had to be staggered in order to accommodate measurement for all samples. There were time constraints which necessitated this approach (See Materials and Methods 4.2.3.).

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Sperm from both NPC at 1 hour (Figures 4-4 a-c) and NPC at 3 hours (Figures 4-5 a-c) were compared to PC determined at 2 hours for selected motility parameters (Figures 4-5 a-c). VCL, VSL, LIN, BCF and VAP were significantly higher ($p<0.0001$) in the NPC at 1 hour when compared to PC at 2 hours. DNCmn, mnALH, mxALH and MAD were respectively significantly lower ($p<0.0001$) in the NPC at 1 hour than for PC at 2 hours.

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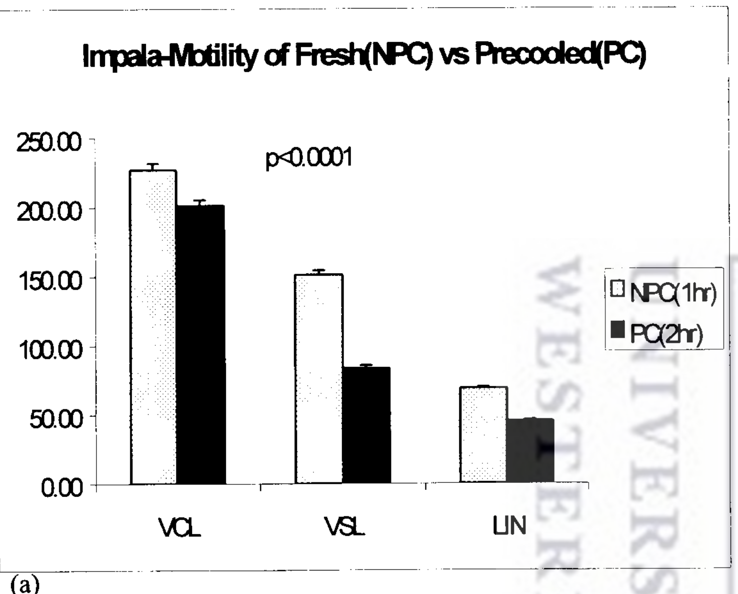
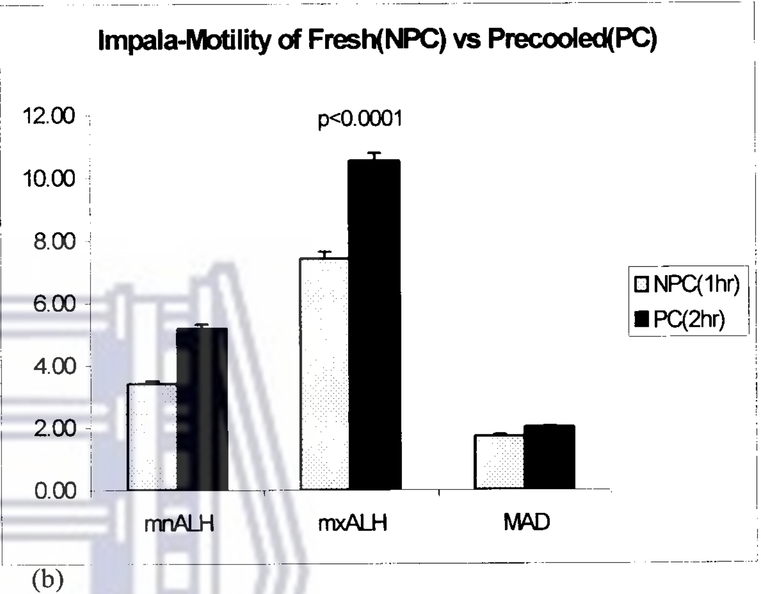


(a)



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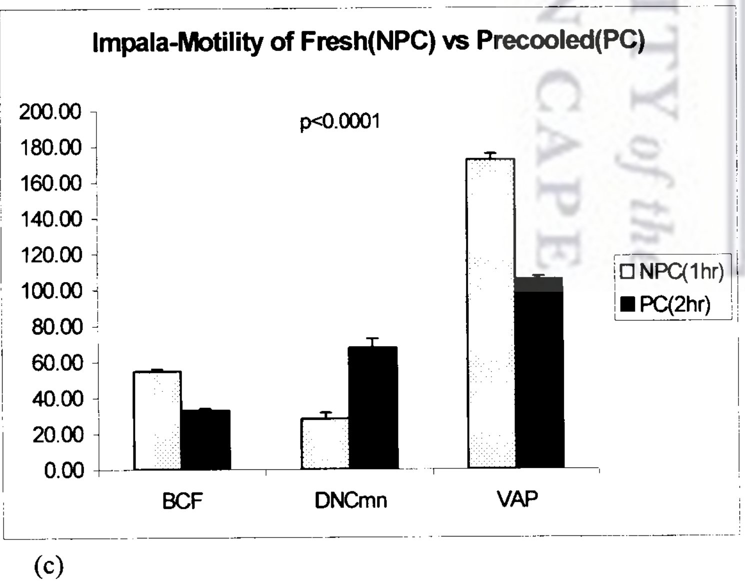

(c)

Fig 4-4

Fig 4-4 (a): Histograms comparing impala cauda epididymal sperm VCL, VSL and LIN at 1 hour fresh (NPC) to 2 hours precooled (PC) sperm. The results show that each parameter respectively differed statistically significantly when1 hour NPC treated was compared to 2 hours PC treated sperm.
Fig4-4 (b): Histograms comparing impala cauda epididymal sperm mnALH, mxALH and MAD at 1 hour fresh (NPC) to 2 hours precooled (PC) sperm. The results show that each parameter differed statistically significantly when 1 hour NPC treated was compared to 2 hours PC treated sperm.
Fig 4-4 (c): Histograms comparing impala cauda epididymal sperm BCF, DNCmn and VAP at 1 hour fresh (NPC) to 2 hours precooled (PC) sperm. The results show that each of these parameters differed statistically significantly, respectively when 1 hour NPC treated was compared to 2 hours PC treated sperm.

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Figures 4-5 (a-c) show that NPC, after 3 hours was significantly lower ($p<0.0001$) than PC after 2 hours for VCL, mnALH, mxALH, MAD and DNCmn respectively. VAP, VSL and LIN were significantly higher when NPC 3 hours was compared to PC at 2 hours.

4.3.2.2 Blue Wildebeest

Most blue wildebeest material arrived on separate days and comparisons of fresh (NPC) and precooled (PC) could be compared at the same time intervals, as there were no time constraints to complete motility measurements.

When comparing motility (Figures 4-6 a-c) of blue wildebeest sperm precooled (PC) for 1 hour to fresh (NPC) sperm at 1 hour, VCL, VSL, LIN, VAP and BCF were significantly higher ($p<0.05$) in the NPC group. There were no differences found in mnALH ($p=0.97$) and mxALH ($p=0.13$) respectively when the PC group was compared to the NPC group.

For the NPC sperm at 3 hours, VCL, VSL, LIN, BCF and VAP were significantly higher ($p<0.05$) and no differences were evident for mnALH ($p=0.071$), mxALH ($p=0.75$), and MAD being significantly lower ($P=0.0045$) when compared to PC at one hour, as shown in figures 4-7 (a-c).

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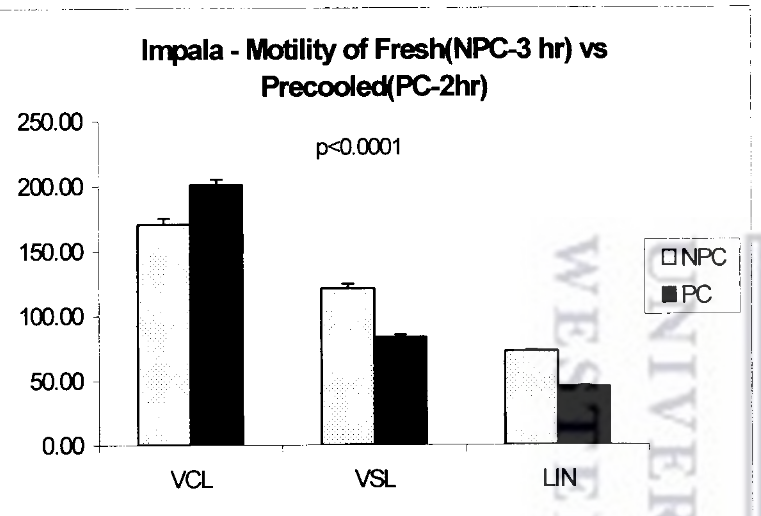

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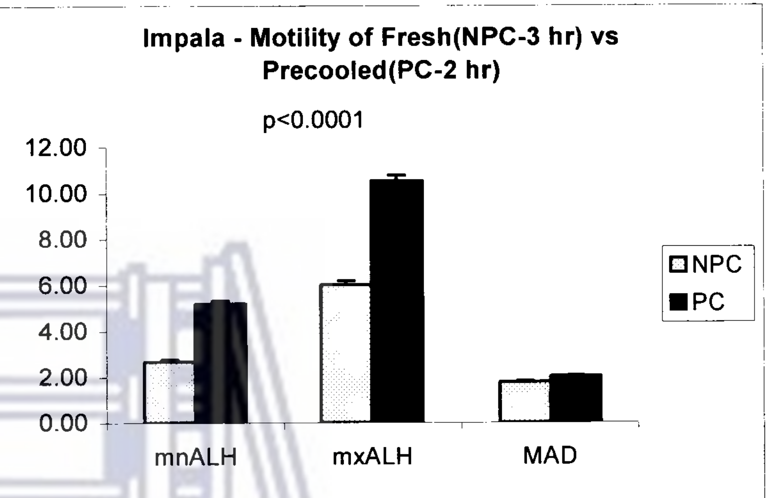

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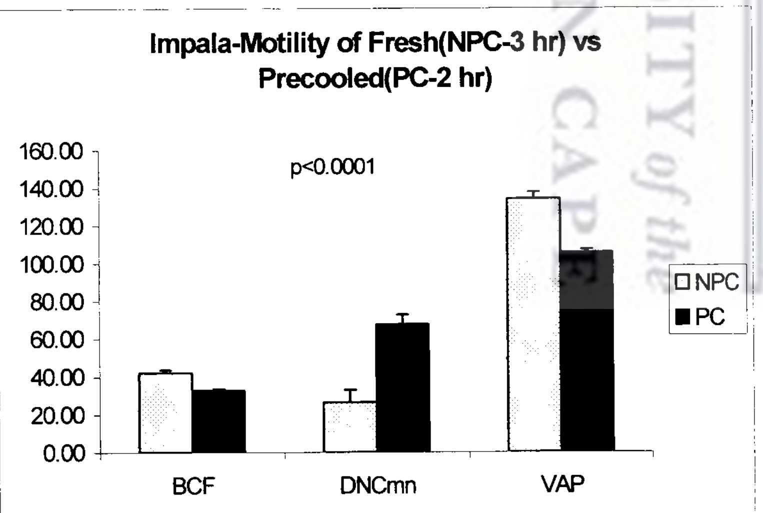

(c)

Fig 4-5

Fig. 4-5 (a) Histograms comparing impala cauda epididymal sperm VCL, VSL and LIN. Fresh (NPC) sperm incubated for 3 hours was compared to precooled (PC) sperm incubated for 2 hours. The results show that each parameter differed statistically significantly when 3 hour NPC was compared to 2 hours PC.
Fig. 4-5 (b) Histograms comparing impala cauda epididymal sperm mnALH, mxALH and MAD. Fresh (NPC) sperm incubated for 3 hours was compared to precooled (PC) sperm incubated for 2 hours. The results show that each parameter differed statistically significantly, respectively for both 3 hour NPC treated and 2 hours PC treated sperm.
Fig. 4-5 (c) Histograms comparing impala cauda epididymal sperm BCF, DNCmn and VAP. Fresh (NPC) sperm incubated for 3 hours was compared to precooled (PC) sperm incubated for 2 hours. The results show that each parameter differed statistically significantly, respectively for both 3 hour NPC treated and 2 hours PC treated sperm.

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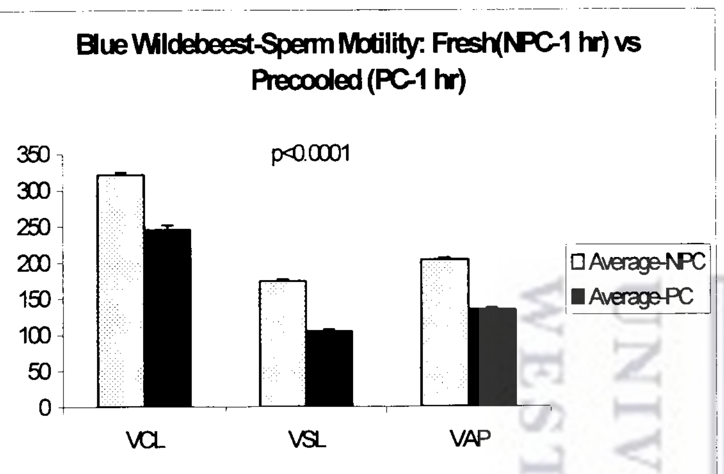
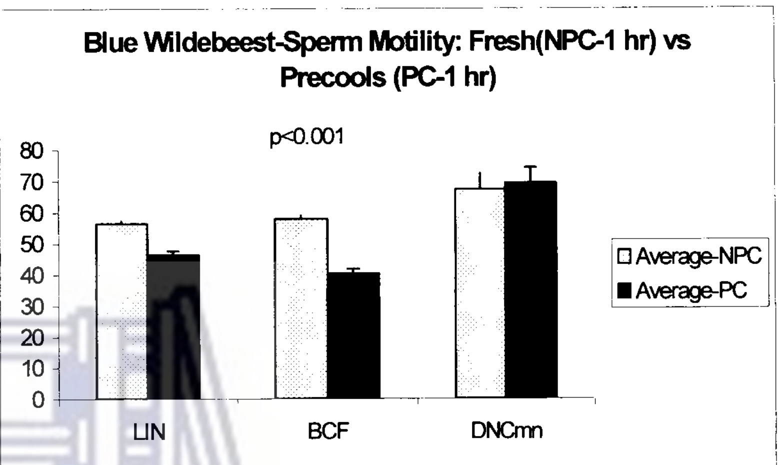


(a)



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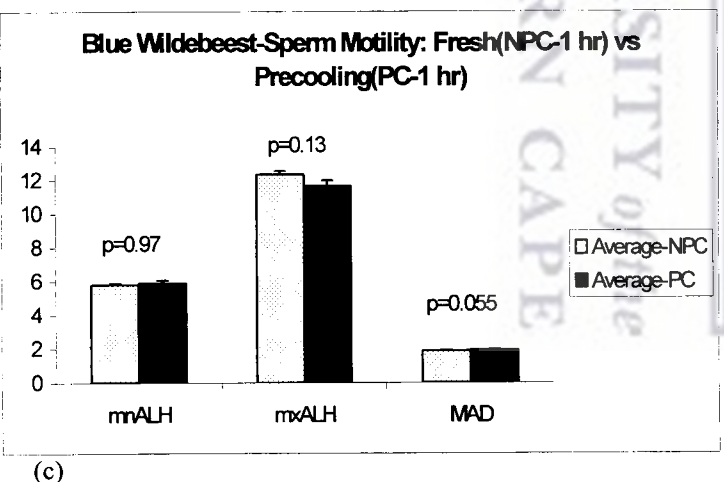

(c)

Fig 4-6

Fig 4-6 (a) Shows histograms of VCL, VSL and VAP when fresh (NPC) sperm incubated for 1 hour was compared to Precooled (PC) sperm incubated for 1 hour. The results show that each parameter differed statistically significantly ($p<0.0001$) when 1 hour NPC was compared to 1 hour PC.
Fig 4-6 (b) Shows histograms of BCF, DNCmn and LIN when Fresh (NPC) sperm incubated for 1 hour was compared to Precooled (PC) sperm incubated for 1 hour. None of the individual parameters were statistically significantly different for either the 1 hour NPC or the 1 hour PC treated sperm.
Fig 4-6 (c) Shows histograms of mnALH, mxALH and MAD when fresh (NPC) sperm incubated for 1 hour was compared to Precooled (PC) sperm incubated for 1 hour. The results show that each parameter differed statistically significantly ($p<0.05$) when 1 hour NPC was compared to sperm from the PC treated group.

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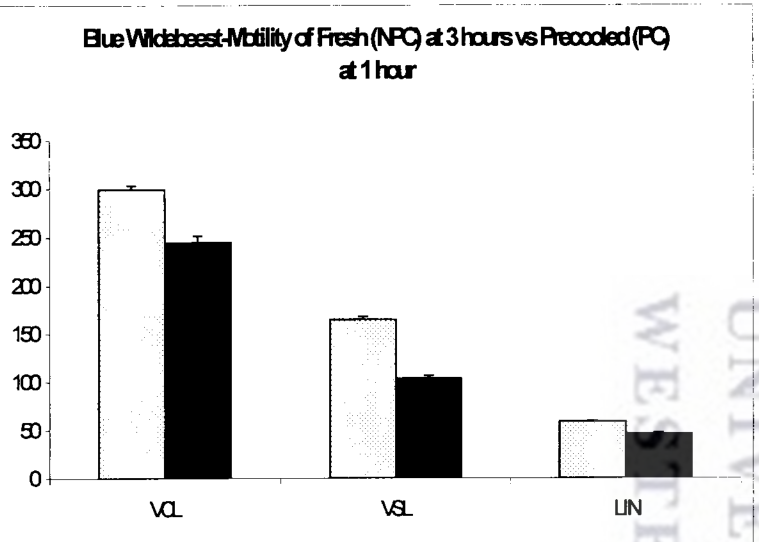
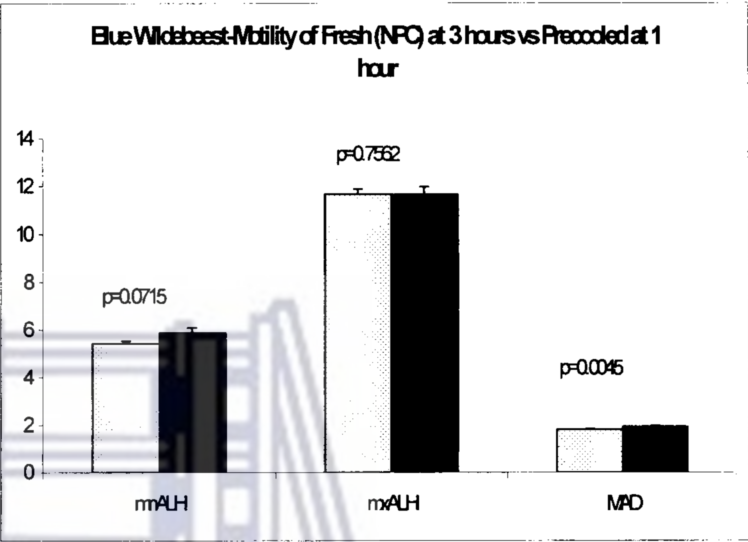


(a)



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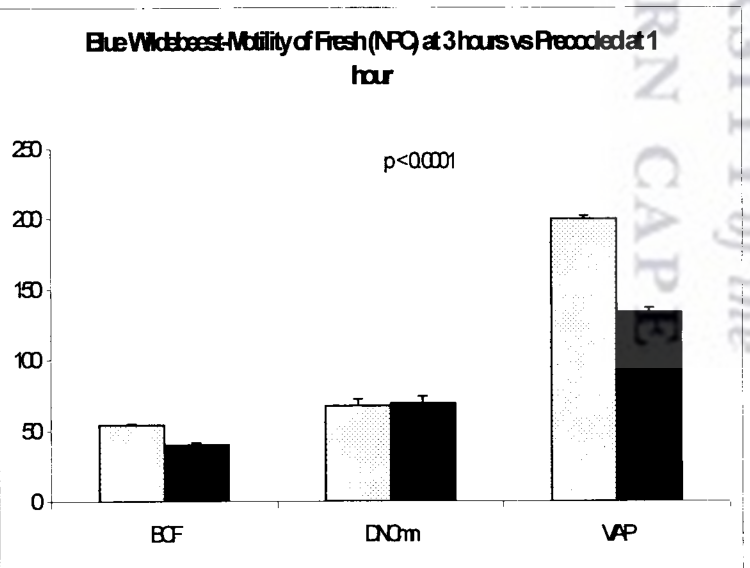

(c)

Fig. 4-7

Fig. 4-7 (a) Histograms comparing blue wildebeest cauda epididymal sperm VCL, VSL and LIN. Fresh (NPC) sperm incubated for 3 hours was compared to Precool (PC) sperm incubated for 1 hour. The results show that the individual parameters differed statistically significantly, respectively for both 3 hour NPC treated and 1 hour PC treated sperm.
Fig. 4-7 (b) Histograms comparing blue wildebeest cauda epididymal sperm mnALH, mxALH and MAD. Fresh (NPC) sperm incubated for 3 hours was compared to Precool (PC) sperm incubated for 1 hour. MAD differed significantly whereas mnALH and mxALH were not statistically significantly different.
Fig. 4-7 (c) Histograms comparing blue wildebeest cauda epididymal sperm BCF, DNCmn and VAP. Fresh (NPC) sperm incubated for 3 hours was compared to Precool (PC) sperm incubated for 1 hour. The results show that the individual parameters differed statistically significantly, respectively for both 3 hour NPC treated and hour PC treated sperm.

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4.3.2.3 A comparison of the effect of precooling on sperm motility in blue wildebeest and impala

In figures (4-8 a-c), VCL, VSL, BCF, VAP, mnALH and mxALH were significantly lower respectively ($p<0.05$) in the impala when compared to blue wildebeest after 2 hours precooling. Figure 4-8 (c) show that MAD was significantly higher in impala than in blue wildebeest ($p=0.01$), but that no differences were evident for DNCmn (Figure 4-8 a) and LIN i.e. Figure 4-8(b) ($p>0.05$) between the two species. The results showed that the sperm of the blue wildebeest had a higher tolerance to the effects of cooling on sperm physiology when compared to impala sperm.

4.4 Sperm Freezing

4.4.1 Sperm Motility

Figures 4-9 a, b, c and d show blue wildebeest sperm motility results obtained from fresh sperm, precooled (PC) sperm, immediately after thawing (IM), and swim-up immediately after thawing (SU). Sperm motion parameters VCL, VSL, LIN, mnALH and BCF were used to describe the changes for each of the above groups. The results indicated that sperm motility recovered during swim-up after thawing. During precooling (PC) and IM, all sperm motion parameters were significantly lower than for both fresh and SU ($p<0.05$) sperm except for mnALH.

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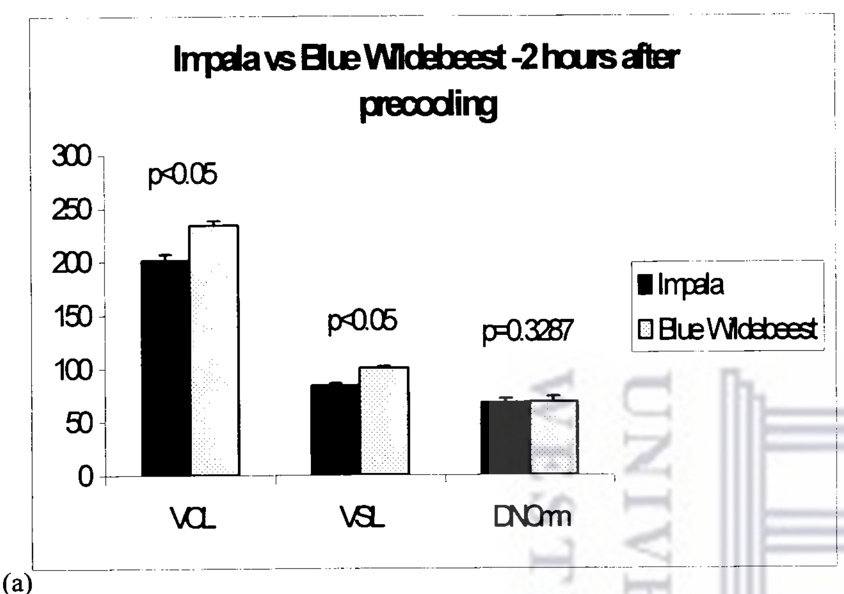
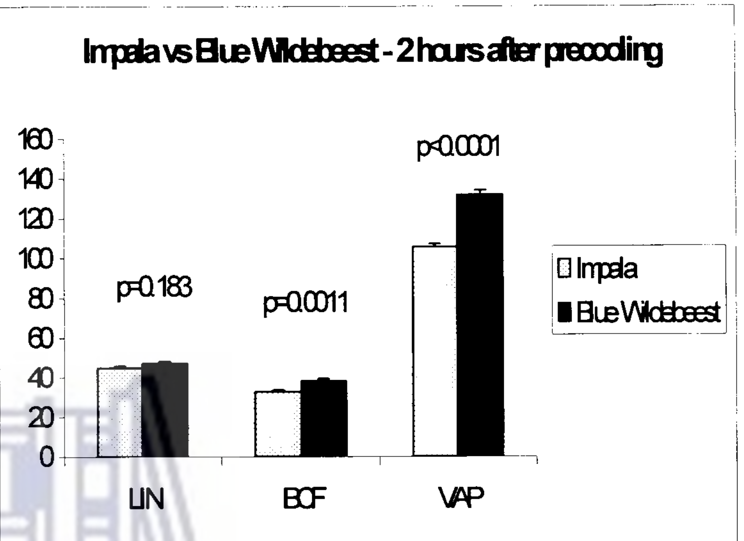


(a)



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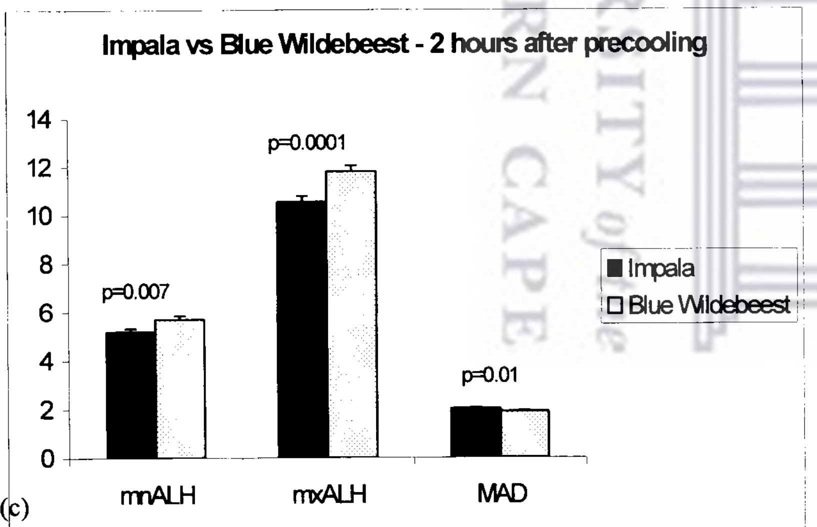

(c)

Fig 4-8 (a): Histograms comparing impala and blue wildebeest VCL, VSL and DNCmn after sperm precooling. Statistically significant differences were found for VCL and VSL, but not DNCmn, respectively..
Fig 4-8 (b): Histograms comparing impala and blue wildebeest LIN, BCF and VAP. Statistically significant differences were obtained when comparing each of the parameters, except LIN.
Fig 4-8(c): Shows histograms comparing impala and blue wildebeest mnALH, mxALH and MAD. The results show that the individual parameters differed statistically significantly for both blue wildebeest and impala precooled sperm.

Fig. 4-8

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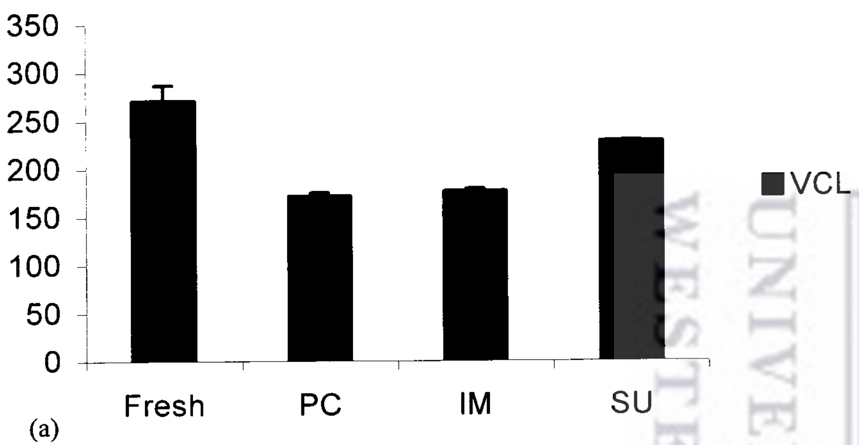
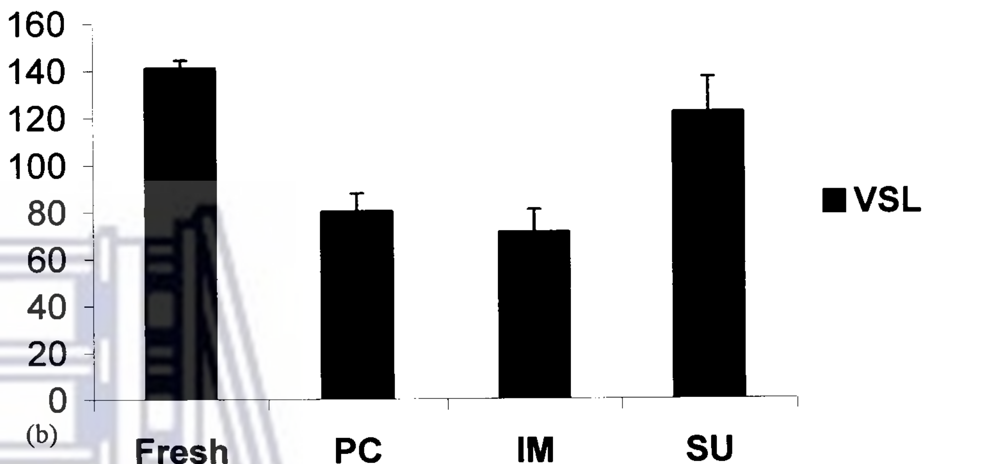
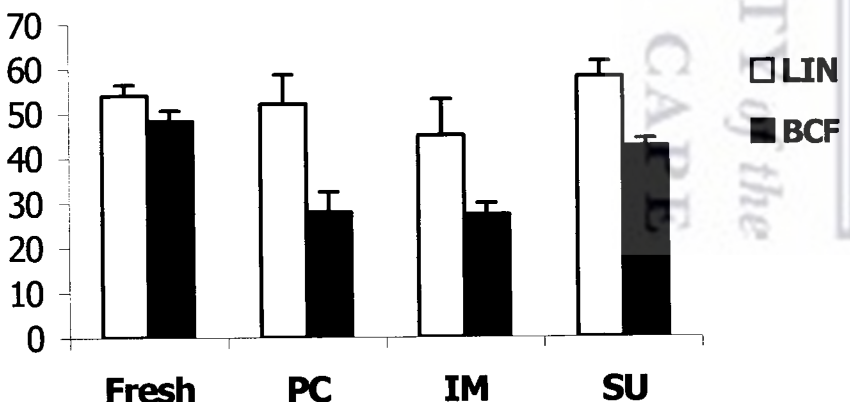


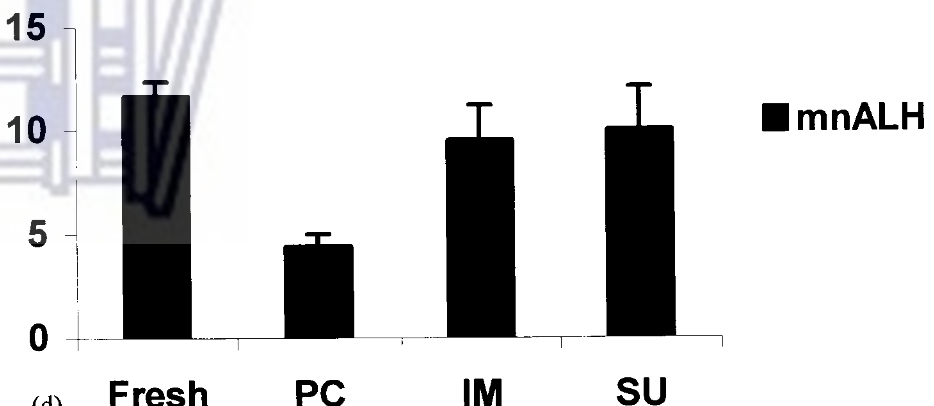
(a)



(b)



(c)



(d)

Fig 4-9 Histograms (±SEM) of selected sperm motility parameters in the blue wildebeest comparing fresh sperm (Fresh) to precooled sperm (PC), sperm immediately after thawing in cryodiluent (IM) and swim-up (SU) treated sperm.

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4.4.2 SYBR-14 – Propidium Iodide Live/Dead staining

The postthaw sperm viability evaluation was 34% live sperm ($p>0.05$ within the group) and 65% sperm which ($p>0.05$ within the group) stained propidium iodide (PI) positive i.e. dead sperm (Figure 4-10).

4.4.3 Morphology

Figures 4-11 (a) and (b) show transmission electron micrographs of fresh and frozen thawed sperm. Figure 4-11(a) is an example of a normal frozen thawed sperm showing a well condensed nucleus with no vacuoles; the plasma membrane adheres tightly to the sperm head, and the acrosomal membrane is intact and sits tightly around the nucleus. Figure 4-11 (b), shows both sperm with an intact membrane and sperm with a swollen membrane. Apart from the swelling of the plasma membrane that takes place, the acrosome has also become dislodged. Figures 4-11 (c) and (d) show further examples of membrane swelling and tearing. These plasma membrane and acrosomal changes have not been observed in examples of fresh sperm and is suggestive that freezing damage has taken place.

4.4.4 Lectin binding assay

Figures 4-12 (a), (b) and (c) show the results of frozen-thawed sperm incubated with Wheat Germ Agglutinin –Fluorescein isothiocyanate (WGA-FITC). In this experiment, staining was done to determine the presence of intact acrosomes. Various staining intensities were described by Mortimer, 1994. From these

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Fig 4-10: Shows the results of a SYBR-14/Propidium iodide viability stain of postthaw outcome in the Blue Wildebeest. SYBR14 stains sperm green at 488nm indicating an intact sperm plasma membrane whereas propidium iodide stains sperm nuclei red at 488nm in sperm with damaged membranes. The sperm sample was incubated for 15 minutes at 37°C and evaluated within two minutes after incubation.

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Fig. 4-11 Transmission electron micrographs of sections through frozen thawed sperm heads of blue wildebeest. (a) is an example of a frozen thawed sperm with intact membranes similar to that seen in Figure 2-30 with the sperm membrane tightly fitted around the sperm nucleus (arrows). (b) and (c) show swelling of the sperm membrane with the plasma and acrosomal membrane clearly distinguished in (c) *arrow heads.* (d) shows damaged sperm membranes and swelling (arrow). These phenomena are similar to the effects of cryopreservation on sperm described by Leverage *et al.* (1972).

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Figure 4-12 (a): Shows frozen-thawed sperm of blue wildebeest with intensely (arrows) stained acrosomes. (b) Shows frozen-thawed sperm from the same species with a lightly stained (arrows) acrosome.

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Figure 4-12 (c): Shows a frozen thawed blue wildebeest sperm with no acrosome (dark or no staining pattern).

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figures, three staining patterns were observed. Figure 4-12 (a) shows intensely stained acrosomes. Fig. 4-12 (b) shows sperm with lightly stained acrosomes and fig. 4-12 (c) shows no staining (dark) of the acrosomal region.

4.5 Discussion

4.5.1 Species specific sperm motility changes after precooling

The data obtained in this study showed species related differences to sperm precooling. Sperm from impala showed a decrease in sperm viability. This was marked by the decrease in VCL, VSL, LIN, VAP, BCF, mnALH and mxALH. Sperm cooling and freezing subject spermatozoa to the combined effects of dilution, cooling, glycerolization, freezing and thawing. The net effect of these processes on sperm function is the loss of fertilising capacity. The latter has been found to be variable depending upon the species (Chauhan *et al.,* 1994). According to Chauhan *et al.* (1994), freezing of bull and buffalo semen led to a decline in ATP concentration as well as the adenylate pool and sperm motility. A positive correlation was observed between ATP concentration and the adenylate pool and with sperm motility (Von Prinzen, 1977; Foulkes and MacDonald, 1979; Kakar and Anand, 1984). According to White (1993), motility and metabolic activity of bull, boar and stallion are irreversibly depressed when exposed to cold shock by rapid cooling.

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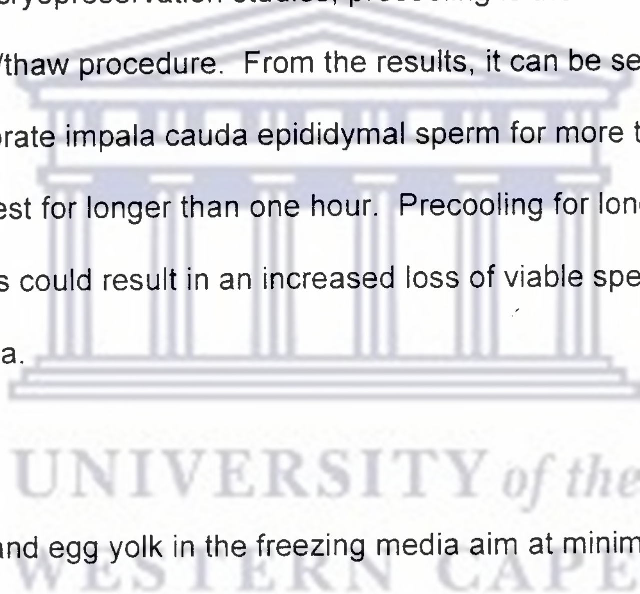
The data for this study further indicated that sperm obtained from impala and blue wildebeest behaved differently in the cryoprotectant medium (Triladyl) during precooling. The significance of this observation is that although these animals belong to the same family, Bovidae, interspecies specific differences were still found. For the impala, sperm motility for most parameters increased or decreased linearly over time. Sperm motility of blue wildebeest changed dramatically after one hour and recovered almost to the same level recorded at 1 hour. For sperm cryopreservation studies, precooling is the most important step in the total freeze/thaw procedure. From the results, it can be seen that there is no need to equilibrate impala cauda epididymal sperm for more than two hours and blue wildebeest for longer than one hour. Precooling for longer than the respective periods could result in an increased loss of viable sperm as indicated by the motility data.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Cryoprotectants and egg yolk in the freezing media aim at minimizing damage to cold shock and maintenance of membrane integrity and sperm viability. According to Kayser *et al.* (1992), damage to sperm from cooling can be classified into two types. These are: a). Direct chilling injury and b). Indirect or latent chilling injury. Briefly, direct chilling injury results in alteration in the plasma membrane and subsequent biochemical and metabolic changes that are immediately evident by altered sperm motion or biochemical test (Varner *et al.*, 1989). Latent damage is not detectable until hours after the cells have reached 0° to 5°C (Kayser *et al.*, 1992).

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4.5.2 Comparison of Fresh or non-precooled (NPC) versus precooled (PC) sperm

The aim of this part of the study was to compare the motility of fresh sperm with that of precooled (glycerolated) sperm at various time intervals. This part of the study investigated the impact of Triladyl, the commercially available cryodiluent for domestic cattle, on cauda epididymal sperm motility. The addition of a cryoprotectant like glycerol to the diluent is to protect the sperm against freeze damage. Other cryoprotectants used in sperm freezing are ethylene glycol, dimethyl sulphoxide (DMSO) and propylene glycol (Holt, 2000). These substances, in high concentrations, can be toxic to sperm. The results obtained in this investigation show that precooling has significantly affected sperm motility in both impala and blue wildebeest. When comparing fresh sperm (NPC) motility at three hours to precooled (PC) sperm motility at two hours in impala, VCL, mnALH, mxALH, MAD and DNCmn were higher in the PC than in NPC group. This could indicate that 7% glycerol and 20% egg yolk found in the cryodiluent (Triladyl) exert a protective and possibly a slightly stimulatory effect on impala epididymal sperm. Results for blue wildebeest showed that precooling sperm in Triladyl did not affect motility in general as indicated by motion parameters DNCmn, mnALH, mxALH and MAD (Fig 4-6) although parameters VCL, VSL, VAP, LIN and BCF were significantly lower in the PC group compared to the NPC group. Species specific differences in response to the cryodiluent exist and need to be taken into account in terms of sperm cryopreservation in order to increase the number of viable sperm.

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4.5.3 Sperm Freezing

Three blue wildebeest were selected for this study. Sperm motility measurements were performed on swim-up samples. Figures 4-9 shows histograms comparing motility data of fresh, precooled, immediately after thawing and swim-up sperm. The results obtained showed that swim-up sperm motility values were significantly higher than both precooled (IM) and postthaw (IM) sperm motility values. It appears from the data that the metabolism and motile architecture of some individual sperm were completely unaffected by freezing at -196°C. From the results, it can be seen that viable blue wildebeest cauda epididymal sperm were recovered after thawing. The sperm swim-up technique would appear to be a useful means of recovering motile sperm following freeze/thawing.

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Membrane integrity plays a significant role in sperm fertilizing ability. Check and Check (1991), cryopreserved sperm and then tested membrane integrity by means of the hypo-osmotic swelling test (HOS). Their results demonstrated that despite preserving reasonable motility after cryopreservation and thawing, the viability and membrane integrity of sperm may drop significantly below fertilising potential. Unfortunately, there was no clear relationship between reduced viability and HOS scores to pregnancy rates for the frozen-thawed sperm. The hypo-osmotic swelling test should provide information regarding sperm membrane integrity and the loss of key membrane components and cellular content. Kakar and Anand (1984) found a positive correlation between the leakage of

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hyaluronoglucosaminidase and acrosin and acrosomal damage. Because of ice crystal formation, damage to the sperm membrane will occur in a certain percentage of sperm which will affect the sperm's viability, motility and fertilising potential.

Howard *et al.* (1986), tested the influence of different cryoprotectants on the post-thaw viability and acrosomal integrity of sperm of the African Elephant (*Loxodonta africana*). Ejaculates were subjected to seven different cryoprotectants i.e. TEST, TRIS, HEPT, PDV-62, TRIL, BF5F and EQ (for explanation and composition of the various media, refer to Howard *et al.*, 1986). It is known that freezing protocols are species specific. Diluents such as TEST, TRIS, HEPT and TRIL were developed for cryopreserving bovine semen, whereas PDV-62 and EQ were formulated for freezing carnivore and equine semen, respectively. Diluent BF5F was developed for boar semen. Their results showed that diluent BF5F was superior to other cryoprotectants and could partly have been due to the presence of the surfactant triethanolamine lauryl sulfate (Howard *et al.*, 1986). Phillips and Lardy (1940) first reported that egg yolk protects sperm from damaging effects of rapid cooling or cold shock. They identified the low density lipoprotein fraction of egg yolk, specifically the phospholipids in the fraction that protects the sperm against cold shock. The susceptibility of sperm to cold shock is linked with a high ratio of unsaturated: saturated fatty acids in the phospholipids and a low membrane cholesterol content (White, 1993). According to White (1993), the high levels of unsaturated

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fatty acids in sperm also make them susceptible to damage from peroxidation which adversely affects motility, metabolism, ultrastructure and fertility. Hydroxynonenal, a product of fatty acid production, depresses the motility and oxygen uptake of ram sperm *in vitro* and may react with –SH groups of the axonemal microtubules. By lowering the environmental temperature, calcium uptake by sperm increases and this may cause a decreased motility due to "calcium intoxication" (Beorlegui *et al.*, 1995). Phospholipids, found in egg yolk, may provide some protection and prevent excessive increase of calcium flux into sperm. It appears that the swim-up method used after freeze/thawing in this investigation may act to wash out substances detrimental to sperm viability. Furthermore, sperm viability appears to be enhanced due to the physiological protective effects of the culture medium Ham's F10.

4.5.4 Transmission Electron microscopy (TEM)

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TEM results of freeze-thaw damage in blue wildebeest were similar to studies done by Aalseth and Saacke (1985). They found that membrane damage (tearing of acrosomal and plasma membranes) was evident and was the cause of decreased motility. Blue wildebeest therefore suffer similar forms of membrane damage following freeze/thawing to that described in other mammals (Leverage *et al.*, 1972)

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4.5.5 Live/dead status of sperm after freeze-thawing

The last part of this study involved the correlation of postthaw live-dead status with the percentage motile sperm. The method involved incubating sperm with a fluorescent probe, which has an affinity for acidic organelles in combination with the nuclear stain, Propidium Iodide (PI). The principle of the experiment was that sperm with intact membranes allowed the dye through, keeping it inside the cell and thus staining the organelles. Live sperm will then stain green under fluorescence conditions whereas sperm with destroyed cell membranes will show a red fluorescence because of the red dye's affinity for nuclear material as it is unhindered by the damaged plasma membrane. This method is, furthermore, effective in glycerol treated sperm as water soluble stains stain sperm with difficulty (Mixner and Saroff, 1954; Aalseth and Saacke, 1986). The author established that 34% of freeze/thaw blue wildebeest sperm was motile and 65% exhibited a red fluorescence. This indicated that virtually all immotile sperm had damaged cell membranes and were dead. Thomas *et al.* (1997) performed similar studies on cryopreserved bovine sperm using LYSO-G (Molecular Probes Inc) and found that the fluorescent dye is retained by the intact sperm membrane. This method provides a precise and reliable procedure for performing postthaw evaluation on cryopreserved sperm.

4.5.6 Lectin Binding

The aim of this part of the study was to determine whether sperm freezing has caused any loss or damage to the acrosome. From the sperm retrieved after

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swim-up, intact acrosomes were present. According to Thomas *et al.* (1997), this method can dually be employed with the SYBR-14 (Live/Dead) stain. It would be possible to isolate viable non-acrosome reacted/damaged sperm. The fluorescent staining method allows for the validation of a postthaw swim-up sample as well as the effect of the freeze/thaw protocol on sperm viability and membrane integrity. It also allows to distinguish between acrosome reacted/intact and damaged acrosomes and therefore the extent of freezing damage.

4.6 Conclusion

Cooling of sperm to 4°C has a species specific effect with respect to motility. The results show that precooling causes most sperm kinematic parameters to change over a period of 1 to 6 hours for impala and blue wildebeest. Precooling also altered most sperm motility parameters when compared to fresh sperm. The decrease in several kinematic parameters after cooling may be due to the toxic side effects of the cryodiluent, glycerol, that has been identified to have toxic side effects on sperm (Holt, 2000).

Generally, blue wildebeest sperm appeared to be more resistant to precooling than impala sperm thus highlighting differences in sperm physiology among species. The results obtained for the blue wildebeest cauda epididymal sperm freezing experiment showed that precooled sperm compared to sperm analysed

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immediately after thawing displayed similar motility characteristics. This emphasizes that precooling may effect sperm physiology to a greater extent than the freeze/thawing procedure. The swim up technique appeared to enhance sperm motility as indicated by selected CASA parameters. Sperm "washed" in a glycerol-free medium may have contributed in restoring sperm motility.

The use of fluorescent probes proved valuable in quantifying postthaw sperm live-dead status or sperm vitality. The dye, SYBR-14 can be used to identify live sperm with low motility or immotile sperm that are alive. Most immotile sperm in blue wildebeest appeared to be dead. Transmission electron microscopy highlighted sperm membrane damage, which appeared to be specifically related to freezing damage.

In conclusion, the results of this study show that the response of sperm to precooling in a glycerolated cryoprotectant diluent is species specific. This has both commercial and gene banking implications. In both cases, it is the number of viable and morphologically intact sperm that will have the greatest fertilizing capacity for the propagation of cryopreserved genes. It also appears that sperm from both impala and blue wildebeest responded well (as indicated by differences obtained in motion parameters) to a commercially available sperm cryodiluent (Triladyl) used in the domestic cattle industry. Due to the species specific differences obtained, further research into wild antelope cauda epididymal sperm may require modification of Triladyl and/or modifications of the protocol for sperm cryopreservation studies.

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CHAPTER 5

General Discussion and Conclusion

For this study, three wild antelope species (impala, blue wildebeest and the blesbok) were selected as a model for studying sperm morphology, sperm motility, and the effects of sperm cooling and cryopreservation on postmortem collected cauda epididymal sperm. Although some information on wild antelope reproduction is available, little is known about their sperm characteristics (Brown *et al.*, 1991; Ackerman, 1995). In this respect, the findings of this study show that there are interspecies differences in almost all the sperm parameters (morphology, motility and response to precooling) measured.

Main Findings of this study

Although all three species belong to the family Bovidae, sperm morphology differed among species regarding the shape of the head. The mammalian sperm head is characteristically flattened (Phillips and Dryden, 1991) and this was prominent in the three species. The sperm of impala, blesbok and blue wildebeest exhibited a dorso-ventrally flattened shape with an acrosome covering about two-thirds of the sperm head and an acrosomal ridge forming the anterior border of the acrosome. The degree of morphological variation in spermatozoa between different mammalian groups is great and a number of workers have used sperm morphology as a marker for phylogenetic and taxonomic studies (Harding *et al.*, 1981; Vitullo *et al.*, 1988; Breed and Inns, 1985; Feito and

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Gallardo, 1982). Impala and blue wildebeest epididymal sperm had a more elongated sperm head than that of blesbok, which was smaller and more rounded. These morphological traits were evident in both light and scanning electron micrographs. The data from head width-length ratios presented are indicative of species specific differences. This suggests that quantitative morphological analysis of normal sperm from impala, blesbok and blue wildebeest could be employed in further understanding the link between closely related species (van der Horst *et al*., 1991). From the morphological data obtained, it was clear that there is a greater similarity in sperm head features and dimensions between impala and blue wildebeest sperm. This was confirmed by sperm head length and width measurements. According to the species classification, the blue wildebeest and blesbok belong to the family Bovidae, subfamily Alcelaphinae whereas the impala also belong to the family Bovidae, but to the subfamily Aepycerotinae. It could therefore have been assumed that sperm morphological features would be similar in the blesbok and the blue wildebeest with impala being different to the other two.

Several abnormalities could be identified at the light microscope level and a more detailed description was possible at the scanning electron microscope level. A variety of head and tail defects were seen that were common among the impala, blue wildebeest and the blesbok. Head defects in bovine sperm have been associated with impaired fertility and sterility (Soley and Coubrough, 1981). Sperm malformations or abnormalities can either be of a primary or secondary

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origin (Pinart *et al.*, 1998). If the malformation occurs within the testis during spermiogenesis, it is considered primary, and secondary if the malformation originates or takes place in the epididymis during sperm maturation (Pinart *et al.* 1998). In the impala, immature sperm were seen as well as sperm with broken necks or non-inserted tails (Mortimer, 1994). Sperm with damaged acrosomes, a proximal cytoplasmic droplet and biflagellation strongly suggested abnormal spermiogenesis in the impala. Blesbok and blue wildebeest displayed similar abnormalities that relate to improper formation of the flagellum during sperm development in the testis. Blue wildebeest sperm revealed very clear surface detail for both the sperm head and the sperm tail. Abnormal sperm obtained from the blue wildebeest showed acrosomal lipping, possibly similar to that described by Coubrough and Soley as retrograde lipping in a subfertile bull, (1977) (TEM sections).. Other deformities with respect to folded tails were noticed in all three species. These were mostly Dag defects (Coubrough and Soley, 1981; Pinart *et al.*, 1998) as shown by folded or coiled tails encapsulated by a membranous sheath.

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Many of the sperm within a semen sample or aspirated epididymal sample are either abnormal or infertile due to various reasons ie primary or secondary type abnormalities. Vitality or live/dead staining methods proved to be the quickest method in assessing, quantitatively, the percentage of normal vs abnormal and live *vs* dead sperm within the population. The results obtained in this study show that there was very little change in the percentage live sperm over a period of six

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hours incubated in capacitating medium. Blue wildebeest had the highest percentage of live sperm after one hour and six hours. This is an indication of two inherent components of sperm, which are (i) susceptibility to the culture medium and (ii) the intact physiology of the sperm (Garner, 1997). The high percentage of viable sperm obtained in this investigation is promising in terms of applications in the field of ART (assisted reproductive technology).

Sperm Motility

For this study, the sperm collected post-mortem from the epididymides of the three antelope species, showed good motility and were incubated over a period of six hours in capacitating medium (Ham's F10 supplemented with 10%fetal calf serum). Sperm motility was quantitatively analysed by means of computer aided sperm motion analysis (CASA). CASA is routinely applied in human fertility laboratories and forms an integral part of assessing the patient's fertility status (Tessler and Olds-Clarke, 1985). Literature found, showed that sperm motility from routine semen evaluation in animal laboratories, were scored by rating sperm motility (Aalseth and Saacke, 1985; Foote and Arriola, 1987; Richardson *et al.*, 1992). Others have shown a positive correlation between sperm motility scoring and quantitative sperm motion analysis (Bornman *et al.*, 1994). With the aid of CASA, sperm swimming patterns can further be quantitated, making sperm motility scoring less subjective and also more descriptive with regards to small deviations in the swimming pattern. CASA has proved to be valuable in relating selected motility parameters with fertility outcome in humans (Oehninger *et al.*,

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2000). In this study, CASA was used to determine whether any species specific differences could be identified, based on selected sperm motility parameters. From the parameters calculated by the Sperm Motility Quantifier (S.M.Q.), it was evident that differences identified based on the change in motility patterns over the six hours incubation period in culture medium could be identified. The results showed that blesbok sperm performed differently and displayed different sperm motility characteristics from that seen in blue wildebeest and impala. One explanation may be that differences seen in sperm motion was a consequence of sperm morphological differences as indicated by different head length-width dimensions. This might not be the case as blesbok was the only one of the three species that displayed motility characteristics similar to that described as hyperactivation (Suarez *et al*., 1991; Tessler and Olds-Clarke, 1985; Suarez, 1988; Ahmad *et al*., 1995). Hyperactivation is a vigorous type of sperm swimming pattern necessary prior to fertilization characterised by a high velocity and low progressiveness or linearity (Suarez, 1996). This sperm motility pattern seen in the blesbok, which differed from the other two antelope species suggests inherent sperm physiological and sperm biochemical differences among the three species (Ahmad *et al*., 1995).

Blesbok sperm velocity and the mean amplitude of lateral head displacement were among the best kinematic parameters to demonstrate differences among the species. These differences in sperm motion parameters among the three species furthermore seem to relate sperm morphology to sperm motility.

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Impala, blesbok and blue wildebeest are all seasonal breeders. In this study, the impact of season on impala sperm motion parameters was investigated. Significant differences were found for the impala comparing samples collected during winter as opposed to samples collected during the summer period. Changes in sperm motility differed significantly for the two collection periods and could be ascribed to changes in endocrine function. Much work, with respect to the effects of seasonality on reproduction, has been done in impala (Brown *et al.*, 1991) and other species, including e.g. the goat (Roca *et al.*, 1992), langur monkey, *Presbytis entellus entellus* (Lohiya *et al.*, 1998), African buffalo, *Syncerus caffer* (Brown *et al.*, 1991) and crossbred rams from the United Arab Emirates (Ibrahim, 1997). All data collected from seasonal breeders show that their semen quality varies from one season to the next. The data found for this part of the study indicated similar trends in seasonality. The importance of this observation has practical implications with regards to sperm cryopreservation. For successful propagation of viable sperm, the prefreeze quality should be of a high standard which demands accurate knowledge of seasonal differences regarding sperm viability parameters.

It was found that the advantages of frozen sperm repositories offer easy access to good quality semen, interactive movement of biological material between living animal populations, thereby maximizing their genetic vigor, and they provide a high level of insurance against the loss of diversity or entire species (Wildt,

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1992). In the domestic livestock industry, semen collected from stud bulls by either electroejaculation or artificial vagina is used in sperm banking (Vishwanath and Shannon, 2000; DeJarnette *et al.*, 2000; Fabbrocini, 2000). According to Holt (2000), the success of bull sperm in reproductive technology has been unmatched in other species such as sheep, pigs and exotic species. Holt (2000), ascribes this to species differences with at least two sources of variation i.e. the physiology and biochemistry of the sperm and secondly, the anatomy and physiology of sperm transport in the female reproductive tract.

The most important step prior to sperm cryopreservation is sperm precooling in cryodiluent. The composition of the diluent is equally important, and only one cryodiluent was used in this study. For this study, the use of a standard protocol and standard bovine cryodiluent was to determine the effects of precooling on sperm motility in blue wildebeest and impala. These two species also had similar sperm morphologies and their motility patterns were similar when incubated over six hours although they belong to different subfamilies. From the results obtained, there were differences in the response of sperm to precooling, or probably to the cryodiluent. Precooling affected the sperm motility when compared to that of fresh sperm. Comparing the performance of the two species, blue wildebeest generally performed at higher velocities and probably higher energy expenditure (as indicated by mean and mxALH, but higher linearity). The results therefore indicate that the outcome of sperm motility to

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precooling has species specific implications and that protocol adjustments be made for each individual species.

Lastly, the effects of cryopreservation on motility and morphology of blue wildebeest sperm were evaluated by means of motility studies and transmission electron microscopy studies. According to the results obtained, postthaw sperm motility could be restored to near prefreeze (non precooled) values. The swim-up technique was used to separate viable from immotile sperm. Sperm membrane intactness was evaluated by means of the transmission electron microscope. Sperm with cryodamage had swollen or noticeable loss of membranes, although some membranes were completely intact, similar to that observed by Leverage *et al*., (1972). In this study, membrane integrity was confirmed with the aid of acidophillic fluoresecent dyes, detecting membrane intactness or damage (Thomas *et al*., 1997). Once membrane intactness was established, thawed sperm were investigated for the presence of the acrosome. Lectin binding experiments confirmed that the acrosomes of some of the sperm were present. The success of the freezing protocol selected will be measured by the postthaw viability of sperm in terms of good motility, membrane integrity and an intact acrosome (Holt, 2000; Richardson, 1992).

Conclusion

In conclusion, sperm from impala, blue wildebeest and blesbok show differences in terms of sperm morphology, motility and the response to cooling. Although,

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blesbok and blue wildebeest belong to the same subfamily and impala to a different subfamily, there were more similarities in terms of morphology and motility between impala and blue wildebeest sperm. It was also clear that the response to sperm "microenvironmental" conditions was different for each species, strongly suggesting species specificity. This information is of value in determining protocols for future investigations on antelope species by other researchers.

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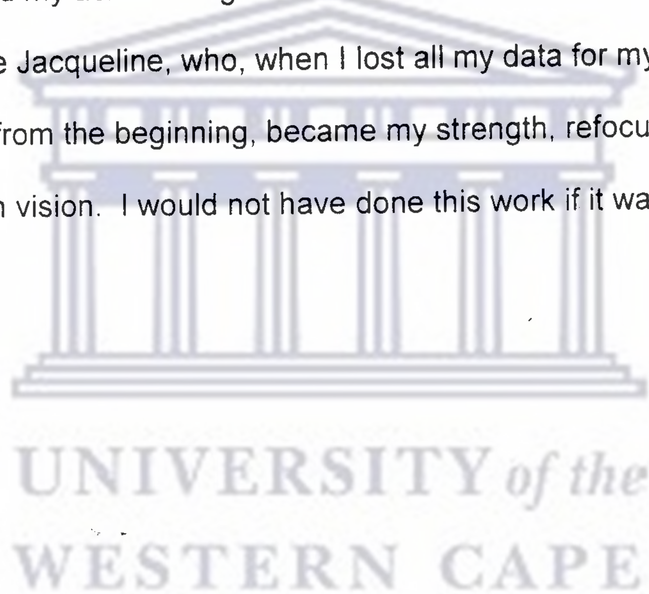

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