CHARACTERIZATION AND CRYOPRESERVATION OF SOUTH AFRICAN UNIMPROVED INDIGENOUS GOAT SEMEN

by

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- To the memory of my father (Thusani Matshaba).

- To my mother (Tebogo Mbenga), sisters (twins Hilda and Helen Mbenga) and brothers (Otsogile and Kesasobaka Mbenga), this is your little present from the first born in return for the love and encouragements during my stay in South Africa in search of a post graduate qualification.

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Declaration

I hereby declare that this dissertation submitted by me to the University of the Free State for the degree, **Magister Scientiae Agriculturae,** is my own independent work and has not previously submitted for a degree to any other university. I furthermore cede copyright of this thesis in favour of the University of the Free State.

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Bright Matshaba
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Chapter 1

General Introduction

South Africa's indigenous goat breeds can be divided into two main groups, namely the improved goats (Boer goat, Kalahari Red goat and Savannah goat) which benefitted from long-term concerted efforts of improvement through animal selection, mainly in South Africa (S.A), and the unimproved indigenous goats (small to medium frame goats from distinct ecotypes) (Campbell, 1995). The unimproved indigenous goats had their origin in the Eastern and Northern regions of Africa, migrated southwards during the great migration of the African tribes, reaching Southern Africa between 700 and 2000 AD (Ramsay *et al*., 2000). Different ecotypes currently prevail in Southern Africa, but these different goat breeds are more concentrated in the areas where the different ethnic groups (tribes) settled. The general appearances (phenotypes) of these indigenous goat breeds tend to support the theories that they originated in different ecosystems. However, the specific genotypes have not been accurately described (Ramsay *et al*., 2000). The unimproved indigenous goats are generically referred to as S.A veld goats. These include the smaller framed Zulu goats with pointed ears, from Kwazulu Natal, as well as the slightly larger Speckled lop-eared goats found in the Eastern Cape Province of South Africa. Other unimproved goat breeds are the medium framed Swazi goat, the small framed pointed eared goats found largely in the Northern Province of S.A and the small framed lop-eared goats found largely in the North West Province of S.A. Campbell (1995), sub-divided these unimproved indigenous goats into the Speckled goats (Eastern Cape), Loskop South indigenous goats (Xhosa goats of the Ciskei), Kwazulu Natal goats (Nguni goats), Delftzijl goats (Tropic of Capricorn) and the Damara goats (indigenous to Namibia and brought into South Africa).

Although the South African unimproved indigenous goats have been commonly farmed by the majority of the small scale farmers in the rural areas of South Africa for many years, where they play a very important socio-economic role, very little is actually known regarding this goat breed or group of breeds (Casey & Van Niekerk, 1988). This is because these animals have previously received little attention from researchers, but are currently enjoying increasing interest from commercial farmers (Campbell, 1995; Sundararaman & Edwin, 2008). This is due to these goats' hardiness and adaptability to the local harsh environmental

conditions, and its outstanding capacity to produce and reproduce efficiently under poor nutritional conditions (Devendra & Burns, 1970; Webb & Mamabolo, 2004). However, as the name portrays, very little animal improvement efforts have been made to improve this breed genetically, unlike the case of the other indigenous (improved) S.A breeds (e.g. Boer, Kalahari Red and Savannah goats), that enjoy international recognition and are currently being exported and found in many regions of the globe (Schwalbach & Greyling, 2000).

There is a need to study them and the potential to genetically improve these unimproved goat breeds through selection. According to Wildt (1992) this could be facilitated by the use of assisted reproductive technologies (ART's), like for example controlled breeding, artificial insemination (AI), and multiple ovulation and embryo transfer (MOET) programmes. The use of ART's could accelerate animal improvement, resulting in higher productive and reproductive performances by facilitating the widespread use of genetically superior bucks on a larger number of females than is possible when using conventional reproduction methods (Rahman *et al*., 2008). From all these and other more advanced technologies like *in vitro* oocyte maturation and fertilization. The use of AI combined with cryopreservation technology has greater potential to make the largest breeding impact in the shortest period of time, and make the best contribution towards the genetic improvement of the unimproved indigenous South African goat under the local conditions. However, the most important limitation to the widespread use of AI at this stage is the poor tolerance of buck semen to the cryopreservation process (Purdy, 2003). In addition, very little information is currently available regarding the basic semen characteristics of the South African unimproved indigenous goat and the ability of their semen to withstand existing cryopreservation protocols.

The aim of this study was therefore to characterize the semen of the South African indigenous unimproved goat, in order to generate baseline data and to test 4 different semen extenders for sperm cryopreservation (for subsequent use in AI), using a standard protocol for goat semen.

Chapter 2

Literature Review

2.1 Introduction

A specific challenge in the cryopreservation of goat semen seems to be the detrimental effects of seminal plasma on the viability of sperm in extenders containing egg yolk or milk (Zhao *et al*., 2008). This is due to an egg yolk coagulating enzyme (phospholipase A) that has harmful interactions with secretions of the bulbourethral gland, reducing the survival rates of the sperm after cryopreservation. This situation is however not observed with egg yolk in cattle seminal plasma and led to several attempts to develop alternative methods of freezing goat semen. Goat semen is currently centrifuged (washed) to eliminate the seminal plasma from the sperm prior to dilution with standard extenders containing egg yolk (e.g. Tris-egg yolk). There however seems to be no need for centrifugation or washing when low concentrations (2%) of egg yolk are used, but this may result in insufficient cryoprotection of the sperm membranes (Baldassarre & Karatzas, 2004).

2.2 Anatomy of the testis

Bearden *et al.* (2004) described the testes, as the primary organs of reproduction in males, as they produce both the male gametes (sperm) and male sex hormones (androgens). Unlike the female, in the male not all the potential gametes are present at birth. At puberty in the male, the germ cells located in the seminiferous tubules undergo continual cell division forming new sperm (spermatogenesis) throughout the normal reproductive life. The testes are covered and supported by the tunica vaginalis, a serous tissue, which is an extension of the peritoneum. This serous coat is established as the testes descend into the scrotum, and is attached along the line of the epididymis.

The outer layer of the testes as such, the *tunica albuginea* testis, is a thin white membrane of elastic connective tissue. Beneath the *tunica albuginea* testis is the parenchyma, the actual functional layer of the testes. Located within these segments of parenchymal tissue, are the seminiferous tubules. The seminiferous tubules originate from the primary sex cords and contain the germ cells (spermatogonia) and the nurse cells (Sertoli cells). These Sertoli cells are larger and less numerous than the spermatogonia. The tight junction at the basement membrane of the Sertoli cells forms the blood-testis barrier. Seminiferous tubules are thus the

site of sperm production and join a network of tubules - the *rete testis*, which connects to 12-15 small ducts, the *vasa efferentia*, which converge to the head of the *epididymis*. The Leydig (interstitial) cells are present in the parenchyma of the testes, between the seminiferous tubules (Senger, 2005).

The testes are enclosed in a two-lobed sac called the scrotum, located in the inguinal region between the hind legs and composed of an outer layer of thick skin, with numerous large sweat and sebaceous glands. This outer layer is also lined with a layer of smooth muscle fibres, the *tunica dartos*, which is interspersed between connective tissue. The *tunica dartos* divides the scrotum into two pouches, and is attached to the *tunica vaginalis* at the bottom of each pouch. The spermatic cord connects the testis to its life support mechanisms, the convoluted testicular arteries and surrounding venous plexus, and nerve trunks. In addition, the spermatic cord is composed of muscle fibres, connective tissue, and a portion of the *vas deferens*. Both the spermatic cord and scrotum contribute to the physical support of the testes and have a joint function in regulating the temperature of the testes. Development of testicular function is essential for the changes observed as puberty approaches (Hafez & Hafez, 2000).

2.3 Puberty

Puberty can be defined as the first mount and/or ejaculation with the release of sperm in males (Delgadillo *et al*., 2007). If defined as the time when fertile sperm are recorded in the ejaculate, the age should be 3 to 5 months in bucks. The sexual development is regulated by the endocrine system and several months before the onset of puberty, pulsatile discharges of LH commence, resulting in the differentiation of the Leydig cells. FSH may synergize in this action, by helping up-regulate the receptors for LH in the Leydig cells. The differentiation of the Leydig cells initially secretes the androgen, androstenedione. As differentiation continues, LH stimulates the production of increasing concentrations of testosterone, which in turn stimulates most other changes associated with approaching puberty (Bearden *et al*., 2004).

2.4 Spermatogenesis

According to Bearden *et al*. (2004) spermatogenesis in farm animals is the process of division and differentiation by which sperm are produced in the seminiferous tubules of the testes and consists of two phases: spermatocytogenesis and spermiogenesis. After formation in the seminiferous tubules, sperm will be forced through the *rete testis* and *vasa efferentia* into the

epididymis, where the sperm are stored while undergoing maturation changes to make the sperm capable of fertilization. From puberty, spermatogenesis will continue as an ongoing process, throughout the life of the male. Spermatocytogenesis involves the mitotic cell division, which results in the production of stem cells and primary spermatocytes, while spermiogenesis is the maturation and formation of sperm (Bester, 2006).

Meiosis during spermiogenesis is a process involving two cell divisions, resulting in spermatids containing a haploid number of chromosomes. Each primary spermatocyte first undergoes a meiotic division, forming two secondary spermatocytes. In this division, the chromosome complement in the nucleus is reduced by half so that the nuclei in secondary spermatocytes contain an unpaired (n) number of chromosomes. Spermiogenesis is then the differentiation of spermatids, which are released as sperm. Spermatids with spherical nuclei differentiate into sperm, and are released from the Sertoli cells into the lumen of the seminiferous tubules. Spermiogenesis is thus the process during which a haploid spermatid undergoes a metamorphosis (change in morphology) to form a mature elongated spermatid or sperm. The number of Sertoli and Leydig cells is related to sperm production, each Sertoli cell supporting a defined number of germ cells. The entire process of spermatogenesis will be complete in 46 to 51 days (Figure 2.1). Spermatogenesis can thus be summarized as follows (Sobti, 2008):

a. An A_2 spermatogonium divides by mitosis, forming an active spermatogonium (A_3) and a dormant spermatogonium (A_1)
b. The active spermatogonium undergoes 4 mitotic divisions, forming 16 primary spermatocytes.
c. Each primary spermatocytes will undergo two meiotic divisions, forming 4 spermatids (a generation of 64 spermatids from the A_3 spermatogonium)
d. The dormant spermatogonium (A_1) will later divide to yield A_2 spermatogonia, which through mitosis form new active (A_3) and new dormant (A_1) spermatogonia.
e. Each spermatid will undergo metamorphosis to form a sperm.

Figure 2.1 depicts a schematic representation of spermatogenesis

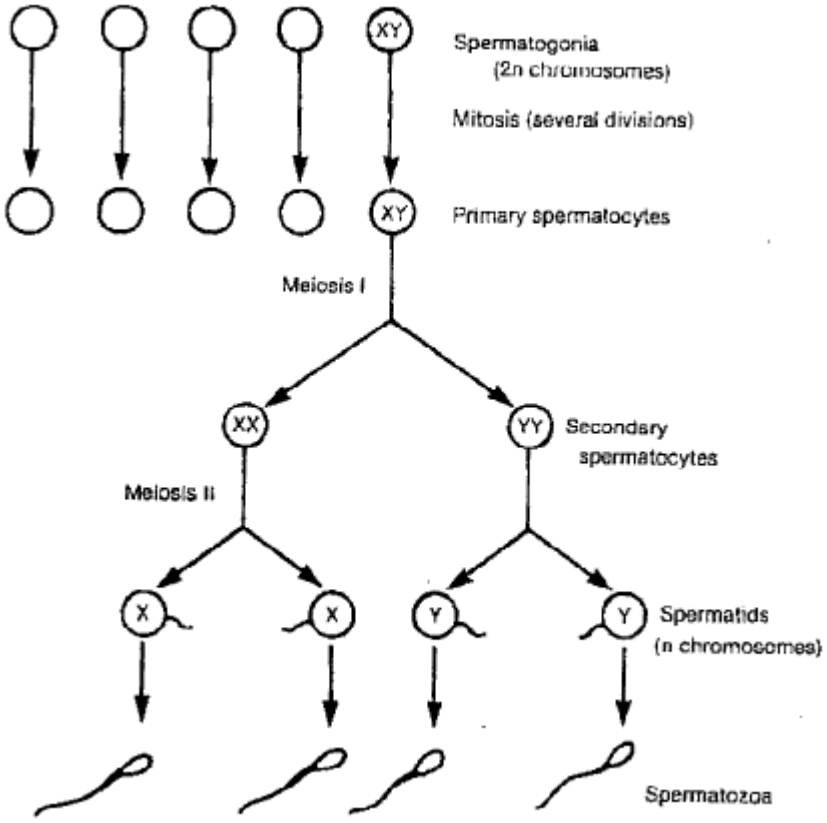

Figure 2.1 Schematic representation of spermatogenesis (Evans & Maxwell, 1987)

2.5 Hormonal control of spermatogenesis

The concerted action of FSH, LH and testosterone is necessary for the maintenance of spermatogenesis.

2.5.1 Follicle Stimulating Hormone (FSH) or Spermatogenesis Stimulating Hormone (SSH)

This hormone (FSH) is also known as spermatogenesis stimulating hormone (SSH). The FSH, together with testosterone, stimulates spermatogenesis in the seminiferous tubules of the testes. Its levels in the male are regulated. The hormone inhibin, which is a protein secreted by the testes, inhibits FSH production by the anterior pituitary, thereby inhibiting FSH secretion and spermatogenesis (Hafez & Hafez, 2000).

2.5.2 Luteinizing Hormone (LH) or Interstitial Cell Stimulating Hormone (ICSH)

In the male, this hormone (LH) is also known as interstitial cell stimulating hormone (ICSH). The LH stimulates the Leydig cells or interstitial cells of the testes (located outside the seminiferous tubules) to produce testosterone. LH is carried from the anterior pituitary by the blood to the interstitial cells (Hafez & Hafez, 2000).

2.5.3 The male sex hormone - testosterone

Testosterone is an androgenic steroid hormone produced by the interstitial cells or Leydig cells, richly supplied with nerves. Testosterone secretion is under endocrine control by a negative-feedback mechanism involving the hypothalamus and anterior pituitary. Low levels of testosterone naturally stimulate the hypothalamus to release GnRH, which in turn is carried by the portal system to the anterior pituitary, where it stimulates the release of LH by the anterior pituitary. The LH then stimulates the Leydig cells in the testes to produce more testosterone. When testosterone levels get too high, the hypothalamus and anterior pituitary are inhibited, and secretion of GnRH and LH suppressed (Sobti, 2008).

2.6 Stimulus for gonadotrophin secretion in the buck

Without GnRH stimulation, the anterior pituitary will not release the two gonadotrophic hormones, FSH and LH. These hormones are necessary for the maturation of the testes and sperm production. It is thought that at the time of puberty the hypothalamus begins to respond to the low levels of circulating testosterone by releasing large amounts of GnRH, which are carried by the portal system to the anterior pituitary, which in turn stimulates the release of LH and FSH (Sobti, 2008).

2.7 Factors affecting semen production and quality

Goats are generally seasonal breeders hence their reproductive activity, semen quantity and quality are affected by seasonality (Zarazaga *et al.*, 2009). Goats successfully mate naturally during autumn when day light length is shorter (short day breeders) allowing for birth at the following spring, optimal for the survival of the young in terms of temperature, feed and water availability. In bucks, a decrease in quantitative and qualitative semen production and sperm fertility during the non-breeding season has been reported (Corteel, 1977). Delgadillo *et al*. (1993) suggested photoperiod or season to be the principal factor influencing seasonality of reproduction in bucks. Other environmental factors such as social stimuli, feed availability and social interaction are also regarded as important regulators of seasonality in

reproduction. Nutrition has been considered an important factor affecting the seasonality of reproduction by Walkden-Brown *et al*. (1994)

2.8 Semen collection

Semen collection is like harvesting any farm crop, as it involves obtaining semen with the maximum number of sperm, of the highest possible quality in each ejaculate (Bester, 2006). It has been recommended that semen collections are performed during autumn/early spring (natural breeding season), to obtain the best quality semen sample for processing and storing. Semen consists of sperm plus secretions from the testis, epididymis, prostate, seminal vesicles and bulbourethral (Cowpers) gland.

2.9 Semen collection techniques

Goat semen is generally collected with the aid of the artificial vagina (AV) or by electrical stimulation (Sobti, 2008).

2.9.1 Electro-ejaculation (EE)

The ejaculation of semen is brought about by inserting a probe or electrode into the rectum of the male and stimulating the nerves of the reproductive system by gradually increasing the electrical current in a rhythmic fashion, for a short period of time. Successful use of this technique requires skill, experience, patience and the knowledge of the individual requirements for stimulation by the male. At present, there are a number of electro-ejaculators available which are either operated by only a battery or a choice of battery or using electrical current. The method of EE is used on males of certain species where the use of the artificial vagina is not possible or impractical. Concern has been expressed regarding animal welfare in the use of the electro-ejaculator (EE) as a semen collection technique (Ortiz-de-Montellano *et al*., 2007). However, this remains the most commonly used technique, particularly for untrained males.

2.9.2 Artificial vagina (AV)

An artificial vagina is a device designed to simulate the female reproductive tract (Donovan *et al.,* 2001).

Plate 2.1 Artificial vagina consisting of a plastic cylinder, rubber cylinder, graduated collecting tube and KY jelly (lubricant)

Prior to collection all parts of the AV should be clean, sterilized and properly assembled. Briefly, an inner rubber sleeve is put into the outer hard cylinder of the AV and both ends of the inner sleeves are deflected over the cylinder, forming a watertight space. Water at a temperature of 43 to 46°C is filled in the space between the sleeve and cylinder before sealing with a rubber stopper. The open side is then lubricated with a small amount of sterile jelly (K.Y. Jelly). The temperature of the AV is very important and should always be checked before attempting semen collection (Silvestre *et al.*, 2004).

The modern AV types are also provided with an air screw, along with the water screw which can be used for blowing air between the two layers to regulate and obtain the desired pressure. The temperature of this AV is equally important and should always be checked before attempting the collection (Bester, 2006).

The warm water in the AV simulates the thermal and mechanical (pressure) stimulation of the vagina over the *glans penis,* necessary to induce ejaculation of the buck. The AV method of collecting semen resembles natural service and is the natural, fastest and most hygienic of the methods available, but requires training of the buck (Wulster-Radcliffe *et al*., 2001).

2.9.2.1 Training of bucks for semen collection with the artificial vagina

Bucks are trained for semen collection using a doe in oestrus as a teaser female. Females are restrained in a stanchion in a neck clamp before the introduction of the buck into the test arena or pen (Silvestre *et al.*, 2004; Bester, 2006). In order to increase the sex drive or libido of the males, the test arena (pen) should be adjacent to the male pen. Bucks must be able to

see the other males mounting the restrained doe (Price *et al*., 1984, Silvestre *et al.,* 2004). Bucks are generally allowed a 5 min interval to ejaculate. After ejaculation or a period of 5 min, whichever occurs first, the male should be removed from the test pen to the adjacent one. After 10 to 15 min, males should be again placed in the test arena. Training is considered completed when males mount and ejaculate regularly when presented with a female teaser (in oestrus or not) in the presence of a collector (Silvestre *et al*., 2004).

2.10 Process of semen collection and transportation to the laboratory

During the semen collection process, when the buck mounts a doe, the penis is gently guided into the AV. Immediately following collection, the ejaculate is transferred to 15 ml test-tube placed in a flask at 37 °C, until semen assessment (Silvestre *et al.,* 2004). It is recommended that the same technician oversees the semen collection process. This should always be performed at the same time and under the same conditions to minimize stress and maximize the quality of the semen (Silvestre *et al.,* 2004; Siudzińska & Łukaszewicz, 2008). Semen concentration, pH and sperm motility should then assessed in the laboratory within 1h after collection (Gacitua & Arav, 2005).

2.10.1 Semen evaluation

Evaluation of semen is a standard practice for evaluating the potential fertility of breeding males, other than directly evaluating their ability to produce progeny. There are many different methods of assessing semen quality and estimating the fertilizing potential of sperm. Some of these techniques are regarded as highly subjective, while others require special laboratory equipment and skills. The quantitative and qualitative characteristics of the semen evaluated include the sperm viability, motility and, as well as the morphology of the sperm. No single semen characteristic can accurately predict the fertility of the semen sample, however by examining various physical characteristics one can estimate the potential fertility (Hafez & Hafez, 2000). The complexity and sensitivity of the sperm cell hinders the goal of researchers to find laboratory assays that could accurately predict the fertilising capacity of a semen sample (Graham & Mocé, 2005).

2.10.1.1 Semen evaluation techniques

Laboratory semen evaluation assays can be classified in several ways. One major difference is between direct and indirect assays. Direct assays evaluate the actual cells individually, while indirect assays measure a component of the entire sample, e.g. the amount of an

enzyme released from the entire semen sample (Graham *et al*., 1980). Although both types of assays can delineate important attributes of the semen sample, this study will focus mainly on the direct assay. Within the category of direct assays, sub-categories of these assays are generally distinguished in which semen is evaluated using manual or automated techniques (Graham & Mocé, 2005). The importance of laboratory semen assays lies in that these help to eliminate poor semen samples from being used in AI and determine which sperm defects are present in semen samples of poor fertility. This is then done by using either manual and/or automated methods (Graham & Mocé, 2005; Mocé & Graham, 2008).

Fresh unstained sperm are generally examined microscopically, to determine the percentage of motile (live) sperm in a semen sample. These estimations can include both the percentage motile cells, as well as progressive motile sperm cells (Graham & Mocé, 2005).

2.10.1.2 Manual/visual sperm analysis

Manual/visual microscopic sperm analyses are conducted by placing a sample of the semen on a microscope slide and visually evaluating it, using specific criteria. These tests use either fresh or fixed, stained or unstained semen and remain the mainstay of the assays conducted by most laboratories. The major limitations of manual analyses are that they can be influenced by human bias (Graham *et al*., 1980), as well as being a time consuming process, either in the semen sample preparation or during the analysis itself, resulting in relatively few (usually ≤ 200) sperm being evaluated per ejaculate (Graham & Mocé, 2005).

2.10.1.3 Automatic sperm analysis procedures

The alternative to manual analyses is to use automated methods, such as the computer automated semen analyzer (CASA) system, which permits the evaluation of sperm motility in a relatively non-biased manner. Computer-assisted sperm analysis is a powerful tool for the objective assessment of sperm motility and the CASA technique has been used to provide precise and accurate information on sperm motion characteristics (Sundaraman & Edwin, 2008). This system utilizes computer technology to simultaneously track individual sperm cells while evaluating various parameters of their movements (e.g. direction, velocity, angle of curvature between the head and tail) and can provide a much more accurate, objective and repeatable measure of both sperm count and motility. CASA provides precise and useful information regarding various sperm motion characteristics like progressive sperm motility, path velocity, progressive velocity, track speed, amplitude and lateral sperm head

displacement and beat cross frequency. In addition, some CASA systems are equipped with the capabilities of evaluating the morphology of the sperm. CASA also yields benefits in terms of accuracy in the semen evaluation, reduction of sperm wastage and time saving in the long term.

2.10.1.4 Definition of sperm motility characteristics

Total sperm motility is generally defined as the ratio of motile cells to the total cell population, expressed as a percentage. Progressive sperm motility (PM) is the number of sperm cells moving in a forward and in a straight-line direction. Straight-line/progressive velocity (VSL) is the velocity on a straight-line distance between the beginning and the end of the track. Curvilinear velocity (VCL) is the velocity over the actual sperm track, which include all movement directions of the sperm. Average path velocity (VAP) is seen as the velocity over a calculated, smoothed path, while straightness (STR) measures the departure of the cell path from a straight line (ratio of VSL/VAP). Linearity (LIN) measures the departure of the sperm cell track from a straight line (ratio of VSL/VCL). The amplitude of lateral head displacement (ALH) is the average time of absolute values in the instantaneous turning angle of the sperm head, along the curvilinear trajectory. Beat-cross frequency (BCF) is the frequency with which the sperm track crosses the smoothed path (King *et al*., 2000; Kozdrowski *et al*., 2007; Sundararaman & Edwin, 2008). ALH reflects the ability of the sperm to penetrate mucus in the uterine cervix and to unite with the oocyte, while VAP, VSL, STR and LIN characterize the velocity of the sperm and are correlated with the fertilizing ability of sperm (Verstegen *et al*., 2002)

Flow cytometry is a powerful tool for evaluating sperm cells. It utilizes technologies that force individual sperm into a confined stream that passes through a laser beam. If the cells have been previously stained, with fluorescent dyes, these cells will fluoresce, and the light from each individual cell can be detected by photomultiplier tubes contained within the equipment. The power of this technology is that approximately 50 000 sperm cells can be counted in a minute. Several different types of dyes can be added to cells at the same time, so that cells can be evaluated for different parameters simultaneously. The staining techniques are all very simple and rapid, air dried sperm cells fixed in a fluorescent fixative or live sperm cells can be recorded and fluorescent probes are currently available to evaluate nearly any cell attribute which one would wish to measure (Ormerod, 2000).

2.10.2 Semen colour and volume

Normal buck semen is greyish white to yellow in colour and varies between bucks and ejaculates of the same buck. Positive correlations have been found between semen volume and semen concentration in bulls (Sarder, 2008). The dense colour (greyish white) indicates a high sperm concentration, with the less dense colour (yellow colour) indicating low sperm concentration. The presence of blood in the semen is indicated by a pink colour of the semen (contamination) and can be due to injury or disease of the penis or reproductive tract. Contaminated semen samples should be discarded (Bester, 2006).

The average ejaculate volume of a buck is 1.0 ml with a range of between 0.5 and 1.2 ml (Hafez & Hafez, 2000). The mean ejaculate volume of the West African Dwarf goat and the Markhoz goat has been found to range from 0.38 ± 0.07 to 0.44 ± 0.07 ml and 0.6 ± 0.03 to 1.2 ± 0.06, respectively (Oyeyemi *et al*., 2000; Talebi *et al*., 2009). The volume as such is determined not only for use in processing of the semen sample, but also to establish the semen production of an individual male. Deviations from the normal distribution, particularly decreasing trends in volume, may indicate a problem due to health factors, or be an indication that the collection procedures for that particular male need to be revised (Bearden *et al*., 2004).

2.10.3 Semen pH

The pH of semen indicates the acidity or alkalinity of the semen sample. Normally, the pH of semen is alkaline because of the secretions of the seminal vesicles (accessory gland). An alkaline pH protects the sperm from the acidity of the vaginal fluid, while an acidic pH indicates problems regarding seminal vesicle function. A pH value outside of the normal pH range (7.2-7.8) is normally harmful to the sperm. The semen pH can be measured using pH-indicator strips, by placing a drop of fresh semen onto the strip and the resultant colour being compared to the colours on a graduated pH scale (Essig, 2007).

2.10.4 Sperm motility

The rate of sperm motility has been defined as the speed at which sperm travels (Gil *et al*., 2001). A percentage of live sperm can be estimated according to their motility (Björndahl, *et al*., 2004). Kozdrowski *et al*. (2007) regard the motility of sperm as one of the most important indicators of the semen quality assessment. Cox *et al*. (2006), reported sperm motility to be related to the migration efficiency of sperm in the cervical mucus (in vitro) or sperm

concentration at the utero-tubal junction and the in vivo fertilization performance of goat sperm. The ability of sperm to migrate through the female genital tract and penetrate or fertilize the oocyte thus depends on the hydrodynamic potential exerted by the flagella bending and the resistance exerted by the secretions present in the lumen of the genital tract. Different rates in the transport of sperm are mainly based on the kinematic properties that define the propulsive strength (Katz *et al*., 1990). Hafez and Hafez (2000) reported sperm motility evaluation to involve a subjective estimation of the viability of the sperm and their motility. Sperm motility is commonly believed to be one of the most important characteristics used when evaluating the fertility potential of ejaculated sperm (Hashida & Abdullah, 2003). It has also been stated that the sperm motility characteristics of goat semen can be useful in the selection and ranking of bucks regarding their potential fertility. Mocé and Graham (2008) referred to this visual estimation of the percentage of motile sperm in a semen sample as the most general laboratory semen assay performed. Sperm motility in general and the characteristics of the sperm motion in particular, could be indicators of sperm quality (Sundaraman & Edwin, 2008). This method of sperm motility is very useful, although it evaluates only one important sperm attribute and it can be accepted to be subjected to human-bias.

It is accepted that sperm motility is extremely susceptible to environmental changes (e.g. excessive warm or cold ambient temperatures) – thus it is essential to protect the ejaculated semen from harmful agents or conditions prior to evaluation. An experienced technician and a properly equipped laboratory are essential for a reliable estimation of the semen motility (Bester, 2006). At present the objective assessment of sperm motility is possible with computer analyses (CASA), which considers many motility properties (Verstegen *et al*., 2002; Klimowicz *et al*., 2008). However, this equipment is expensive and is generally not used in routine semen evaluation procedures. The rate of sperm motility in commercial operations is frequently assessed subjectively on a scale of 1 to 5. This can be done as accurately as when estimating the percentage of motile sperm, but has little value for evaluating semen quality. During the subjective measurement of sperm motility a cell is generally considered to be motile if its tail is moving, i.e. even if it does not demonstrate progressive movement. Another problem is the overestimation of the subjectively evaluated sperm motility due to a high sperm concentration and sperm speed. The CASA system however eliminates these human errors. This is why CASA instruments generally report

lower motility values for mass and progressive motility, than the visual estimates (Klimowicz *et al*., 2008).

The longevity of sperm motility in a fresh semen sample (room temperature of 20 to 25°C), and in extended semen (room temperature, or refrigerated temperatures of – 4 to 6°C) include the parameters of sperm motility considered by Hafez and Hafez (2000). Post-thaw sperm motility is determined by using a phase-contrast microscope (x400) on a warm stage (38°C). A threshold of 50% post-thaw sperm motility is accepted as the industry standard for frozen semen, post-thaw, suitable for AI (Gil *et al*., 2001).

It is recommended that individual sperm motility should be evaluated in at least 200 individual sperm to give a reliable average result. The score and criteria used include the following (Loskutoff & Chrichton, 2001):
0 = no sperm movement
1 = head movement only (no forward sperm progression)
2 = slow forward sperm progression (usually with laboured head movement)
3 = fast forward sperm progression
4 = faster forward sperm progression
5 = fastest, linear forward sperm movement

2.10.4.1 Progressive sperm motility

The motility of a semen sample is generally expressed as the percentage of cells mobile under their own power. Semen quality is then monitored by evaluating the progressive motile sperm (Sundaraman & Edwin, 2008). The progressive motile sperm are those cells that are moving or progressing from one point to another, in a more or less straight line. Other types of motility include circular and reverse movements occurring due to sperm tail abnormalities and a vibrating or rocking movement that is often associated with the ageing of the sperm cell. Progressive motility is the most important individual sperm quality test, as fertility is highly correlated with the number of progressive motile sperm inseminated. The percentage motility of a semen ejaculate can range from 0% to 80% (Bearden *et al*., 2004).

2.10.5 Semen concentration (sperm density)

Semen concentration is expressed as the number of sperm cells per ml and must be known for each ejaculate to be used in order to maximize the number of AI units containing a given

number of motile sperm per unit (AI dose)**.** Sperm concentration is positively correlated with fertilization rate, although higher sperm concentrations definitely enhance of the success rate for embryonic development (Brown & Knouse, 1973; Wolf & Inoue, 2005). Garner *et al*. (2001) reported that contrast analyses showed sperm viability to be significantly decreased as the sperm concentration decreased. Normally buck semen concentration ranges between 1326.3 ± 335.3 and 1744.3 ± 459.6 x 10^6 sperm/ml for Boer goat bucks (Almeida *et al*., 2007) or 2.5 x 10^9 to 5.0 x 10^9 sperm/ml for goat breeds in general (Hafez & Hafez, 2000). The haemocytometer is generally used for exact cell counts. This entails a microscope slide calibrated with precise volume chambers. A semen sample of the ejaculate is diluted at a fixed rate with water (to kill the sperm cells) and thus render them immobile. The number of sperm cells in a chamber are counted under the microscope and multiplied by the dilution factor used. This is a very accurate technique, although very time consuming (Loskutoff & Crichton, 2001).

There exist other methods to determine the sperm density of a sample like the spectrophotometric or colorimetric method. The advantage of these methods is that they are accurate and fast to implement. Photometers as such are however not accurate when using contaminated semen samples, and the addition of cloudy extenders prior to determination of sperm concentration can also influence the results obtained (Hafez & Hafez, 2000).

The Spermacue™ (Minitüb, Germany) is a small compact and accurate photometer developed for measuring semen concentration. It can be calibrated for bovine, canine, equine, porcine, and small ruminant species. Cleaning the apparatus is simple, and maintenance is minimal. Spermacue™ is generally accepted as the preferred photometer in animal reproduction laboratories around the world (*http//www.minitube.com*). It determines semen concentration in both raw and diluted semen samples and requires <10µl raw ejaculate in the disposable micro-cuvette. The self-loading micro-cuvette also ensures accurate sample volume. It has a LED light source for stable calibration and the digital display shows the semen concentration in million sperm/ml and the final reading is based on the average of multiple readings. The machine automatically resets to zero after each sample. It is 110V or battery operated, making it portable (Rigby *et al*., 2001)

2.10.6 Sperm morphology

Sperm morphology seems to be one of the most important qualitative characteristics of semen and can also serve as an indicator of some disorders in the process of spermatogenesis (Kuster *et al*., 2004). Morphologic assessment of the sperm is an integral component in the analysis of semen and is an important part of any breeding buck soundness examination (Kuster *et al*., 2004). Sperm abnormalities have been found to negatively affect the motility, the survival and fertilization rates in several species. Although general classification systems for the morphology of sperm from different species have been reported, the classification categories are different for the various species and the adoption of a uniform system within each species is necessary (Graham & Mocé, 2005). Several dye exclusion techniques have been developed over time to distinguish between live and immotile sperm or dead sperm. The underlying principle in which these techniques are based on, is that sperm with structurally intact cell membranes (supposedly live sperm) are not stained and therefore do not absorb the stain, while dead sperm with disintegrating cell membranes, absorb the stain (Björndahl *et al*., 2004). The results of a semen morphology evaluation are generally recorded as the percentage normal and abnormal sperm, with an indication of the type of abnormality. It is normal that some sperm from an ejaculate are morphologically abnormal, but when this percentage becomes excessive, the fertilization rate may decrease (Sarder, 2008).

Differential semen staining: Eosin is referred to as a differential stain, as it cannot pass through living cell membranes. A background stain such as nigrosin, opal blue or fast blue provides a good contrast making the unstained sperm heads more visible. The partial stained and totally stained sperm then represent the dead cells, whereas the unstained sperm represent the live cells (Bearden *et al.*, 2004). The eosin-nigrosin stain is commonly used in the laboratory, where it also allows the analysis of the sperm structure. It is an effective and simple technique of staining, in addition to allowing sperm to be readily visualized. It is also the so-called “live-dead” stain, allowing assessment of membrane integrity at the same time as the morphology (Björndahl *et al*., 2003). The stain produces a dark background in which the sperm stand out as light coloured cells. Live sperm exclude the eosin stain and appear white in colour, whereas “dead” sperm (those with loss of membrane integrity) take up the eosin and appear pinkish in colour. These live sperm can then be categorized into different forms, for example morphological normal, or with defects in the acrosome (crooked or loose). The sperm head (bulb, small, enlarged, looped), the sperm neck (broken at different angle in relation to head), mid-piece and sperm tail (swelling, looping, partial or totally

lacking) are other abnormalities (Łukaszewicz *et al*., 2008). Success in the evaluation of sperm morphology also depends on the stain preparation techniques, stain type and staining methods (Bilgili *et al*., 1985)

Sperm abnormalities are generally divided into primary and secondary abnormalities or in some classification systems major and minor abnormalities (Kebede *et al*., 2007). These classification systems utilizing major and minor categories put less emphasis on where defect arises, but emphasizes their overall effect on subsequent fertility. Therefore, major sperm defects are those associated with impaired fertility, while minor defects have less effect on the fertility rate and can usually be compensated for by adding more sperm cells to the insemination dose (Chenoweth, 2005). It is unlikely that cryopreservation induces major changes in the morphology of sperm (Graham & Mocé, 2005), although poor handling techniques or sub-optimal cooling and freezing conditions may induce irreversible changes such as acrosomal damage or reflex of the sperm tail (Saacke, 2000). Abnormal sperm may also be classified into the following 5 categories: Loose sperm heads, abnormal sperm heads, abnormal sperm tail formations, abnormal sperm formations, and abnormal sperm tail formations with a distal cytoplasmic droplet (Hafez & Hafez, 2000). Loskutoff and Crichton (2001) classify sperm abnormalities as follows:

Primary sperm abnormalities (those occurring during spermatogenesis in the seminiferous epithelium of the testis). These primary defects are more severe than secondary or tertiary abnormalities and include the following:

Sperm head:

- Microcephalic (small heads). Macrocephalic (large/swollen heads), Double heads
- Abnormal acromosomes

Mid-piece of the sperm cell:

- Swollen, elongated, abaxial

Tail of the sperm cell:

- Double, short tails

Secondary sperm abnormalities (those occurring during maturation in the epididymis or after detachment from the seminiferous tubule). These are considered less serious. These abnormalities include the following:

Sperm head:

- Detached, loose/damaged acrosomes

Mid-piece of the sperm cell:

- Bent, protoplasmic droplets

Tail of the sperm cell:

- Bent, shoe-hook, protoplasmic droplets

Tertiary sperm abnormalities (those resulting from poor handling of the semen – post-ejaculation or consequence of the environment) and finally include the following:

- Reacted (dead sperm) acromosomes (due to cold shock or exposure to ultra violet rays/light
- Coiled sperm tails (resulting from non-iso-osmotic solution)

Primary and secondary sperm abnormalities have been negatively correlated to fertility (Saacke & White, 1972). A schematic representation of these abnormalities, adopted from Loskutoff and Crichton (2001) are outlined on the next page in Figure 2.2.

2.11 Semen quality

Yamashiro *et al*. (2006) considered a buck semen sample of 0.75 ml ejaculate volume, a sperm motility of more than 80% and concentration of more than 3 x 10^9 sperm/ml to be intrinsically of high quality. The limiting factor in the semen fertility is the inability of a single sperm to penetrate the *zona pellucida* of the ova. The quality of stored semen is then often affected by the handling procedures such as e.g. dilution, centrifugation, dilution in semen extender and freezing technique of the semen (Bustamante Filho *et al*, 2009).

2.11.1 Post-ejaculation viability of sperm cells and sperm preservation

Sperm viability generally refers to its capacity to remain alive (the number of live sperm divided by the total sperm population), while sperm preservation refers to the viable storage of semen for extended periods of time (Holman, 2009).

2.11.2 Factors affecting the viability of post-ejaculation sperm cells

Temperature, pH, osmotic pressure, sperm concentration, hormones, gases and light are factors affecting the rate of sperm cell metabolism, the rate at which the sperm can convert and utilize the energy substrates of the seminal plasma to remain active and alive (Bearden *et al*., 2004).

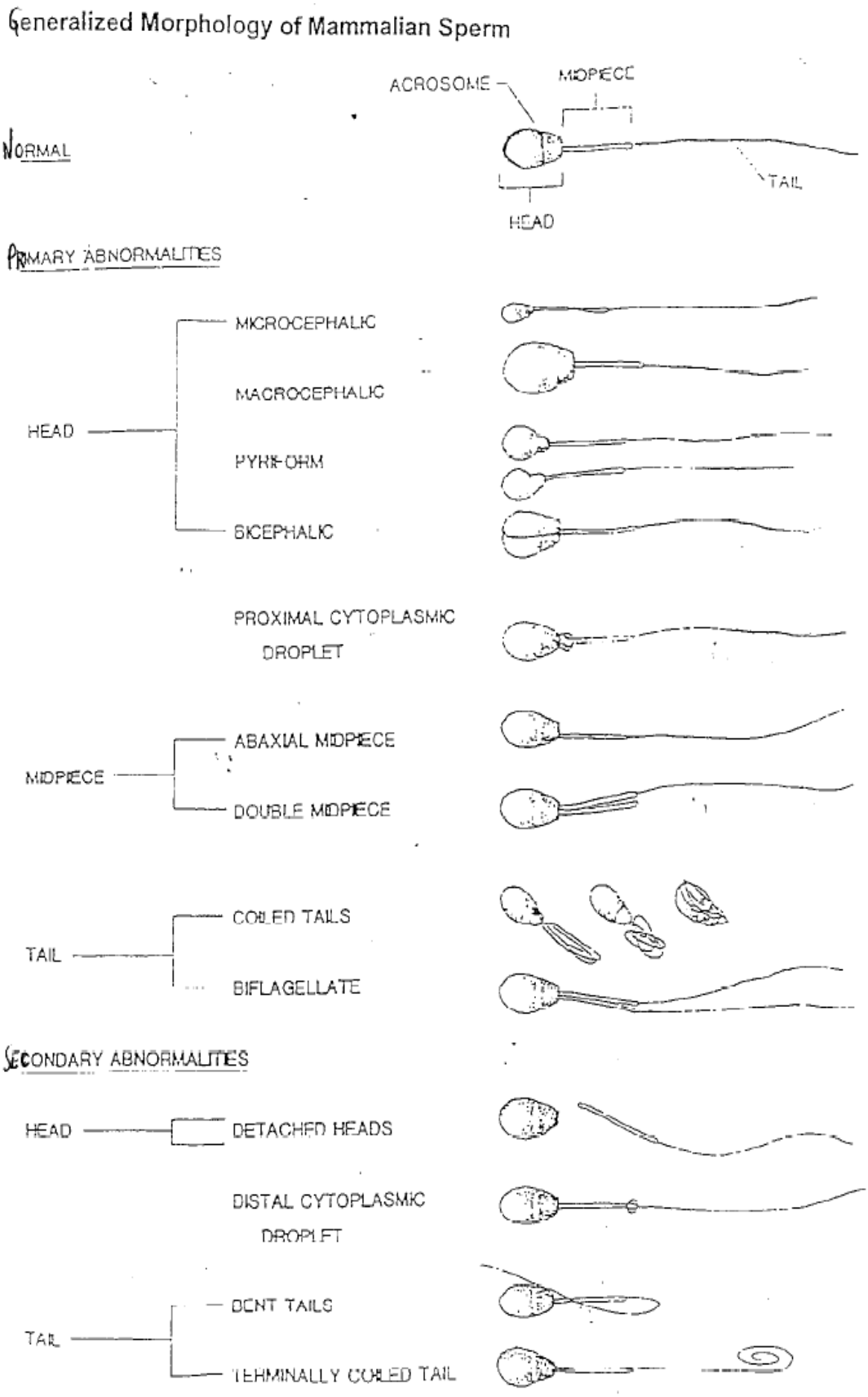

Figure 2.2 A schematic representation of sperm abnormalities (Loskutoff & Crichton, 2001)

a. Temperature

Metabolic rates increase and the life span of sperm decreases as the temperature of the semen increases. When the ambient temperature rises above 50°C, sperm suffer an irreversible loss of motility. If maintained at body temperature (or a few degree below), sperm will survive for only a few hours, due to the exhaustion of available energy substrates in the cell. Reducing the temperature of the semen will slow the metabolic rate and extend the fertile life-span of the sperm cells, provided that precautions are taken to protect sperm against cold shock and freezing (Bearden *et al*., 2004). However, a severe temperature reduction may also cause irreversible damage to the sperm cell. The most critical range for cold shock of sperm occurs when the semen temperature is reduced from 15ºC to 0°C. Both egg yolk and milk contain lecithin and lipoproteins, which protect the sperm against cold shock (Salamon & Maxwell, 2000).

b. Semen pH

A pH of approximately 7.0 (6.9 to 7.5 for different species) fall into the optimum activity ranges of most of the enzymes in the sperm cell. Therefore, a higher metabolic rate is expected when the pH of semen is maintained near neutrality (7.0). However the pH of semen could deviate toward alkalinity or acidity, and then the metabolic rates are increased or reduced respectively. So for example, a decrease in semen pH may arise due to build up of lactic acid or a combination of factors (Bearden *et al*., 2004).

c. Osmotic pressure

Semen maintains maximum metabolic activity when diluted with isotonic diluents, also called extenders. Either hypotonic or hypertonic extenders will reduce the metabolic rate, but neither will extend the life of the semen. Both hypotonic and hypertonic extenders will alter the transfer of water through the semi-permeable cell membrane of the sperm, disrupting the integrity of the cell. It is therefore important that only isotonic extenders are used (Bearden *et al*., 2004).

d. Semen concentration or sperm density

Semen concentrations are generally expressed as the number of sperm cells per ml, and increasing the concentration above that found in the normal ejaculate decreases the sperm metabolic rate. Potassium is a natural metabolic rate inhibitor and is the principal cation in

the sperm cell. Therefore by increasing the sperm concentration, the metabolic activity of the sperm will be reduced, due to an increase in potassium concentration (Bearden *et al*., 2004).

e. Steroid hormones

Testosterone and other androgens generally decrease the metabolic rate of the sperm cell, but the concentrations (testosterone) found in the male reproductive system show no permanent effect (Bearden *et al*., 2004). Oestrogens appear to produce the reactive oxygen species (ROS), especially hydrogen peroxide, at levels that significantly disrupt the DNA structure in sperm (Anderson *et al*., 2003).

f. Gases

Low concentrations of carbon dioxide stimulate the aerobic metabolism of sperm. If the partial pressure of carbon dioxide exceeds 5 - 10%, the metabolic rate of the sperm cell is depressed. Oxygen on the other hand is necessary for aerobic metabolism. However, too high levels of oxygen are toxic and will decrease the metabolic rate of the sperm (Bearden *et al*., 2004).

g. Light

Semen should be protected from light and never be exposed to direct sunlight. The use of gold-coloured fluorescent light tubes in the laboratory is vital for the protection from the harmful light rays. Light intensities that are normally found in the laboratory can decrease the metabolic rate, motility, and fertility of sperm. The harmful effect is observed especially if the semen is in contact with oxygen (Bearden *et al*., 2004).

h. Antimicrobial Agents

In the absence of specific infectious disease organisms, antibiotics are beneficial by reducing the competition of other bacteria commonly present in the semen. Most of the antibiotics and particularly some of the fungicidal agents are extremely toxic to sperm (Ahmad & Foote, 1986). At levels compatible with sperm, other antimicrobial agents are not very effective in combating microbial contaminants. Penicillin (1000 IU per ml of diluent) and streptomycin (1000µg per ml diluent) have been generally used to control both pathogenic and non-pathogenic bacteria in semen since the late 1940's. The recommended antibiotics that control bacterial growth and their concentrations per ml undiluted semen or per ml non-glycerol

portion of the diluent is 500µg/ml Gentamicin; 100µg/ml Tylosin; 300/600 µg/ml Linco-Spectin (300µg/ml Lincomycin and 600µg/ml Spectinomycin) (Bearden *et al*., 2004).

2.12 Preservation of sperm cells

Semen can be stored either fresh or frozen (cryopreserved). The two main methods of preservation are refrigeration and freezing. The storage of cryopreserved sperm is associated with a reduction in cell viability and fertilizing capacity, while the quality of the stored semen is affected by the handling procedures such as dilution, centrifugation, dilution in the semen extender and cryopreservation as such (Bustamante Filho *et al*., 2009). In addition there are many other minor factors related to the pre-freezing procedures, e.g. the straw freezing position, sperm concentration, sperm washing procedures, equilibration method and equilibration time. There are several reports showing that successful sperm cryopreservation of goat semen requires the removal of seminal plasma, and the dilution with skimmed milk could result in a higher conception rate than the dilution with egg-yolk buffers (Gordon, 2004). Some constituents of the goat seminal plasma damage sperm during cryopreservation, but its presence during the thawing process improves semen quality and the conception rates in boars with a poor post-thaw semen quality (Okazaki *et al*., 2009).

2.12.1 Storage of semen at reduced temperatures (refrigeration)

Refrigeration is the process by which semen is stored at low temperatures (4-5ºC) for at least 48h (Dondero *et al*., 2006). The sperm must however not be subjected to cold shock during the storage at low temperatures. When sperm is cooled to a temperature close to 0°C, irreversible damage can be induced. Egg yolk was found (and more likely its high molecular weight, low-density lipoprotein fraction) to have the ability to reduce the loss of acrosomal enzymes, thus preventing degenerative changes in the acrosome and providing protection against cold shock during liquid storage (Salamon & Maxwell, 2000).

2.12.1.1 Liquid semen storage

Liquid-stored semen can be an alternative to frozen-thawed sperm for use in AI, as the freezing of sperm can be an expensive process (Zhao *et al*., 2008). Freshly collected semen can be maintained in an unfrozen state in extenders and often stored for up to 2-3 days. This can be referred to as fresh extended semen (Vadnais, 2007). Semen that is to be stored at above 0ºC needs to be maintained at approximately 5°C in a refrigerator. Thus cooling is accomplished by placing a tube with the pre-diluted semen (35°C) in a container of water at

the same temperature (Bearden *et al.,* 2004). The main methods of storage of semen in a liquid state are the storage at low temperatures (0-5ºC) or 10-15°C, and ambient temperature, by reversible inactivation of the sperm (Salamon & Maxwell, 2000)

2.12.2 Cryopreservation of semen

Cryopreservation of semen has become a valuable tool for the long term (years) preservation of genetic material of endangered species or sires of superior breeding value (Schäfer-Somi *et al*., 2006). The success of any protocol for semen cryopreservation may be evaluated post-thawing by evaluating the sperm characteristics. Classically, sperm motility, viability, acrosomal membrane integrity, as well as other in vitro assays have been used to assess the success of cryopreservation and fertilizing potential (Purdy, 2003). The sperm cells are easily damaged after ejaculation, and the seminal plasma helps modify their in vitro viability. However, opinions differ regarding the elimination of seminal plasma before the processing of semen for storage. In practice ejaculates are used either devoid of, or containing seminal plasma (Leboeuf *et al*., 2000). Many modifications have been developed in semen cryopreservation techniques, with the goal being improving sperm viability following thawing (Barbas & Mascarenhas, 2009).

Cryopreservation exposes sperm to stressful elements, leading to a reduced cell viability (Bustamante Filho *et al*., 2009) and often causes ultra structural, biochemical and structural damage of the sperm cell, resulting in decreased motility and viability (Kozdrowski *et al*., 2007). The destabilization of the sperm membranes leads to premature acrosome reactions, shortens the life span of the cell and reduces fertility. Inevitably, semen cryopreservation results in a reduction of semen quality, mostly due to cold shock occurring when the temperature is decreased from 15°C to 4°C, as well as freezing damage (Pegg, 2002). Freezing and thawing of semen in a base isotonic diluent cause gross modifications in the plasma membrane, including a break in the head and detachment at the head and tail. This kind of damage has also been reported in refrigerated ram sperm, including specific alterations in the tail (Aisen *et al*., 2005)

The semen cryopreservation process generally evokes osmotic stress twice on the sperm cell, i.e. once during freezing and again during thawing. During freezing, ice crystals begin to form in the solution outside the cell. Intracellular water is then expelled by osmosis causing cellular dehydration. During thawing, an influx of extracellular water occurs, causing the

membrane to swell, inducing a second osmotic stress (Vadnais, 2007). It is important to evaluate semen before cryopreservation to ensure the sperm is initially viable (Sargent & Mohun, 2005).

Differences have also been recorded between individual males, regarding the freezability and fertility of their semen. Bucks could thus be classified as good or bad freezers (Leboeuf *et al*, 2000). The fact that males can often be classified as good or bad freezers implies that certain characteristics of membrane structure, which may be genetically determined, predispose towards survival of sperm under cryopreservation stress (Watson, 2000).

A simple sperm cryopreservation model has been developed using a chemically defined medium (Modified Ringer's Solution: RPS), with mature goat sperm derived from the caudal epididymis as part of the model. The procedure is generally based on the systematic manipulation of different rates of cooling, freezing and the maximum freezing temperature using a computer-controlled programmable freezer. Data generated using this model can be easily analyzed, as the medium does not contain complex substances such as egg yolk, skimmed milk or milk whey (Kundu *et al*., 2000; 2002)

Kundu *et al*. (2001) also observed that amino acids and dimethyl sulfoxide have an additive effect in augmenting the cryoprotecting potential of glycerol, suggesting that the mechanism of action is different from that of glycerol. Alanine showed maximal cryoprotection potential, and a dextran was able to cryoprotect the cells from the damaging action of the ice crystals by not entering the sperm cells, because of its high molecular mass (Kundu *et al*., 2002).

2.12.2.1 Diluents (extenders)

The purpose of a cryopreservation diluent is to supply the sperm cells with sources of energy, protect the cells from temperature-related damage, and maintain a suitable environment for sperm to survive temporarily (Purdy, 2006). A number of diluents have been evaluated in the past for the freezing of goat semen, e.g. reconstituted skim cow milk, sodium citrate-glucose yolk, lactose yolk, saccharose ethylenediaminetetraacetic acid, $CaNa_2$ yolk, raffinose yolk, Spermasol yolk and Tris- yolk (Leboeuf *et al*., 2000). All extenders used for semen preservation in domestic farm species must have the appropriate pH and buffering capacity, suitable osmolality and should protect the sperm cells from any cryogenic injury (Salamon & Maxwell, 2000).

Salamon and Maxwell (2000), reported milk to be an acceptable semen diluent, because of its protein fraction. This may act as a buffer against changes in pH and could also function as a chelating agent against any heavy metals present. Cow's milk is generally preferred above the milk of other species. Commercially available ultra-heat-treated (UHT) "long-life" skim milk has proven to be a satisfactory diluent for fresh semen. An advantage of this UHT milk is that it is sterile, and thus does not require heating and may be used directly as a diluent, without further treatment. A freshly opened carton of UHT milk must be used on the same day.

Tris based extenders, normally used for semen freezing (buck and ram) contain fructose or lactose as sugars, compared to other semen extenders (Purdy, 2006). Tris-based semen extenders according to Barbas and Mascarenhas (2009) should contain buffers, carbohydrates (glucose, lactose, raffinose, saccharose or trehalose), salts (sodium citrate, citric acid), egg yolk and antibiotics. Buffer solutions e.g. phosphate or a 3.2% 2,9-trisodium citrate dehydrate solution adjusted to a pH of 6.9 (by the addition to citric acid), have been commonly used in combination with egg yolk. The addition of citric acid is usually unnecessary, as the egg yolk component (20% by volume) has sufficient buffering capacity to return the pH to neutral (Hafez & Hafez, 2000).

2.12.2.1.1 Egg yolk

Egg yolk is generally accepted to be an effective diluent in semen diluents for the protection of the sperm cell against cold. However, it is difficult to produce semen diluents of uniform quality, because of individual quality differences inherent to egg yolk (Matsuoka *et al.*, 2006). Egg yolk (EY) or milk and glycerol represent the indispensable compounds of practically all media used for bull semen preservation in the liquid or frozen state. Egg yolk and milk, being products of animal origin, represent a potential risk for contamination of the semen and the composition is not uniform (Bergeron & Manjunath, 2006). Cabrera *et al*., (2005) observed certain detrimental effects of egg yolk coagulating enzyme (EYCE) in Canary buck semen cryopreservation thus suggesting sperm washing before dilution. Corteel (1981) however, considered sperm washing as a complex and time consuming process causing loss of sperm cells. Furthermore, it did not appear to improve the fertility of the sperm cells in dairy goats (Corteel *et al*., 1975), and fibre-producing goats (Ritar & Salamon, 1983). Egg yolk coagulating enzyme (EYCE), a phospholipase secreted into the seminal plasma by the bulbo-urethral gland hydrolyses the egg yolk lecithin into fatty acids and

lysolecithin, which is toxic to buck sperm (Leboeuf *et al*., 2000; 2003). Until now, the use of the Tris-egg yolk cryopreservation diluents, such as the one described by Ritar and Salamon (1983), is recommended, as it is easy and practical to use (Rahman, *et al*., 2008).

Ritar and Salomon (1983) further recommended 1.5% egg yolk, in the final concentration of the diluent for use, without prior removal of seminal plasma. The diluent utilized by Tuli *et al*. (1991) contained 16.8% egg yolk as a final concentration. Thus not all Tris-egg yolk diluents are the same, and caution should be exercised concerning the components of the diluent. Particularly as higher concentrations of egg yolk (>1.5%) decrease the post-thaw sperm viability of buck semen samples not washed free of the seminal plasma prior to cryopreservation (Ritar & Salamon, 1983). Aboagla andTerada (2004) concluded that the addition of egg yolk to the semen extenders plays a major role during the freezing stage of goat semen cryopreservation, and that the addition of trehalose significantly improves its cryoprotective effect. Furthermore neither glycerol nor egg yolk alone could increase the percentage of intact acrosomes. The combination of these two major cryoprotectants, then significantly increased the percentage of intact sperm acrosomes.

2.12.2.1.2 The effect of different diluents on certain semen parameters

Hafez and Hafez (2000) reported that the semen extender used may alter the sperm motility, usually by increasing the velocity measures. After initial extension, a high percentage of sperm may exhibit a circular motility pattern, which usually ceases after 5–10 minutes in the extender. It would appear as if the sperm cells are reflecting light when excessive fluid is present between the slide and the cover slide, emerging while the sperm move forward. In the case of less fluid the sperm cells may appear to move in a two-dimensional pattern. Sperm swimming in a tight circular motion could indicate cold shock. Oscillatory motion may indicate aged or dying cells. Infertility or sub-fertility in males may be correlated to the semen motility patterns. Several procedures have been developed for the objective evaluation of sperm motility e.g. time-lapse photomicrography, frame-by-frame playback videomicrography, spectrophotometry, and computerized sperm analyses (CASA).

2.12.3 Cryoprotective agents

Cryoprotectants are included in a cryopreservation medium to reduce the physical and chemical stresses derived from cooling, freezing and thawing of the sperm cells (Purdy, 2006). A cryoprotectant is generally defined as a substance that is used to protect biological

tissue from freeze damage (due to ice formation). The use of a cryoprotectant agent (CPA) is thus very important to avoid intracellular ice formation. Goat semen cryoprotectants are generally divided into two categories, namely non-permeating cryoprotectants (milk or egg yolk) or permeating cryoprotectants (glycerol, ethylene glycol, or dimethyl sulfoxide). Permeating CPA's, depending on the concentration used, are toxic and induce sperm membrane damage and decrease sperm motility (Medeiros *et al*., 2002). Commonly used cryoprotectants in semen cryopreservation include DMSO (dimethyl sulfoxide), ethylene glycol, glycerol, propylene glycol, sucrose or trehalose (Li *et al*., 2005).

Semen freezing (cryopreservation) involves a precise process which suspends sperm in a mixture designed to dehydrate the cell, so that ice crystals do not form within (and rupture it), when the cell is frozen. Glycerol is the typically common dehydrating agent for most mammalian sperm and embryos, while DMSO is used for fish and amphibian gametes; and ethylene glycol for certain embryo freezing.

2.12.3.1 Glycerol

Glycerol has been reported by Medeiros *et al*. (2002) and Salamon and Maxwell (2000) to be the most commonly used cryoprotective agent in diluents for freezing semen. Glycerol is regarded as a non-permeating CPA, hence exerting an extracellular effect due to a osmotic stimulation and cell dehydration mechanism thus decreasing the volume of intracellular water available for freezing. The intracellular effect of glycerol, exerted through its ability to permeate the cell membrane, is a decrease in the intracellular osmotic stress effect of dehydration. This occurs by replacing intracellular water necessary for the maintenance of cellular volume, the interaction with ions and macromolecules, and lowering the freezing point of water. Glycerol as a cryoprotectant has yielded successful results in the cryopreservation of goat semen (Leboeuf *et al*., 2000; Sundararaman & Edwin, 2003; Peterson *et al*., 2006; Purdy 2006). However, it is somewhat toxic to sperm (Holt, 2000), and may induce osmotic damage (Purdy, 2006). Glycerol has been regarded as a superior cryoprotectant, as it penetrates the sperm membrane rapidly (El-Alamy & Foote, 2001).

While glycerol offers protection to the sperm, it may also cause structural damage during the pre-freezing process. It must therefore be added to semen to protect it during the freezing and thawing processes. It was suggested that glycerol should be added not earlier than 20 to 30 min before the freezing of the semen. In semen frozen by the slow conventional method, and

using mainly hypertonic extenders, glycerol is frequently used within the range of 6-8%. Upper levels cause damage to the sperm cells, lowering the post thawing survival of the sperm and lower levels offer poor protection (Barbas & Mascarenhas, 2009). The best results have been obtained using 4-6% glycerol, and a freezing rate of 10-100°C/min (Byrne *et al*., 2000; Anel *et al*., 2003)

Glycerol can be added to the semen in the separate diluent fractions (two-step dilution) or by the single addition of the diluent containing glycerol (one-step dilution). The damage to sperm results from the selective freezing of free water, both inside and outside the cells. Therefore glycerol binds water and decreases the freezing point of the solution and less ice is formed in its presence at any temperature (Bearden *et al*., 2004).

2.12.4 Processing and freezing of semen

The processing of buck semen often starts firstly with the washing of the buck semen with a Ringer's solution, even when skim milk is used as a diluent. This washing is accomplished by adding 20 parts of the Ringer's solution (at the same temperature as the semen), for each part of freshly collected semen. Then centrifuging the semen for 10 minutes at 1000 G and aspirating the supernatant to be discarded. After repeating this procedure of washing 1 ml of Ringer solution is used to re-suspend the sperm, before further processing. If skim milk is used as a diluent, it must be heated to 95°C for 10 minutes and cooled prior to use. Glycerol at a concentration of 7% is generally added to only half of the diluent, resulting in a concentration of 3.5% in the final dilution. Penicillin and streptomycin should be added to the non-glycerol portion of the diluent at a rate of 2000 to 4000 units and micrograms respectively/ml as an antibiotic.

The semen is diluted to the half desired final volume with a fraction of the diluent containing no glycerol, and slowly cooled (2 h) to 5ºC. After the diluted semen has reached a temperature of 5°C, an equal volume of the glycerol – containing diluent (also at 5°C), is slowly added drop-wise to the diluted semen (Bearden *et al*., 2004). The semen can then be packaged into 0.25 ml straws. A period of 4 h should be allowed for equilibration before freezing.

Traditionally equilibration has been regarded as the total time that the sperm remains in contact with glycerol, before the freezing process. This is the period during which the

glycerol penetrates the sperm cell to establish a balanced intracellular and extra-cellular concentration (Salamon & Maxwell, 2000). The semen equilibration at 5°C generally varies for cryopreservation in different species and irrespective of the freezing protocol employed.

Diluted buck semen has been reported to be cooled to 4-5°C over a 1.5-4 h period, and then frozen in either pellets or straws (Leboeuf *et al*., 2000; Barbas and Mascarenhas, 2009). The equilibration of extended semen at 5°C is an essential step in many of the protocols for the cryopreservation of mammalian sperm. This equilibration facilitates the cryoprotectant glycerol, present in the semen diluent to exert a beneficial effect in terms of minimizing the degree of structural damage to sperm during freezing (Sundararaman and Edwin, 2008).

According to Sargent and Mohun (2005), there are three methods of freezing semen currently being implemented. These comprise a slow (Styrofoam box), medium (dry ice/ethanol bath) or fast (liquid nitrogen) procedure. The Styrofoam box is a common box used for shipping. Slow freezing using the Styrofoam box has consistently given the best results in a trial reported by Sargent and Mohun (2005). The dry ice/ethanol bath is a small container with a magnetic stirrer placed on a rack inside the smaller container. The fast freezing method on the other hand implies simply submerging the semen straws into liquid nitrogen.

Associated with the method used for freezing, comes the method or form of packaging of the semen. Freezing of sperm in pellets on dry ice is rapid and inexpensive, but inventory management is problematic, as the actual semen samples cannot be labelled. For pellets, once the semen sample is cooled, semen aliquots of 0.1-0.3 ml are dispensed into indentations on a block of dry ice (solid carbon dioxide; -79°C) and frozen for 2-4 min. The pellets are then plunged into liquid nitrogen for storage (Chemineau *et al*., 1991).

The freezing of semen in straws is relatively more expensive and laborious than the pellet technique, but each straw sample can be labelled for accurate inventory management. Cryopreservation may be done over liquid nitrogen vapours or in a programmable bio-freezer. When using a Styrofoam box, the rack containing the straw samples is placed into the liquid nitrogen vapour at a height of 3-4 cm above the liquid for 7-8 min, and the straws are then plunged in liquid nitrogen for storage (Evans & Maxwell, 1987). In the straw method, filled semen straws are arranged horizontally 4-5 cm above the liquid nitrogen vapours for a variable period of time (10-20 min), with good post thawing results (Byrne *et al*., 2000;

Leboeuf *et al*., 2000). Chemineau *et al*. (1991) reported straw size to determine the freezing height above the liquid nitrogen. It was suggested that 0.5 ml straws should be frozen at 4 cm for 3 min, and then plunged into the liquid nitrogen, while 0.25 ml straws should be placed 16 cm above the liquid nitrogen for 2 min, then lowered to 4 cm for 3 min, and then plunged into the liquid nitrogen for storage.

The freezing rate suggested for buck semen by Bearden *et al*. (2004) is much slower than that used for bull semen and necessitates the use of alcohol and dry ice, at the following cooling rates (Bearden *et al*., 2004):

From 5°C to 0°C	30 minutes
From 0°C to -5°C	10 minutes (½°/min.)
From -5°C to -10°C	5 minutes (1°/min.)
From -10°C to -17°C	3 minutes (2°/min.)
From -17°C to -79°C	16 minutes (4°/min.)

The cooled semen is then transferred to liquid nitrogen for indefinite storage.

Programmable freezers are convenient for the freezing of large quantities of semen straws, controlling the rate of freezing. These are frequently used at AI centres (Purdy, 2006; Barbas & Mascarenhas, 2009). The benefit of any programmable freezer is that the freezing curve can be customized, e.g. 4 to -5°C at a rate of 4°/min, -5 to -110°C at a rate of 25°/min and 110 to -140°C at a rate of 35 °C/min, before the semen straws are plunged into the liquid nitrogen (Purdy, 2004). Freezing rates often vary according to the AI centre. So for example, the following cooling rates can be used, 4 to -5°C at 20°C/min, -5 to -110°C at 55°C/min and -110 to -140°C at 35°C/min, followed by the immersion into the liquid nitrogen (Byrne *et al*., 2000; Leboeuf *et al*., 2000). Slow freezing methods are used to avoid ice crystal formation, allowing water to leave the cells by osmosis (Fisher & Fairfull, 1986). Barbas and Mascarenhas (2009) reported that the use of faster cooling rates (15-60ºC/min) yield best sperm survival after freezing-thawing. Best results were obtained at a rate of 10-100ºC/min (Byrne *et al*., 2000; Anel *et al*., 2003)

Ritar *et al*. (1990a; b) reported semen frozen in pellets to yield superior sperm motility following thawing (39%), compared to semen frozen in straws. However, sperm motility was the same for sperm frozen in 0.25 ml straws (33% post-thaw motility) and 0.5 ml straws (34% post-thaw motility). The differences in post-thaw sperm motility, viability and fertility

may be attributed to the different cooling rates utilized in the pellet and straw method (Ritar, 1993).

In general all semen cryopreservation protocols have a number of potentially sperm damaging stress factors. Firstly, it involves the change in temperature, secondly the osmotic ant toxic stress presented by the exposure to molar concentrations of cryoprotectants and thirdly, the formation and dissolution of ice in the extra-cellular environment (Watson, 2000). Cytoplasmic sperm disruption through intracellular ice formation may be further compounded by the growth of ice crystals during the thawing (recrystallization) process, as suggested by Holt (2000).

The success of semen cryopreservation has been reported as being dependent on the rate of dilution of the semen to a large degree. Originally, the semen was diluted to protect the sperm during cooling, freezing and thawing. However, the rate of dilution was often changed for technical reasons – such as to increase the number of females that could be inseminated with each ejaculate (insemination doses), or to standardize the number of sperm in each dose of frozen/thawed semen (Salamon & Maxwell, 2000)

There is still a considerable difference of opinion between researchers concerning the freezing of buck semen (Bearden *et al*., 2004). The difference of species in the ability of their sperm to survive the freeze-thawing process is generally related to their tolerance to osmotic stress. Frozen semen must be stored at a temperature of at least below -76°C. However liquid nitrogen with a temperature of -196°C, is the refrigerant of choice (Guthrie *et al*., 2002). Leboeuf *et al.* (2000) stated that a comparison between methods and protocols for freeze-thawing of semen are difficult, because of the different parameters evaluated, and the lack of uniformity in the methodology used.

2.13 Thawing of semen

In the freeze-thawing procedures, the warming phase (thawing) is just as important to survival of the sperm as the cooling phase (Salamon & Maxwell, 2000). Sperm that survive cooling to -196°C still face the challenge when warming and thawing, and must thus traverse the critical temperature zones twice (-15°C to -60°C). Thus, frozen-thawed sperm are partially capacitated and capable of achieving fertilization over a shorter period of time (Leboeuf *et al*., 2000). The thawing of semen samples is determined by the method used to

freeze the semen. So for example sperm pellets should be thawed in a dry test tube at 37°C, while the thawing of semen straws may be performed using various methods (Evans & Maxwell, 1987). Traditionally, a straw is thawed by placing it in a water bath 37°C for 12-30s (Deka & Rao, 1987). Attention to the thawing temperature and timing becomes much more critical at temperatures greater than 37°C in the water bath, as these high temperatures can result in tremendous sperm mortalities if not performed correctly (Tuli *et al*., 1991). Purdy (2006) concluded that the buck semen straws should be thawed at 37°C in a water bath for 20-30s.

If thawed goat semen has good motility, namely > 40% individual motility (IM) and < 20% sperm abnormalities, it could be used for AI (Barbas *et al*., 2006). Ram sperm cryopreserved in a Tris-egg yolk extender has been reported to attain up to 72.0 ± 1.5% total sperm motility , 29.0 ± 1.1% progressive motility, 73.0 ± 0.4% straightness, 43.0 ± 0.5% linearity and 29.9 ± 1.3% sperm viability (da Silva Maia *et al*., 2009)

2.13.1 Other factors affecting post-thaw sperm motility

a. Effect of semen concentration

Recent studies on different species, including aquatic organisms have shown the importance of standardization of sperm concentration for freezing protocols (Yang *et al*., 2007). This is especially relevant to the optimal concentration of the cryoprotectant. Dong *et al*. (2008) concluded that semen concentration may affect the semen freezing outcome and freezing in higher concentrations may yield a better post-thaw sperm motility in monkeys.

b. Effect of semen washing through centrifugation

Sperm washing can help to remove the dead sperm (thus improve motility) and also eliminate decapacitation activity of the seminal plasma (VandeVoort, 2004). However, semen washing reduces the sperm numbers. No significant differences were recorded by Dong *et al*. (2008) between washed and unwashed semen samples, but the washed sperm showed higher progressive motility in a short incubation time of 1h after thawing.

c. Effect of equilibration time

Equilibration of the diluted semen prior to freezing plays an important role in the sperm survival during the cryopreservation process. The effective equilibration time is defined as

the interval required for a semen sample to acclimate to 4ºC, prior to freezing. In a study with rhesus monkeys it was stated that equilibration method has no major effect on post-thawing sperm motility (Dong *et al*., 2008).

d. Effect of straw freezing position

Dong *et al.* (2008) suggested that the straw position does not affect the semen freezing outcome and concluded that straw position has no effect on the post-thawing sperm motility.

Chapter 3

General Materials and Methods

3.1 Study site

This study was conducted at the Agricultural Research Centre (ARC), Irene campus (25° 55' S; 28° 12' E), Republic of South Africa (RSA). The centre is located in the highveld region of RSA, situated at an altitude of 1525m above sea level. The climatic conditions and ambient temperatures range from hot days to cool nights in summer to moderate winter days with cold nights and an average annual rainfall of 464mm (Webb *et al*., 2004).

3.2 Experimental animals

A total of 10 young, mature (2-3 years of age) unimproved indigenous goat bucks (mean body weight 38.1 ± 9.3 kg) were trained for semen collection using the artificial vagina (AV) method. The study was conducted between April (autumn) and August (winter), 2009. During this period the bucks grazed on natural pastures and received fresh water *ad libtum.* Additional supplementation (300–350 g concentrate or 1kg maize silage/animal/day - depending on the availability) was also provided. The experimental animals were cared for and handled in accordance with standard protocols and guidelines for the Care and Use of animals of the Agricultural Research Council.

3.2.1 Training of bucks for semen collection by the artificial vagina (AV) method

Bucks were trained to mount and ejaculate in the AV daily (7 days a week), before the onset of the study. Training for semen collection by the AV method was executed for a period of 4 weeks, using a doe in oestrus as a teaser for semen collection. Briefly, a female was detected in oestrus and restrained in a neck clamp before the introduction of the buck into the testing arena or pen (Silvestre *et al.*, 2004; Bester, 2006). In order to improve the libido of the bucks, these were placed in a pen adjacent to the semen collection arena prior to semen collection, so that the bucks were able to observe the other males mounting the restrained doe (Price *et al*., 1984; Silvestre *et al.*, 2004). Bucks were allowed a 5 minute period to attempt to mount and ejaculate. After ejaculation or a period of 5 minutes, whichever occurred first, the buck was moved to the adjacent pen and after 10 to 15 minutes, males that did not ejaculate at their previous attempt, were again placed in the collection arena. Training was considered

successful when males mounted and ejaculated into the AV at regular intervals i.e. 4 consecutive days when presented to any restrained female as a teaser, even if the doe was not in oestrus, in the presence of the same semen collector (Silvestre *et al*., 2004).

3.3 Semen collection, temporary incubation (holding) and transportation to the laboratory

From the initial group of 10 indigenous bucks, only 7 animals were successfully trained for semen collection with the aid of an AV. These bucks were then utilized during the first stage of this study, to characterize the semen of the unimproved indigenous S.A goat. Semen was collected twice a week from each buck (Gacitua & Arav, 2005; Sundararaman & Edwin, 2008) with the aid of an AV, filled with water at 41 to 46°C (Silvestre *et al.*, 2004; Yamashiro *et al*., 2006). As previously mentioned, to stimulate the bucks, a doe in oestrus was used. During the semen collection process, when a buck mounted a doe, the penis was gently guided into the AV. To minimize stress and maximize the quality and quantity of the ejaculate, the collection procedure was always carried out under the same conditions i.e. by the same person, at the same time of the day (8:00 to 10:00), in the same pen and using the same equipment (Siudzińska & Łukaszewicz, 2008). Immediately following collection and macroscopic evaluation, the ejaculates were transferred into screw top conic plastic tubes, placed and kept in a thermo-flask at 37 °C, until semen quality assessment (Silvestre *et al.*, 2004) within a period of 1h (Gacitua & Arav, 2005).

3.4 Semen evaluation (macroscopic and microscopic)

Macroscopic (semen volume, colour, concentration and pH) evaluation was performed immediately after collection. The microscopic (sperm motility, velocity, morphology and viability) analyses were conducted in the Germplasm Conservation and Reproduction Biotechnologies (GCRB) laboratory of the ARC.

3.4.1 Macroscopic semen evaluation

The ejaculated semen volume (ml), colour and pH were recorded immediately after collection in the collection arena. The semen volume was measured directly from the graduated collection tubes, while the semen pH was measured manually with the aid of Neutralit® pH-indicator strips (Merck KGaA, 64271 Darmstadt, Germany) by pipetting a drop (10 µl) of fresh semen onto the strip and spreading it gently. The resultant colour of the strip was then compared to the colour code of the graduated pH scale to obtain a pH reading.

Plate 3.1 Pictures of the S.A unimproved indigenous bucks used in this study

Plate 3.2 Artificial vagina + graduated collecting tube

Plate 3.3 Thermo-flask and screw top conic plastic tube

3.4.2 Semen concentration

In the GCRB laboratory of the ARC, the sperm concentration of the semen sample was determined with the aid of a spectrophotometer (SpermaCue®, Minitüb, Germany). A volume of 20 µl undiluted fresh semen was pipetted into a microcuvette (HemoCue AB, Ängelholm, Sweden), which was then inserted in the spectrophotometer to give an automated sperm concentration reading ($x10^6$ sperm/ml).

Plate 3.4 SpermaCue®

3.4.3 Microscopic semen evaluation

In the GCRB laboratory, semen samples were analyzed for sperm motility rate and membrane integrity within 1h after collection, using standard recommended laboratory procedures (Choe *et al.* 2006). Microscopic evaluation, both manual/visual sperm analyses and automated sperm analyses (CASA - computer assisted sperm analysis) were conducted as follows:

3.4.3.1 Sperm motility evaluation using CASA

For the automated sperm motility rate analyses, a Sperm Class Analyzer®-SCA® (V.4.0.0.1 Animal/Veterinary Microptic S.L, Barcelona, Spain) was used to analyze the sperm motility [% immotile/static or motile, (progressive or non-progressively motile sperm)] and sperm velocity parameters (static, slow, medium, rapid, curvilinear, straight-line, average path, linearity, straightness and wobble velocities). The sperm washing solution (BO) developed by Bracket and Oliphant (1975), was prepared fresh as described in Protocol 3.1, and warmed to 37°C on a warm plate (Precision Scientific Company, Chicago 47, USA), before adding the semen sample. A total volume of 10 µl semen was diluted with 500 µl of BO medium to perform the sperm swim-up technique. This technique allows only sperm with the ability to swim from the bottom (where sperm are deposited) to the surface of the medium to be analyzed (superior sperm). The sperm solution was then incubated in the MCO-20 AIC Sanyo CO_2 incubator (Sanyo Electric Biomedical Co., Ltd, Japan), adjusted to 37°C for 5 minutes. Following the incubation period, 5 µl of this semen solution collected from the

surface of the solution was then pipetted onto a pre-warmed bevel-edged, frosted end microscope glass slide (Thermo Scientific Menzel-Gläser, Germany) and gently covered with a microscope cover glass (Menzel gläser, Germany) and evaluated under x10 magnification using a SCA® microscope, imaged on a monitor. The generated data were saved onto a Microsoft excel sheet for later statistical analysis.

Plate 3.5 Sperm Class Analyzer®

Plate 3.6 Warm plate

Plate 3.7 MCO-20 AIC Sanyo CO_2 incubator

The Brackett and Oliphant sperm washing solution (BO-W) as originally described by Brackett and Oliphant (1975) was prepared in four steps as follows:

Step 1: Preparation of Solution A (10 x BO stock solution with storage time of 30 days)

Component	**Molecular Weight**	**mM**	**g/100ml**
NaCl	58.44	112.00	6.5453
KCl	74.56	4.02	0.2997
$NaH_2PO_4.H_2O$	137.99	0.83	0.1145
$MgCl_2.6H_2O$	203.30	0.52	0.1057
$CaCl_2.2H_2O$	147.02	2.25	0.3308
Glucose	181.16	13.90	2.5181
Antibiotics (penicillin) or Streptomycin (0.02g/ml)		50 mg/ml	5 ml 2.5 ml
0.5% Phenol Red			1.5 ml
Ultra pure water (Sabax)			Add up to 100 ml
Total volume			100 ml

This solution was filtered using a craft suction unit (Rocket) and a 250 ml filter system. In the preparation of the BO stock solution A, it is important that the $MgCl_2.6H_2O$ and $CaCl_2.2H_2O$ be dissolved in water, separately. Water must be added to the tube first, and then the components are added. The components should also always be stored at 4ºC.

Step 2: Preparation of solution B (1xBO working solution with a maximum storage time of 2 weeks)

Component	**Molecular Weight**	**mM**	**g/100 ml**
$NaHCO_3$	84.01	37.0	0.3108
Na-Pyruvate	110.04	1.25	0.0138
BO stock solution A			10 ml

The components of the working solution were made up to 100 ml with Milli-Q produced ultrapure water (Sabax), and then the solution was filtered.

Step 3: Preparation of sperm washing stock solution

This entailed the addition of 0.1553g caffeine **(**MW of 194.20) to 80 ml of solution B. It is important that the caffeine is dissolved, before the addition of the BSA. Check pH (7.2 to 7.4)

after adding caffeine. The remaining 20 ml is used in the preparation of IVF maturation media.

Step 4: Preparation of sperm washing solution (BO-W)
Remove 12 ml of the sperm washing stock solution, and add 0.042g BSA to the remaining 68 ml. It is once again important that the BSA must dissolve on its own before filtering-do not shake the solution. Thereafter filter the solution.

Plate 3.8 Chemical balance used to weigh the ingredients of the sperm washing solutions used in this study

3.4.3.2 Sperm structural morphology (normal or abnormal) and viability (live or dead)
Sperm viability or the percentage live sperm was determined using the new improved eosin/nigrosin stain (pH=8.39; osmolarity=411, Batch no: 2009/03), manufactured by the Onderstepoort Faculty of Veterinary Sciences' Pharmacy (60 µl eosin/nigrosin and 6 µl semen prepared in a thin smear). This staining method indicates the viability (percentage live or dead) of the sperm cells and allows a good evaluation of sperm morphology (normal or abnormal sperm morphology) (Björndahl *et al*., 2003).

The sperm smears were evaluated on the same day of semen collection, using slide preparations with the aid of a fluorescent microscope (BX 51TF), using an oil immersion objective (x100 magnification). A total of 100 sperm/slide were evaluated and counted for each animal per semen collection, with the aid of a DBC.6 Model Laboratory counter (Han Lien International Corp). The gross structural normal/abnormalities for each ejaculate was recorded as a percentage of the total number of cells evaluated (Hidalgo & Dorado, 2009).

In the evaluation of the percentage live sperm using the nigrosin/eosin stain, the live sperm fluoresced white or transparent, while the dead sperm, fluoresced red (Bearden *et al.* 2004).

Live sperm were further categorized as morphologically normal or abnormal with the sperm abnormalities being determined using two different sets of criteria. The first set of criteria used the location of the abnormality on the sperm such as e.g. the head (i.e. bulb, small, enlarged, looped head, etc), mid-piece and tail (i.e. swollen, looping, partially or totally lacking the tail, etc), as described by Łukaszewicz *et al*. (2008).

Plate 3.9 Fluorescent microscope (BX 51TF) used in this study to evaluate the sperm

In the second set of criteria, sperm abnormalities were classified as primary, secondary or tertiary abnormalities, according to the severity of the abnormality or lesions as described by Loskutoff and Crichton (2001). The following criteria were used:

1) Primary (more crucial) sperm abnormalities included the following:
    a. Sperm head: microcephalic (small heads), macrocephalic (large/swollen heads), double heads and abnormal acromosomes.
    b. Mid-piece of the sperm cell: swollen, elongated and abaxial.
    c. Tail of the sperm: double and short.
2) Secondary (less crucial) sperm abnormalities include the following:
    a. Sperm head: detached, loose and damaged acrosome.
    b. Mid-piece of the sperm cell: bent and protoplasmic droplets.
    c. Tail of the sperm cell: bent, shoe-hook and protoplasmic droplets.
3) Tertiary (those resulting from poor handling of the semen – post-ejaculation or as a consequence of manipulation and other environmental factors). Tertiary sperm abnormalities, included the following:
    a. Reacted (dead) acrosome.
    b. Coiled sperm tail.

One hundred sperm from different parts of the thin smear were individually evaluated and each type of sperm abnormality (primary, secondary or tertiary) was expressed as a percentage of the total number of sperm cells evaluated.

3.5 Semen cryopreservation

Semen was extended using one of 4 different extenders. The semen extenders used were namely, 1.5% Egg Yolk-Tris (Ritar & Salomon, 1983); 15% Bovine Serum Albumin-Tris (Matsuoka *et al*., 2006); Ovixcell® and Bioxcell® (Gil *et al*. 2003). Ovixcell® and Bioxcell® are commercially available extenders supplied by IMV (L'Aigle, France). The selections of the other extenders were based on reports from the literature. The 1.5% egg yolk and 15% BSA extenders were based on reports that 1.5% egg yolk-Tris is considered easy to use and does not require sperm washing prior to use, while the 15% Bovine Serum Albumin (BSA), in comparison with other concentrations (0, 0.3,1,5,10 and 15%) recorded the highest sperm motility of 58.3 ± 6.7% (Ritar & Salamon, 1983; Matsuoka *et al*., 2006).

Tris is the general abbreviation used for the organic compound tris (hydroxymethyl) aminomethane. The yolk of a freshly laid chicken egg was used to prepare the 1.5% Tris-egg yolk extender. Each extender was prepared in 2 fractions; a faction A without glycerol as a cryoprotective agent (CPA) and a fraction B with 12 % glycerol (v/v). Fraction A was warmed to 37 ºC prior to addition to semen while Fractions B was kept at 5 ºC prior to use, and added to fraction A also at 5 ºC.

Only buck semen samples of high quality (i.e. >70% live sperm ; >70% normal sperm; >70% individual total motility) and a density of 80-500 × 10^6 sperm/ml (Ritar *et al*., 1990a; Gil *et al*., 2001) were pooled and used for the sperm cryopreservation trial in this study. Semen was pooled as the bucks generally produced relatively low ejaculate volumes and also to eliminate individual buck differences in terms of semen quantity and quality (Karagiannidis *et al*., 2000).

The pooled semen samples were respectively divided into 4 aliquots. Each aliquot was then diluted at a 1:1 ratio at room temperature with the Fraction A (CPA-free) extenders at 37 ºC and equilibrated for 1.5 h at 5°C in a refrigeration unit (Recam International, South Africa), inside the laboratory. Each of the 4 aliquots (extended solution A) was divided into two halves and placed into a different test tube. One of the 2 tubes per treatment was further

diluted 1:1 with fraction A (CPA–free), while the other tube was diluted 1:1 with fraction B (containing glycerol at 12% as a CPA). Fraction B containing glycerol, was added after 1.5 h equilibration drop-wise (while mixing gently), at 5°C, into the respective test tube containing semen diluted in extender A in order to prevent morphological injury to the sperm at room temperature (Barbas & Mascarenhas, 2009). Further equilibration was performed for another 1.5 h, making the entire equilibration time 3h (Purdy, 2006), and the final glycerol concentration in the four extenders containing CPA 6% (Kundu *et al*., 2000).

Plate 3.10 The walk-in refrigeration unit used in this study to conduct semen cryopreservation

The semen straws (0.25 ml), sterile water, polyvinyl alcohol powder (PVA) and the soft tissue paper were all cooled down to 5°C, prior to be used. Straws were filled with semen following the 3h equilibration period, with the diluted semen samples being aspirated into the straws (at least 2 straws for each one of the extender treatments**;** thus 4 extenders with or without 6% glycerol as a cryoprotectant, making a total of 8 treatments) and the straws sealed with PVA powder. After loading, all straws were placed into the straw holder and frozen in a programmable freezer (CBS freezer 2100 series, America), using a customized freezing curve (Table 3.1). The freezing protocol was concluded after approximately 7.5 minutes, the semen straws were plunged into a polystyrene container filled with liquid nitrogen. These were then transferred into goblets and preserved in a liquid nitrogen tank (-196ºC) for 7 days, pending further sperm analyses (Barbas *et al*., 2006).

Table 3.1 Freezing rates used to cryopreserve the semen of the unimproved indigenous bucks (Purdy personal communication 2004, IMV Corporation, USA)

From Temperature (ºC)	To Temperature (ºC)	Rate of cooling (ºC/min)
5°C	-5°C	4°C/min
-5°C	-110°C	25°C/min
-110°C	-140°C	35°C/min

Plate 3.11 The programmable freezer used in this study

Plate 3.12 Semen straws in a hanger (loaded inside the liquid nitrogen) ready to be transferred into a storage tank

Plate 3.13 The Liquid Nitrogen Tank used in this study

3.6 Thawing of cryopreserved semen straws and post-thaw semen analyses

Seven days after being cryopreserved, one semen straw from each treatment (semen extender) was thawed in a water bath (Ju-Labortechnik, Germany) at 37°C for 30 seconds (Deka & Rao, 1987; Purdy, 2006). Both ends of the sealed straws were cut with a pair of scissors and the semen emptied into a plastic screw top conical tube.

Plate 3.14 The water bath used in this study

To test the effect of incubation time on all these sperm parameters measured post-thaw, semen was analyzed immediately after thawing (0 minutes) and following an interval of 30 and 60 minutes for sperm motility, using the SCA® system (CASA). During this period,

semen samples were incubated at 37°C in an MCO-20 AIC Sanyo 5% CO_2 incubator (Sanyo Electric Biomedical Co.,Ltd, Japan). All semen samples were analysed for sperm motility, velocity, viability and morphology by pipetting a droplet and analyzing it using the same criteria and methodology as described for the fresh semen samples (paragraph 3.4.3).

3.7 Statistical analysis

Data was analyzed using the statistical program GenStat® 2003 (Payne *et al*., 2007). In each of the following chapters, the specific statistical analysis methods used will be described in more detail.

Chapter 4

General characterization of the South African unimproved indigenous goat semen

4.1 Introduction

The South African unimproved indigenous goat breed is comprised of a number of different ecotypes found in various parts of Southern Africa. Amongst these are the Speckled goats (Eastern Cape), the Loskop South indigenous goats (Xhosa goats of the Ciskei), the Kwazulu Natal goats (Nguni goats) and the Delftzijl goats (Tropic of Capricorn) (Campbell, 1995). The South African unimproved indigenous goats are generally found amongst the majority of small scale rural farmers in South Africa (Casey & Van Niekerk, 1988). However, very little is known regarding this breed. In the past the breed received very little attention from researchers. Nevertheless, this goat breed has been enjoying increasing interest from commercial farmers over the last few years (Campbell, 1995; Sundararaman & Edwin, 2008). This could be ascribed to its great attributes, including hardiness and adaptability to the local harsh environmental conditions and its outstanding capacity to produce and reproduce efficiently under sub-optimal nutritional conditions (Devendra & Burns, 1970; Webb & Mamabolo, 2004). However the reproductive characteristics of the South African unimproved indigenous goat are not very well known. According to current knowledge, no previous report exists in the literature regarding the semen characteristics of this genotype. This information is of paramount importance for future studies regarding the preservation (refrigeration for short term or cryopreservation for long term periods, respectively) of semen for AI utilization.

There is currently a need to document the basic semen characteristics (ejaculate volume, pH, sperm concentration, viability, structural morphology, velocity and motility) of this indigenous breed to serve as baseline data for preliminary studies on semen preservation for artificial insemination (AI) use. The use of this assisted reproductive technique (ART) has the potential to accelerate the genetic improvement of the breed, with similar benefits as those observed in the Boer goat breed (Schwalbach & Greyling, 2000). The aim of this study was thus to evaluate the semen of the South African unimproved indigenous goat, by evaluating its basic characteristics, namely the ejaculate volume, semen pH, sperm concentration, sperm

viability (percentage live sperm), structural sperm morphology (normal or abnormal), sperm motility (static, non progressive or progressive) and velocity (static, slow, medium or rapid) parameters.

4.2 Materials and Methods

A detailed description of the materials and methods used in this study is set out in Chapter 3 (General Materials and Methods) e.g. site where trials were conducted, care and training of the animals. This section will refer specifically to the materials and methods used in this part of the study.

4.2.1 Semen Collection

Seven South African unimproved indigenous bucks that were successfully trained for semen collection with the aid of an artificial vagina (AV) were used in this study. Semen was collected from each buck twice weekly (Gacitua & Arav, 2005; Sundararaman & Edwin, 2008) with the aid of an AV filled with water at 41 - 46°C (Silvestre *et al.,* 2004; Yamashiro *et al*., 2006). A doe in oestrus was restrained in a neck clamp to stimulate the bucks for semen collection. Six ejaculates were obtained from each buck in a period of 3 weeks between April and May 2009 (during the natural breeding season). During the semen collection process, when the buck mounted a doe, the penis was gently guided into the AV. To minimize stress and maximize the quality of the ejaculates, collections were always carried out under the same conditions, i.e. by the same person, at the same time of the day (8:00 – 10:00), in the same pen and using the same equipment (Siudzińska & Łukaszewicz, 2008).

4.2.2 Semen evaluation (macroscopic and microscopic), incubation/holding and transportation to the laboratory

Immediately following semen collection, the ejaculate was macroscopically evaluated and transferred into plastic screw top conic tubes, placed and stored in a thermo-flask and maintained at 37 °C (Silvestre *et al*., 2004), until sperm assessment (electronic/automated and microscopic analyses) at the Germplasm, Conservation and Reproduction Biotechnologies Laboratory (GCRB) of the ARC, within 1h (Gacitua & Arav, 2005).

4.2.3 Semen volume and pH

The ejaculate volume (ml) and semen pH were recorded immediately after collection. The semen volume being measured using a graduated collection tube. The semen pH was measured manually with the aid of Neutralit® pH-indicator strips (Merck KGaA, 64271 Darmstadt, Germany) by pippeting a drop (10 µl) of fresh semen onto the strip and spreading it gently. The resultant colour of the strip was then compared to the colour code of the graduated pH scale to obtain a pH reading.

4.2.4 Sperm cell concentration

In the laboratory, the sperm concentration of the semen was determined with the aid of a spectrophotometer (SpermaCue®, Minitüb, Germany). A volume of 20 µl undiluted fresh semen was pipetted into a microcuvette (HemoCue AB, Ängelholm, Sweden), which was then inserted into the SpermaCue to give an automated sperm concentration reading in $X10^6$ sperm/ml.

4.2.5 Sperm viability and morphology

Sperm viability (percentage live sperm) was determined using the new improved nigrosin/eosin (N/E) stain (pH=8.39; osmolarity=411; manufacture date: 30.04.09; Batch no: 2009/03), manufactured by the Onderstepoort Faculty of Veterinary Sciences' pharmacy (60 µl eosin/nigrosin and 6 µl semen aliquots). The vital staining method was indicative of the live or dead status of the sperm cells and allowed a good evaluation of the sperm morphology (normal or abnormal) of the sperm (Björndahl *et al*., 2003).

When using this staining technique, live sperm fluoresce white and the dead sperm red by absorbing the stain (Bearden *et al*., 2004). Live sperm were further categorized into morphologically normal or abnormal sperm. Abnormalities were recorded using two different sets of criteria. The first criteria used the location of the abnormality such as head, mid-piece and tail, as described by Łukaszewicz *et al*. (2008). Abnormalities were then also classified as primary, secondary or tertiary, according to the seriousness of the lesion as described by Loskutoff and Crichton (2001).

4.2.6 Sperm motility analysis by CASA

For automated sperm analyses, a Sperm Class Analyzer®-SCA® (V.4.0.0.1 Animal/Veterinary Microptic S.L, Barcelona, Spain) was used to analyze the sperm motility (percentage immotile/static sperm or motile, but not progressive and progressively motile sperm) as well as certain velocity parameters (static, slow, medium, rapid, curvilinear, straight-line, average path, linearity, straightness and wobble velocities). Progressive sperm movement measured by computer assisted sperm analysis (CASA) is generally expressed by progressive velocity (VSL), path velocity (VAP) and track speed (VCL). The sperm washing solution (BO) was warmed to 37°C on a warm plate of a slide warmer (Precision Scientific Company, Chigago 47, USA), before adding the semen. A total volume of 10 µl of semen was diluted with 500 µl of BO medium for swim-up and incubated in the MCO-20 AIC Sanyo CO_2 incubator (Sanyo Electric Biomedical Co.,Ltd, Japan), adjusted to 37°C for 5 minutes. Following the incubation period, 5 µl of this semen solution was pipetted onto pre-warmed, bevel-edged, frosted-end, microscope glass slides (Thermo Scientific Menzel-Gläser, Germany), gently covered with a microscope cover glass (Menzel gläser, Germany) and evaluated under x10 magnification of the CASA microscope, throwing an image on the monitor. The results were saved on a Microsoft excel sheet.

4.2.7 Statistical analysis

Data were analyzed using the statistical program GenStat® 2003 and are presented as the mean and standard deviation (SD), for each buck (for 6 collections over a 3 week period). The overall mean (± SD) for all parameters considered were also calculated. Analysis of variance (ANOVA) for unbalanced data was used to compare the semen from the different unimproved indigenous bucks in terms of semen volume, semen pH, sperm concentration, morphology and motility. The data was normally distributed with homogeneous treatment variances, and comparisons were done at the 5% level of confidence.

4.3 Results and Discussion

Table 4.1 sets out the mean (± SD) values for ejaculate volume, semen pH and sperm concentration for each one of the 7 experimental animals evaluated.

Table 4.1 The mean (± SD) ejaculate volume, semen pH and sperm concentration of the South African unimproved indigenous goat during the natural breeding season

Buck	Volume (ml)	pH	Concentration (x10^6 sperm/ml)
1	0.3^b ± 0.2	7.1^a ± 0.6	672.6^a ± 310.0
2	0.4^b ± 0.2	7.7^a ± 0.4	618.2^a ± 174.1
3	0.7^a ± 0.2	7.7^a ± 0.4	688.0^a ± 78.7
4	0.4^b ± 0.1	7.5^a ± 0.6	715.0^a ± 266.3
5	0.5^b ± 0.1	7.2^a ± 0.3	683.7^a ± 244.2
6	0.3^b ± 0.3	7.5^a ± 0.0	826.0^a ± 1.4
7	0.4^b ± 0.1	7.8^a ± 0.4	578.0^a ± 140.0
Overall average	0.5 ± 0.2	7.5 ± 0.2	681.7 ± 74.6
SEM	0.2	0.5	214.7
Probability	0.016	0.374	0.908
CV%	43.0	6.1	31.7

SEM = standard error of the mean
CV = coefficient of variation
$^{a-b}$Means with different superscripts within the same column, differ significantly at $P < 0.05$

In general, the mean values observed for semen volume and pH, as well as for sperm concentration, were very similar between all the experimental animals used. The exception was for the semen volume of buck number 3 (Table 4.1), which produced a significantly higher ejaculate volume than any of the other animals evaluated. This animal however recorded similar semen pH and sperm concentration values to all other bucks evaluated in this trial.

The mean ejaculate volume has been found to vary between breeds, and it is affected by the age of the buck, method of collection and between the seasons of the year (Hashida & Abdullah, 2003; Silvestre *et al*., 2004; Daramola *et al*., 2007). So for example, the mean ejaculate volumes differ between 0.75 ml in Zarabi goats, 1.56 ml in Boer goats to 2.0 ml for Saanen bucks and 2.1 ± 1.0 ml in Korean Native bucks (Greyling & Grobbelaar, 1983; Gacitua & Arav, 2005; Barkawi *et al*., 2006; Choe *et al*., 2006; Talebi *et al*., 2009). From the values quoted in the literature, it is evident that the S.A unimproved indigenous goat evaluated in the current trial recorded a relatively lower ejaculate volume when compared to the other breeds. Positive correlations have been recorded between semen volume and semen concentration by Sarder (2008). In goats only a few does can be inseminated with one ejaculate. Contrary, high semen volume is a problem in that it dilutes the total sperm present, thus decreasing the sperm concentration and this is especially evident in pigs.

It is evident from the literature that semen pH varies amongst breeds, age of the buck, method of collection, method of determination and between different seasons of the year. Semen pH varied from 6.4 in the Boer goat to 7.0 ± 0.4 for Korean Native bucks and 7.3 in West African Dwarf buck-kids (Greyling & Grobbelaar, 1983; Barkawi *et al*., 2006; Choe *et al*., 2006; Daramola *et al*., 2007; Talebi *et al*., 2009). The pH values recorded in this study are in agreement with other reports. Mammalian semen is quoted to normally have a pH in the range of 7.2 to 7.8 (Prins, 1999). Furthermore, Fukuhara and Nishikawa (1973) reported the oxygen uptake of goat sperm to be maximal between a pH of 7.2 and 7.5, and sperm cell motility to be optimal between semen pH values of 7.0 and 7.2. Normally the pH of semen is slightly alkaline, because of the seminal vesicle secretions. This also protects the sperm from the acidity of the vaginal fluids in the female's reproductive tract. An acidic pH could suggest problems with seminal vesicle function. A pH value outside of the normal pH range has been found to be harmful to sperm (Essig, 2007).

It has also been found that the sperm concentration of goats vary amongst breeds, age of the buck, between different seasons of the year, method of collection and technique used for determination (Silvestre *et al*., 2004; Gacitua & Arav, 2005; Barkawi *et al*., 2006; Choe *et al*., 2006;. Daramola *et al*., 2007; Talebi *et al.,* 2009). It has been recorded to differ between 4.2 ± 0.5 x 10^6 sperm/ml in Majorera goats, to 1326.3 ± 335.3 x 10^6 sperm/ml for Boer goat bucks and 4.04 x 10^9 sperm/ml in the Serrana breed (Barbas *et al*., 2006; Almeida *et al*., 2007; Batista *et al*., 2009). A high sperm concentration is positively correlated to its fertilization ability. In addition, higher sperm concentrations definitely enhance a higher percentage of embryonic development (Wolf & Inoue, 2005; Brown & Knouse, 1973). Garner *et al*. (2001) reported that contrast analyses showed that sperm viability significantly decreased as sperm concentration decreased, thus less viable sperm cells may be obtained from semen samples with a low sperm concentration. The concentration values obtained in this study were comparable to those obtained by Batista *et al*. (2009), who also used the SpermaCue method to determine sperm concentration in Majorera goats. This endorses the effectiveness of this method for the determination of sperm concentration in goats. Almeida *et al*. (2007) used the improved Neubaur haemocytometer and obtained sperm concentration results similar to those reported by Greyling and Grobbelaar (1983) for the Boer goat, which are approximately double that recorded in the current study for the unimproved S.A indigenous goat. It is unclear why the unimproved indigenous goats have a relatively lower sperm concentration. It is important to conduct more studies on this breed to comfirm or reject these results.

The percentage (mean ± SD) normal and percentage live the sperm for the different South African unimproved indigenous bucks, as well as the overall averages recorded during the 6 collections over the 3 week period of this study, are set out in Table 4.2.

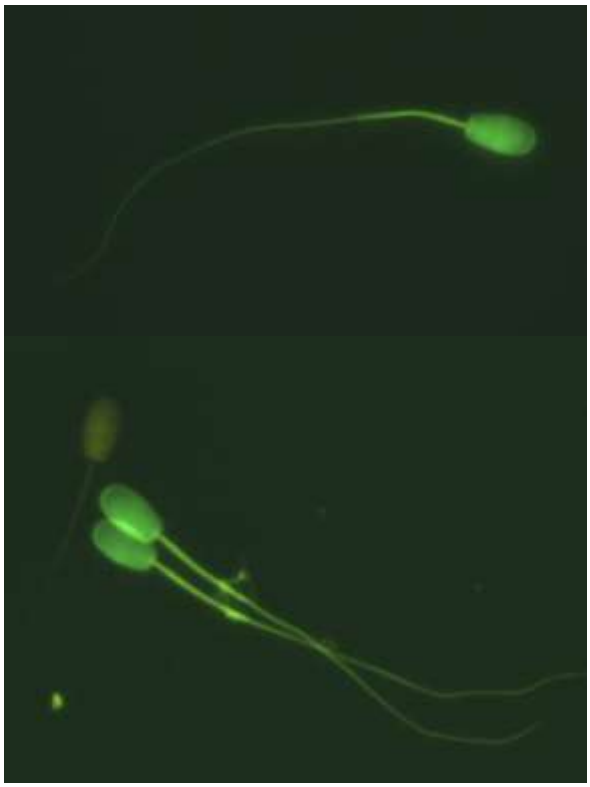

Plate 4.1 An example of a live sperm

Table 4.2 The mean (± SD) percentage live and percentage normal sperm in the semen of the South African unimproved indigenous bucks during the natural breeding season

Buck	Live sperm (%)	Normal sperm (%)
1	$79.7^{ab} \pm 6.8$	$84.7^{a} \pm 4.1$
2	$60.3^{c} \pm 7.3$	$69.5^{c} \pm 9.6$
3	$76.5^{ab} \pm 7.0$	$82.8^{a} \pm 4.3$
4	$72.8^{b} \pm 11.7$	$72.6^{bc} \pm 6.1$
5	$79.3^{ab} \pm 4.0$	$79.3^{ab} \pm 8.5$
6	$86.5^{a} \pm 0.7$	$86.5^{a} \pm 0.7$
7	$78.5^{ab} \pm 9.2$	$77.5^{abc} \pm 7.8$
Overall average	76.3 ± 8.2	79.0 ± 6.3
SEM	7.8	6.7
Probability	0.002	0.005
CV%	10.5	8.5

SEM = standard error of the mean and CV = coefficient of variation of each experiment
$^{a-c}$Means with different superscripts within the same column, differ significantly at $P < 0.05$

The South African unimproved indigenous bucks used in this study recorded an overall average percentage of 79.0 ± 6.3% for normal and 76.3 ± 8.2% for live sperm, which comply with the means for good quality semen. So for example, Gil *et al*. (2001) reported semen with >70% live and >70% normal sperm as being semen of good quality and which is in line with the findings of the current study. The normal sperm recorded differed between 76.2 ± 9.3 % in Boer goats to 81.1% for Serrana bucks and the percentage live sperm varied between 51% in West African Dwarf buck-kids, to 88.2 ± 5.6% in Korean Native bucks and 90.7 ± 0.8% in Markhoz bucks (Barbas *et al*., 2006; Barkawi *et al*., 2006; Choe *et al*., 2006; Almeida *et al*., 2007; Daramola *et al*., 2007; Talebi *et al*., 2009).

Buck number 2 in the current trial recorded a significantly ($P < 0.05$) lower percentage normal as well as live sperm, compared to all the other bucks evaluated. The values for this buck also fall outside the range quoted in the literature (i.e. lower than 70% live and 70% normal sperm) and could most possibly be attributed to the effect of sperm concentration, age and secretory activities of the accessory sex glands may affect these semen characteristics (Tomar *et al*., 1985; Leon *et al*., 1991; Garner *et al*., 2001). Contrast analyses have shown that sperm viability significantly decreased as the sperm concentration decreased (Garner *et al.,* 2001). Age has been recorded to have a significant effect on the sperm abnormalities, except for proximal cytoplasmic droplets (Tomar *et al*., 1985). In addition, impaired spermatogenesis is generally induced by impaired secretory activities of the sex glands (Leon *et al*., 1991). The mean percentage live and normal goat sperm tend to vary between breeds, age of the buck, method of determination and between different seasons of the year (photoperiod).

Sperm morphology seems to be one of the most important qualitative characteristics of semen and its assessment is an integral component in the analysis of semen and is an important part of any breeding soundness examination (Kuster *et al*., 2004). Sperm viability tests are used in cases of low motility to determine the presence of live sperm vs. static sperm and sperm morphology is considered as a predictor of successful fertilizing ability. Poor sperm morphology has been indicated to be an indicator of decreased fertility in goats. Thus, animals with few normal sperm, fail to fertilize without the aid of micromanipulation (Chandler *et al*., 1988).

In Table 4.3 the sperm abnormalities of the S.A unimproved indigenous buck's semen are further described in terms of their locations and expressed as a percentage of the total number of sperm cells evaluated.

Plate 4.2 Sperm with a tail abnormality

Table 4.3 The mean (± SD) head, mid-piece and tail sperm abnormalities of semen in South African unimproved indigenous bucks during the natural breeding season

Buck	Head (%)^{ns}	Mid-piece (%)^{ns}	Tail (%)^{ns}
1	5.3 ± 6.6	2.3 ± 1.0	7.7 ± 5.2
2	5.2 ± 6.1	6.0 ± 3.9	17.7 ± 8.6
3	2.8 ± 4.0	3.7 ± 1.6	10.7 ± 6.4
4	4.0 ± 4.2	6.0 ± 3.4	18.2 ± 8.5
5	2.3 ± 1.5	6.7 ± 2.3	11.7 ± 6.5
6	5.5 ± 0.7	4.5 ± 0.7	3.5 ± 0.7
7	4.5 ± 6.4	3.0 ± 4.2	15.0 ± 9.9
Overall average	4.2 ± 1.3	4.6 ± 1.7	12.1 ± 5.4
SEM	5.1	2.7	7.2
Probability	0.955	0.160	0.088
CV%	120.1	59.2	56.7

SEM = standard error of the mean
CV = coefficient of variation
ns = no significant differences between means in the same column ($P > 0.05$)

As it is evident in Table 4.3, similar means were recorded for all the different types of sperm abnormalities (head, mid-piece and tail) in all the unimproved indigenous bucks evaluated. This indicates that all bucks used had very similar sperm morphologies, which gives credibility to the overall average abnormalities obtained in this study as an indicative value for the breed. Though there was no statistical difference among the bucks, a large numerical difference was observed on tail abnormalities for buck 6 and this can be associated with the large variation amongst individual bucks. Mean sperm abnormalities have been found to vary

amongst breeds, technique of determination and between different seasons of the year. The overall mean percentage of sperm abnormalities for the head, mid-piece and tail were 4.2 ± 1.3, 4.6 ± 1.7 and 12.1 ± 5.4%, respectively (Table 4.3). Sperm abnormalities recorded for other breeds are 9.3% sperm head abnormalities in Serrana goats, 7.5 ± 4.0 % mid piece abnormalities and 35.0 ± 5.9% for Boer goats (Barbas *et al*., 2006; Almeida *et al*., 2007). The sperm head abnormalities recorded in the current study were less than the 9.3% observed by Barbas *et al*. (2006). Most of the sperm abnormalities in this study were situated on the tail, although they were not significantly different from those from the other locations (head or mid-piece). Poor semen morphology has been accepted as an indicator of decreased fertility in goats, thus a high percentage of abnormal sperm impairs the fertilization potential. In general, these results are in line with those cited in the literature (Chandler *et al*., 1988).

In Table 4.4 the sperm abnormalities, as classified according to the severity of the lesions are set out. More crucial, less crucial and sperm injuries resulting from improper handling of the semen (post-ejaculation) or as a consequence of manipulation and other environmental factors were classified as primary, secondary and tertiary lesions, respectively. Each type of sperm abnormality (primary, secondary or tertiary) was then expressed as a percentage of the total number of sperm cells evaluated (Loskutoff & Crichton, 2001; Kebede *et al*., 2007).

Table 4.4 The mean (± SD) sperm structural abnormalities in the ejaculate of the South African unimproved indigenous bucks during the natural breeding season

Buck	Primary abnormalities (%)^{ns}	Secondary abnormalities (%)^{ns}	Tertiary abnormalities (%)^{ns}
1	0.8 ± 1.6	6.8 ± 4.4	6.2 ± 3.3
2	0.7 ± 2.4	13.7 ± 5.1	13.5 ± 6.2
3	0.8 ± 0.8	7.5 ± 4.2	8.8 ± 2.9
4	0.2 ± 0.5	12.8 ± 4.0	15.2 ± 9.3
5	0.3 ± 0.6	11.0 ± 4.0	9.3 ± 5.5
6	0.5 ± 0.7	7.0 ± 4.2	6.0 ± 2.8
7	2.5 ± 2.1	8.0 ± 11.3	12.0 ± 1.4
Overall average	1.0 ± 0.8	9.5 ± 2.9	10.1 ± 3.6
SEM	1.5	4.2	5.5
Probability	0.496	0.166	0.131
CV%	160.3	49.9	53.4

SEM = standard error of the mean
CV = coefficient of variation of each experiment
ns = no significant differences between means in the same column (P > 0.05)

Similar means were recorded in the different individual unimproved indigenous bucks used in this study in terms of primary, secondary and tertiary sperm abnormalities. Mean overall abnormalities have been reported to differ between breeds, age of the buck, method of determination and between the different seasons of the year (photoperiod). In West African Dwarf buck-kids, an average of 1.2% primary abnormalities were recorded (Daramola *et al*., 2007), which is in line with the findings of this study. The overall abnormalities recorded in this study were approximately 20% of the total sperm cells. Similarly, a total of 11.9 ± 0.2% sperm abnormalities were reported for Alpine goats and 13.5 ± 0.3% sperm abnormalities for Zarabi goats (Karaginnidis *et al*., 2000; Barkawi *et al*., 2006), respectively. Primary and secondary abnormalities have been negatively correlated to fertility rates (Saacke & White, 1972). It is evident that a high proportion of major sperm abnormalities is detrimental to the fertilization potential. Sarder (2008) reported a negative correlation between percentage abnormal sperm and percentage motile sperm.

The results in terms of sperm morphology (total abnormalities) using both systems (location or severity) are very similar and in line with those reported in the literature for other goat breeds. Furthermore, these results are also indicative of general good semen quality with high fertilization potential in the S.A unimproved indigenous goat.

The sperm motion characteristics recorded by the CASA system in the semen of the unimproved indigenous goat are summarized in Table 4.5.

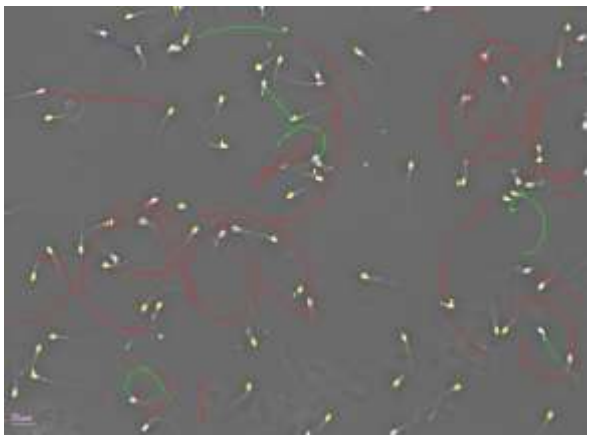

Plate 4.3 Image of sperm motility tracts using the CASA system

Table 4.5 The mean (± SD) sperm motion characteristics in the ejaculates of the South African unimproved indigenous buck during the natural breeding season

Buck	Static (%)^{ns}	NPM (%)^{ns}	PM (%)^{ns}	Slow (%)^{ns}	Medium (%)^{ns}	Rapid (%)^{ns}
1	56.3 ± 39.3	24.8 ± 22.2	18.9 ± 17.8	3.5 ± 2.4	7.2 ± 6.4	32.9 ± 33.8
2	39.8 ± 20.5	28.9 ± 15.7	31.3 ± 7.0	4.4 ± 5.9	6.7 ± 5.2	49.1 ± 20.7
3	35.3 ± 28.8	22.6 ± 19.2	42.8 ± 24.5	5.6 ± 3.7	3.7 ± 3.0	56.6 ± 26.3
4	23.1 ± 6.1	28.9 ± 5.0	48.0 ± 4.9	6.4 ± 3.8	4.4 ± 2.7	66.1 ± 4.9
5	16.7 ± 12.9	40.2 ± 9.4	41.6 ± 6.0	2.1 ± 2.2	6.1 ± 1.8	73.6 ± 18.3
6	13.7 ± 6.2	52.9 ± 6.9	33.5 ± 13.1	6.3 ± 3.6	8.6 ± 1.0	71.5 ± 8.9
7	31.2 ± 5.0	24.2 ± 11.4	44.7 ± 16.4	6.5 ± 2.8	5.2 ± 1.0	57.2 ± 6.3
Overall average	30. 9 ± 14.7	32.1 ± 10.9	37.3 ± 10.0	4.9 ± 1.7	6.0 ± 1.7	58.2 ± 14.1
SEM	23.0	15.1	14.8	4.0	4.0	21.2
Probability	0.239	0.274	0.110	0.743	0.648	0.184
CV%	69.8	51.2	39.5	82.0	70.6	37.5

Static = non-motile; NPM = motile but non-progressive motility; PM = Progressive motility;
SEM = standard error of the mean
CV = coefficient of variation
ns = no significant differences between means in the same column ($P > 0.05$)

Similar sperm motility values were recorded in the ejaculates of all indigenous bucks in terms of sperm motion characteristics as determined by the CASA system. The S.A unimproved indigenous goat has recorded 31 % static sperm and total motility of 69 %. Relating to the findings in the literature, these results are more or less in line with other findings though they highly differ from some on the progressive motility. The overall average progressive sperm motility recorded in this study was 37%, which is much lower than the 72.1% reported for Boer goats. The 78.9% reported for Zarabi goats and 83.9% reported for the Angora (Markhoz) breed (Barkawi *et al*., 2006; Sundararaman & Edwin, 2008; Talebi *et al*., 2009). In general, the S.D for motility parameters are very high, indicating great differences between ejaculates of the same buck. This can be attributed to animal variation and probably human error based on the fact that CASA is a relatively new system at Germplasm, Conservation and Reproduction Biotechnologies Laboratory of the Agricultural Research Centre. Staff operating the system is still under training and is not fully familiar with the system.

Sperm motility is believed to be one of the most important characteristics to estimate the fertilisation potential of ejaculated sperm and can also be useful in the selection and ranking of bucks regarding their potential fertility (Hashida & Abdullah, 2003). Cremades *et al*. (2005) considered sperm motility to be important for certain steps of sperm transport within the female reproductive tract, and primarily for the penetration of the zona pellucida of the oocyte. Sperm motility, in general and characteristics of sperm motion in particular, are good indicators of the quality of sperm. Generally, by evaluating the proportion of progressively motile from a sample, the quality of semen can be monitored (Sundararaman & Edwin, 2008). The progressive motile sperm being defined as one progressing from one point to another in a more or less straight line. Only the progressively motile and rapid moving sperm are said to stand a chance to fertilize the oocytes, under natural conditions (Boyle e*t al*., 1992). The ability of the sperm to swim into a medium has been extensively used in the selection of motile sperm cells for in vitro fertilization (Tanphaichitr *et al*., 1987).

In Table 4.6 the mean sperm values for sperm velocity and linearity parameters of the different individual unimproved indigenous bucks, as determined by the CASA analysis, are set out.

Table 4.6 The mean (± SD) values for sperm velocity and linearity parameters in the semen of unimproved indigenous bucks during the natural breeding season

Buck	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)
1	152.2^{a} ± 36.8	87.2^{a} ± 28.6	119.4^{a} ± 40.0	56.3^{a} ± 8.4	73.2^{ab} ± 1.7	76.9^{a} ± 10.8
2	176.5^{a} ± 30.0	120.8^{a} ± 25.3	154.7^{a} ± 29.8	68.5^{a} ± 8.6	78.5^{a} ± 8.0	87.4^{a} ± 4.5
3	193.3^{a} ± 21.2	130.8^{a} ± 27.6	158.4^{a} ± 18.9	67.1^{a} ± 9.5	81.8^{a} ± 9.1	81.9^{a} ± 3.4
4	196.4^{a} ± 13.8	138.7^{a}±10.8	168.6^{a} ± 13.1	70.7^{a} ± 4.0	82.3^{a} ± 1.9	85.8^{a} ± 3.5
5	205.5^{a} ± 15.6	129.0^{a} ± 2.2	177.5^{a} ± 3.6	63.0^{a} ± 4.3	72.7^{ab} ± 1.1	86.6^{a} ± 4.9
6	179.1^{a} ± 38.9	99.2^{a} ± 26.6	147.6^{a} ± 18.1	55.1^{a} ± 2.9	66.6^{b} ± 9.9	83.2^{a} ± 8.0
7	203.8^{a} ± 38.9	141.2^{a} ± 49.7	164.1^{a} ± 37.5	68.2^{a} ± 11.4	84.8^{a} ± 10.9	80.2^{a} ± 3.1
Overall verage	186.7 ± 18.9	121.0 ± 20.4	155.8 ± 18.7	64.1 ± 6.2	77.1 ± 6.5	83.2 ± 3.7
SEM	26.4	24.5	24.9	7.6	6.7	5.6
Probability	0.124	0.056	0.087	0.066	0.040	0.123
CV%	14.2	20.0	16.0	11.7	8.6	6.7

VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness and WOB = wobble, CV = coefficient of variation

SEM = standard error of the mean

$^{a-b}$Means with different superscripts within the same column, differ significantly at $P < 0.05$

In general, the sperm parameters recorded for VCL, VSL, VAP, LIN and WOB were very similar between all the bucks used. The exception was the STR (straightness) of sperm for buck number 6, which was significantly lower ($P < 0.05$) than that of all the other animals evaluated. However, this animal showed similar sperm motility values regarding VCL, VSL, VAP, LIN and WOB parameters, to those of all the other experimental animals. From the values of table 4.6 it is evident that the variance component for all motility parameters considered are relatively high. This indicates relatively high differences between ejaculates of the same buck and poor repetition of values recorded between ejaculates.

Progressive sperm velocity (VSL), path velocity (VAP), linearity (LIN) and straightness (STR) generally express the progressive movement of sperm as measured by CASA (computer assisted sperm analysis) and are correlated with the fertilizing ability of the sperm (Verstegen *et al*., 2002). In the literature, more attention has been focussed on the LIN and STR motility. The LIN measures the deviation of the cell track/trajectory from a straight line and STR measures the departure/deviation of the cell path from a straight line. The overall sperm linearity and straightness recorded in this trial were 64.1 ± 6.2% and 77.1 ± 6.5% respectively. The results for linearity (LIN) are in line with the ones reported by Sundararaman and Edwin (2008) and Anel *et al*. (2003) who recorded 61.70 to 62.80% for the Boer goat and 62.5% for Churra rams, respectively.

Goats have been characterised as short day breeders i.e. their highest breeding activity and fertility is in autumn and as photoperiod increases (spring and early summer), qualitative and quantitative semen production decreases, abnormal spermatozoa increases thus fertility decreases (Delgadillo *et al*., 1993; Corteel, 1977; Talebi *et al*., 2009). Nutrition is considered to be an important factor affecting seasonality of reproductive functions in bucks. Thus, poor nutrition reduces reproduction mostly in extensive or semi-extensive systems, where food availability is highly dependent on the season. This attribute has led researchers to link nutrition with the accountability for seasonal reproductive patterns (Walkden-Brown *et al*., 1994; Zarazaga *et al.*, 2009). The differences between breeds have been associated with the latitudes where they are raised, with those far from the equator affected most by season (Corteel, 1977; Corteel, 1981; Tuli *et al.*, 1991). Therefore, these factors can be associated with the poor results obtained from this study considering the fact that the trial extended into the transition period.

4.4 Conclusions

This was a preliminary study on the semen characteristics of the South African unimproved indigenous breed and these accounts for the limited references or citations on the breed. The 7 bucks used are assumed to be good representatives of the South African unimproved goat breed. In general, most of the semen quantity and quality values obtained from the individual experimental animals were very similar amongst the bucks.

South African unimproved indigenous bucks have shown to produce a lower semen volume, sperm concentration, and less progressively motile sperms compared to the European, Asian breeds and also the S.A Boer goat. This can be attributed to factors such as age, different ecotypes that the bucks originated from (breeds), the season of the year in which the study was conducted, method of semen collection and semen handling. Results for sperm morphology shown an indication of good quality semen for the S.A unimproved indigenous goats, but these are contradicted by the sperm motility results. This could be attributed to the laboratory error (human factor). Sperm pH, sperm viability, % normal, severity and the location of abnormalities as well as velocity parameters were in line with values from the literature for other goat breeds. This indicates effective semen collection and evaluation techniques.

Chapter 5

The efficiency of four different extenders for semen cryopreservation in the unimproved indigenous South African goat

5.1 Introduction

Semen cryopreservation is generally defined as the freezing of sperm cells, to preserve them indefinitely, for future use (Dinnyes *et al*., 2007). This technique has facilitated the widespread use of superior males and resulted in accelerated genetic improvement of livestock, particularly dairy cattle (Gordon, 2004). However, goat semen does not freeze satisfactorily, compared to semen from, for example the bull or ram. In general, all sperm cryopreservation protocols include temperature reduction, cellular dehydration, freezing and ultimately thawing (Medeiros *et al*., 2002). Each of these steps may contribute to reduce the cryopreserved sperm quality after thawing (Lebouef & Salamon, 2000). The freezing or cryopreservation technique is thus seen as one of the most crucial factors that affect the post-thaw quality of cryopreserved goat semen, and ultimately its fertilization capacity, as measured by the conception rate following AI (Choe *et al.,* 2006).

There are indications that frozen-thawed sperm have much more structural damage, when compared to fresh sperm and that this cell damage severely decreases both the sperm motility and the fertilizing ability (Matsuoka *et al,* 2006). To ensure acceptable fertilization success, not only must suitable extenders, sperm dilution rates, cooling and thawing rates be implemented, but an intricate knowledge of the sperm physiology of the species is essential in order to maximize the post-thawing recovery of sperm and consequently its fertilizing ability (Purdy, 2006).

Specific problems in the cryopreservation of goat semen are related to the sensitivity of the sperm to chilling or low temperatures, and the detrimental effects of certain seminal plasma components (the interaction between these and some components of the semen extender), resulting in poor post thaw sperm viability (Purdy, 2003). One of the most important seminal plasma components contributing to this poor cryopreservation ability in goats is an enzyme known as egg yolk coagulating enzyme (EYCE), a phospholipase A, originating from secretions of the bulbo-urethral gland. This enzyme hydrolyses the egg yolk lecithin (in the

extender) to fatty acids and lisolecithin enzyme which are toxic to goat sperm (Leboeuf *et al*., 2003). The EYCE, which is not present in bull semen, has forced research into the development of alternative semen extenders for buck semen cryopreservation. In order to overcome this limitation with egg yolk, goat semen is often both centrifuged and washed to partly eliminate the toxic effects of this enzyme in the seminal plasma prior to dilution with the standard semen extenders containing high egg yolk concentrations (e.g. 20% egg yolk). Another alternative is the use of extenders with low-egg yolk concentrations (e.g. 2%). However, these low concentrations may result in insufficient cryoprotection of the sperm cell membranes and also result in poor sperm viability, post- thawing (Baldassarre & Karatzas, 2004). There are also reports indicating that successful cryopreservation of goat semen requires the total removal of seminal plasma. The subsequent dilution of the semen with skimmed milk may result in higher post-thaw conception rates, when compared to following dilution with buffers containing egg yolk (Gordon, 2004).

Washing of sperm is regarded as a complex and time consuming process that also induces cell damage and causes loss of sperm (Corteel, 1981). Ritar and Salamon (1983) and Corteel *et al*. (1975), found that sperm washing does not appear to improve the fertility of the sperm in dairy and fibre-producing goats. In addition Cabrita *et al*. (2001) reported that semen extenders containing products of animal origin may also pose a risk of carrying infectious disease caused by virus or bacteria. For this reason, there has recently been a movement towards trying to remove all components of animal origin, including egg yolk, milk and even bovine serum albumin (BSA) which has been used as a substitute for egg yolk in semen extenders for buck sperm cryopreservation. A soybean-lecithin-based extender (Andromed; Minitüb, Tiefenbach, Germany) that was originally developed for bovine semen cryopreservation has been successfully utilized for buck semen (Janett *et al*., 2005).

Another egg-yolk free extender, Biociphos-Plus® (IMV, France), in which the egg-yolk is replaced by a sterile soybean extract to reduce the risk of contamination derived from animal borne substances is commercially available and has been used in bull semen preservation with success (Thun *et al.*,2002). Gacitua and Arav (2005) used another egg-yolk free extender, Andromed® which was found to be effective in cryopreserving buck semen. An animal protein free commercial extender (Ovixcell®, IMV Technologies, France) also developed for ram semen, and is to be tested in this study.

The aim of this trial was thus to evaluate and compare the efficiency of four different semen extenders for the cryopreservation of the unimproved indigenous South African goat sperm.

5.2 Materials and Methods

5.2.1 Selection of experimental animals

From the group of 7 bucks that were successfully trained for semen collection with the aid of the AV and used to characterize the semen of the unimproved South African goat (Chapter 4), only 5 bucks (*n = 5*) were selected to serve as semen donors for sperm cryopreservation. One buck produced relatively poor semen samples, while the other became reluctant to mount and ejaculate into the AV regularly during the second experiment. For these reasons, these two bucks were excluded from this part of the study. Only buck semen samples of high quality (i.e. >70% live sperm; >70% normal sperm; >70% motile sperm) and a sperm concentration of >500 × 10^6 sperm/ml, were pooled and used in the cryopreservation study (Ritar *et al*. 1990a; Gil *et al*. 2001). The semen samples were pooled due to the relatively low volumes of ejaculates obtained from bucks in general and to eliminate individual differences in terms of semen quantity, quality and cryotolerance (Karagiannidis *et al*., 2000).

5.2.2 Semen collection

Semen was collected twice a week on 7 occasions during a period of one month from each buck (at ±4 days intervals), during the natural mating season. For more details on the experimental animals used, the general management, semen collection and handling techniques, please refer to Chapter 3 (General Materials and Methods).

5.2.3 Semen processing

Pooled semen was extended using one of four different extenders, namely Tris-1.5% egg yolk (Ritar & Salomon, 1983), Tris-15% BSA (Matsuoka *et al*., 2006), Ovixcell® (Nedambale personal communication 2009, ARC, RSA) and Bioxcell® (Gil *et al*. 2003). The latter two semen extenders are both commercially available and ready to be used, available from IMV (L'Aigle, France). Each one of these 4 extenders was prepared in two fractions: Fraction A (without glycerol as a cryoprotectant-CPA) and Fraction B (containing 12% glycerol as a CPA). Fraction A was warmed to 37 ºC prior to use, while Fraction B was cooled to 5 ºC, prior to use.

Initially, the pooled semen samples were divided into 4 aliquots of similar volume. Each aliquot was then diluted at a 1:1 ratio at room temperature with one of the four Fraction A's (CPA-free) extenders at 37 ºC and equilibrated for 1.5 h at 5 °C in a refrigeration unit (Recam International, South Africa). After a period of 1.5 h of equilibration at 5 °C, each one of the four aliquots (extended solution A) was further sub-divided into two halves and placed into two different test tubes (8 test tubes in total). One of the 2 test tubes per extender containing semen diluted in Fraction A was further diluted 1:1 with the same extender's fraction A (CPA–free) and served as control treatments, while the other tube was diluted 1:1 with fraction B (containing glycerol at 12%) of the respective extender, to evaluate the effect of adding glycerol at 6% to the extended semen.

The experimental outlay of this trial is set out in Table 5.1. Fraction B containing glycerol, was added drop-wise (while mixing gently), at 5°C, into the test tube containing semen diluted in the respective extender A in order to prevent injury (shock) to the sperm (Barbas & Mascarenhas, 2009). The extended semen was subjected to another period of 1.5 h of equilibration at 5ºC, making the entire equilibration time 3h (Purdy, 2006), and the final glycerol concentration in the four extenders containing CPA at 6% (Kundu *et al*., 2000).

Table 5.1 Experimental outlay using four semen extenders with or without 6% glycerol

Extender	Glycerol	
	0%	6%
Tris - 1.5% egg yolk	T_1	T_2
Tris – 15% Bovine Serum Albumin	T_3	T_4
Ovixcell	T_5	T_6
Bioxcell	T_7	T_8

T_1 to T_8 = treatments 1 to 8

The reason why the Tris-1.5% egg yolk treatment was selected, is that it was considered easy to use as it does not require sperm washing and it gave the best results in goats, according to Ritar and Salamon (1983), when compared to other higher egg yolk concentrations. The Tris-15% BSA was also selected as it gave the best results in cryopreserved ram semen according to Matsouka *et al*. (2006), when compared to other BSA concentrations. Both Bioxcell® and Ovixcell® were chosen for the study due to the fact that these commercial extenders are free from any products of animal origin. Bioxcell® (developed for bull semen) has produced very good post-thaw results in ram semen (Gil *et al*., 2003), while Ovixcell® (developed for ram semen) is a recently developed extender, offered to a few animal reproductive laboratories for a preliminary evaluation in sheep and goats (Nedambale personal communication 2009, ARC, RSA).

5.2.4 Semen freezing/cryopreservation protocol

Straws (0.25ml), water, polyvinyl alcohol powder (PVA) and soft paper tissue were all cooled down to 5°C prior to the loading of the straws. Straws were loaded with the extended semen at the end of a total of 3 h equilibration period by aspiration. At least two straws for each one of the 8 semen extender treatments considered (4 different extenders with or without 6% glycerol making a total of 8 treatments) were filled with extended semen and sealed with PVA. Straws of different colours were used in order to identify the 8 different treatments. After loading and sealing, all the straws were frozen simultaneously in a programmable freezer (CBS freezer 2100 series), using a customized freezing curve (Table 3.2). When the freezing process was concluded, after approximately 8 minutes, the straws were plunged into a polystyrene container filled with liquid nitrogen before being transferred into goblets and preserved in a liquid nitrogen tank (-196ºC) for 7 days, whilst pending further sperm analyses (Barbas *et al*., 2006).

5.2.5 Semen thawing and evaluation

After 7 days of storage in liquid nitrogen (-196ºC), one semen straw from each one of the 8 treatments collected and cryopreserved at each of the 7 collections occasions (replications), was thawed by placing the frozen semen straw into a water bath at 37°C for 30 seconds (Deka & Rao, 1987; Purdy, 2006). Following drying of the straw, both of the sealed ends were cut using a pair of scissors, and the thawed semen emptied into a sterile conical plastic tube from which semen samples were then drawn, for microscopic evaluation.

Semen samples (both immediately after extending into the 4 different extenders without glycerol and post-thawing) for each one of the eight treatments considered and from each of the seven collections were evaluated for sperm viability (percentage live or dead) and morphology (live and normal sperm) using standard laboratory procedures (Loskutoff & Crichton, 2001; Bearden *et al*., 2004) Sperm motility and velocity parameters were assessed by Computer assisted sperm analysis (CASA) using a Sperm Class Analyzer®. More details on the criteria and methodology used for sperm analysis are set out in the General Materials and Methods in Chapter 3.

To test the effect of incubation time on post-thaw sperm motility, semen was analyzed immediately after thawing (0 minutes), 30 minutes and 60 minutes after incubation at 37°C in an MCO-20 AIC Sanyo CO_2 incubator (Sanyo Electric Biomedical Co., Ltd, Japan), using the Sperm Class Analyzer®.

5.2.6 Statistical analysis

Data were analyzed with the aid of the statistical program GenStat® 2003, using a complete randomized design (CRB), replicated 7 times. An analysis of variance (ANOVA) was used to compare the data obtained from the fresh semen extended with the 4 different Fraction A extenders. ANOVA procedures were also used to compare the data obtained from the post-thawed semen between 8 groups at 3 different times i.e. 0, 30 and 60 minutes of incubation post-thawing. The data were acceptably normal with homogeneous treatment variances. Thus testing was performed at the 5% significance level of confidence. Differences between treatment means were also compared (multiple comparisons), using the Fisher's projected t-test for least significant differences (LSD) at the 5% level of confidence (Snedecor & Cochran, 1980).

5.3 Results and Discussion

The structural sperm morphology (normal or abnormal sperm) and viability (live or dead sperm) parameters for the fresh semen of the South African unimproved indigenous goat recorded 5 to 10 minutes after semen dilution with the 4 different semen extenders (Fraction A) at room temperature are set out in Table 5.2. No significant differences were recorded in the structural morphology and viability of the sperm immediately after being diluted with one of the 4 extenders (Fraction A) considered. However, there was a decrease of approximately 20% in the percentage live and normal sperm immediately after semen dilution compared to

the fresh undiluted pooled semen sample (Table 4.2). This reduction was mainly related to the reduction in sperm viability, although without any significant changes in the morphology. These results thus indicate that all 4 semen extenders evaluated in this study were equally capable of preserving fresh semen for a short period of time (prior to semen freezing). All however had a similar minor detrimental effect on buck sperm viability. The addition of BSA at 15% to the Tris diluent resulted in similar sperm viability results, to those of the Bioxcell® and Ovixcell® and Tris-egg yolk (at 1.5%) diluents.

These results suggest that for short-term sperm preservation, it could be recommended to use BSA, instead of egg yolk in the Tris extenders, with similar results. Thus eliminating possible detrimental effects caused by the EYCE. In addition, Bioxcell® and Ovixcell® could also be used for short-term preservation of the fresh sperm for the same reasons, but these diluents are too expensive.

The CASA sperm motion characteristics recorded for the unimproved South African buck sperm, shortly after dilution, are depicted in Table 5.3. No significant differences were recorded between extenders following semen dilution, but there was a slight decrease of about 4% in the extended semen's sperm motility, compared to that of fresh semen (Table 4.5).

Ovixcell® and Bioxcell extenders recorded a higher progressive motility and rapid velocity sperm, even though not significantly different from the Tris based extenders. Regarding the sperm velocity (of the motile sperm), no significant differences were recorded in terms of the rapid and slow sperm velocities, between extenders. However, semen extended in Tris-BSA showed higher medium sperm velocity ($P < 0.05$), compared to all other extenders used in this study. Cremades *et al*. (2005) reported sperm motility to be important for sperm transport within the female reproductive tract, and primarily for the penetration of the zona pellucia of the oocyte. Thus sperm motility, in general and the characteristics of the sperm motion in particular, may be used as indicators of the quality of sperm (fertilizing ability). In this study Tris BSA proved to be more effective to maintain medium velocity in goat sperm preserved fresh for a short period. It is therefore assumed that it lead to a higher potential fertilizing ability. The BSA can therefore be considered a suitable replacement for egg yolk which is considered detrimental to goat sperm preservation with Tris based extenders.

Table 5.2 Mean (± SE) structural sperm morphology and viability of fresh South African unimproved indigenous goat semen diluted with 4 different extenders

Extenders	% Live and normal Sperm [ns]	% Dead sperm [ns]	% Sperm abnormalities [ns]		
			Head	Mid-piece	Tail
Tris-BSA	59.2 ± 5.1	36.4 ± 4.1	0.2 ± 0.2	0.8 ± 0.4	3.8 ± 0.9
Tris-Yolk	49.2 ± 6.4	47.2 ± 7.6	1.2 ± 1.2	0.4 ± 0.2	2.0 ± 0.6
Bioxcell®	57.8 ± 5.3	40.0 ± 5.8	0.0 ± 0.0	0.4 ± 0.4	2.2 ± 0.6
Ovixcell®	54.4 ± 2.9	42.0 ± 3.6	0.4 ± 0.2	0.6 ± 0.4	2.6 ± 0.4
SEM	4.3	4.7	0.5	0.3	1.2
Probability	0.388	0.444	0.416	0.759	0.723
LSD	n/a	n/a	n/a	n/a	n/a
CV%	20.6	29.7	308.3	146.6	120.4

Tris-BSA = Tris-15% Bovine Serum Albumin
Tris-yolk = Tris-1.5% egg yolk
SE = standard error
SEM = standard error of the mean
LSD = t-test least significant difference at the 5% level
CV = coefficient of variation
ns = no significant differences between means in the same column ($P > 0.05$)

Table 5.3 The mean (± SE) sperm motion characteristics of the South African unimproved buck as determined by CASA, following fresh semen dilution using 4 different semen extenders

Extender	Static sperm (%)^{ns}	Non-progressive motile sperm (NPM) (%)^{ns}	Progressive motile sperm (PM) (%)^{ns}	Slow velocity sperm (%)^{ns}	Medium velocity sperm (%)	Rapid velocity sperm (%)^{ns}
Tris-BSA	36.8 ± 4.9	21.8 ± 4.0	41.5 ± 4.0	7.1 ± 2.1	10.2^{a} ± 2.6	46.0 ± 5.8
Tris-Yolk	39.7 ± 8.1	16.8 ± 3.2	43.5 ± 7.1	5.5 ± 1.4	5.4^{b} ± 1.4	49.4 ± 8.6
Bioxcell®	32.9 ± 5.6	19.0 ± 2.4	48.6 ± 5.2	6.6 ± 1.3	4.6^{b} ± 0.6	55.9 ± 4.4
Ovixcell®	30.3 ± 5.8	19.5 ± 3.4	50.2 ± 7.2	5.3 ± 1.4	4.2^{b} ± 0.8	60.3 ± 5.7
SEM	5.8	3.0	5.6	1.5	1.5	5.8
Probability	0.673	0.717	0.657	0.812	0.030	0.332
LSD	n/a	n/a	n/a	n/a	4.291	n/a
CV%	43.7	41.8	32.2	64.6	63.6	29.2

Tris-BSA = Tris-15% Bovine Serum Albumin
Tris-yolk = Tris-1.5% egg yolk
SE = standard error
SEM = standard error of the mean
LSD = t-test least significant difference at the 5% level
CV = coefficient of variation
$^{a-b}$Means with different superscripts within the same column, differ significantly ($P < 0.05$)
ns = no significant differences between means in the same column ($P > 0.05$)

Table 5.4 Mean (± SE) sperm velocity and linearity parameter values determined by CASA for fresh diluted semen of South African unimproved buck semen using 4 different semen extenders

Extender	Curvilinear velocity (VCL) (µm/s)^{ns}	Straight-line velocity (VSL) (µm/s)^{ns}	Average path velocity (VAP) (µm/s)^{ns}	Linearity (LIN) (%)^{ns}	Straightness (STR) (%)^{ns}	Wobble (WOB) (%)^{ns}
Tris-BSA	185.5 ± 15.1	143.4 ± 12.8	165.7 ± 15.1	77.3 ± 2.9	86.7 ± 2.0	89.1 ± 2.0
Tris-Yolk	195.2 ± 18.0	147.1 ± 15.4	170.2 ± 15.4	75.1 ± 2.2	85.8 ± 1.8	87.5 ± 1.7
Bioxcell	206.1 ± 7.4	161.6 ± 6.0	184.5 ± 6.6	78.5 ± 1.4	87.6 ± 1.0	89.6 ± 0.7
Ovixcell	216.3 ± 12.5	163.6 ± 14.4	187.3 ± 12.6	75.1 ± 3.2	86.7 ± 2.7	86.5 ± 2.0
SEM	12.8	11.7	12.0	2.4	1.8	1.6
Probability	0.377	0.533	0.517	0.670	0.919	0.487
LSD	n/a	n/a	n/a	n/a	n/a	n/a
CV%	16.9	20.2	17.9	8.1	5.6	4.7

Tris-BSA = Tris-15% Bovine Serum Albumin
Tris-yolk = Tris-1.5% egg yolk
SE = standard error
SEM = standard error of the mean
LSD = t-test least significant difference at the 5% level
CV = coefficient of variation
ns = no significant differences between means in the same column ($P > 0.05$)

The mean CASA sperm velocity and linearity parameters of buck semen diluted with the 4 different extenders are set out in Table 5.4. No significant differences were recorded for any of the sperm velocity or linearity parameters determined using CASA, between the 4 different extenders.

From a practical point of view most researchers focus their analyses on sperm linearity and straightness, as these are the best indicators of potential fertility (Dorado *et al*., 2007). The sperm motion parameters [straight-line/progressive velocity (VSL), linearity (LIN) and beat-cross frequency (BCF)] generally contribute to the overall sperm mobility phenotype (King *et al.*, 2000). Sperm VSL has also been highly correlated with fertility rate in rats (Moore & Akhondi, 1996).

According to Hashida and Abdullah (2003), amplitude of lateral head displacement (ALH) has been noted to be a good penetration predictor in older (≥ 2.5 to 4.5 years of age) bucks. Contrary, curvilinear velocity (VCL) and straightness (STR) were found to be good penetration predictors in younger, but mature (≥ 1.0 to 2.5 years of age), bucks (Hashida, 2005).

The population of the sperm cells moving forward and in a straight line was recorded to be above 85% in all the treatment groups. Linearity of the sperm movement ranged from 75.1 ± 2.2% to 78.5 ± 1.4% and straightness from 85.8 ± 1.8% to 87.6 ± 1.0%. The straight line velocity (VSL) again ranged from 143.4 ± 12.8 to 163.6 ± 14.4µm/s. These results are in line with those reported by Dorado *et al*. (2007), using fresh Florida buck semen. However these results are better than those reported for Boer goat semen extended in 20% egg yolk and 7% glycerol by Sundararaman and Edwin (2008).

In Table 5.5, the post-thaw viability and morphology of the cryopreserved sperm of unimproved indigenous S.A goats using the 4 different semen extenders (with or without glycerol), are set out. Following the freeze-thawing processes, semen evaluation results clearly indicated that cryopreservation considerably reduces the viability of goat sperm, when compared to fresh semen. A reduction of about 30 to 45% in the percentage live and normal sperm occurred and similarly, the same increase in the percentage dead sperm was recorded. However, no significant differences were recorded regarding the morphology between fresh and frozen sperm, as well as between the four different extenders used, following

cryopreservation. Therefore it can be considered that cryopreservation did not induce any significant gross structural abnormalities to the sperm cells, but it has reduced significantly ($P < 0.05$) the number of live cells in a semen sample post-thawing. This seems to indicate that the induced sperm damage sustained presumably affects more the function (physiological processes), than the physical morphology (structure). If structural sperm damage is sustained, it is probably more in the micro-structure (i.e. organelles and acrosome), rather than in the macro-structure of the sperm cell (head, tail and mid-piece). Obtaining 20 to 40% live and normal sperm post thawing is a relatively good achievement, when comparing results of similar studies elsewhere.

Glycerol-free Tris-egg yolk extender recorded the highest ($P < 0.05$) percentage dead sperm, while Bioxcell® recorded the highest ($P < 0.05$) percentage live and normal sperm (39.6 ± 5.7%), although not different from that of the Bioxcell® with glycerol (32.0 ± 3.4%) or Ovixcell® without glycerol (37.1 ± 3.1%). The addition of glycerol (6%) to Tris-based extenders also had significant beneficial effects. Contrary, the addition of glycerol (6%) had a detrimental effect on Ovixcell® a commercial semen extender which already contains an unknown cryoprotectant. Tris-BSA, as well as Tris-Yolk without glycerol and Ovixcell® with 6% glycerol recorded lower ($P < 0.05$) percentages of live and normal sperm cells, and consequently a higher percentage of dead sperm compared to Bioxcell. Unexpectedly, the glycerol-free Tris-BSA recorded similar percentages of live sperm values to that of Ovixcell® with 6% glycerol. These results obtained also demonstrate that Tris based extenders with glycerol (6%) provide comparable cryoprotection to the Ovixcell® and Bioxcell® semen extenders (without 6% glycerol).

Sperm morphology is generally considered to be a good predictor of successful fertilizing capacity (Łukaszewicz, 1988). Generally, frozen-thawed semen with a total of < 20% sperm abnormalities is said to be acceptable for artificial insemination (Barbas *et al*., 2006). Subsequently, poor sperm morphology has been correlated with a decreased fertility rate in goats. Although animals with few normal sperm, fail to fertilize without the aid of micromanipulation, this doesn't necessarily mean that they have sperm with abnormal chromosomes (genetic information). These animals can produce normal fertile offspring when defective sperm are injected artificially into viable oocytes (Burruel *et al*., 1996; Yanagimachi, 1998).

Table 5.5 The mean (± SE) sperm structural morphology and viability of frozen-thawed semen of unimproved South African goats, using 4 different extenders, with or without glycerol (6%)

Extender	Live and normal Sperm (%)	Dead sperm (%)	Sperm abnormalities (%)[ns]		
			Head	Mid-piece	Tail
*Tris-BSA	19.1^{de} ± 3.3	77.7^{ab} ± 3.0	0.7 ± 0.7	0.3 ± 0.2	2.1 ± 1.1
Tris-BSA + Gly	28.2^{abc} ± 5.5	67.5^{c} ± 5.4	0.1 ± 0.2	0.2 ± 0.2	3.1 ± 1.8
*Tris-Yolk	14.3^{e} ± 3.2	84.6^{a} ± 3.3	0.1 ± 0.1	0.6 ± 0.2	0.4 ± 0.2
Tris-Yolk + Gly	25.2^{bcd} ± 2.3	67.7^{c} ± 4.2	1.6 ± 1.2	1.8 ± 0.9	3.6 ± 1.9
*Bioxcell®	39.6^{a} ± 5.7	58.6^{c} ± 3.5	0.1 ± 0.1	1.1 ± 1.0	3.3 ± 0.6
Bioxcell® + Gly	32.0^{abc} ± 3.4	65.6^{c} ± 3.7	0.1 ± 0.1	0.1 ± 0.1	1.7 ± 0.7
*Ovixcell®	37.1^{ab} ± 3.1	60.7^{c} ± 3.1	0.3 ± 0.2	1.0 ± 0.6	0.9 ± 0.3
Ovixcell® + Gly	25.9^{cd} ± 4.6	69.6^{bc} ± 5.7	0.0 ± 0.0	0.9 ± 0.7	3.0 ± 1.1
SEM	4.0	4.0	0.5	0.6	1.0
Probability	0.001	<0.001	0.097	0.271	0.061
LSD	11.5	11.4	n/a	n/a	n/a
CV%	29.2	12.9	295.1	187.5	99.7

Tris-BSA = Tris-15% Bovine Serum Albumin
Tris-yolk = Tris-1.5% egg yolk
Gly = 6% glycerol
*Glycerol free
SE = standard error
SEM = standard error of the mean
LSD = t-test least significant difference at the 5% level
CV = coefficient of variation
[a-e]Means with different superscripts within the same column, differ significantly ($P < 0.05$)
ns = no significant differences between means in the same column ($P > 0.05$)

Glycerol is regarded as the most generally used cryoprotectant in diluents for the freezing of livestock semen (Salamon & Maxwell, 2000; Medeiros *et al*., 2002). Glycerol exerts an intracellular effect by replacing the intracellular water and decreasing the freezing point of the intracellular fluid content thereby reducing freeze damage in the cell by preventing ice crystal formation (Holt, 2000; Bearden *et al*., 2004).

The percentage live sperm post-thawing obtained in this trial with the use of 1.5 % egg yolk-Tris, with (25.2 ± 2.3%) or without (14.3 ± 3.2%) glycerol at 6% was found to be much higher than the 3.0 ± 2.7% reported by Choe *et al*. (2006), when using 20% egg yolk and 7% glycerol on Korean native bucks semen. Da Silva Maia *et al*. (2009) recorded a value of 29.9 ± 1.3% post-thaw live sperm in rams when using Tris-egg yolk extender (using 10.0% egg yolk and 7.0% glycerol). Gil *et al*. (2003) recorded 38.0±8.2% viable ram sperm post thawing, which is in line with the 39.6 ± 2.1% obtained in the current study using Bioxcell®. Barbas *et al*. (2006) have also obtained a much higher percentage live sperm (44.5%) and percentage normal sperm (64.9%) post thawing with Serrana bucks, using a semen extender described by Evans and Maxwell (1990).

The results of this study indicate that the type of extender used for the cryopreservation of buck semen has a major effect on the post-thaw sperm cell viability. Cryopreservation dramatically reduces the percentage of live sperm, when compared to sperm in a fresh semen sample. Surprisingly, Tris-based control semen extenders (without glycerol as a cryoprotectant) still recorded a considerable number of viable or live sperm (14 to 19%), following semen freezing and thawing. This indicates that BSA and egg yolk have some cryoprotective ability (Salamon & Maxwell, 2000).

The effect of semen cryopreservation is that it induces capacitation-like reactions in the sperm, which destabilizes the acrosomal head membrane thereby rendering it more fusigenic (premature competence to fertilize the oocyte) and reduces the fertilizing ability (Graham & Mocé 2005; Vadnais & Roberts, 2007). These alterations may not affect sperm morphology but reduce the lifespan and the ability to interact with the female reproductive tract and sperm fertility (Medeiros *et al*., 2002).

Table 5.6 The mean (± SE) sperm motion characteristics of post-thawed semen of South African unimproved indigenous buck extended using 4 different extenders, with or without glycerol (6%)

Extender	Static sperm (%)	Non-progressive motile sperm (%)	Progressive motile sperm (%)	Slow velocity sperm (%)	Medium velocity sperm (%)	Rapid sperm velocity (%)
*Tris-BSA	$91.3^{ab} \pm 2.0$	$3.9^{cd} \pm 0.9$	$5.3^{cd} \pm 1.5$	$1.1^{c} \pm 0.4$	$1.2^{bc} \pm 0.4$	$6.3^{bc} \pm 1.5$
Tris BSA + Gly	$86.2^{abc} \pm 2.7$	$5.9^{bcd} \pm 1.4$	$6.7^{bcd} \pm 1.4$	$1.7^{bc} \pm 0.5$	$2.3^{ab} \pm 0.5$	$9.7^{abc} \pm 2.1$
*Tris-Yolk	$96.6^{a} \pm 1.1$	$1.7^{d} \pm 0.6$	$1.6^{d} \pm 0.7$	$0.4^{c} \pm 0.2$	$0.0^{c} \pm 0.0$	$3.0^{c} \pm 1.0$
Tris-Yolk + Gly	$83.2^{bcd} \pm 2.8$	$8.2^{abc} \pm 0.3$	$8.3^{abcd} \pm 2.1$	$3.0^{ab} \pm 0.7$	$2.3^{ab} \pm 0.5$	$11.1^{ab} \pm 2.3$
*Bioxcell®	$79.7^{cd} \pm 3.1$	$9.7^{ab} \pm 1.7$	$10.6^{abc} \pm 1.9$	$2.9^{ab} \pm 0.6$	$1.9^{ab} \pm 0.6$	$15.5^{ab} \pm 2.6$
Bioxcell® + Gly	$74.6^{d} \pm 3.5$	$11.2^{a} \pm 1.4$	$14.3^{a} \pm 2.6$	$3.7^{a} \pm 0.4$	$2.8^{a} \pm 0.3$	$19.0^{a} \pm 3.4$
*Ovixcell®	$77.3^{cd} \pm 3.7$	$9.4^{ab} \pm 1.3$	$13.3^{ab} \pm 2.6$	$3.0^{ab} \pm 0.6$	$2.0^{ab} \pm 0.4$	$16.7^{a} \pm 3.5$
Ovixcell® + Gly	$77.9^{cd} \pm 3.0$	$10.3^{ab} \pm 1.7$	$11.8^{abc} \pm 2.2$	$2.9^{ab} \pm 0.6$	$2.9^{a} \pm 0.6$	$16.3^{a} \pm 3.0$
SEM	3.7	1.7	2.5	0.6	0.5	3.4
Probability	0.123	0.111	0.191	0.043	0.469	0.424
LSD	10.6	4.8	7.1	1.6	1.5	9.7
CV%	11.9	68.4	79.7	89.7	94.4	71.4

Tris-BSA = Tris-15% Bovine Serum Albumin
Tris-yolk = Tris-1.5% egg yolk
*Glycerol free
Gly = 6% glycerol
SE = standard error
SEM = standard error of the mean
LSD = t-test least significant difference at the 5% level
CV = coefficient of variation
[a-d]Means with different superscripts within the same column, differ significantly ($P < 0.05$)
ns = no significant differences between means in the same column ($P > 0.05$)

The CASA sperm motion characteristics for the S.A unimproved indigenous buck semen following freeze-thawing are set out in Table 5.6. Following the freeze-thawing processes, Bioxcell® and Ovixcell® (without glycerol) recorded the highest percentages of progressively motile and rapid moving sperm, when compared to the Tris-based extenders. The progressive motility, medium and rapid velocities of sperm are considered to be the most useful parameters when estimating the potential fertilizing ability of sperm. This discussion will thus be concentrated on these more meaningful parameters.

The highest post-thawing progressive (forward movement) sperm motility (14.3 ± 2.6%) was recorded in the Bioxcell® treatment group. These motility results compare favourably to those recorded by Kozdrowski *et al*. (2007) using the same Tris-Yolk extender on French Alpine bucks (e.g. 10.7 ± 3.5% for rapid sperm movement and 6.1 ± 1.8% for progressively motile sperm). Da Silver Maia *et al*. (2009) recorded 29.0 ± 1.1% progressively motile sperm, 47.0 ± 1.5% rapid sperm, 9.0 ± 0.3% slow sperm and 19.0 ± 1.5% static sperm post thawing using Tris-egg yolk on Santa Inês ram semen. These values are higher than those recorded for the unimproved indigenous South African bucks in the present study.

In the current study Tris-egg yolk (without glycerol) recorded approximately 3.4% live and motile sperm. This is very low, as one would expect all the sperm to be dead after freezing and thawing, due to the lack of a cryoprotectant. It is suggested that although the purpose of an extender for cryopreservation (e.g. egg yolk) is to supply the sperm with sources of energy, it must also have the ability to protect the sperm from cryo-damage. While maintaining a suitable environment for sperm to survive temporarily (Aboagla & Terada. 2004; Bergeron & Manjunath, 2006; Purdy, 2006). Egg yolk generally contains a fraction that preserves the sperm motility, by activating the sperm plasma membrane (Okamura *et al*., 2005).

A review article by Barbas and Mascarenhas (2009), reported that in domestic species, the acceptable number of motile sperm in a good quality frozen-thawed semen sample is about 50% of the motility of the corresponding value in the fresh semen sample. However the number of motile sperm per insemination dose varies between species. In the ram for example, frozen-thawed sperm may have a high proportion (40-60%) of motile sperm, although only about half (20-30%) remain biologically functional (Anel *et al*., 2003). On the other hand a sperm cell may be classified as motile, but it may be damaged, and this will

reduce its fertilizing ability (Medeiros *et al*., 2002). It has also been reported that if frozen semen is of high quality following thawing, namely > 40% sperm motility, it can be used for transcervical, laparoscopic or *in vitro* AI (Barbas *et al*., 2006). Consequently, a threshold of 50% post-thawing sperm motility is used as an industry standard (benchmark) for semen suitable for AI in cattle (Gil *et al*., 2001).

The mean CASA velocity and linearity parameters in post-thawed S.A unimproved indigenous buck sperm are summarized in Table 5.7. Following the freezing-thawing processes, significant ($P < 0.05$) differences were recorded in most sperm velocity and linearity parameters amongst the 8 treatments (extenders) considered.

The sperm linearity and straightness percentage declined for all the extenders considered post-thawing, when compared to those of fresh extended semen. Anel *et al*. (2003) reported the STR and LIN parameters to be the most common primary indicative variables for predicting the post-thawed percentage motile sperm. The VSL, VAP, LIN and STR parameters generally express the progressive sperm movement, as measured by a computer assisted sperm analysis system (CASA) and are correlated with the fertilizing ability of the sperm (Verstegen *et al*., 2002). In general Tris-based extenders without glycerol recorded a low sperm linearity and straightness percentages (i.e. less than 50%).

The sperm linearity for Ovixcell® reduced from 75.1 ± 3.2% to 63.5 ± 4.0%, and for Bioxcell® it decreased from 78.5 ± 1.4% to 62.2 ± 3.0%. The straightness of sperm movement for Ovixcell® decreased from 86.7 ± 2.7% to 72.7 ± 4.5% and for Bioxcell®, from 87.6 ± 1.0% to 75.6 ± 2.7%. These results are higher than those reported by Kozdrowski *et al*. (2007) using the same glycerol and egg yolk concentrations on French Alpine goat semen. These results are however in line and even better than the results reported by Sundararaman and Edwin (2008) using 20% egg yolk and 7% glycerol in Boer goat semen and optimistic expectations of fertilization ability can be deduced from them.

Table 5.7 Mean (± SE) values of sperm velocity and linearity parameters for frozen-thawed South African unimproved buck semen extended with 4 different extenders, with or without glycerol (6%)

Extender	Curvilinear velocity (VCL) (µm/s)^{ns}	Straight-line velocity (VSL) (µm/s)^{ns}	Average velocity path (VAP) (µm/s)^{ns}	Linearity (LIN) (%)	Straightness (STR) (%)	Wobble (WOB) (%)
*Tris-BSA	154.5 ± 19.3	86.3 ± 12.3	119.0 ± 15.0	48.6^{ab} ± 5.5	63.4^{bc} ± 6.9	65.3^{a} ± 6.2
Tris BSA + Gly	158.5 ± 8.0	92.7 ± 8.7	120.4 ± 8.3	58.3^{a} ± 4.6	75.2^{a} ± 4.4	75.5^{a} ± 3.0
*Tris-Yolk	118.9 ± 26.3	65.8 ± 14.2	85.5 ± 18.1	33.8^{b} ± 7.2	43.9^{c} ± 8.8	43.0^{b} ± 8.7
Tris-Yolk + Gly	161.0 ± 13.3	82.0 ± 9.5	122.1 ± 11.5	51.5^{ab} ± 4.1	67.9^{ab} ± 4.7	75.0^{a} ± 2.4
*Bioxcell®	163.5 ± 8.2	105.3 ± 9.6	136.2 ± 9.3	62.2^{a} ± 3.0	75.6^{a} ± 2.7	81.9^{a} ± 2.3
Bioxcell® + Gly	169.7 ± 8.0	111.2 ± 7.3	141.8 ± 7.6	64.7^{a} ± 2.4	77.7^{a} ± 2.0	83.1^{a} ± 2.0
*Ovixcell®	161.1 ± 11.0	107.1 ± 8.2	141.3 ± 10.0	63.5^{a} ± 4.0	72.7^{ab} ± 4.5	83.2^{a} ± 4.3
Ovixcell® + Gly	152.6 ± 9.5	98.3 ± 8.3	127.9 ± 8.4	64.3^{a} ± 3.2	76.6^{a} ± 3.4	83.7^{a} ± 1.8
SEM	23.6	14.4	18.1	6.3	8.0	7.6
Probability	0.155	0.498	0.128	0.431	0.322	0.093
LSD	n/a	n/a	n/a	18.1	22.5	21.6
CV%	24.7	34.9	25.5	28.0	21.7	15.3

Tris-BSA = Tris-15% Bovine Serum Albumin
Tris-yolk = Tris-1.5% egg yolk
*Glycerol free
Gly = 6% glycerol
SE = standard error
SEM = standard error of the mean
LSD = t-test least significant difference at the 5% level
CV = coefficient of variation
$^{a-c}$Means with different superscripts within the same column, differ significantly ($P < 0.05$)
ns = no significant differences between means in the same column ($P > 0.05$)

In Table 5.8 the effects of incubation time post-thawing and semen extender on the unimproved S.A indigenous buck sperm motion characteristics are set out. In general, Tris based extenders recorded better sperm motility results after 30 minutes of incubation, while Bioxcell® and Ovixcell® recorded better results immediately post-thawing. These results may suggest that frozen semen extended in Bioxcell® and Ovixcell® should be used for AI as soon as possible after thawing, while semen extended in Tris based extenders should be used at approximately 30 minutes post-thawing. However, this still needs to be tested and verified in practice.

Sperm progressive motility and rapid velocities are related to potential fertilizing ability, thus in Table 5.8, the research focussed only on these two parameters. Although starting at very low percentages, sperm extended in Tris based extenders showed an improvement that matched the better performing Bioxcell® and Ovixcell® extenders, 30 minutes following incubation for both parameters before declining again. Bag *et al*. (2003) recorded 50.3% sperm with rapid velocity immediately post-thawing and 46.2% an hour later, for native Malpura and Bharat Merino breeds on semen, cryopreserved in a TEST-yolk-glycerol extender and incubated at 37ºC. In line with these results, Joshi *et al*. (2005) recorded 48.7% sperm with rapid velocity, immediately post-thaw and 41.3% an hour later for Garole ram semen, using the same extender as Bag *et al*. (2003). Although not further incubated, semen from 3 adult Sarda male goats was assessed at 37ºC immediately post-thawing by Berlinguer *et al*. (2009). The latter recorded progressive sperm motilities of 19.6 ± 11.3%, 20.4 ± 6% and 25.7 ± 9.3%, and rapid velocities of 45.4 ± 12%, 47.3 ± 4.5% and 49.7 ± 15.8%.

In this study, the progressive motility results for Bioxcell® (18.4 ± 6.4) are comparable to those reported by Berlinguer *et al*. (2009) for the Sarda bucks (19.6 ± 11.3), using a Tris-based extender (4% glycerol and 20% egg yolk). However, the results for rapid sperm velocity recorded in this study are much lower, correspondingly to about half of those reported in the literature for post-thawed buck sperm (Bag *et al*., 2003; Joshi *et al*., 2005; Berlinguer *et al*., 2009). The possible reasons explaining the relatively poor tolerance to cryopreservation of the unimproved S.A indigenous buck sperm observed in this study could be due to several factors. The fact that the unimproved S.A indigenous bucks recorded a lower sperm concentration (about half of that of most other goat breeds) and the relatively high dilution rate used in the first steps of the cryopreservation process could attribute to this phenomenon.

Table 5.8 Mean percentages (± SE) of progressive motile and rapid velocity moving sperm post-thawing, at different incubation times

Extender	Sperm motility parameters					
	% Progressive motility			% Rapid velocity		
	0 min	30 min	60 min	0 min	30 min	60 min
Tris-BSA + Gly	$5.8^{cd} \pm 2.0$	$8.4^{bcd} \pm 3.6$	$6.0^{cd} \pm 1.7$	$7.7^{c} \pm 3.3$	$13.4^{abc} \pm 4.9$	$8.0^{c} \pm 2.4$
Tris-Yolk + Gly	$8.8^{bcd} \pm 4.4$	$11.9^{abcd} \pm 4.0$	$4.1^{d} \pm 1.8$	$12.2^{bc} \pm 4.5$	$14.8^{abc} \pm 4.2$	$6.5^{c} \pm 2.9$
Bioxcell®	$15.5^{ab} \pm 3.1$	$10.2^{abcd} \pm 3.2$	$6.1^{cd} \pm 2.7$	$20.6^{ab} \pm 5.4$	$15.8^{abc} \pm 4.3$	$10.2^{bc} \pm 3.0$
Ovixcell®	$18.4^{a} \pm 6.4$	$14.2^{abc} \pm 2.7$	$7.4^{bcd} \pm 2.9$	$25.2^{a} \pm 7.8$	$14.3^{abc} \pm 4.3$	$10.5^{bc} \pm 4.6$

Tris-BSA = Tris-15% Bovine Serum Albumin
Tris-yolk = Tris-1.5% egg yolk
Gly = 6% glycerol
Min = minutes
SE = standard error
[a-d]Means for the same sperm motility parameter (i.e. Progressive motility & rapid velocity sperm) with different superscripts within the same columns (extender) and rows (incubation time), differ significantly ($P < 0.05$)

Based on the post-thawing sperm viability, motility and velocity results recorded in this study, one can assume that cryopreserved semen (using Tris-based extenders with glycerol at 6%, Bioxcell® or Ovixcell®), from unimproved S.A bucks could be used in *in vitro* or even for AI using the laparoscopic method.

To current knowledge, there is no previous work reported in the literature on goat semen cryopreservation using either Bioxcell® or Ovixcell® and this has limited the discussion of this part of the study to a large extend. Although the latter performed equally or better than the Tris based extenders, it has been suggested that sperm motility of recently thawed samples is not a good indicator of the success achieved when using *in vitro* fertilization. Roth *et al*. (1999), considered longevity after sperm incubation a more reliable parameter to predict fertilizing potential of semen sample.

5.4 Conclusions

The results of this study revealed that the semen cryopreservation of the unimproved S.A goat drastically reduces its sperm viability and motility parameters. Consequently its potential fertilizing ability is severely reduced. Damage to the sperm seems to affect their physiology, rather than their physical appearance, as the results of the analyses of both sperm morphology of fresh and cryopreserved (post-thawed) samples are very similar. A small reduction in the sperm viability was noticeable immediately after dilution, in all 4 semen extenders. However, most of the sperm losses seem to occur during the freezing and thawing processes. In addition this experiment also demonstrated that there were no major differences amongst the semen extenders used regarding the morphology of S.A unimproved indigenous buck sperm (both fresh and post-thawing). Nevertheless, Bioxcell® and Ovixcell® seemed to be the more effective extenders to be used for goat semen cryopreservation purposes as these extenders demonstrated higher sperm viability, motility and velocity post thawing.

The Tris-BSA extender with glycerol at a 6% level recorded lower sperm viability and motility, when compared to Bioxcell® and Ovixcell®, however it was similar to the egg yolk-Tris extender (with glycerol at 6% level). These findings suggest that BSA at 15% can be used to replace egg yolk in the preparation of Tris-based goat semen extenders with similar results. These results further prove that it is essential to add glycerol as a cryoprotectant to the Tris-based extenders used to freeze goat semen.

The present study also suggests that post-thawed buck semen should be used for insemination after thawing at approximately 30 minutes of incubation (at 37ºC), if cryopreserved in Tris-based extenders. However, semen must be used immediately after thawing when Bioxcell® and Ovixcell® extenders are used. More research on cryopreservation is warranted to develop a protocol that produces better post-thaw sperm viability, motility and acceptable fertilizing ability.

Chapter 6

General conclusions and recommendations

6.1 General Conclusions

In general, the South African unimproved indigenous bucks produce an ejaculate with a lower semen volume, sperm concentration, and progressively motile sperm compared to the Boer goat and most of the European and Asian goat breeds. However, semen pH, sperm viability and morphology (percentage normal, severity and location of abnormalities) as well as motility (velocity and linearity) parameters are in line with values from the literature for other goat breeds.

The results of the semen cryopreservation study revealed that sperm viability and motility parameters decline slightly following extension and decline drastically following cryopreservation. This experiment also demonstrated that there are no differences between the four semen extenders used in this trial, regarding their effect on the morphology of South African unimproved indigenous buck sperm. This suggests that the damage to the sperm seem to be more physiological than physical. Nevertheless, Bioxcell® and Ovixcell® seemed to be more effective extenders to be used for semen cryopreservation of the unimproved South African goat, as these extenders demonstrated a higher post-thaw sperm viability and motility, compared to the Tris-based extenders.

The findings of this study also suggest that BSA can be used with acceptable results, to replace egg yolk in the preparation of Tris-based goat semen extenders in order to avoid the detrimental effects of the egg yolk coagulating enzyme (EYCE) on the sperm. In addition, the results prove that it is essential to add glycerol as a cryoprotectant to Tris-based extenders.

Post thaw sperm forward progression and velocity reduced with incubation time for Bioxcell® and Ovixcell® extenders, but improved up to 30 minutes after thawing in Tris-based extenders, before declining again. These results suggest that frozen-thawed semen extended in Tris based extenders should be used for AI at approximately 30 minutes post-thawing, while semen extended and frozen in Bioxcell® and Ovixcell® must be used as soon as possible after thawing.

6.2 Recommendation

1. Further studies are required on the unimproved S.A goat breed, in order to better characterize their semen quantitatively and qualitatively.
2. Buck semen cryopreservation in this breed should be investigated further in order to develop cryopreservation protocols and semen extenders which can result in better post-thaw viability of the sperm.
3. An increase in the number of experimental animals is recommended for future studies.
4. Other semen extenders, thawing temperatures, cooling rates, concentrations of egg yolk and glycerol need to be evaluated.
5. Fertility tests of fresh, diluted and frozen semen samples are crucial in future studies.
6. The investigation of the effect of age of the buck and season on the quality of the semen and its cryopreservation ability is also recommended for future studies.
7. The evaluation of the effect of the sperm washing technique prior to extension is also of importance in future research.

CHARACTERIZATION AND CRYOPRESERVATION OF SOUTH AFRICAN UNIMPROVED INDIGENOUS GOAT SEMEN

by

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Abstract

Semen from 7 South African unimproved indigenous bucks that were successfully trained from a group of 10 bucks for semen collection with the aid of an artificial vagina (AV) was characterized and then cryopreserved, using different semen extenders. Semen was collected twice a week and evaluated macroscopically for ejaculate volume and pH immediately after collection. Within 1h of collection, semen was further analysed electronically for sperm concentration. Thin semen smears were stained with eosin/nigrosin and evaluated under a fluorescent microscope for viability (percentage live or dead) and morphology (percentage normal or abnormal). In addition semen samples were evaluated using the computer assisted sperm analysis (CASA) for sperm motility (static, non progressive and progressive), velocities (static, slow, medium, rapid, VCL, VSL and VAP) and linearity (LIN, STR and WOB) parameters using a Sperm Class Analyser® (SCA®).

Four different semen extenders, namely: Tris-1.5% yolk, Tris-15% BSA, Ovixcell® and Bioxcell® (IMV, L'Aigle, France) were used to cryopreserve pooled semen samples, with and without 6% glycerol thus making a total of 8 treatments. Immediately after dilution and after thawing, semen samples were compared through the evaluation of viability, morphology, motility, velocity and linearity parameters, using the same methodology used for fresh semen. Semen was then incubated at 37ºC and analysed for motility and velocity parameters after 30 and 60 minutes of incubation.

Regarding the fresh semen samples, the South African unimproved indigenous bucks recorded an overall average ejaculate volume of 0.5 ± 0.2 ml, pH of 7.5 ± 0.2 and sperm concentration of 681.7 ± 74.6 x 10^6 sperm/ml. On average, bucks recorded 79.0 ± 6.3% normal and 76.3 ± 8.2% live sperm cells in the ejaculates. The average percentage of sperm abnormalities on head, mid-piece and tail were 4.2 ± 1.3%, 4.6 ± 1.7%, and 12.1 ± 5.4%, respectively. The overall sperm abnormalities recorded were 1.0 ± 0.8%, 9.5 ± 2.9% and 10.1 ± 3.6% for primary, secondary and tertiary abnormalities, respectively. The mean static, non-progressively motile (NPM), progressively motile (PM), slow, medium and rapid sperm cells recorded were 30.9 ± 14.7%, 32.1 ± 10.9%, 37.3 ± 10.0%, 4.9 ± 1.7%, 6.0 ± 1.7% and 58.2 ± 14.1%, respectively.

Viability of goat sperm following fresh semen dilution with the four different semen extenders was similar, however a reduction of approximately 20% in the percentage live and normal sperm was recorded (5-10 minutes after dilution), when compared to the fresh undiluted pooled semen sample. Similar motility parameters were recorded shortly after fresh semen dilution using the 4 different extenders. A slight decrease of approximately 4% in the extended semen's sperm motility was observed, when compared to that of fresh undiluted semen. For the sperm velocity parameters, semen extended in Tris-BSA showed significantly higher medium sperm velocity.

Following freezing-thawing, a drastic reduction in the percentage live and normal sperm was recorded in all treatments. Bioxcell® without glycerol recorded the highest number of live and normal sperm. The Bioxcell® and Ovixcell® extenders recorded the highest percentage linearity and straightness movement of the sperm. In general, cryopreservation reduced the sperm cell viability and motility parameters. In addition no effect of extender on the morphology of South African unimproved indigenous buck sperm was observed. Sperm motility and velocity results showed that sperm extended in Bioxcell® and Ovixcell® recorded

higher values immediately post-thawing, while the Tris-based extenders recorded the highest values after 30 minutes of incubation, before declining rapidly.

The South African unimproved indigenous bucks seem to produce a lower semen volume (ejaculate), sperm concentration, and percentage progressively motile sperms, compared to the European, Asian and Boer goat breeds. The results demonstrate that Bioxcell and Ovixcell are suitable extenders to induce high post-thawing viability, motility and velocity of buck sperm.

Key words: Unimproved Goat; Semen; CASA; Characterization; Cryopreservation; Extenders

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