Characterisation and cryopreservation of semen from the indigenous Namaqua Afrikaner sheep breed, in comparison with the Dorper and Dohne Merino breeds

By

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Declaration

I, ***Phutiane Thomas Letsoalo***, declare that this dissertation has not been submitted to any University, and that it is my genuine work steered under the supervision of Dr M.A. Snyman and Prof V. Muchenje. All support towards the construction of this work and all the references contained herein have been fully endorsed.

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Abstract

The aim of this study was to characterise and cryopreserve semen of the indigenous Namaqua Afrikaner breed, and to compare it to that of Dorper and Dohne Merino sheep, whose semen is commercially frozen on a large scale. The study was conducted between January and August 2015. September 2013-born Namaqua Afrikaner (12), Dohne Merino (12) and Dorper (9) rams were used in the study. The rams were kept under kraal conditions with adequate shade, and they received a high protein, high energy diet. Originally it was envisaged to collect semen samples using the artificial vagina (AV) method, which proved to be problematic with the Namaqua Afrikaner rams. Semen samples were subsequently collected twice a week by either AV (Dohne Merino and Dorper) or electro-ejaculation (EE; all three breeds). Macroscopic sperm traits were assessed and sperm concentration determined immediately after collection. Each semen sample was diluted with Triladyl® (1:3) and subsequently frozen in liquid nitrogen vapour in straws. Frozen straws were thawed and evaluated at 7, 30 and 90 days after cryopreservation. A droplet (0.5 ml) from each thawed sample was assessed microscopically for post-thaw motility and percentage live sperm. Nigrosin-Eosin smears were prepared of fresh, diluted and frozen-thawed semen samples for determination of percentage live sperm. Data of all the traits were analysed with the GLM and CHI-SQUARE procedures of the SAS statistical package. Breed had a significant effect on ejaculate volume of fresh semen collected either via electro-ejaculation or artificial vagina. Dorper rams (1.37 ± 0.08 ml) and Dohne Merino rams (1.20 ± 0.08 ml) produced ejaculates with higher *(P* <0.05*)* semen volume than Namaqua Afrikaner rams (1.09 ± 0.08 ml) when using the EE. With the AV method, Dohne Merino rams (1.46 ± 0.08 ml) produced a higher *(P* <0.05*)* ejaculate volume than Dorper rams (1.22 ± 0.08 ml). Motility of the frozen-thawed semen samples was lower than that of the fresh and diluted samples for all breeds and collection methods. Furthermore, motility 3 hours after thawing was also lower than motility assessed immediately after thawing for all breeds and collection methods. Motility of frozen-thawed semen collected with an AV and evaluated at 7, 30 and 90 days after freezing was significantly higher than that collected via EE. Dorper rams had the lowest sperm concentration (1.10 ± 5.29x10^9 sperm/ml) and the Namaqua rams the highest sperm concentration (1.22 ± 5.20x10^9 sperm/ml) (*P* <0.05). The AV samples had a higher (*P* >0.05) sperm concentration (1.20 ± 3.68x10^9 sperm/ml) than the EE samples (1.11 ± 3.51x10^9 sperm/ml). The percentage live sperm in the fresh semen samples did not differ

among Namaqua Afrikaner (67.76 ± 1.94%), Dohne Merino (68.59 ± 1.94%) and Dorper (72.82 ± 1.98%) rams. The percentage live sperm for all three breeds dropped considerably after freezing to 17.76 ± 2.03%, 17.86 ± 2.03% and 22.72 ± 2.07% respectively. It remained constant for all the breeds from 7 until 90 days after freezing, indicating that length of storage should not have an effect on percentage live sperm for semen collected via AV or EE. Percentage live sperm of the frozen-thawed semen of the Namaqua rams was lower than that of the Dorper rams, indicating that the Namaqua semen collected via EE did not freeze as well as that of the Dorper semen. In conclusion, neither fresh nor frozen-thawed Dorper and Dohne Merino semen collected via EE did differ significantly. Furthermore, except for semen volume, Dorper and Dohne Merino semen collected via AV did not differ significantly whether evaluated as fresh or frozen-thawed semen. However, both Dorper and Dohne Merino rams produced semen with higher motility and a higher percentage live sperm post-thaw when the semen samples were collected via an AV than via EE. From these results it can therefore be postulated that if Namaqua semen were collected via AV it could have a higher post-thaw percentage live sperm than if semen was collected via EE. Advanced further studies are necessary to investigate the reason for the lower post-thaw survival rate of sperm of the Namaqua Afrikaner rams. This is necessary as semen stored in a cryobank for breeding and conservation purposes for this endangered breed has to be of high quality. Such resources as cryobanks are expensive and funds cannot be wasted on preserving inferior samples that could not fertilize an ovum when needed. The low percentage of live sperm obtained with the frozen-thawed samples in this, as well as other studies on indigenous breeds, is an indication that further research is needed into more suitable freezing protocols. It can be concluded that Namaqua Afrikaner semen cannot be frozen successfully for the purpose of storage in a cryobank, when using a freezing protocol based on Triladyl® as extender. Furthermore, any increase in post-thaw survival rate of sperm will be beneficial and it is therefore suggested that all efforts be made to solve the problem of the Namaqua Afrikaner rams that do not want to ejaculate into an artificial vagina.

Keywords: Cryopreservation; Indigenous breeds; Semen volume; Semen motility; Percentage live sperm; Triladyl®

Declaration

I, ***Phutiane Thomas Letsoalo*** hereby declare that, the work contained in this thesis is my own original work. I understand what plagiarism is and I am aware of the University's policy in this regard, I did not refer to work of current or previous students, lecture notes, handbooks or any other study material without proper referencing. I can affirm that where other people's work has been used that the work has been properly acknowledged and referenced. I furthermore cede copyright of this thesis in favour of University Fort Hare and Grootfontein Agricultural Development Institute.

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%	Percentage
AI	Artificial insemination
AnGR	Animal Genetic Resources
AV	Artificial vagina
BCS	Body condition score
CASA	Computer Assisted Sperm Analyzer
ºC	Degree centigrade
DARS	Dohne Agricultural Research Station
DBS	Dorper Breeders' Society
DMBS	Dohne Merino Breeder's Society
DMSO	Dimethyl sulfoxide
EE	Electro-ejaculator
FAO	Food and Agriculture Organisation
FSH	Follicle stimulating hormone
GADI	Grootfontein Agricultural Development Institute
GnRH	Gonadotropin-releasing hormone
ICSH	Interstitial cell stimulating hormone
EY	Egg-yolk
EYCE	Egg-yolk coagulating enzyme
LH	Luteinising hormone
$Na_3C_6H_5O_7$	Sodium citrate
PVCP	Polyvinyl Chloride Powder
SSH	Spermatogenesis stimulating hormone
VCV	Vaginal collection vial
VS	Vitrification solution

1. General introduction

The Namaqua Afrikaner is one of the oldest sheep breeds in South Africa. The original Afrikaner sheep migrated south with the Khoikhoin people from the more north-western parts of Africa and entered South Africa between 200 and 400 AD, therefore it is regarded as a national heritage (Epstein, 1960; Ramsay *et al*., 2001).The Namaqua Afrikaner is well known for its capability to survive the harsh South African climate (Epstein, 1960). The breed has a slim body with long legs, well adapted for walking long distances in search of food and water (Burger *et al*., 2013). The breed was facing extinction when the Department of Agriculture bought one of the last purebred herds from Mr P.J. Maas from Namies, Springbok. This herd has been kept at the Carnarvon Experimental Station since 1966. In 1991, part of this herd was transferred to the Grootfontein Agricultural Development Institute (GADI). Since August 1995, the GADI herd is run at the Karakul Experimental Station near Upington (Snyman *et al*., 1996c).

Campbell (1995) estimated that only 2000 Namaqua Afrikaner sheep are left in the country. In 1997, an attempt was made to establish a breeding interest group for Namaqua and Ronderib Afrikaner sheep. A request was made in the popular press for people owning these sheep to contact Grootfontein. A small number of people owning Namaqua sheep made contact and most of them only had a few of these sheep. This indigenous fat tailed sheep breed of South Africa is listed as an endangered sheep breed on the FAO's endangered species list (FAO, 2007). It is therefore evident that this breed should be regarded as endangered and steps towards its protection and conservation should be taken as a priority.

Conservation of endangered species and breeds is very important for the maintenance of biological diversity, and modern breeding management of some domestic farm animals. There is global consent about the decline in domestic animal diversity in the world and the necessity to conserve genetic diversity (Hiemstra *et al*, 2005). For effective conservation of endangered indigenous breeds it is important that comprehensive knowledge of the breed's characteristics is documented (Qwabe *et al*., 2013; Groeneveld *et al*., 2010). The demand for animal and animal products is increasing worldwide due to an alarming growing human population. This has resulted to a large number of genetically pure livestock breeds being listed on the list of endangered species or in worse scenario being extinct (Roldan &

Gomendio, 2008). Hoffman and Scherf, (2005) has indicated that there are approximately 30 animal species which have been domesticated, of which 30% are at risk of extinction. Indigenous breeds in general demonstrate low production figures when compared to commercial stock, however, they may hold potential due to years of adaption to the pressures of the specific local environment (Qwabe, 2011). Adaptive traits that are usually associated with the indigenous breeds include: tolerance to various diseases, tolerance to extreme temperatures and humidity, tolerance to change in the availability of feed, adaptation to low capacity management and ability to survive, produce and reproduce for long period of time (Scherf, 2000). The conservation of these indigenous breeds could contribute to current or future traits of interest and therefore it should be considered essential for maintaining future breeding options (Groeneveld *et al*., 2010).

Cryopreservation would allow indefinite storage of biological material without weakening over a time estimate of at least thousands of years, but perhaps much longer (Hiemstra, 2011). Hiemstra, (2011) further reported that this practice is generally believed to be only viable or efficient in few animal species. Cryopreservation can be defined as the storage or conservation of Animal Genetic Resources (AnGR) and other important biological materials that will be used for the development or establishment of future generations of the specie or breed (Hiemstra *et al*., 2005; Boa-Amponsem & Minozzi, 2006). This method has proven to be very efficient, in that it allows large quantities of genetic material to be stored in a limited amount of space (Ruane, 2000). Hiemstra *et al.* (2005) indicate that restoration of an extinct breed is possible by using the cryopreserved genetic material of that particular breed. Hiemstra *et al.* (2005) further reported that problems occurring in the living populations, such as decreasing numbers or inbreeding can be alleviated by the use of unrelated genetic material from the cryo-bank. In farm animals, trends within breed diversity are as important as between-breed diversity to be able to cope with changing requirements and future demands in breeding and selection (Hiemstra *et al.*, 2005). A small effective population size in rare or endangered breeds requires monitoring of within-breed diversity and conservation programmes to maintain within-breed diversity (Hiemstra *et al.*, 2005).

Developments in cryobiology and different reproductive technologies have increased the possibilities for gene banking in the past few decades (Hiemstra, 2011). Various animal resources such as semen and embryos can be collected or harvested and then be preserved for later use in most of the farm animals. Preservation of these animal resources has proven to be

a key issue in developing some of the reproductive technologies, which has provided the possibility to conserve some of the important genes from rare or valuable breeds (Felip *et al*., 2009).

Characterisation of indigenous farm animal genetic resources such as the Namaqua Afrikaner sheep is the primary step in a conservation program. The genetic diversity that exists between the breeds can be properly conserved and utilised if it is well evaluated genetically. Snyman *et al*. (2010) reported that several South African sheep and goat breeds have been characterised on a molecular level. These include studies on a number of sheep breeds by Buduram (2004), on three commercial sheep breeds and three indigenous goat populations by Visser *et al*. (2004) and on Nguni sheep by Kunene *et al.* (2009). Further studies included genetic characterization of commercial goat populations (Pieters, 2007), Meatmaster sheep (Peters *et al*., 2010) and Namaqua Afrikaner sheep (Qwabe, 2011). Ruane (2000) also added that characterisation permits us to identify the main features, both the strengths and weaknesses, of the available genetic resources and this understanding be used to develop breeding programmes for sustainable use of the genetic resource to disseminate the positive traits identified in the population It can also play a significant role regarding issues of access to and benefit-sharing of agricultural genetic resources, as well as the conservation thereof. The main tool in conservation of these genetic resources is the gene bank through cryopreservation, whereby the material is thawed whenever it is needed (Ruane, 2000).

Extinction of mammalian species is part of the natural process of evolution and is irreversible, but is now appearing at much higher rate than speciation because of human activities such as territorial damage, over-hunting, or competitiveness with introduced herbivores (Holt and Pickard, 1999). Cryopreserved semen can provide opportunity to recover a lost breed through at least six generations of back-crosses, starting with a group of females of another breed (Ollivier & Renard, 1995). However, differences in generation interval and reproductive rates between species may also have an effect in the decision-making of conservation programmes. Some species such as birds and pigs are possible to regenerate very quickly with inexpensive, sometimes less cultured methods, compared to other species (Hiemstra *et al.,* 2005). There is a general understanding of the importance of biodiversity in the world, although very few people are aware of the need to preserve the diversity in domestic livestock. With the extinction of each farm animal breed, there is a risk

of losing genetic traits of vital importance to our agricultural future or to medical progress (Dohner, 2001).

1.1 Justification

Indigenous breeds are evidently well adapted to their natural environment, that being the prevailing climate and fodder varieties, as well as being adapted to main stressors covering parasites and diseases (Burger *et al*., 2013; Cloete *te al*., 2013a; FAO, 2003; Niang *et al*., 2014). Such an indigenous sheep breed in South Africa is the fat-tailed Namaqua Afrikaner, an endangered species livestock genetic resource (Burger *et al*., 2013; Epstein, 1960; Snyman *et al*. 1993; FAO, 2000; ARC, 2013). The Namaqua Afrikaner breed is one of the few indigenous breeds for which scientific production and reproduction norms are available (Snyman *et al.*, 1993; Snyman *et al.*, 1996a, 1996b, 1996c, 1996d; Snyman & Jackson-Moss, 2000; Snyman *et al*., 2002). The current herd at the Carnarvon Experimental Station was used for these studies.

Numerous causes have contributed to the severe impairment of indigenous genetic resources and even extinction of indigenous breeds (Qwabe, 2011). These include the use of exotic breeds, changes in breeder's inclinations due to short-term socio-economic influences, degradation of the ecosystem in which the breeds were developed as well as natural disasters such as drought and diseases (FAO, 1998; FAO, 2000; Qwabe, 2011). Therefore, there is a need to characterise indigenous breeds in order to understand the existing diversity to facilitate the development of rational utilisation and conservation strategies for these breeds (Hanotte & Jianlin, 2005).

Ever since the Namaqua Afrikaner was regarded as endangered, it is very important that this breed must be protected. The Department of Agriculture has decided to intervene in this course and through workshops held in the Northern Cape (May 2007) and Pretoria (November 2007), an agreement was reached that the DNA bank program of GADI should be extended, which by that time only included Angora goats. Subsequently the following program was initiated: "Establishment of the South African Biological Reserve for Small Stock research and conservation". This program includes three main projects, which respectively deal with the establishment and maintenance of:

- Live herds of animals (conservation and research)
- Cryopreservation bank (primarily conservation, secondary research)
- Blood and DNA bank (genomic research).

The endangered Namaqua Afrikaner breed is included in all the three projects. A project establishing a cryopreservation bank for the South African Namaqua Afrikaner sheep breed was started in 2008 (Snyman, 2008). The aims of this project were to collect, freeze and cryopreserve 1 000 embryos from Namaqua Afrikaner ewes and 10 000 semen samples from Namaqua Afrikaner rams. Currently, only 307 embryos have been cryopreserved (Snyman, 2016).

No available literature on the freezing of Namaqua Afrikaner semen could be found. Although the Namaqua Afrikaner was included together with three other indigenous sheep breeds, the Damara, Pedi and Zulu sheep in the study of Munyai (2012), the Namaqua Afrikaner was not included in the trials involving the actual freezing of the semen. Total sperm motility of the Namaqua Afrikaner rams in the study of Munyai (2012) was 37.1%, compared to 74.9% of that of the Pedi rams, which had the highest total sperm motility. Semen samples with a motility of less than 50% are usually not frozen in practice (Shipley *et al*., 2007). However, in the study of Munyai (2012), only two rams per breed were used for semen sample collection. It is well known that there is large variation in semen quality traits among rams within breeds, and even within flocks (Thurston *et al.,* 2002). Furthermore, semen samples were obtained with the electro-ejaculation method in the study of Munyai (2012). This method, as also indicated by Bopape *et al.* (2015) and Munyai (2012), was said to be not the most acceptable method to obtain semen for freezing purposes. As concluded by Munyai (2012), further investigations using more rams and different semen collection techniques, such as the artificial vagina, should be conducted.

Before Namaqua Afrikaner semen could therefore be frozen for the Namaqua Afrikaner cryopreservation bank, further research into the phenotypic characterisation and freezing ability of Namaqua Afrikaner ram semen should be done. In this study, more animals were used and semen was collected via the artificial vagina as well as through electro-ejaculation. The aim of this project was therefore to characterise and cryopreserve semen of the indigenous Namaqua Afrikaner breed, and to compare it to the Dorper and Dohne Merino

breeds, which semen is being frozen commercially on a large scale. Semen evaluation of sheep in South Africa has been well documented in the Dorper (Bester et al. 2004; Fourie et al. 2002 & 2004; Malejane et al., 2014; Skinner, 1971; Schoeman & Combrink, 1987) as well as the Dohne Merino sheep breeds (Eastern Dohne Central Nucleus).. The Dohne Merino is another of the important South African dual-purpose breeds for which semen are being frozen successfully on a large commercial scale. These two breeds will be used as benchmark for comparison of Namaqua semen. Therefore, this current study was well-versed by Munyai (2012) recommendations that more studies can be done with the use of more rams, different semen collection technique and on long term effect (storage) on semen, and by the inadequacy of Namaqua Afrikaner studies where semen was cryopreserved when attained via AV.

The objectives of the study were to:

- Characterise fresh semen collected through an artificial vagina and with electro-ejaculation from Namaqua Afrikaner rams and compare these characteristics with semen collected through an artificial vagina and with electro-ejaculation from Dorper and Dohne Merino rams
- Evaluate viability of frozen-thawed semen of Namaqua Afrikaner semen and compare it with frozen-thawed semen from Dorper and Dohne Merino rams.
- Compare semen collection methods (artificial vagina and electro-ejaculation)

1.2 Hypothesis

- There are no differences in the characteristics of fresh semen from the indigenous Namaqua Afrikaner rams and the semen from Dorper and Dohne Merino rams.
- There are no differences in the viability of frozen-thawed semen of the indigenous Namaqua Afrikaner and the semen of Dorper and Dohne Merino rams.
- There are no differences in the characteristics of fresh or frozen-thawed semen collected via artificial vagina or electro-ejaculation.

2. Literature review

2.1 Introduction

Conservation and development of local indigenous breeds are important. Not only are they more resilient to climatic stress, local parasites, diseases, and represent a unique source of genes for improving health and performance traits of industrial breeds (Cardellino, 2006), but they are also able to utilise low quality food sources. It is also important to develop and use local breeds that are already adapted to their environments, most of which are harsh, with very limited natural and managerial input. Over the past 15 years, 300 out of 6 000 breeds identified by FAO have become extinct (Cardellino, 2006). Large numbers of indigenous breeds that are important for food security are no longer being improved or utilized in a sustainable manner and are in danger of being lost or diluted by crossbreeding (Cardellino, 2006).

Hiemstra *et al*. (2005) indicated that the universal diversity in domestic animals is considered to be under threat, with a large number of domestic animal breeds endangered worldwide, in a critical status or already extinct. With a total number of 6 379 domestic animal breed populations, 9% are said to be in critical condition and 39% endangered (FAO, 2007). There is worldwide consent on the global decline in domestic animal diversity and the need to conserve genetic diversity.

There are two strategies that can be used to conserve genetic diversity, namely *in situ* and *ex situ*. Usually, *in situ* conservation or conservation by utilization is preferred as a method to conserve breeds. A breed has to advance and adapt to changing environments, and efforts should be promoted to create a need for products or functions of the breed Hiemstra *et al*. (2005). Conservation without further development of the breed or without expected future use is not a desirable strategy as there would be no incentive for such an expensive activity. Nevertheless, in addition to *in situ* conservation, methods or techniques to maintain live animals outside their production or natural environment *(ex situ* live) or through cryopreservation of germplasm *(ex situ)* are set up to preserve rare breeds as well as the more widely used commercial breeds. Furthermore, cryopreservation of germplasm is a very good *ex situ* strategy to conserve existing allelic diversity for future use (Hiemstra *et al.*, 2005).

2.2 Semen cryopreservation

Cryopreservation is an artificial disruption of the post-ejaculatory maturation process of the spermatozoa. Even with the best preservation methods to date, the cryopreservation process still causes harmful damage to the spermatozoa. Furthermore, freezing and successive thawing procedures render the remaining surviving spermatozoa physiologically different from spermatozoa before cryopreservation (Lemma, 2011). Spermatozoa are sensitive to any form of stress in their *in vivo* environment, this is even more so under *in vitro* conditions and leads to deteriorating sperm quality. This results in poor fertility from the frozen-thawed semen as compared to fresh semen (Lemma, 2011).

Semen can be defined as a liquid cellular suspension containing spermatozoa and secretions from the accessory organs of the male reproductive tract. Hafez & Hafez (2000) defined the fluid portion of the ejaculate as seminal plasma. Polge *et al*. (1949) reported that spermatozoa were the first mammalian cells to be successfully cryopreserved. The success of this was credited to the unanticipated discovery by Polge *et al.* (1949) and co-workers of the cryoprotective effect of glycerol. Semen is regarded as the most important biological material, although embryos and, to a lesser degree, oocytes and somatic cells, have also received considerable research attention. It can easily be collected and preserved through slow freezing protocols with long-term storage needing liquid nitrogen (Hiemstra *et al*., 2005). This practice is commonly used in cattle, sheep, goat and horse production (Boa-Amponsem & Minozzi, 2006).

Hiemstra (2011) indicated that freezing and thawing leads to a significant decrease of the percentage of motile sperm or membrane-intact sperm. In addition, the enduring sperm may have reduced fertilizing ability and longevity. Hence, the fertility of frozen–thawed semen is generally lower compared to the fresh semen. Nevertheless, in some circumstances, e.g. in cattle, the same level of fertility can be attained provided that the sperm quantity is sufficiently increased to make up for the lower fertility of frozen-thawed semen (Hiemstra, 2011) . Harvesting of biological materials for the gene bank and production of live offspring following thawing of formerly cryopreserved material is made possible through a variety of reproductive technologies, such as artificial insemination using semen, or multiple ovulation and embryo transfer using embryos (Ruane, 2000).

Cryopreservation practice is commonly used in the livestock industry and it is regarded as a safe and feasible strategy to be used in domestic animals. There is, however, a considerable difference between animals in terms of efficiency and efficacy of how they respond to this practice (Hiemstra *et al*., 2005). Regardless of that, cryopreserved semen is successfully used worldwide in artificial insemination (AI) programs (Cassou, 1964). Conservation or preservation of semen is regarded as the best strategy in support of breeding programs of small populations or during the need to preserve/conserve genetic diversity in commercial populations (Hiemstra *et al*., 2005). Ollivier and Renard (1995) reported that cryopreserved semen can be used to recover a lost breed through at least six generations of back-crosses, starting with a group of females of another breed.

2.3 Reproductive physiology in the ram

2.3.1 Testes

The testes can be defined as the primary organs of the male reproduction system (Bearden *et al*. 2004). They produce both the male gametes (sperm) and male sex hormones (androgens). Unlike the female, the male does not possess all the potential gametes at birth but these develop throughout his reproductive life. At puberty in male, the germ cells located in the seminiferous tubules undergo continual cell division forming new sperm cells throughout the normal reproductive life (Bearden *et al*. 2004). Testes are enclosed in a two lobed sac called the scrotum. It is 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. The outer layer which is called *Tunica dartos* intersperses between the connective tissue and divides the scrotum into two pouches; it is also attached to the *tunica vaginalis* at the bottom of each pouch (Hafez & Hafez, 2000).

For normal sperm production to occur, the testes have to be at a temperature several degrees below normal body temperature. The ram has large sweat glands in the skin of the scrotum and a system of muscles that raise or lower the testes into the body for temperature regulation (Schoenian, 2016).

The *epididymis* is a tightly coiled mass of thin tubules located inside the scrotum and posterior to the testis. It carries sperm from the testes to the *ductus deferens* in the male reproductive system. Sperm cells mature as it passes through the epididymis so that it is ready to fertilize an egg by the time it enters the *ductus deferens* (Hafez & Hafez, 2000). The entire

mass of the *epididymis* is actually a single, six-meter tubule that has been coiled upon itself so tightly that the entire mass of it is only around 4 cm long (Hafez & Hafez, 2000). Figure 2.1 shows a cross-sectional part of the testes.

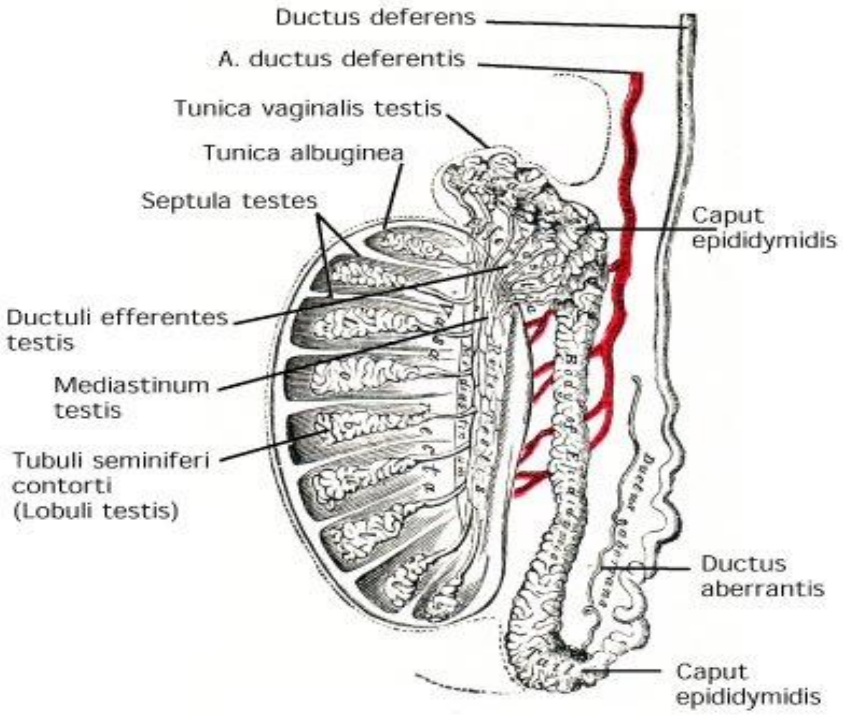

Figure 2.1. Cross-section through testes
(http://veterinary-online.blogspot.co.za/2013/01/reproductive-organs-in-animals.html)

It is the spermatic cord that connects the testis to its life support mechanisms, the convoluted testicular arteries and the surrounding venous plexus and nerve trunks (Hafez & Hafez, 2000). 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. Hafez & Hafez (2000) indicated that the development of testicular function is essential for the changes observed as puberty approaches. Puberty can be specified as the first mount and ejaculation with the release of sperm in males (Delgadillo *et al*., 2007; De Souza *et al*., 2011).

2.3.2 Sperm production

Spermatozoa are formed in the seminiferous tubules of the testis through a process called spermatogenesis (Hafez & Hafez, 2000). After formation in the seminiferous tubules, the sperm cells are forced through the *rete testis* and *vasa differentia* into the epididymis where they are stored while undergoing maturation changes that make the sperm capable of

fertilization (Hafez & Hafez, 2000; Munyai, 2012).

Cloete *et al*. (2000) indicated that the high significant correlation between testicular weight and age indicates that approximately 80% of the variation in testicular weight is associated with bodyweight in rams. However, Salisbury *et al*. (1978) reported that the relationship between testis size and bodyweight decreases after puberty. After puberty, the effect of body weight and growth on semen production is still important for some time, but as maturity is reached other factors begin to play a more important role and can change the relationship of body weight and semen production. There is a decrease in testicular tone over time (Coulter *et al*., 1997; Steyn, 2006).

2.3.2.1 Spermatogenesis

Spermatogenesis in farm animals can be defined as the process of division and differentiation by which sperm are produced in the seminiferous tubules of the testes and consists of two phases, namely, Spermatocytogenesis and Spermiogenesis (Bearden *et al*., 2004; Bester, 2006. During the process the germ cell progress from the diploid to haploid state and then change shape to become fully developed spermatozoa. Spermatogenesis is highly dependent on optimal conditions (temperature) for the process to occur efficiently and it is critical in reproduction. This process starts at puberty and continues until the animal dies. The process can take 40-49 days to be completed (Salisbury *et al*., 1978; Cameron *et al*., 1988).

2.3.2.1.1 Spermatocytogenesis

Spermatocytogenesis is the male form of gametocytogenesis and involves stem cells division to produce precursors of sperm cells (Bearden et al. 2004). The stem cells involved are called spermatogonia and are a specific type of stem cell known as gametogonia. The other two types of cells found along the basement membrane of the seminiferous tubules are Sertoli cells which are larger, less in number and are somatic cells which play a supporting role during both spermatocytogenesis and spermiogenesis (Evans & Maxwell, 1987). The spermatogonia which are usually small rounded and more in numbers are regarded as the potential gametes (Bester, 2006).

After they migrated to the embryonic testes, the primordial germ cells undergo a number of mitotic cell divisions before forming the genocytes (Evans & Maxwell, 1987). Before puberty these genocytes differentiate into A0 (stem cells), A1 (dormant) and A2 (dormant)

spermatogonia along the basement membrane of the seminiferous tubules. A2 spermatogonium will divide, forming either dormant (A1) spermatogonium (Figure 2.2), starting a new generation of developing germ cells (Evans & Maxwell, 1987). The active spermatogonia will undergo four mitotic divisions in bulls and rams thereby eventually forming 16 primary spermatocytes. With rams these mitotic divisions are completed on day 15-17 (Evans & Maxwell, 1987; Bester, 2006; Malejane *et al*., 2014).

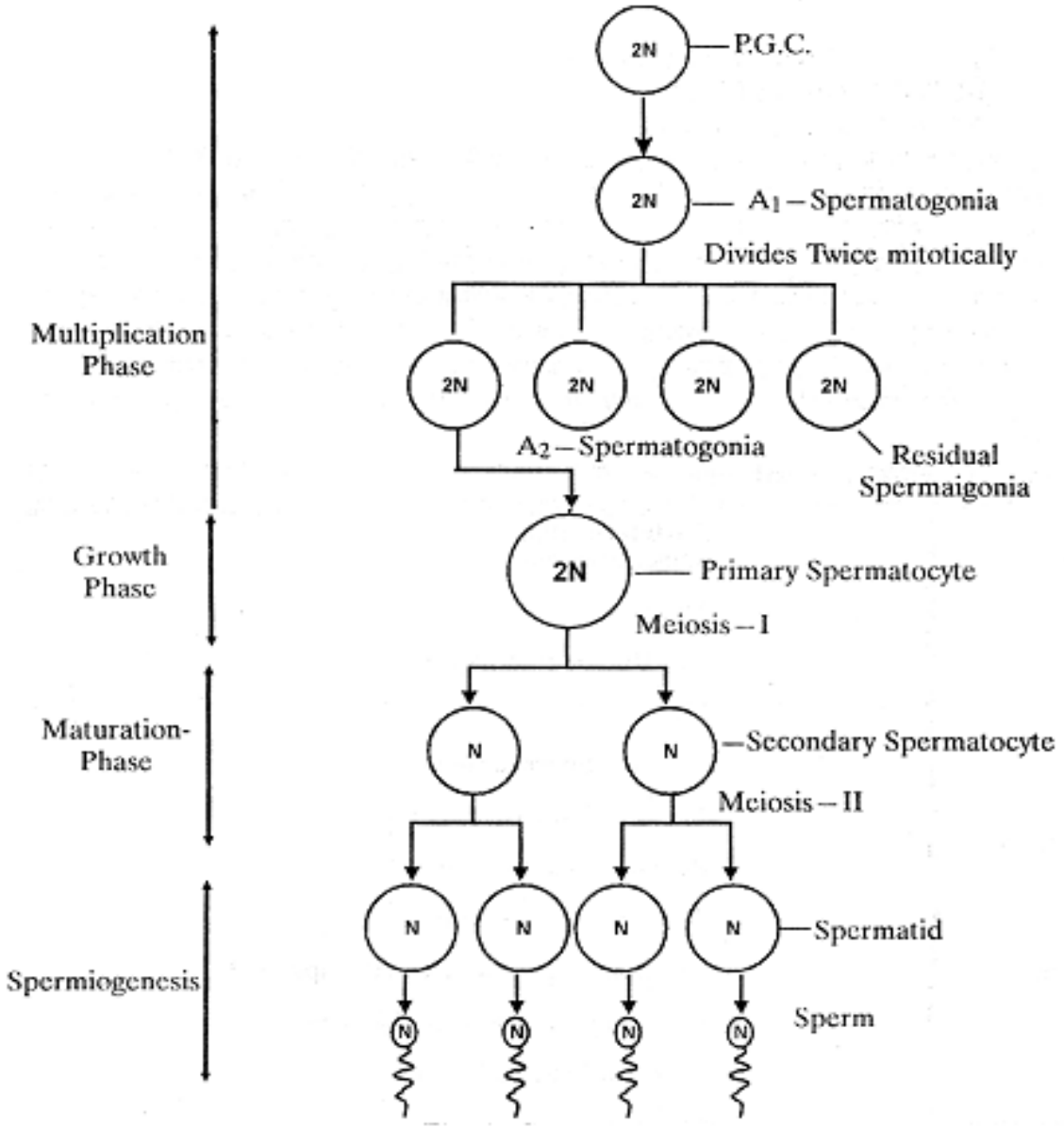

Figure 2.2. Schematic representation of Spermatogenesis (http://sfsoo.com/diagram/diagram-of-spermatogenesis)

2.3.2.1.2 Meiosis

Bearden *et al*. (2004) defined meiosis as a process involving two cell divisions resulting in spermatids containing a haploid number of chromosomes. All the primary spermatocytes go through a meiotic division and as a result two secondary spermatocytes are formed. During the division the chromosome number is reduced into a haploid so that the nuclei in the secondary spermatocytes contain parallel or haploid number of chromosomes (Bearden *et al*. (2004). In rams this phase takes approximately 15-17 days. After their formation, secondary spermatocytes again divide into two spermatids. Four spermatids are formed from each primary spermatocyte or 64 from each active A3 spermatogonium in bulls and rams (Bearden *et al*., 2004).

During the division A1 spermatogonia divide through mitosis to form A2 spermatogonia. The possible yield of spermatids is higher than is usually realised (Bearden *et al*., 2004). After a resting period or dormant state of some weeks, the dormant (A1) spermatogonium divides forming A2 spermatogonia which also divides to form a new active (A3) and new dormant (A1) spermatogonia. A0 spermatogonia (reserve stem cells) do occasionally divide forming a new A0 and A1 spermatogonia. The formation of dormant spermatogonia from A2 spermatogonia is the key process in maintaining the continuity of spermatogenesis and as such not restricting the supply of potential gametes within the testes (Bearden *et al*., 2004; Bester, 2006).

2.3.2.1.3 Spermiogenesis

Spermiogenesis can be defined as a metamorphological stage within the spermatogenesis process whereby, the spermatids undergoes a metamorphosis then released as spermatozoa (Sobti, 2008). During this process the nuclear material will locate in a certain area of the cell thereby forming the head of the sperm, while the rest of the cell stretches to form a tail. The cap (acrosome) around the head of the sperm will then form from the Golgi apparatus of the spermatids (Bester, 2006).

When the cytoplasm from the spermatid is removed during the formation of the tail, a droplet will form on the neck of the sperm. The mitochondria from the spermatid will be formed in a spiral around the upper one-sixth of the tail thereby forming the mitochondrial sheath (Bester, 2006). Spermatids with spherical nuclei changes into sperm and are then released from the Sertoli cells into the lumen of the seminiferous tubules and through to the rete testis. The

progressive motility after maturation and the absence of cytoplasm makes sperm cells unique to all other cells. This process of spermiogenesis can be completed in 15-17 days in rams (Bester, 2006).

2.3.3 Hormonal mechanism of spermatogenesis

Production of sperm cells and the androgens (testosterone) that take place in the testis is regulated by specific hormones. The hormones that regulate these processes are termed gonadotropins, and during the initiation of spermatogenesis they are pumped into the blood stream by the pituitary gland located at the base of the brain, thus they are endocrine hormones (Evans & Maxwell, 1987).

This hormone production process by pituitary gland is also regulated by other centres in the brain (hypothalamus) which can be stimulated by the environmental factors. Without the support of gonadotropins, the production of sperm cells and the male hormones (androgens) by the testes cannot take place. The major gonadotropins sustaining and regulating spermatogenesis are follicle stimulating hormone and luteinizing hormone (Evans & Maxwell, 1987).

2.3.3.1 Follicle stimulating hormone (FSH)

The hormone FSH is also known as the spermatogenesis stimulating hormone (SSH) (Hafez & Hafez, 2000). Follicle stimulating hormone and testosterone stimulates spermiogenesis within the seminiferous tubules of the testes. Schoeman and Combrink (1987) indicated that at puberty the level of FSH in the blood reaches its peak and causes hypertrophy of the Sertoli cells and an increase in the diameter of the seminiferous tubules.

This hormone has been reported to have a critical role in regulating Spermiogenesis (Hafez & Hafez, 2000), the process that controls the formation of normal mature sperm with fertilising ability. There is however, a difference in the way in which FSH controls spermiogenesis in different species of different ages (Moudgal & Sairam, 1998). Hafez & Hafez (2000) reported that the hormone inhibin, a protein secreted by the testes, inhibits FSH production by the anterior pituitary thereby inhibiting FSH secretion and spermatogenesis.

2.3.3.2 Luteinizing hormone (LH)

Luteinizing hormone in males is also referred to as interstitial cell stimulating hormone (ICSH) (Hafez & Hafez, 2000). This hormone stimulates the Leydig cells (interstitial cells) of the testes that are located outside the seminiferous tubules to produce testosterone (Laing, 1955). The produced testosterone then acts on the seminiferous tubules to enhance spermatogenesis (Evans & Maxwell, 1987). An increase in both volume and the activity of the Leydig cells is reported to be influenced by the secretory pattern of LH (Foster *et al*., 1978). However, (Cole & Cupps, 1969) indicated that it might be possible that it is through testosterone secretion that LH has an effect upon the seminiferous tubules.

2.3.3.3 Testosterone

Testosterone can be defined as the principal male sex hormone belonging to the class known as the androgens. Thus it is an androgenic steroid hormone produced by the Leydig cells (interstitial cells) which are richly supplied with nerves. Its secretion is endocrine in that, it is regulated by a negative-feedback mechanism involving the hypothalamus to release gonadotropin-releasing hormone (GnRH) (Evans & Maxwell 1987). As a result GnRH is carried by the portal system that lies between the two areas of the brain to the anterior pituitary gland, where it stimulates the release of LH by the anterior pituitary (Evans & Maxwell 1987).

The result is that the anterior pituitary gland releases FSH and the LH which targets the testes. FSH then induces the seminiferous tubules to produce spermatozoa and a feedback hormone called inhibin (Cole & Cupps, 1969). LH promotes the production of testosterone by the interstitial cells of the testes. The inhibin and testosterone initiate a feedback on the anterior pituitary to inhibit the production of FSH and LH, and on the hypothalamus to inhibit the production of GnRH. Evans & Maxwell (1987) indicated that when these inhibin and testosterone levels drop, GnRH, FSH and LH production increases once again. Figure 2.3 illustrates the overall mechanism of the endocrine hormones regulating reproduction in males.

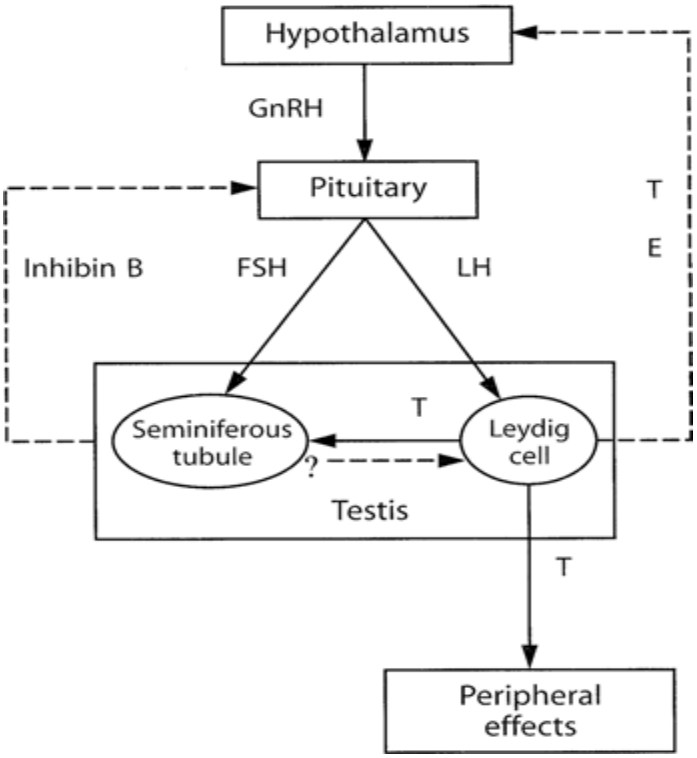

Figure 2.3. Schematic representation of the endocrine hormones regulating reproduction in rams (https://researchgate.net/publication/235703542/Theroleoftestosteroneinspermatogenesis)

2.4 Environmental factors affecting semen quality

Factors such as inadequate nutrition, high ambient temperatures and aging of the animal have a negative effect on the overall semen production (Sonderquist *et al*. 1996). However, an extended photoperiod in small stock, frequent semen collection and certain genetic factors could possibly stimulate sperm production in a positive way (Flowers, 1997). Sonderquist *et al*. (1996) has emphasised that a thorough knowledge about these factors is important in all artificial insemination (AI) centres.

2.4.1 Age and breed

Generally age of the ram affects the quality of the semen during collection. Ejaculates collected from yearling rams have proven to contain spermatozoa with a high percentage of abnormalities and lower percentages of motile cells compared to adult rams (Lymberopoulos *et al*., 2008). This theory was supported by Al-Ghalban *et al*. (2004), where in goats the percentage of abnormal sperm was lower in mature bucks as compared to the percentage in bucks at puberty. However, several studies suggested that an aging ram is associated with a decline in quality of certain semen parameters (Centola & Eberly, 1999).

Skinner *et al*. (1971) reported that the Dorper ram lambs have shown an increase in sperm concentration after the age of 140 days. Testicular weight and diameter of the seminiferous tubules also increased with age (Skinner *et al*., 1971) resulting in increased sperm concentration. There is a correlation between age, scrotal circumference and ejaculate volume of the ram up to 5 years of age (Burfening & Rossi, 1992).This theory was supported by Osinowo *et al*. (1988) and Toe *et al*. (2000), that the genital system of the ram undergoes maturational changes during that period. However, frozen-thawed semen of mature rams was reported to have a lower sperm motility (37%) in comparison to that of young rams (45%) (Lymberopoulos *et al*., 2008). Therefore, Matshaba (2010) recommended further investigation regarding the effect of age of the ram and season on the quality of semen and its cryopreservation ability.

Sheep breeds according to Mahoete (2010) have shown differences in semen quantity and quality during and after the breeding season. These differences between breeds and individual rams in semen characteristics make it necessary to perform semen evaluation of each breed in order to quantify semen characteristics of the different breeds. This is supported by Matshaba (2010) through the recommendation that semen cryopreservation of indigenous breeds should be investigated further so as to be able to develop cryopreservation protocols and semen extenders which will result in better post-thaw viability of the sperm.

2.4.2 Season

Thongtip *et al*. (2008) reported that seasonality affected semen quality in bulls, boars, stallions and rams. Cryopreservation can also be affected by the season of collection. Hafez & Hafez (2000) indicated that semen collected during the breeding season freezes better than semen collected during the non-breeding season. Seasonal variation in the thyroid activity and seminal characteristics has also been observed in Iranian fat-tailed rams (Zamiri & Khodaei, 2005). Thongtip *et al*. (2008) went further in suggesting that the thyroid gland may be involved in seasonal transition of reproductive activity in the ram. Thyroidectomised rams have given low semen quality with a decreased sperm concentration and motility and an increased percentage of abnormal sperm (Brookes *et al*., 1965).

According to Schwab *et al*. (1987) bulls produces higher volume of semen, sperm concentration and number of sperm *per* ejaculate during winter. Menendez-Buxadera *et al*.

(1984) also reported semen quality to be higher in winter in Zebu bulls as compared to Holstein and Criollo bulls. However, these results are in contrast with the findings of Fuente *et al*. (1984), who obtained the lowest semen quality during winter in Galician Blonds bulls, the results that might be attributed to the nutritional prominence of the bulls. It is however, evident that season of collection significantly affects semen production (Graffer *et al*., 1988; Stalhammar *et al*., 1988). Cole & Cupps (1969) reported that high summer temperatures decrease the fertility in sheep and metabolism of spermatocytes is severely influenced when the scrotal temperature is artificially increased to 41 °C for 3 hours.

Sheep and goats are generally seasonal breeders, thus the semen quality will be affected by season as they mate naturally during autumn when the day light length is decreasing, and that allows birth at the following spring (Zarazaga *et al*., 2009). Spring allows optimal conditions for survival of the young in terms of temperature, feed and water availability (Zarazaga *et al*., 2009).

Corteel (1977) reported a decrease in quantitative and qualitative semen production and sperm fertility during the non-breeding season in rams. Delgadillo *et al*. (2007) suggested photoperiod or season as the principal factor influencing seasonality of reproduction in rams. However, Chemineau *et al.* (2004) established that Creole female goats and Black Belly ewes are capable of showing seasonality under the photoperiodic changes of the temperate zone even though they do not originate from these zones. A marked seasonality in the ovulatory activity of these two breeds was induced when females were exposed to a simulated temperate photoperiod (Chemineau *et al.*, 2004). Factors such as social stimuli, feed availability and social interaction are also regarded as important regulators of seasonality in reproduction (Delgadillo *et al*. 2007). Walkden-Brown *et al*. (1994) regard nutrition as an important factor affecting the seasonality of reproduction. However, temperature is expected to influence sperm production and quality due to site and morphology of organ where spermiogenesis takes place.

2.4.3 Ambient temperature and testicular thermoregulation

Spermiogenesis will be negatively affected by temperatures that are above that of the normal body temperature (Kastelic *et al*., 1996). For normal semen production to occur the scrotum should be able to maintain the temperature lower than that of the body temperature. In maintaining the necessary thermoregulation for spermatogenesis, the ram has large sweat

glands in the skin of the scrotum, as well as a system of muscles that raise or lower the testes closer to the body as a mechanism to regulate the temperature (Kastelic *et al*., 1996).

If ambient temperature is high the conditions required for normal spermatogenesis cannot be maintained, as a result the spermatogenic tissues degenerate (Folch, 1984). Several physiological mechanisms play an important role in testicular thermoregulation. These include the regulation of blood flow, the control of the testis position relative to the body by scrotal musculature, sweating, counter-current heat exchange in the vascular cone, and overall radiation of heat from scrotal surface (Cook *et al*., 1994). Kastelic *et al*. (1996) reported that the scrotum and testes have complementary temperature variances that contribute to testicular thermoregulation.

Cook *et al*. (1994) described the counter-current heat exchange within the vascular cone functions as the transfer of heat from the warm blood flowing down the testicular artery towards the testis, to the cooler blood returning from the testis through the testicular venous system. Vascular cone also plays an important function in the radiation of heat from the scrotum, as the scrotal skin overlying the vascular cone is usually the warmest area on the scrotum (Acevedo, 2001). Bulls with abnormal high scrotal temperature patterns have a lower percentage of sperm showing normal head, tail and acrosome morphology but exhibit a higher percentage of sperm cells with proximal droplets than bulls with normal scrotal temperatures (Lunstra & Coulter, 1996). Food availability and ambient temperature determine energy balance, and variation in energy balance is the ultimate cause of seasonal breeding in all mammals and the proximate cause in many (Bronson, 2009).

2.4.4 Nutritional effect on semen quality and fertility

Smith & Akinbamijo (2000) reported that rams that graze on pastures of fluctuating quality may have testes which double or halve in size during the year as a result of seasonal variation in quality of the pasture. Improvement in nutritional intake of both protein and energy during the two month period before mating increased the testicular size and subsequent sperm production by as much as 100% (Fernandez *et al*., 2004).

The effects of underfeeding in mature animals may be rectified as there are transitional, but the same cannot be said with young animals, because of the permanent damage caused to the germinal epithelium of the testes (Hafez & Hafez, 2000). Underfeeding was reported by

Schwalbach *et al*., (2006) to have significant negative effect on the testicular development and semen quality in young Boer goat rams. Generally, the severity of most nutritionally induced changes to reproductive function in adult rams is temporary, but that can vary from a slight effect on seminal characteristics and libido to infertility (Brown, 1994).

Studies by Oldham *et al*. (1978) and Hotzel *et al*. (1998) proved that spermatogenesis in rams is sensitive to an increase in protein intake. They further indicated that the effect relate to an increase in testicular size due to an increase in the volume of the seminiferous epithelium and the diameter of the seminiferous tubules. The testicular size is controlled by nutrition to an extend that well fed rams in spring possess larger testes as compared to poorly fed rams in autumn (Martin *et al*., 2012) of the same age and breed. However, irrespective of feeding levels and protein intake, the fertility of semen was the same, but the concentration of sperm cells was higher in bulls that were on high plate of protein diet (Cole & Cupps, 1969). Nutritional effects are manifested by increasing the frequency of the pulses of LH and possibly FSH (Lindsay *et al*., 1984; Hotzel *et al*., 2003). But the energy components of the diet, especially the fatty acids appear to play an important role in reproductive responses following changes in nutrition. For an example fatty acids can stimulate the GnRH dependent pathways that initiate changes in testicular functioning (Boukhliq & Martin, 1997; Blache *et al*., 2002).

Fourie *et al*. (2004) indicated that an intensively fed ram's increase in testicular size might be observed as acceptable, but the ram's reproductive efficiency may be hampered by excessive scrotal fat deposition, especially in the neck of the scrotum. This interfered with the testicular thermoregulatory mechanisms essential for optimal sperm production. This is in agreement with Labuschagné (2001) who concluded that the feeding of high-energy diets to young bulls resulted in fat accumulation in different parts of the body, including the scrotum and thereby negatively affecting the process of spermatogenesis. Hafez (1993) also suggested that rams should not be allowed to be become too fat, or have a body condition score (BCS) of above 4, as they proved to be sexually inactive and are even more prone to heat stress.

It was also found that sperm reserves of bulls fed on a medium rather than on high energy diet to be superior. The proportion of progressive motile sperm cells being lower in high energy fed bulls (Coulter *et al*., 1997). Furthermore, Mwansa and Makarechian (1991) reported that bulls on a high energy diet throughout the performance testing period produced

semen of reduced quality. The effects of nutrition on scrotal circumference need to be considered when evaluating bulls and rams for reproductive soundness or when using scrotal circumference as a selection criteria (Pruitt & Corah, 1986; Fourie *et al*., 2004).

2.5 Semen Collection

Semen collection is often compared to harvesting any farm crop; it is a collection of the maximum number of sperm cells, of the highest possible quality in each ejaculate (Bester, 2006). To obtain the best quality semen for processing and storing, it is recommended that collections should be performed during autumn or early spring. This statement is in agreement to D'Alessandro and Martemucci (2003) as their results showed that season has a significant influence on semen freezability in Leccese ram, with the best performance occurring in the summer and autumn period, corresponding to the reproductive season in temperate zones. The quality and quantity of semen can be influenced negatively by the use of improper techniques during collection (Bearden & Fuquay, 1980).

2.5.1 Techniques used for semen collection

In sheep and goats the artificial vagina (AV) and electro-ejaculation (EE) are commonly used methods for semen collection. Mahoete (2010) reported that there is another method of collection called the vaginal collection vial (VCV) developed by Wulster-Radcliffe *et al*. (2001b). The VCV technique makes use of a glass vial of roughly 9 cm long, bent over at 10° angle. The bend assists in securing the vial being kept in place during mating. It is inserted into the vagina of the ewe about ten minutes before mating (Wulster-Radcliffe *et al*. 2001b). Different from the AV, VCV does not require training of rams, which is often time consuming and could take up to 3 weeks, depending on the individual ram (Wulster-Radcliffe *et al*., 2001a; Ortiz-de-Montellano *et al*., 2007).

2.5.1.1 Artificial Vagina

This method is easy to use and the collections are relatively clean and with significant volumes (Salisbury *et al*., 1978). AV can be described as a device which is designed to mimic the female reproductive tract (Donovan *et al*., 2001). AV is made of a rigid cylinder of plastic and walled with rubber sleeves on the inside and the ends of the sleeves are folded over the plastic cylinder to form a watertight space. A graduated class tube is fitted at one end for semen collection. The space between the sleeve and the cylinder is filled with water at a

temperature of 43-46 ºC before closing it with a rubber stopper.

Before collection it is recommended that all the parts to be used be clean, sterile, and properly assembled. Silvestre *et al*. (2004) and Bester (2006) emphasised the importance of temperature within the AV before attempting semen collection. The temperature in the AV resembles body temperature while the pressure provides the mechanical stimulation of the vagina over the glans penis (Donovan *et al*., 2001).

2.5.1.1.1 Training of rams for semen collection using an artificial vagina

Wulster-Radcliffe *et al*. (2001a) indicated that as much as it resembles natural service and is the most hygienic of the methods available, it does however, require training of the ram. Rams are trained for semen collection by using a ewe that is preferably in oestrus as a teaser female. The ewe will be restrained in a stanchion in a neck clamp before the introduction of the ram into the pen (Silvestre *et al*., 2004; Bester, 2006). In order to enhance the libido of the ram, the pen should be close to the male pen so that the other rams in the pen that have to be trained can be able to see the ram mounting the restrained ewe in order to be stimulated as well (Price *et al*., 1984; Silvestre *et al*., 2004). When the ram mounts its penis is deflected into the AV thereby ejaculating naturally. The semen collector should always make sure that the temperature of the AV is correct.

Rams are generally allowed 5 minutes to ejaculate. After ejaculation or a period of 5 minutes, depending which comes first, it should be removed from the pen into the adjacent pen. After a period of 10 to 15 minutes, the ram should again be placed within the test pen. Training of the rams is considered to be complete when they can mount and ejaculate regularly when exposed to a ewe teaser whether in oestrus or not, in the presence of a collector (Silvestre *et al*., 2004).

2.5.1.2 Electro-Ejaculation

Bester (2006) reported that the first person to use this method was Gunn (1936) in Australia. This method is carried out by stimulating the spinal cord between the 4^{th} lumbar and the 1^{st} sacral vertebrae. This is through the insertion of a probe or electrode into the rectum of the ram, stimulating the nerves of the reproductive system by gradually increasing the electrical current in a rhythmic pattern for a short period of time. The use of this technique requires skill, experience, patience and knowledge of the whole process (Salisbury *et al*., 1978).

During the use of this method, the electric current produce general tetanic contractions of all body muscles and a slight and temporary motor inability of the hindquarters and hind limbs at the end of collection. This method is mainly used in males of certain species where the use of AV is not possible or impractical (Carter *et al*., 1990).

2.5.1.3 Artificial vagina (AV) vs. Electro-ejaculation (EE)

The use of EE is regarded as the fastest and more convenient method with a higher semen volume, but with low sperm concentration compared to AV but it is stressful to the animal (Mattner & Voglmayr, 1962; Salamon & Morrant, 1963; Wulster-Radcliffe *et al*., 2001b; Marco-Jimenez *et al*., 2005). Matthews *et al*. (2003) reported that semen collected using an AV produces a higher sperm concentration, but with similar volume and sperm morphology when compared to that of semen collected using EE. Carter *et al*. (1990) also found that repeatability of the volume of the ejaculate obtained, sperm concentration, total sperm number, percentage of normal sperm cells and wave motion were marginally higher with the AV method than with the use of EE.

Concerns have been raised regarding animal welfare in the use of EE method over the years (Ortiz-de-Montellano *et al*., 2007). But this method is still commonly used, more especially with untrained males. Sundararaman *et al*. (2007) concluded that the advantage of this method is that training is not required prior to semen collection, more ejaculates are collected in a short space of time and that semen from superior sires incapable of mounting due to injuries or old age can still be collected. Bopape *et al*. (2015) recorded a higher semen volume and total sperm motility using EE method compared to AV method, however AV resulted in higher sperm concentration, total, rapid sperm motility and normal sperm compared with the EE technique.

Bopape *et al.* (2015) indicated that the success of conservation of semen can depend on the semen collection method and that the use of AV seems to be the most suitable method for collecting semen. This results concur with Jiménez-Rabadán *et al.* (2012)'s findings where AV gave better results for sperm quality after thawing, and suggested that the breeding season might also be a factor that influence semen quality. This was also observed by Carter *et al*. (1990) during the comparison of AV and EE methods in rams where the repeatability of ejaculate volume collected, sperm concentration, total sperm number, the percentage of

normal sperm and wave motion proved to be slightly higher when using the AV method. In addition to difference due to different methods used to collect semen, there are several other factors that affect semen quality such as temperature, pH and light.

2.5.2 Factors affecting the viability of spermatozoa after semen collection

2.5.2.1 Temperature

Bearden & Fuquay (1980) reported that the metabolic rate of the sperm cell increases when the temperature of the semen increases while the life span of the cells decreases, thus showing the degree of sensitivity to temperature. Beraden & Fuquay (1980) the most reported indication of cold shock is an irreversible loss of motility of the spermatozoa. Reduction in the temperature of semen slows down the metabolic rate and increases the fertile life of sperm cells, when precautions are taken to protect it against the cold shock (Evans & Maxwell, 1987; Pérez-Pé *et al*., 2001).

2.5.2.2 Semen pH

Most enzymes in the sperm cells function at an optimum pH of approximately 7.0, thus a higher metabolic rate will be expected when the pH of semen is maintained closer to neutrality (Bester, 2006). The metabolic rate will therefore be reduced if the pH of semen deviates towards alkalinity or acidity. Study in this area has established the importance of diluting semen in a buffered medium that accommodates changes in pH so that absolute fertility of semen can be preserved (Bearden & Fuquay, 1980)

2.5.2.3 Light exposure

Exposing the semen to light after collection can decrease the metabolic rate, motility and the fertilizing capabilities of the sperm cells. Bester (2006) recommended that semen should never be exposed to direct sunlight as the ultraviolet light could be detrimental to the spermatozoa, and as such emphasised the use of a Styrofoam box in transporting the semen to the laboratory following collection to guard against the effect of direct sunlight. This recommendation is in agreement with Bearden & Fuquay (1980), that exposure to light cause a photochemical reaction in the semen, thereby causing the production of hydro-peroxide which can be damaging to the sperm cells.

2.6 Evaluation of semen

Bester (2006) defined spermatozoa as unique cells because of their uniformity and function. Furthermore mature sperm cells are described as a terminal cell which would not undergo further division or differentiation as it is an end product of complex developmental processes (Bester 2006). Examination of semen is a standard procedure for examining or assessing the potential fertility of breeding males, other than direct examination of their ability to produce progeny (Hafez & Hafez, 2000). There are different methods used in assessing semen quality in estimating their potential fertility. However, there is no single characteristic that can accurately predict the fertility of a semen sample; it is only by examining various physical characteristics that one can estimate the potential fertility (Hafez & Hafez, 2000). Semen can be examined using a light microscope to estimate the sperm viability and the percentage motile sperm cells prior to cryopreservation and again at post-thaw before attempting to use it through AI (Rowe *et al*., 1993). Evans & Maxwell (1987) reported that the quality of spermatozoa in an ejaculate is dependent on the volume and concentration of the semen sample. It is however, the complexity and sensitivity of the spermatozoa that hinder the objective of researchers in finding laboratory assays that could accurately predict the fertilising capacity of a semen sample (Graham & Mocé, 2005).

2.6.1 Subjective assessment of semen

2.6.1.1 Volume of the ejaculate

Volume of semen collected can be assessed using either a calibrated collecting glass tube or even more accurately using calibrated pipettes (Evans & Maxwell, 1987). An average ejaculate volume of ram semen was reported to be 1.1 ml with a range of between 0.5 and 1.2 ml (Seremak *et al*., 1999; Hafez & Hafez, 2000). Generally the ejaculate volume of semen from mature rams varies from 0.5 ml to 2 ml (Evans & Maxwell, 1987). Gil *et al*. (2003) reported semen volumes between 0.75 and 2ml with an AV on Polish long sheep ram.

Semen volume will decrease with frequent collection of semen from the rams three or more times per day or even for an extended period of time (Hafez and Hafez, 2000). Hafez & Hafez (2000) further reported that age of the ram and body condition, season of the year, skill of the technician involved and the frequency of collection could affect the ejaculate volume. Matthews *et al*. (2003) reported no significant differences between semen collected from Dorper rams with the use of AV or EE in terms of volume.

2.6.1.2 Sperm colour and concentration

Generally ram semen colour differs from milky-white to pale creamy (Bag *et al*., 2002). Bag *et al*. (2002) described a pink colour of the semen sample as an indication of presence of blood and suggested that this can be due to injury or disease of the ram's reproductive tract, while a grey or brown colour of the semen sample reflects contamination or infection of the ram's reproductive tract. Urine can also be present within semen samples collected with an electro-ejaculator, while a yellowish discoloration with strong odour can be an indication of presence of urine (Bag *et al*., 2002). Contaminated semen samples will negatively influence sperm fertilising ability, therefore they should be discarded (Bester, 2006).

Hafez & Hafez (2000) reported that there is a correlation between the colour of the semen and the concentration of the ejaculate. The density or viscosity of the semen sample reflects the number of spermatozoa present in the sample. Table 2.1 shows how sperm concentration varies based on semen sample colour.

Table 2.1. Assessment of sperm concentration according to the colour of the semen sample

Ejaculate colour	Number of spermatozoa (x10^9) per ml
Thick creamy	5.0 (4-6 x 10^9)
Creamy	4.0 (3.5-4.5 x 10^9)
Thin creamy	3.0 (2.5-3.5 x 10^9)
Milky	2.0 (1-2.5 x 10^9)
Thin milk	0.7 (0.4-1.0 x 10^9)
White	Insignificant

Source: Hafez & Hafez (2000); Evans & Maxwell (1987)

Hafez (1993) defined sperm concentration as the number of sperm cells per millilitre (ml) of a semen sample. The sperm concentration within the sample can be determined physically by the use of a haemocytometer or spectrophotometer (Evans & Maxwell, 1987). Loskutoff & Crichton (2001) indicated that the number of sperm cells lying in a chamber is counted under the microscope and multiplied by the dilution factor used. They further indicated that the method is very accurate, yet time consuming and suggested that other methods such as trophotometric or colorimetric can be used instead.

Hafez & Hafez (2000) however, disagree with the use of photometers suggesting that they are not accurate when using contaminated semen samples, and that the addition of cloudy extenders prior to sperm concentration determination could tamper with the results. There is however, a new developed photometer (Spermacue^{tm}) which is accurate in measuring semen concentration. It can be calibrated for bovine, canine, equine, porcine and small ruminant species, and it automatically resets to zero after each sample (Rigby *et al*., 2001).

2.6.1.3 Sperm motility

Sperm motility is usually believed to be one of the most important characteristics when assessing the fertilising capability of ejaculated sperm (Hashida & Abdullah, 2003). Good wave motion can be seen in the collection glass with the naked eye. This assessment is regarded as the simplest test for motility assessment for fresh undiluted semen. Sperm motility assessment relates to the subjective microscopic estimation of the viability of the sperm cells (Hafez & Hafez, 2000). Sundararaman & Edwin (2008) suggested that generally sperm motility and the characteristics of the sperm motion in particular, could be indicators of sperm quality. Deterioration in sperm motility characteristics was detected with an increase in storage time (Kasimanickam *et al*., 2007).

With a microscope, a scoring system from 0 to 5 (Table 2.2) can be used to assess each semen sample, where 0 indicates no movement at all and 5 indicating a very good wave motion (Hafez & Hafez, 2000; Loskutoff & Chrichton, 2001). Even though this form of assessment is subjective, with practice an accurate estimate of the motility of semen sample could be achieved (Evans & Maxwell, 1987). In a study by Matshaba (2010), results revealed that sperm viability and motility parameters decline slightly following dilution and a drastic decline following freezing.

Table 2.2. Assessment of sperm motility score card and description

Score	Class	Description
5	Very good	Dense, very rapidly moving waves; 90% or more of the spermatozoa are active
4	Good	Vigorous wave movement but not as rapid as for score 5; 70-85% of sperm cells are active
3	Fair	Only small, slow moving waves; 45-65% of sperm cells are active

Score	Class	Description
2	Poor	No waves are formed but some movement of sperm is visible; 20-40% of sperm cells are alive but with poor motility
1	Very poor	Only about 10% of spermatozoa show signs of life. Only weak movement
0	Dead	All sperm cells are motionless

Source: Hafez & Hafez (2000); Loskutoff & Chrichton (2001)

2.6.1.4 Sperm morphology

Morphological assessment of the sperm cell forms an integral part in the analysis of semen and is considered a very important part of any breeding ram soundness examination (Kuster *et al*., 2004). It was reported that the aging of the sperm cells resulted in morphological changes, even in semen which is kept under controlled temperatures (Salisbury *et al*., 1978). Graham & Mocé (2005) suggested that even though the general classification systems for the morphology of sperm from various species have been reported, the classification categories are different for those species and adoption of a uniform system within each species is necessary.

Numerous dye exclusion techniques have been developed over time to differentiate between live and immotile sperm or dead sperm (Björndahl *et al*., 2004). The underlying principle in which these methods are used is that sperm cells with structurally intact cell membranes are not stained and as such they don't absorb the stain, while dead sperm with disintegrating cell membranes absorb the stain (Björndahl *et al*., 2004). The Eosin-Nigrosin stain is commonly used in the laboratory and is referred to as a differential stain because of its inability to pass through living cell membranes (Bearden *et al*., 2004). The use of Eosin-Nigrosin is simple and effective and allows sperm to be readily seen. It is also called "live-dead" stain, which allows assessment of the membrane integrity at the same time as the morphology of the sperm cell (Björndahl *et al*., 2003).

Sperm cells that are alive appear to be white in colour as they don't absorb the Eosin stain while dead cells appear to be pinkish in colour. The live sperm can then be categorised into different forms, e.g. morphologically normal or with defects in acrosome (crooked or loose). Other abnormalities include sperm head (bulb, small, enlarged or looped), the sperm neck

(broken at various angle in relation to head), mid-piece and sperm tail (swelling, looping, partial or totally lacking) (Łukaszewicz *et al*., 2008). Abnormalities are usually divided into primary and secondary types of abnormalities or major and minor abnormalities; in some classification systems (Kebede *et al*., 2007). Gil *et al*. (2003) regard semen with less than 10% abnormalities as normal in sheep. Success in the assessment of sperm morphology depends on the stain preparation techniques, stain type and staining methods (Bilgili *et al*., 1985).

2.6.2 Objective assessment of semen

Rowe *et al*. (1993) indicated that most of the laboratories make use of the light microscope as a standard semen evaluation method as compared to the use of the Computer Assisted Sperm Analysis (CASA) system to estimate sperm survival and percentage of motile sperm. Although useful, the light microscope method considered manual, can have major limitations because of influences from human bias (Graham *et al*., 1980). Graham & Mocé (2005) further indicated that the use of the microscope method can be time consuming either in semen sample preparation or during the analysis itself, which often results in few semen samples being evaluated. More objectivity and repeatability in the assessment of sperm motility can be achieved with the aid of the CASA (Rowe *et al*., 1993)

2.7 Cryopreservation of semen

The general objective of cryopreservation is the storage of gametes of certain individual animals that were selected for the improvement of the herd, preserving the endangered or prevent the extinction of breeds. Generally all the protocols used in the freezing of the semen do have potential damaging stresses to the spermatozoa (Purdy, 2006). Watson (2000) reported sudden changes in temperature, submission to osmotic and toxic stresses as a result of exposure to molar concentrations of cryoprotectants and finally the formation and dissolution of ice in the intracellular and extracellular environment. These damaging effects of cooling and freezing on the sperm membrane differ among domestic animals and they are influenced by several components (Medeiros *et al*., 2002; Lemma, 2011). Boar sperm has proven to be the most sensitive, followed by bull, ram and stallion sperm, while rabbit, human and rooster sperm are least sensitive to cold shock (Parks, 1997).

There are numerous factors that can have an impact on the ultimate number of the spermatozoa count which will survive the collection, processing, freezing and the thawing

processes. These factors can in turn be influenced by other factors. The degree to which the collected semen sample is diluted during processing can be prejudiced by the type of extender used. The extender should be added very slowly to the semen sample to prevent shock to the sperm cells. The process of freezing the extended semen, the rate of cooling down, as well as the ultimate temperature at which semen will be stored all might impact the fertilizing capabilities of the frozen-thawed semen. Storage method maybe determined by packaging of the frozen semen as well as the period that semen has to be stored (Van Staden, 2010). Semen quality was found to deteriorate as a result of cooling, freezing, thawing and the adding of cryoprotectants (Fernandez-Santos *et al*., 2006). Marco-Jiménez *et al*. (2005) also indicated that procedures used during the preservation of semen cause damage to the sperm plasma membrane.

Diluted ram semen can be cooled to 5 ºC over a 1.5 - 4 hour period and then frozen in either pellets or straws (Leboeuf *et al*., 2000; Barbas & Mascarenhas, 2009). There are still differing opinions among researchers concerning the freezing of ram semen as semen from other species differ in their ability to survive the freeze-thawing process and this is generally related to their tolerance to osmotic stress (Bearden *et al*., 2004). Frozen semen is supposed to be stored at a temperature of at least -76 ºC, while with liquid nitrogen it is stored at a temperature of -196 ºC (Guthrie *et al*., 2002). Leboeuf *et al*. (2000) however, stated that a comparison of methods and protocols for freeze-thawing of semen is still difficult, because of the different parameters evaluated and the lack of uniformity in the methodologies used.

2.7.1 Semen cryopreservation techniques

There are two techniques that are extensively used in germ cells and tissue preservation; these being slow freezing and vitrification (Papadopoulos *et al*., 2002). There are, however, damaging factors that are associated with both these methods and are related to chilling injury, cryoprotectant toxicity, osmotic stress and crystal formation (Zhang & Rawson, 1995; Mazur, 1985).

2.7.1.1 Slow freezing

This method involves cooling of the germ cells and tissue cells in a cryoprotectant fluid and storing them in canisters of liquid nitrogen at sub-zero temperatures (Rajakumar, 2012). Rajakumar (2012) reported further that the only limiting factor or criticism in the use of this method is that there can be ice crystal formation inside the stored germplasm and tissue cells,

damaging them and consequently compromising their viability for transfer or use. Woelders & Malva (1998) however, reported that slow cooling is needed in order to allow sufficient efflux of water to minimize the chance of intra cellular ice formation. They further reported that as the cooling continues, ultimately the viscosity of the unfrozen fraction becomes too high for any further crystallization. An amorphous solid will be formed in the absence of ice crystals with that being referred to as vitrification.

2.7.1.2 Vitrification

Rajakumar (2012) reported that unlike slow freezing, this method produces a glass-like solidification of living cells that resist the formation of ice crystals, which prove to be potentially damaging during the cooling and warming periods. The absence of ice crystal formation can be attributed to high concentration mediums used as protectants; however, these high concentrated mediums may cause damages due to abrupt osmotic changes, the extremely low water potential and chemical toxicity (Mazur, 1963; Muldrew & McGann, 1994).

Rall (1987) indicated that embryos can be treated with a 25% vitrification solution at room temperature and then cooled at 4 ºC before being transferred to a 50% vitrification solution and ultimately 100% vitrification solution and packed accordingly before being put into liquid nitrogen. High percentages of cryoprotectant agents reduce osmotic effects while lower temperatures and shorter time periods proved to prevent the damage by chemical toxicity (Polge *et al*., 1949; Li *et al*., 2006).

2.7.2 Factors affecting cryopreservation of semen

2.7.2.1 Importance of extenders/diluents

Dilution of semen in an appropriate media is one of the important factors affecting sperm endurance during cryopreservation (Rasul *et al*., 2000). Diluents are used to increase ejaculate volume of the semen without affecting semen quality and they preserve the fertility of the sperm for the longest period of time (Hafez & Hafez, 2000; Van Staden, 2010). The purpose of a semen cryopreservation extender is usually to supply the spermatozoa with a source of energy, to provide protection for the cells from temperature related damages, while maintaining a suitable environment for the spermatozoa to survive temporarily (Royere *et al*., 1996). A great range of extenders combining various elements have been suggested and used for extending semen. Milk and milk-based extenders are known to be practical and efficient

in protecting spermatozoa of different species (Varner *et al*., 1989; Batellier *et al*., 2001). The semen extender used may alter the sperm motility, usually by increasing the velocity measures (Hafez & Hafez, 2000).

An ideal medium should have a pH between 6 and 8, with preference of 7 , maximum water solubility and minimum solubility in all other solvents, minimum salt effects, minimum buffer concentration, least temperature effect, well behaved cation interactions, greater ionic strengths and chemical stability (Bates 1961; Good *et al*., 1966; Good and Izawa, 1972; Keith and Morrison, 1981). For an extender to have a positive effect on the quality of semen it should contain an energy source for it to protect the spermatozoa from cryogenic injuries (Salamon & Maxwell, 2000; Oláh *et al*., 2012).

Tris-based semen extenders used for freezing contains fructose or lactose as sugars, compared to other semen extenders (Purdy, 2006). According to Barbas and Mascarenhas (2009) they should contain buffers, carbohydrates (glucose, lactose, raffinose, saccharose or trehalose), salts (sodium citrate), citric acid, egg yolk and antibiotics. These provide an energy substrate for the spermatozoa and to maintain osmotic pressure of the diluents (Berlinguer *et al*., 2007; Filiberto *et al*., 2011) while antibiotics control proliferation of any microbial contaminants (Salamon & Maxwell, 2000).

Tris-citric acid based extender was suitable for the cryopreservation of buffalo spermatozoa in terms of post-thaw motility and survivability as it was suggested that zwitterion buffers, largely Tris-citric acid may provide the most acceptable buffering system to improve the post-thaw freezing ability and consequently may also improve the fertility of buffalo spermatozoa (Ahmad *et al*., 1986). Graham *et al*. (1972) indicated that zwitterion buffers have a pH closer to the p*K*a (acid dissociation constant). Also the p*K*a is least influenced by temperature as compared to other buffers.

2.7.2.2 Temperature

Cooling is a period of adaptation of spermatozoa to decreased metabolism. Diluted semen is cooled slowly to avoid potential cold shock. The cold shock is assumed to be damaging the function of membrane proteins that are essential for structural integrity or ion metabolism (Watson, 2000). Hurried cooling can be negative in that it depresses the rate of fructose breakdown, oxygen uptake, and ATP synthesis by the sperm which results in the loss of

energy supply and motility (Blackshaw and Salisbury, 1957; Wales and White, 1959). White (1993) further suggested that cold shock may increase calcium uptake by sperm. Some (Marshall, 1984) however, think that faster cooling will not generate problems if semen is extended in an idyllic buffering system Parks (1997) observed that cooling cattle bull spermatozoa from body temperature to 5 °C performed at a rate of 10 °C/h has minimum deleterious effects.

Under such circumstances adequate protection is provided for bull semen by equilibration of semen with a glycerol containing diluent, the glycerol offers protection when ram semen is cryopreserved, but it has proven to be less effective than in bull semen (Bearden & Fuquay, 1980; Bester, 2006). Presence of glycerol prevents the crystallization of water within the sperm cells, which ultimately allows the spermatozoa to be frozen rapidly (Holt, 2000). Results obtained by Matshaba (2010) emphasised the importance of glycerol addition as a cryoprotectant to Tris-based extenders.

2.7.2.3 Semen pH

Bearden & Fuquay, (1980) found that it is important to dilute semen with buffer mediums that accommodate changes in pH, so as to enhance the fertility of the semen (Bearden & Fuquay, 1980; Bester, 2006). For an extender to have a positive effect on the quality of semen it should have the correct pH (6.7 to 7.0) and buffering capacity.

2.7.2.4 Osmotic pressure

Semen and diluents should be isotonic as spermatozoa maintain their maximum metabolic activity when semen is diluted with an isotonic extender (Bearden & Fuquay, 1980). Furthermore, the sperm membrane is semi-permeable and as such hypotonic and hypertonic extenders will inhibit water transfer through the membrane and thus disrupting the integrity of the cell which result to clumping and ultimately the death of the sperm cell (Latif *et al*., 2005; Bearden & Fuquay, 1980). Swanson (1949) observed that bovine sperm are more sensitive to hypertonic solutions of sodium citrate ($Na_3C_6H5O_7$) than to hypotonic solutions.

2.7.2.5 Concentration of spermatozoa

Dilution of semen in a moderate buffered isotonic solution containing fructose will not affect the metabolic rate greatly, but it will extend the potential lifespan of the sperm cell (Bearden & Fuquay, 1980). And such dilutions are usually done prior to lowering the temperature of

the semen sample; however, care must be taken when diluting the semen samples to prevent shock to the sperm (Bearden & Fuquay, 1980). However, the final dilution ratio of semen to the extender used in the AI straws depends on the sperm concentration after semen collection (Karoliina, 2009). Dong et al. (2008) said that semen concentration may affect the semen freezing outcome and freezing in higher concentrations may yield a better post-thaw motility in monkeys.

2.7.3 Cryoprotective agents

A cryoprotectant can be defined as a substance that is used to protect biological tissue from freezing damage. Its use is very important to avoid intracellular ice formation. They are used in the cryopreservation medium to reduce the physical and chemical stresses derived from cooling, freezing and thawing on the spermatozoa (Gao *et al*., 1997; Purdy, 2006). Glycerol and egg york were amongst the first primary extenders to be used for freezing semen (Garner *et al*., 1999 ; Curry, 2000; Holt, 2000; Medeiros *et al*., 2002), and today many extenders use glycerol as the main cryoprotectant.

Ram semen cryoprotectants are usually divided into two categories, namely; non-permeating (milk or egg yolk) and permeating cryoprotectant (glycerol, ethylene glycol and dimethyl sulfoxide). The latter are favoured if the exposure time prior to cooling can be reduced, and they are likely to diffuse rapidly out of the cell thus preventing osmotic injury (Kasai, 1996). Commonly used cryoprotectants in semen cryopreservation include dimethyl sulfoxide (DMSO), ethylene glycol, glycerol, propylene glycol, sucrose or trehalose (Aisen *et al.*, 2005; Li *et al*., 2005; Bucak *et al*., 2007; Soylu *et al*., 2007; Uysal & Bucak, 2007; Forouzanfar *et al*., 2010; Tonieto *et al.*, 2010; Câmara *et al*., 2011; Moustacas *et al*., 2011; Budai *et al.*, 2014; Mata-Campuzano *et al.*, 2015). Various commercial extenders are also available (Marinez-Pastor *et al*., 2009; Akçay *et al*., 2012).

2.7.3.1 The effect of Triladyl®

Triladyl® is a commercial egg-yolk based extender which is mostly used during the cryopreservation of semen of different animal species. The use of Tris-egg yolk cryopreservation diluents is recommended as it is regarded as easy and practical to use (Rahman *et al*., 2008). They further recommend 1.5% egg yolk in the final concentration of the diluent for use without prior removal of seminal plasma. The diluent used by Tuli *et al*. (1991) contained 16.8% egg yolk as a final concentration. That was an indication that not all

Tris-egg yolk diluents are the same and caution should be exercised concerning the components of the diluent. Aboagla & Terada (2004) indicated that the addition of egg yolk to the semen extenders plays a major role during the freezing stage of ram semen cryopreservation and that the addition of trehalose significantly improves its cryoprotective effect.

The effects of Triladyl® extender in semen is favourable for ram (Hegedűšová *et al*., 2012) and bison semen as compared to custom made tris citric acid extenders (Lessard *et al*., 2009; Hussain *et al*., 2011). The use of Triladyl® as reported by Marinez-Pastor *et al*. (2009) indicates that this diluent has been used for cryopreservation of wild ruminants' semen. This report concurs with Asher *et al*. (2000), who reported its use in semen of chital deer collected by electro ejaculation. With bovine semen Carballo (2005) observe sperm motility was higher after thawing when Triladyl® was used.

Rekha *et al*., (2015) recorded higher percentage of spermatozoa with increased motility, viability, functional integrity and normal morphology in Triladyl® extended post-thawed semen compared with tris-fructose-egg-yolk (TFEY) extended semen within each pre-freezing sperm concentration. Even though the basic components of Triladyl® and TFEY extenders are similar (tris based) and delivered an equivalent buffering system during cryopreservation, they resulted in comparable ($P > 0.05$) sperm quality, and that might be as a result of the quantitative differences in the configuration of Triladyl® elements (Rekha *et al*., 2015). Rekha *et al*. (2015) concluded that the optimal concentration of sperm from indigenous ram for cryopreservation is 400 x 10^6/ml, and that regardless of diluents Triladyl® proved to be better compared with tris-fructose-egg-yolk.

2.7.3.2 Effect of Egg yolk and Glycerol

Egg yolk (EY) is a common constituent used in the diluents of semen to protect the spermatozoa against cold shock and gives protection during the freezing and thawing process. It is however, difficult to produce semen diluents of uniform quality, because of individual quality differences inherent to EY (Matsuoka *et al*., 2006). Although EY is sometimes replaced partially by substances with similar activity such as soybean lecithin, it is still likely to remain an important component of diluents used for the freezing of ram semen, particularly due to its protective effect on the plasma membrane (Salamon & Maxwell, 2000). It delays the aging of ram spermatozoa (Watson & Martin, 1976).

Bergeron & Manjunath (2006) reported that since EY and skimmed milk are products of animal origin, they represent a potential risk for contamination of the semen and the composition is not uniform. Cabrera *et al*. (2005) observed certain detrimental effects of egg yolk coagulating enzyme (EYCE) during cryopreservation of Canary buck semen , thus suggested that sperm washing before dilution is important. However, Corteel (1981) considered sperm washing as a complex and time consuming exercise causing a loss of spermatozoa. Furthermore, the washing did not appear to have enhanced the fertility of the spermatozoa in dairy goats (Corteel *et al*., 1975), and fibre producing goats (Ritar & Salamon, 1983). Leboeuf *et al*. (2000; 2003) observed that the 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 ram semen.

Glycerol has been reported by Medeiros *et al*. (2002) and Salamon & Maxwell, (2000) to be the most commonly used cryoprotective agent in diluents for semen cryopreservation It is regarded as a permeating cryoprotectant agent, hence exerting an extracellular effect due to an osmotic stimulation and cell dehydration mechanism thus decreasing the volume of intracellular water available for freezing and an increased survival rate to cryopreserved cells (Holt, 2000). The damage to the spermatozoa results from the selective freezing of free water both inside and outside the cells (Bearden *et al*., 2004). Thus glycerol binds water and decreases the freezing point of the solution and less ice is formed in its presence at any temperature (Bester, 2006).

Glycerol gave effective results in the cryopreservation of ram semen as a cryoprotectant (Sundararaman & Edwin, 2003; Peterson *et al*., 2006). It is however, to a smaller degree toxic to sperm (Holt, 2000) as it may induce osmotic damage (Purdy, 2006). Glycerol is used at a reasonably high concentration which can be damaging to spermatozoon viability at higher temperatures, hence it is added after the semen has been cooled (Fahy, 1986). Berndtson & Foote (1969) observed a decrease in pre-freeze motility of sperm as the level of glycerol in the extender was increased from 0 to 12%. This demonstrated the toxicity of glycerol towards fresh semen. The toxicity of glycerol with increase in concentration in semen also differs between species (Holt, 2000). In the study conducted by Soltanpour and Moghaddam (2013) it was reported that the diluents containing of 7% glycerol and 20% egg-yolk had better sperm protection ability than extender containing 5% glycerol and 5% egg-yolk according to sperm motility, pH and sperm viability. It was also evident in Beran *et al*. (2012) study that

the extenders containing egg yolk [AndroMed (47.4 %), Triladyl (48.4 %), and Bioxcell (41.5 %)] reached higher levels of sperm activity in both sodium citrate and a physiological solution at the beginning of the test (P <0.05-0.01) after determining their effect on traits of sperm activity after thawing.

2.7.4 Freezing method

2.7.4.1 Freezing in pellets

Freezing the semen in pellets is quick and economical, but inventory management is problematic because the actual semen samples cannot be labelled (Evans & Maxwell, 1987) due to its natural state (frosty). With pellets once the semen sample is cooled, the aliquots of 0.1-0.3 ml are dispensed into indentations on a block of dry ice (solid carbon-dioxide, -79 ºC) and frozen for 2-4 min and thereafter the pellets are plunged into liquid nitrogen at -196 ºC (Evans & Maxwell, 1987; Chemineau *et al*., 1991; Abd-Allah *et al*., 2007).

Semen frozen in pellets yielded superior sperm motility following thawing (39%) as compared to semen frozen in straws (Ritar *et al*., 1990). The differences in post–thaw sperm motility, viability and fertility may be attributed to the different cooling rates used in the pellet and straw method (Ritar, 1993).

2.7.4.2 Freezing in straws

Freezing of semen in straws is relatively more expensive and laborious than the pellet technique, but each straw can be labelled for accurate inventory management (Purdy, 2006). This technique is done over liquid nitrogen vapours or in a programmable bio-freezer. Evans & Maxwell (1987) indicated that when using programmable freezers, large quantities of semen straws can be frozen. Purdy, (2006) explained that the freezer replicate pellet freezing by placing the semen straws in a freezer at a temperature of -80 ºC for a period of 7-15 minutes and then plunging the semen straws into liquid nitrogen.

Straws are filled with semen and arranged horizontally 4-5 cm on a rack above the liquid nitrogen vapours for a variable period of time (15-20 minutes) give good post-thawing results (Byrne *et al*., 2000). It was also suggested that straws of 0.5 ml be frozen at 5 cm above the liquid nitrogen, while 0.25 ml straws be placed 15 cm above the liquid for a time of 2 minutes then lowered 4 cm for 3 minutes and thereafter plunged into the liquid nitrogen for storage (Chemineau *et al*., 1991; Leboeuf *et al*., 2000; Abd-Allah *et al*., 2007). Rey (1957) reported

that dropping the semen straws directly into nitrogen result in poor post-thaw motility or to some extend the death of all spermatozoa.

2.7.5 Thawing of cryopreserved semen

There are a few methods or techniques that could be used to thaw frozen semen; rolling of the semen straw between the palms of the hands (Macpherson & Penner, 1972), air thawing (Bean, 1972), thawing in the reproductive tract of a cow (Davidovic *et al*., 1972) and warm water thawing (Aamdal & Anderson, 1968; Robbins *et al*., 1972, 1973; Almquist & Wiggins, 1973). The latter method is used commercially (Rodriguez *et al*., 1975; Foote, 1975, 1999).

Thawing of semen samples is determined by the method used to freeze the semen. For example semen cryopreserved in pellet form should be thawed in a dry test tube at 37 ºC, while the thawing of semen straws may be achieved using various methods (Evans & Maxwell, 1987). Generally, a semen straw is thawed by putting it in a water bath at a temperature of 37 ºC for a period of 12 to 30 seconds (Deka & Rao, 1987; Purdy, 2006). Temperature and timing become much more precarious at temperatures higher than 37 °C, as these high temperatures can result in tremendous sperm mortalities if thawing is performed incorrectly (Tuli *et al*., 1991).

Salamon & Maxwell (2000) reported that in the freeze-thaw procedure, the warming phase (thawing) is just as important to the survival of the spermatozoa as the initial cooling phase during freezing. It would appear that sperm cells that survive a cooling to -196 ºC still face the challenge when warming and thawing and as such they need to transverse the critical temperature zones twice (-15 to -60 ºC). The thawing rate would thus be dependent on whether the cooling rate was sufficiently high to induce intracellular freezing, or low enough to produce cell dehydration. If the cooling rate was high then fast thawing will be required to prevent recrystallization of any intracellular ice present in the sperm cell (Fiser *et al*., 1987). Sperm thawed at a fast rate are also exposed for a short period of time to the concentrated solute and cryoprotectant and the restoration of the intracellular and extracellular equilibrium is more rapid than with slower thawing (Fiser *et al*., 1987). This was emphasized by Hammerstedt *et al.* (1990) who indicated that the rate of semen thawing should be adjusted for different freezing rates so as to ensure optimal survival rates of sperm cells.

Becker *et al*. (1977) reported that post-thaw bovine sperm motility and acrosome

conservation are increased when the semen packaged in straws are thawed rapidly. Furthermore, these rates should be precisely timed to avoid higher internal straw temperatures which may result in pH fluctuation and consequently protein denaturation and cell death (Becker *et al*., 1977). The injury caused by warming up occurs when the sperm cells passes through the temperature region of -5 °C to15 °C (Kumar *et al*., 2003). Water bath temperatures between 4 °C and 75 °C can be used to effectively thaw semen; however, the temperature selected for the water bath depends on the desired rate of thawing (Lemma, 2011). Some semen thawing procedures include the addition of warmed extender to the semen straw to aid the process of thawing, which will also increase the volume and assist to maintain viability of the sperm cells (Lemma, 2011). Thawing extenders may be used for semen stored in pellets, vials or straws and are added as part of the thawing procedure (Lemma, 2011).

Evans & Maxwell (1987) reported that it is a general practice that ram semen is thawed at 38 to 42 ºC for a period of 15-30 seconds, but thawing at higher temperatures (60-75 ºC) may possibly produce similar post-thaw sperm motility, acrosome integrity and fertility of the sperm cell. For bull semen thawing in a 35 °C water bath for at least 30 sec is recommended by most AI societies (Marshall, 1984). These different thawing procedures have implication for conception rate from cryopreserved semen.

2.8 Conception rate with cryopreserved semen

Even though the artificial insemination of sheep has been investigated for many years, much research is still being carried out to find ways of improving the pregnancy rates that can be achieved when using cryopreserved semen (Motamedi-Mojdehi *et al*., 2013). Furthermore, for many indigenous or locally important breeds of sheep, not much is known about the ideal conditions for the dilution, cooling, freezing and thawing of their semen, nor how these affect pregnancy rates. Thomas *et al*. (1998), Saacke *et al*. (2000), Larsson and Rodriguez-Martinez (2000), Muller (2000) and Evenson *et al*. (2002) reported that many tests of sperm motility, morphology, acrosomal status, defective sperm organelles and DNA metabolism have been correlated with fertility. Therefore, sperm quality tests after semen processing and different thawing procedures needs to be verified for semen from indigenous sheep breeds.

Deneke *et al*., (2010) reported that pregnancy rates depends largely upon the individual animal and the quality of semen produced, the minimum standard set for semen quality prior

to acceptance of freezing protocol, the thawing protocol, post-thaw semen quality control, the numbers and reproductive ability of the females used for insemination, the timing of the insemination, the number of inseminations, the number of inseminations per cycle and the number of cycles and inseminations per pregnancy.

Lightfoot and Salamon, (1970) and Salamon and Maxwell, (1995) have shown that the low fertility rates following cervical insemination of frozen thawed semen are ascribed to the damage on the spermatozoa during the freeze thaw process resulting in impaired sperm transport, viability and fertilization capacity and increased embryonic mortality.

Watson (2000) reported that most mammalian species show a reduction in fertility after cervical insemination with frozen semen, which is correlated with the damage that occurs during cryopreservation that reduces motility to 40 to 50%, thereby requiring an increase in the number of sperm needed to achieve adequate fertility. However, Maxwell *et al*. (1999) have indicated that the harmful effects of the freeze thaw process can be defeated by re-suspension of thawed spermatozoa in a diluent containing ram seminal plasma; this would present practical difficulties under field conditions. Conception rates for frozen semen at best can reach values approaching those of natural service but may also result in a complete failure despite that the protocol used had minimal negative effect during freezing of semen (Pickett & Amann, 1993). It is then very difficult to predict the rate of conception with any conviction and it is also very difficult to compare with precision the pregnancy rates obtained in different research work using frozen semen.

Variable results have been reported for conception rates and lambing percentages in sheep following cervical or intra-uterine insemination with frozen-thawed semen. A mean conception rate of 58% has been reported for cervical AI with frozen thawed semen comprising large numbers of ewes under field conditions (Olesen, 1993). D'Allesandro *et al*. (2001) reported lambing percentages between 54.8 and 81.2 % in Leccese dairy ewes. Fukui et al. (2007) obtained conception percentages ranging from 65.5% to 66.7% and lambing percentages from 62.1% to 66.7% after intra-uterine insemination with frozen-thawed semen in Suffolk ewes. Similar results were reported by Pualenz *et al*. (2007) for Norwegian Crossbred ewes employing cervical insemination. Donovan *et al*. (2004) also used cervical insemination in Norwegian and Irish breeds and obtained conception percentages of 36% to 45% with frozen-thawed semen, compared to 66% to 86% with fresh semen. Within *vitro*

fertilization, Silva *et al*. (2011) obtained 58% to 87.5% fertilised ova.

2.9 Artificial insemination (AI)

According to Hiemstra *et al*. (2005) there has been a steady improvement in the practice and approach of AI in several animal species during the past few decades and also regarding the knowledge on the viability of sperm in the female genital tract. However, there are big differences between species in insemination methods and pregnancy rates using either fresh or frozen semen. In cattle and pigs, present AI infrastructure allows easy collection and future use of semen, but only in cattle has the use of frozen semen replaced the use of fresh semen. In pig production, difficulties in using frozen semen (reduced fertility, high freezing, storage and transport cost) are still greater than the advantages of AI (Hiemstra *et al*., 2005).

Molinia *et al*. (1996) discovered that the difference in pregnancy rates between surgical and non-surgical AI was superior with frozen semen than with fresh semen; 70% versus 20% pregnancy with non-surgical AI (with $180x10^6$ sperm) vs. surgical AI (with $10x10^6$ sperm). It is assumed that frozen-thawed sperm are less motile and lack stamina to transverse the highly glutinous cervical mucus, but phagocytosis of the sperm by leukocytes is also considered a cause of the reduced fertility (Hiemstra *et al*., 2005). Advancement in developing a non-surgical technique to reach the oviductal end of the uterine horns as closely as possible would improve the efficiency and ease of use of cryopreserved semen in sheep (Hiemstra *et al*., 2005).

2.10 Semen freezing in indigenous breeds

Munyai (2012) indicated that there were very few studies conducted on the semen quality of indigenous South African breeds, such as the Damara, Namaqua Afrikaner, Pedi and the Zulu and the potential of their gametes to be preserved. In his study Munyai (2012) recorded a low percentage (32%) of live sperm in fresh Namaqua Afrikaner semen collected via EE compared to Damara (58.8%), Pedi (59.3%) and the Zulu (64.3%) sheep. Furthermore, the sperm concentration recorded by Munyai (2012) ranged from 0.9 to $1.3x10^9$ sperm/ml, which was low when compared to other studies (Evans & Maxwell, 1987). Semen volume of the indigenous breeds recorded by Munyai (2012) was lower (0.4 to 0.9 ml) than that reported by Gil *et al*. (2003) who reported an ejaculate volume of 0.75 to 2 ml in mature Polish long-wool sheep rams and 0.5 to 0.7 ml in young yearlings rams.

Through the use of the Computer Assisted Semen Analyser (CASA), Munyai (2012) observed that there were no breed effects on the total motile sperm, although the Namaqua Afrikaner (37.1%) recorded lower motility than Damara and Pedi rams. Pedi (52.7%) rams, however, recorded a significantly higher proportion of progressive motile sperm cells compared to Damara (36.4%), Namaqua Afrikaner (17.4%) and the Zulu sheep (32.6%). Mahoete (2010) reported a lower percentage live sperm in Zulu rams when compared to Pedi and Merino rams.

The Namaqua Afrikaner was not included in the actual semen freezing trials of Munyai (2012). In the report of Mahoete (2010), the post-thaw percentage live sperm ranged from 26.7% to 45.8% as compared to the fresh semen which ranged from 75% to 76.7% for Pedi, Zulu and Merino rams. Of the three breeds, the Pedi had the lowest percentage live sperm post-thaw. No sufficient literature on frozen semen or use of an artificial vagina in Namaqua Afrikaner rams or other indigenous breeds could be found. It is therefore necessary to investigate the semen characteristics of this breed, in order to evaluate the viability of a cryopreservation bank for the Namaqua Afrikaner breed.

3. Materials and methods

3.1 Experimental location

This study was conducted at the Grootfontein Agricultural Development Institute (GADI) near Middelburg (31° 29′ 38″ S, 25° 1′ 2″ 'E) in the Eastern Cape Province, Republic of South Africa (RSA) under kraal conditions. The average annual rainfall is approximately 350 mm of which 246 mm on average occurs in summer, 60 mm in autumn and 19 mm in winter. The rainfall is highest during the period October to March, with peak levels during February and March (Worldweatheronline, 2014; Weatherbase, 2015). Frost occurs from April to September. Mucina & Rutherford (2006) described the veld type as Eastern Upper Karoo.

3.2 Experimental animals

Namaqua Afrikaner, Dohne Merino and Dorper rams were used for semen collection in this study

3.2.1 Namaqua Afrikaner sheep

As already mentioned, the Namaqua Afrikaner is one of the oldest indigenous fat-tailed sheep breeds in South Africa. This herd has been kept at the Carnarvon Experimental Station since 1966. In 1991, part of this herd was transferred to GADI. Since August 1995, the GADI herd has been at the Karakul Experimental Station near Upington (Snyman et al., 1996c).

Figure 3.1. Namaqua Afrikaner rams

3.2.2 Dorper sheep

The Dorper is a mutton breed developed in the 1930s by the Department of Agriculture of South Africa at GADI, by crossing a Dorset Horn ram and Blackhead Persian ewes (Nel, 1993).

Figure 3.2. Dorper rams

Research on the Dorper continued and on 19 July 1950, 28 farmers and 11 officials formed the Dorper Breeders' Society. Currently, there are 600 breeders represented by 10 clubs in the various parts of South Africa and Namibia. According to Marais and Schoeman (1990), the Dorpers grew from 2.6 million in 1963/64 to 6.6 million in 1990. It is currently estimated that the number of Dorpers in South Africa is over 7 million.

3.2.3 Dohne Merino sheep

The Dohne Merino is a synthetic, dual-purpose breed developed by the South African Department of Agriculture using Peppin-style Merino ewes and German Mutton Merino sires. The progeny were interbred and selected for high fertility, rapid lamb growth rate, and fine Merino wool under commercial rangeland conditions (McMaster, 2005).

Figure 3.3. Dohne-Merino rams

The development of this breed was initiated and executed by Mr. J.J.J. Kotze at the Dohne research station in 1951. The breed was named after the Dohne Agricultural Research Station (DARS) situated in Stutterheim in the Eastern Cape (McMaster, 1991). The Dohne Merino is one of the leading woollen breeds in South Africa. Currently there are 31 000 registered ewes on the Dohne Merino Breeders' Society database.

3.2.4 Source of experimental animals

Rams from the above mentioned three breeds were used for this study, i.e. dual purpose (Dohne Merino), meat breed (Dorper) and indigenous breed (Namaqua Afrikaner). September 2013-born rams were used. Twelve Namaqua Afrikaner rams and twelve Dohne Merino rams were obtained from the Carnarvon Namaqua Afrikaner flock and the Grootfontein Dohne Merino stud, respectively. Ten Dorper rams were bought from a local farmer. On their arrival at GADI, both the Namaqua Afrikaner and Dorper rams were put in a quarantine facility for a period of a week and two weeks respectively before being allowed to go into their respective pens in Blikkiesdorp. Blood samples were taken from both groups for Brucella tests and they received the treatments as per the standard Grootfontein biosecurity standard operating procedures. One Dorper ram was stolen before the trial commenced.

3.3 Experimental procedures

The study was conducted between January and August 2015. The GADI veterinarian carried out a genital soundness evaluation before the start of the trial to ensure that all rams were genitally sound and produced semen. The rams were kept in Blikkiesdorp in kraals with adequate shade facilities. Water was provided on an *ad libitum* basis. The rams of each breed were kept in a separate kraal. The rams received the diets as summarised in Table 3.1 from the beginning of January 2015.

The rams were adapted to the pelleted diet as follows: They received lucerne hay *ad libitum* plus 100 g pellets/ram/day for three days. Thereafter the pellets were increased with 100 g/ram/day until the desired amount of 1.5 kg or 3% of body weight was reached. Lucerne hay was decreased to an amount of 500 g/ram/day when the full amount of pellets was reached. Half of the daily feed portion was provided in the morning and half in the afternoon. The composition of the high protein high energy diet is given in Table 3.2.

Table 3.1. Experimental layout

Item	Dorper	Dohne Merino	Namaqua Afrikaner
Diet	High protein diet + lucerne hay	High protein diet + lucerne hay	High protein diet + lucerne hay
Embavit ® (Vitamin A,D,E)	Every 2 months starting January	Every 2 months starting January	Every 2 months starting January
Embamin T.E.® (Co, I, Se, Zn,	Every 2 months starting January	Every 2 months starting January	Every 2 months starting January

Table 3.2. Composition of the high protein high energy pelleted diet

Feedstuff	Percentage
Lucerne	37.5
Maize	45
Cotton seed oil cake meal	16
Feed lime	1
Ammonium chloride	0.5
Nutrient	**Percentage**
Crude protein	16.1
Energy (TDN)	68.5
Crude fibre	13.6
Ca	0.94
P	0.39
Urea	0

Embamin T.E. (Reg.No. V8189 Act.36/1947) were prepared according to the label prescription by mixing 1 ℓ Embamin T.E.™ with 9 ℓ of clean water. Each ram received 1 ml of the diluted Embamin T.E.™ per 4.5 kg body weight orally according to the schedule indicated in Table 3.1.

Embavit (Reg.No. V8189 Act.36/1947) were prepared according to the label prescription by mixing 500 ml Embavit ™ with 2 ℓ of clean water. Each ram received 1 ml of the diluted Embavit ™ per 5.0 kg body weight orally according to the schedule indicated in Table 3.1.

The rams were exercised daily by letting them walk at a fast pace for at least an hour from the beginning of the trial until the middle of January. From then onwards, they were exercised twice a week. The rams were also cared for and handled in accordance with standard protocols and the guidelines of GADI.

3.4 Training of the rams for semen collection using the AV

As mentioned in the objectives of the study, it was envisaged to collect semen samples using the artificial vagina (AV) method. However, during the training period it was discovered that the Namaqua Afrikaner rams refused to use the AV. Different attempts were made to condition them to the AV but none could tempt the Namaqua Afrikaner rams to use the AV. Consequently the electro-ejaculation method (EE) was included in the study. Semen samples from all three breeds were obtained with EE, and from the Dohne Merino and Dorper rams via AV.

The Dorper and Dohne Merino rams were trained to mount a teaser ewe as shown by Figure 3.5 and to ejaculate in the AV as illustrated by Figure 3.4 with a calibrated collecting tube attached to the end. As 21 rams had to be trained, training took place on a daily basis (5 days/week) for a period of 8 weeks before the start of semen collection. A teaser ewe was injected with 17β-Estradiol-17-cypionate ($C_{26}H_{36}O_3$) to induce the oestrus cycle scent 24 hours before training. The ewe was then put on a stanchion with a neck clamp to have it stationed in a way that will appeal to the ram to mount. The ram was then steered to the teaser ewe and as it mounts, its penis was guided away from the teaser ewe and into the AV which was kept warm (34 ºC) by water in between the covering sleeves and the outer covers of the AV (Silvestre *et al*., 2004).

Figure 3.4. Artificial Vagina

Figure 3.5. Ram being trained

All the rams to be used on a specific day were kept in an adjacent pen in view of the other ram that was mounting a restrained ewe as a way of trying to upsurge their libido (Price, 1984; Silvestre *et al*., 2004). Rams were allowed 5 minutes to try to mount and ejaculate. After ejaculation or a period of 5 minutes, depending whichever came first, the ram was taken back into the adjacent pen and kept there for a second attempt after 10 to 15 minutes. The training was considered successful when rams managed to mount and ejaculated into the AV at regular intervals, i.e. 4 successive days when presented to a restrained teaser ewe, even when the ewe was not injected (Silvestre *et al*., 2004).

3.5 Semen collection with the AV

Semen samples were collected twice a week by either AV (Dohne Merino and Dorper) or EE (all breeds). During semen collection with the AV, the method describe above was followed. In trying to relieve stress and maximise the quality and quantity of the ejaculate, the collection procedure was always carried out under the same conditions i.e. same collector, same time of collection, same pen and using the same techniques. Immediately after collection the samples were taken to the laboratory where macroscopic and microscopic assessments were done.

3.6 Semen collection using EE

Semen was collected from all rams with the EE (Figure 3.6) over the trial period. Only six

rams from each group was selected per day for collection. Semen was collected twice in a week by the GADI veterinarian with assistance from the farm workers. The nature of this process was rather intensive as animals needed to be handled with caution and consideration to avoid injuries both on the animal and the worker as well.

Figure 3.6. Electro Ejaculator

The process was done through the insertion of a probe or electrode Figure 3.6 into the rectum of the ram, stimulating the nerves of the reproductive system by gradually increasing the electrical current in a rhythmic pattern for a short period of time (Carter *et al*., 1990). Although this method has been a concern for a number of years with regard to animal welfare (Ortiz-de-Montellano *et al*., 2007), it is still used in animal industry especially with the untrained animals. The ejaculates were collected in a calibrated tube in the same way as with the AV. During the process one worker held the penis of the ram and directed its gland into the warmed tube. There was a need to guard against any physical contamination and to ensure that collections were enough.

3.7 Semen Evaluation

Evaluation was carried out through both macroscopic and microscopic methods. Macroscopic evaluation was done directly after the semen sample was collected. During the evaluation care was taken to guard against cold shock as it can be detrimental to the sperm cells. The glassware for collection and handling was well cleaned, sterilised, dried and warmed (minimum 34 °C) as illustrated by Figure 3.7.

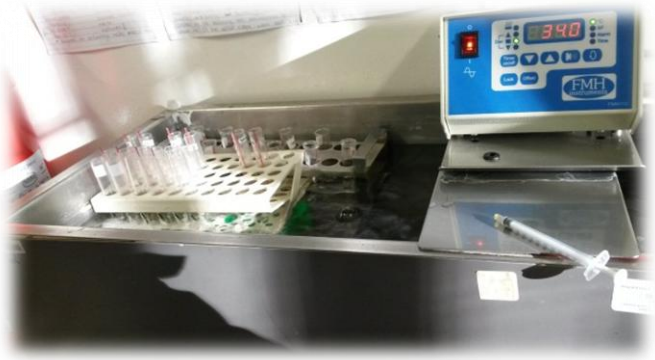

Figure 3.7. Water-bath with min Temp of 34 degree Celsius

Each semen sample was assessed for volume, sperm concentration (density), colour, motility and morphology. Directly after collection, the semen volume was recorded while still in the calibrated collecting tube. The sample was then dispensed into a glass tube which was then immersed into a water-bath at 34 °C through the use of tube holders.

3.7.1 Volume

Directly after collection, the semen volume was recorded while still in the calibrated collecting tube. Figure 3.8 illustrate the calibrated semen in a collecting tube.

Figure 3.8. Calibrated collecting tube

3.7.2 Colour and semen density

The colour and density of the fresh semen sample was assessed and classified according to the scale summarised in Table 3.3.

Table 3.3. Assessment of sperm concentration according to colour of the semen sample

Ejaculate colour	Number of spermatozoa ($x10^9$) per ml
Thick creamy	5.0 (4-6 x 10^9)
Creamy	4.0 (3.5-4.5 x 10^9)
Thin creamy	3.0 (2.5-3.5 x 10^9)
Milky	2.0 (1-2.5 x 10^9)
Thin milk	0.7 (0.4-1.0 x 10^9)
Watery	Insignificant

Source: Hafez & Hafez (2000); Evans & Maxwell (1987)

3.7.3 Sperm concentration

The sperm concentration was determined physically through the use of a haemocytometer (WHO, 2010). This method is regarded as the simplest, most convenient and cheapest means of accurately determining the number of cells in a sample/suspension. Before assessment for concentration the counting chamber, which is mirror-like, and cover-slip were all well cleaned with lens paper. Figure 3.9 shows the instrument haemocytometer.

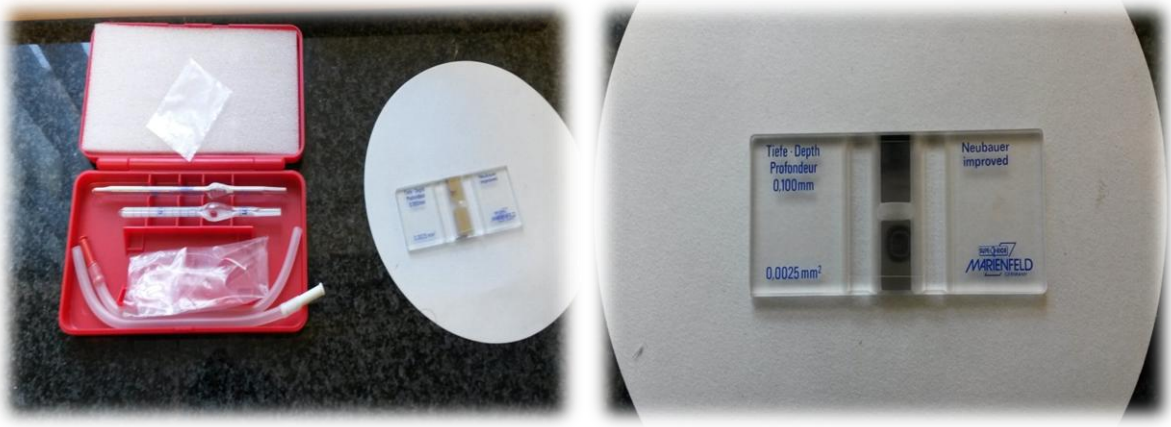

Figure 3.9. Haemocytometer

The spermatozoa were killed through dilution of the subsample with tap water in order to count 100 to 400 sperm cells within the chosen 5 squares. A dilution rate of 9.9 ml tap water and 0.1 ml fresh semen (1:100 dilution factors) was used. Figure 3.10 illustrates the dilution of semen sub-sample.

Figure 3.10. Dilution of sub-sample

After dilution, 15 µl of the sample was pipetted under the cover slip of each side of the haemocytometer and placed carefully on a horizontal plate to allow the semen to set for a maximum period of 2 to 3 minutes to avoid them floating. It was then placed under the microscope with an x40 objective lens. Sperm cells were counted in 5 of the 25 chosen squares on both sides and the averages were then taken. Care was taken not to overload the two chambers, as that would have given an inaccurate count of the cells. A manual counter clicker was used during counting. The means from the total 5 squares in both chambers were then recorded as a final count. The following equation was used to calculate the cell concentration of the total count of the 5 counted squares:

$$\textbf{Total cells/ml} = \textbf{Total cells counted} \times \frac{\textbf{Dilution factor}}{\textbf{Number of Squares}} \times \textbf{10 000 cells/ml.}$$

3.7.4 Sperm motility

Assessment of sperm motility was done subjectively as good wave motion can be seen in the collection glass with the naked eye. A droplet of fresh semen was evaluated for motility under a microscope as displayed by Figure 3.11, while Table 3.4 summarised the scales used.

Figure 3.11. Microscope

Table 3.4. Scale of assessment of semen motility

Score	Class	Description
5	Very good	Dense, very rapidly moving waves; 90% or more of the spermatozoa are active
4	Good	Vigorous wave movement but not as rapid as for score 5; 70-85% of sperm cells are active
3	Fair	Only small, slow moving waves; 45-65% of sperm cells are active
2	Poor	No waves are formed but some movement of sperm is visible; 20-40% of sperm cells are alive but with poor motility
1	Very poor	Only about 10% of spermatozoa show signs of life. Only weak movement
0	Dead	All sperm cells are motionless

Source: Hafez & Hafez (2000)

3.7.5 Semen smears

For fresh semen, smears were prepared immediately after semen collection. Eosin-Nigrosin solution was used to prepare the smears for determination of percentage live and dead sperm cells. The smears were labelled on the frosted part of the microscope slide with the identity number of the ram, date and time of collection of the semen sample. The counting was done through the use of a microscope. A drop of emersion oil was placed on the surface of a smeared slide then the oil gently smeared to cover the area. The slide was then placed under the microscope at x40 magnification. The counting on the slide was done from the top, middle and the bottom and the average of the three sections was taken as the total count.

Figure 12 shows how the counting on the slide was done.

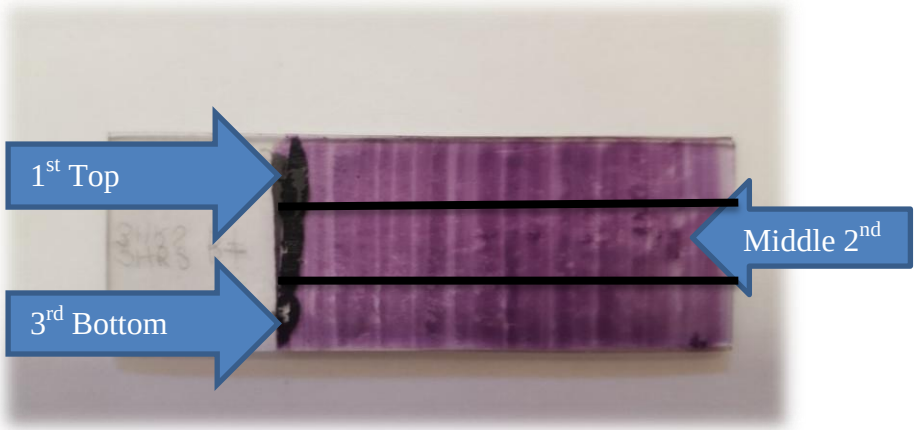

Figure 3.12. Eosin-Nigrosin Smears

3.8 Dilution of semen sample

After all the above evaluations were done, the semen sample was diluted with Triladyl® which was prepared according to the ratios on the label prescription. An amount of 60 ml clean fresh water was added to 20 g of Triladyl® and then to 20 g of fresh whipped egg yolk. The diluent was used to dilute the volume of semen obtained at the ratio of one part of semen to three parts of diluent (1:3) then mixed gently at 34 °C. A droplet of the diluted semen sample was evaluated for motility under a microscope, using the scale as summarised in Table 3.4. Eosin-Nigrosin smears were also prepared from the diluted semen samples for determination of percentage live and dead sperm cells.

3.9 Freezing of the semen

Only semen samples of 0.8 ml to 2.0 ml and had a motility of 3 or above were used for further processing for freezing, while those samples that measured less than 0.8 ml or had a below 3 motility were discarded.

The diluted samples were packed into 0.25 ml straws and sealed with polyvinyl chloride powder (PVCP) and cooled for 3 to 4 hours at a temperature of 5 °C. Figure 3.13 illustrates how the straw where placed on the rack and just before they were plunged into the goblets.

Figure 3.13. Straws on rack before plunging into goblets

After cooling, the straws were placed horizontally on a rack about 5 cm above the surface of liquid nitrogen (LN_2) in a Styrofoam cooler-box for about 15 min. The straws were then plunged into goblets that were immersed into liquid nitrogen in a basin and into the canisters and immersed into the liquid nitrogen tank at -196 °C for storage.

A total of 600 straws were frozen by the end of the trial, totalling 240 AV straws and 360 EE straws.

3.10 Evaluation of frozen semen

Evaluation of the frozen-thawed semen was done at three different time intervals; one week, one month and then after three months. Straws were drawn out of the liquid nitrogen and plunged into a basin of warm water (minimum 34 °C) for 15 seconds to thaw. The straws were then wiped dry with a paper towel. The ends of the straws were then cut-off using a pair of scissors to allow the contents to drip into a test tube that was placed on a test-tube rack in the warm water-bath.

A droplet (0.5 ml) from each thawed semen sample was assessed for post-thaw motility, using the scale as summarised in Table 3.4. Percentage live and dead sperm were also assessed under the microscope using a droplet of the frozen-thawed semen sample. Furthermore, Eosin-Nigrosin smears were also prepared from the frozen-thawed semen samples for determination of percentage live and dead sperm cells.

3.11 Statistical analysis

Statistical analyses of the data were performed using linear (GLM; SAS, 2009) and categorical (CHI- SQUARE; Preacher (2001)) procedures.

The following linear model was fitted for sperm motility, semen volume, sperm concentration and percentage live sperm:

$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$

Where

Y_{ijk} = trait of the k^{th} animal of the j^{th} collection method of the i^{th} breed,

μ = overall mean,

α_i = fixed effect of the i^{th} breed,

β_j = fixed effect of the j^{th} collection method used,

$(\alpha\beta)_{ij}$ = Interaction between fixed effect of the i^{th} breed and j^{th} collection method used,

e_{ijk} = random error with zero mean and variance $I\sigma^2_e$.

For the interactions, only the Dorper and Dohne Merino data were included.

Chi-square procedures, incorporating Yates' correction for continuity, (Preacher (2001) were used to obtain frequency distribution tables for semen density and semen colour for the different breeds as well as collection methods.

4. Results

4.1 Effect of collecting semen via electro-ejaculation on semen traits of different breeds before and after freezing of the semen

The effect of breed on semen traits measured and assessed on fresh and diluted semen collected via electro-ejaculation is summarised in Table 4.1

Table 4.1. The effect of breed on semen traits (± s.e.) measured and assessed on fresh and diluted semen collected via electro-ejaculation

Traits	Dohne Merino	Dorper	Namaqua
Volume (ml)	1.20^{ab} ± 0.08	1.37^{a} ± 0.08	1.09^{b} ± 0.08
Sperm concentration ($x10^9$ sperm/ml)	1.14 ± 5.20	1.10 ± 5.29	1.22 ± 5.20
Motility of fresh sample	4.85 ± 0.07	4.81 ± 0.07	4.70 ± 0.07
Motility after dilution with Triladyl®	4.13 ± 0.10	4.24 ± 0.10	4.15 ± 0.10
% Live sperm fresh sample	68.59 ± 1.94	72.82 ± 1.98	67.76 ± 1.94
% Live sperm after dilution with Triladyl®	63.41 ± 2.11	64.92 ± 2.15	63.41 ± 2.11

[a,b] Values with different superscripts differ significantly between breeds (P <0.05).

The Dorper rams produced a higher semen volume (P <0.05) compared to the Namaqua Afrikaner rams (1.37 ± 0.08 ml vs 1.09 ± 0.08 ml). The mean sperm concentration of the fresh semen did not differ significantly (P >0.05) among the breeds. The sperm motility of the fresh semen (before and after dilution) also did not differ (P >0.05) among the breeds. Nor did the percentage live sperm (before and after dilution) differ among the breeds (P >0.05).

The distribution of the three breeds over the different semen density categories is summarised in Table 4.2 for semen samples collected via electro-ejaculation.

Table 4.2. Distribution of semen samples collected via electro-ejaculation of the three breeds over the different semen density categories

Breed	Watery	Milky	Thin cream	Creamy	Thick cream
Dohne Merino	0	9	12	7	2
Dorper	1	9	11	4	4
Namaqua	0	6	6	10	8

Chisq = 11.412; DF = 8; P-value = 0.179

Yates' chi-sq = 6.5; Yates' P-value = 0.591

Semen samples of the Dohne Merino and Dorper rams collected via the EE were mostly categorised as Thin Cream. The majority of the Namaqua Afrikaner semen samples collected via the EE were categorised as Creamy.

The distribution of the three breeds over the different semen colour categories is summarised in Table 4.3 for semen samples collected via electro-ejaculation. For all three breeds, the colour of the majority of the semen samples was categorised as Creamy.

Table 4.3. Distribution of semen samples collected via electro-ejaculation of the three breeds over the different semen colour categories

Breed	White	Creamy
Dohne Merino	8	22
Dorper	5	24
Namaqua	5	25

Chisq = 1.167; DF = 2; P-value = 0.558

Yates' chi-sq = 0.519; Yates' P-value = 0.771

The effect of breed on motility of frozen-thawed semen samples, subjectively evaluated under a microscope, from semen collected via electro-ejaculation is summarised in Table 4.4. Frozen-thawed semen from Dorper rams displayed a higher motility (P <0.05) than that of the Namaqua Afrikaner rams (1.55 ± 0.14 vs 1.03 ± 0.14) when evaluated one month after freezing. There were no significant differences observed (P >0.05) in sperm motility among the breeds during the other evaluation periods.

Table 4.4. The effect of breed on motility (± s.e.) of frozen-thawed semen samples, subjectively evaluated under a microscope, from semen collected via electro-ejaculation

Traits	Dohne Merino	Dorper	Namaqua
Motility 1W(0)	1.53 ± 0.15	1.93 ± 0.15	1.58 ± 0.15
Motility 1W(3)	0.80 ± 0.09	0.81 ± 0.09	0.88 ± 0.09
Motility 1M(0)	1.32^{ab} ± 0.14	1.55^{a} ± 0.14	1.03^{b} ± 0.14
Motility 1M(3)	0.78 ± 0.10	1.03 ± 0.10	0.78 ± 0.10
Motility 3M(0)	1.53 ± 0.15	1.57 ± 0.15	1.37 ± 0.15
Motility 3M(3)	0.73 ± 0.93	0.79 ± 0.94	0.68 ± 0.93

[a,b] Values with different superscripts differ significantly between breeds (P <0.05).

1W(0) = Evaluated 1 week, directly after thawing; 1W(3) = Evaluated 1 week, 3 hours after thawing; 1M(0) = Evaluated 1 month, directly after thawing; 1M(3) = Evaluated 1 month, 3 hours after thawing; 3M(0) = Evaluated 3 months, directly after thawing; 3M(3) = Evaluated 3 months, 3 hours after thawing

The effect of breed on percentage of live sperm of frozen-thawed semen collected via electro-ejaculation is presented in Table 4.5. Percentage live sperm was determined under a microscope using a droplet of frozen-thawed semen.

Table 4.5. The effect of breed on percentage of live sperm (± s.e.) of frozen-thawed semen collected via electro-ejaculation (microscope; using a droplet of frozen-thawed semen)

Traits	Dohne Merino	Dorper	Namaqua
% Live 1W(0)	17.86 ± 2.03	22.72 ± 2.07	17.76 ± 2.03
% Live 1W(3)	10.03 ± 1.61	12.57 ± 1.64	11.10 ± 1.61
% Live 1M(0)	15.8 ± 1.72	20.17 ± 1.75	16.57 ± 1.72
% Live 1M(3)	9.33^{a} ± 1.59	13.97^{b} ± 1.61	10.67^{a} ± 1.58
% Live 3M(0)	22.20 ± 2.01	23.41 ± 2.05	20.36 ± 2.01
% Live 3M(3)	12.73 ± 1.89	14.86 ± 1.92	13.63 ± 1.89

[a,b,] Values with different superscripts differ significantly between breeds (P <0.05).

1W(0) = Evaluated 1 week, directly after thawing; 1W(3) = Evaluated 1 week, 3 hours after thawing; 1M(0) = Evaluated 1 month, directly after thawing; 1M(3) = Evaluated 1 month, 3 hours after thawing; 3M(0) = Evaluated 3 months, directly after thawing; 3M(3) = Evaluated 3 months, 3 hours after thawing

The mean sperm survival rate (percentage live sperm) of the Dorper rams (13.97 ± 1.61 %) post-thawing was significantly higher (P <0.05) than in the Dohne Merino and Namaqua Afrikaner rams (9.33 ± 1.59 and 10.67 ± 1.58 % respectively) when evaluated one month after freezing and three hours after thawing. There were no observed significant differences (P >0.05) during the other evaluation stages.

The effect of breed on percentage of live sperm of frozen-thawed semen collected via electro-ejaculation is presented in Table 4.6. Percentage live sperm was determined under a microscope from semen smears.

Table 4.6. The effect of breed on percentage of live sperm (± s.e.) of frozen-thawed semen collected via electro-ejaculation (microscope; counted from semen smears)

Traits	Dohne Merino	Dorper	Namaqua
% Live 1W(0)	26.92^{ab} ± 2.64	29.84^{a} ± 2.68	20.19^{b} ± 2.64
% Live 1W(3)	24.07^{ab} ± 2.17	27.78^{a} ± 2.21	21.12^{b} ± 2.17
% Live 1M(0)	24.56^{a} ± 2.70	33.97^{b} ± 2.75	19.47^{a} ± 2.70
% Live 1M(3)	23.13^{a} ± 2.23	30.13^{b} ± 2.27	17.79^{a} ± 2.23
% Live 3M(0)	28.96^{a} ± 2.71	28.96^{a} ± 2.76	20.58^{b} ± 2.71
% Live 3M(3)	23.75^{a} ± 2.44	25.52^{a} ± 2.48	15.41^{b} ± 2.44

^{a,b} Values with different superscripts differ significantly between breeds (P <0.05).
1W(0) = Evaluated 1 week, directly after thawing; 1W(3) = Evaluated 1 week, 3 hours after thawing; 1M(0) = Evaluated 1 month, directly after thawing; 1M(3) = Evaluated 1 month, 3 hours after thawing; 3M(0) = Evaluated 3 months, directly after thawing; 3M(3) = Evaluated 3 months, 3 hours after thawing.

Dorper rams had a higher (P <0.05) percentage live sperm of frozen-thawed semen than Namaqua Afrikaner rams after a week of evaluation, both directly and after three hours of evaluation. At the one month evaluation, a higher percentage live sperm (P <0.05) was observed in the Dorper compared to both the Dohne Merino and Namaqua Afrikaner rams. At the three-month evaluation, both the Dorper and Dohne Merino rams had a significantly higher percentage live sperm (P <0.05) that the Namaqua Afrikaner rams.

The trends in percentage live sperm from the fresh semen stage until three months after freezing are depicted in Figure 4.1 for the three breeds for semen collected via electro-ejaculation.

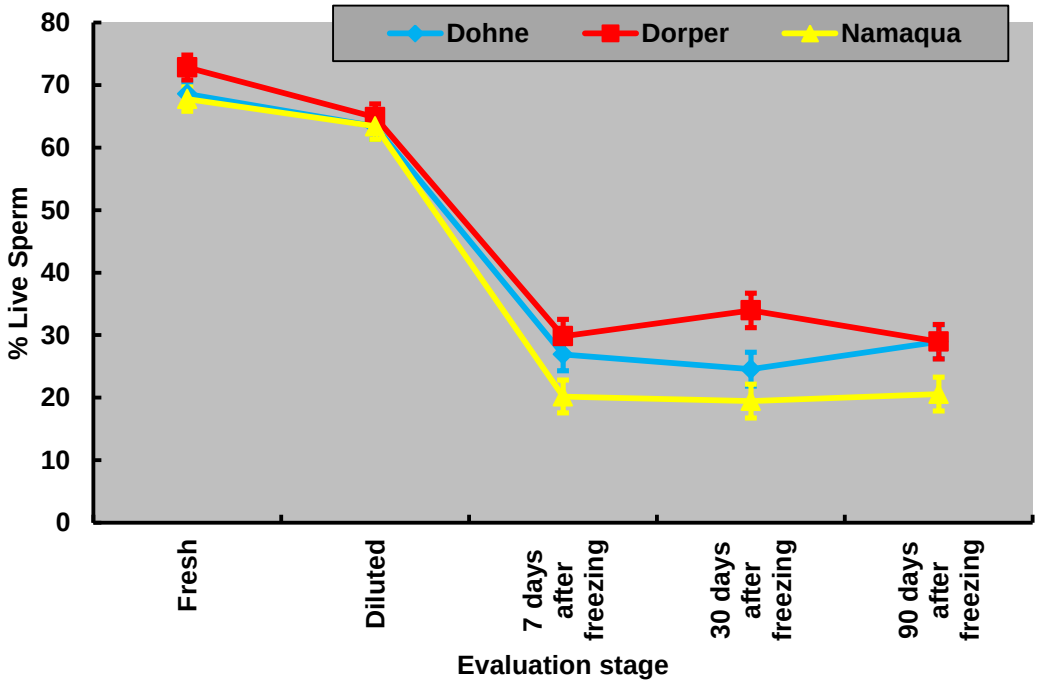

Figure 4.1. Percentage live sperm at different stages for the three breeds for semen collected with electro-ejaculation

From Figure 4.1 it can be seen that percentage live sperm of all breeds dropped considerably after freezing. It remained constant for all breeds from one week until 3 months after freezing, indicating that length of storage should not have an effect on percentage live sperm.

4.2 Effect of collection of semen with an artificial vagina on semen traits of Dohne Merino and Dorper rams before and after freezing of the semen

The effect of breed on semen traits measured and assessed on fresh and diluted semen collected with an artificial vagina is summarised in Table 4.7.

Table 4.7. The effect of breed on semen traits (± s.e.) measured and assessed on fresh and diluted semen collected with an artificial vagina

Traits	Dohne Merino	Dorper
Volume (ml)	1.46 [a] ± 0.08	1.22[b] ± 0.08
Concentration (x10^9 sperm/ml)	1.21 ± 5.01	1.19 ± 5.40
Motility of fresh sample	4.74 ± 0.07	4.78 ± 0.08
Motility after dilution with Triladyl®	4.03 ± 0.09	4.04 ± 0.10
% Live sperm fresh sample	67.07 ± 2.19	64.38 ± 2.32
% Live sperm after dilution with Triladyl®	66.29 ± 2.18	68.37 ± 2.30

[a,b] Values with different superscripts differ significantly between breeds (P <0.05).

The Dohne Merino rams produced a higher semen volume (P <0.05) compared to the Dorper rams (1.46 ± 0.08 vs 1.22 ± 0.08 ml). The mean concentration of the fresh semen did not differ significantly (P >0.05) between the breeds. Similarly, sperm motility and percentage live sperm of the fresh semen (before and after dilution) also did not differ (P >0.05) between these breeds.

The distribution of the three breeds over the different semen density categories is summarised in Table 4.8 for semen samples collected with an artificial vagina.

Table 4.8. Distribution of semen samples collected via artificial vagina of the three breeds over the different semen density categories

Breed	Watery	Milky	Thin cream	Creamy	Thick cream
Dohne Merino	0	2	12	11	4
Dorper	0	1	10	11	3

Chisq = 0.364; DF = 4; P-value = 0.985

Yates' chi-sq = 0.091; Yates' P-value = 0.999

Breed differences occurred in density of semen samples. Density of the semen samples of the Dohne Merino rams collected via an AV were mostly categorised as Thin Cream, followed by Creamy. In the Dorper rams, samples were mostly categorised as Creamy, followed by Thin cream.

The distribution of the three breeds over the different semen colour categories is summarised in Table 4.9 for semen samples collected with an artificial vagina. Colour of all semen samples of both the Dohne Merino and Dorper rams that were collected via the AV were categorised as Creamy.

Table 4.9. Distribution of semen samples collected via artificial vagina of the three breeds over the different semen colour categories

Breed	White	Creamy
Dohne Merino	0	29
Dorper	0	25

Chisq = 0; DF = 1; P-value = 1

Yates' chi-sq = 0.019; Yates' P-value = 0.890

The effect of breed on motility of frozen-thawed semen, subjectively evaluated under a microscope, collected with an artificial vagina is summarised in Table 4.10. No significant differences were observed between the Dorper and Dohne Merino rams in motility of frozen-thawed semen collected with an AV.

Table 4.10. The effect of breed on motility (± s.e.) of frozen-thawed semen, subjectively evaluated under a microscope, collected with an artificial vagina

Traits	Dohne Merino	Dorper
Motility 1W(0)	2.09 ± 0.16	2.02 ± 0.17
Motility 1W(3)	1.22 ± 0.12	1.22 ± 0.13
Motility 1M(0)	2.33 ± 0.14	1.94 ± 0.15
Motility 1M(3)	1.40 ± 0.10	1.26 ± 0.11
Motility 3M(0)	2.43 ± 0.15	2.22 ± 0.16
Motility 3M(3)	1.21 ± 0.11	1.26 ± 0.11

1W(0) = Evaluated 1 week, directly after thawing; 1W(3) = Evaluated 1 week, 3 hours after thawing; 1M(0) = Evaluated 1 month, directly after thawing; 1M(3) = Evaluated 1 month, 3 hours after thawing; 3M(0) = Evaluated 3 months, directly after thawing; 3M(3) = Evaluated 3 months, 3 hours after thawing.

The effect of breed on percentage of live sperm of frozen-thawed semen collected with an artificial vagina is presented in Table 4.11. Percentage live sperm was determined under a microscope using a droplet of frozen-thawed semen. No significant differences were observed between the breeds in percentage of live sperm in frozen-thawed semen collected with an AV.

Table 4.11. The effect of breed on percentage of live sperm (± s.e.) of frozen-thawed semen collected with an artificial vagina (microscope; using a droplet of frozen-thawed semen)

Traits	Dohne Merino	Dorper
% Live 1W(0)	26.86 ± 2.34	30.60 ± 2.52
% Live 1W(3)	18.90 ± 1.87	23.24 ± 2.01
% Live 1M(0)	27.38 ± 1.90	23.44 ± 2.01
% Live 1M(3)	18.00 ± 1.62	16.00 ± 1.75
% Live 3M(0)	26.45 ±1.85	25.08 ± 2.00
% Live 3M(3)	16.07 ± 1.58	16.00 ± 1.70

1W(0) = Evaluated 1 week, directly after thawing; 1W(3) = Evaluated 1 week, 3 hours after thawing; 1M(0) = Evaluated 1 month, directly after thawing; 1M(3) = Evaluated 1 month, 3 hours after thawing; 3M(0) = Evaluated 3 months, directly after thawing; 3M(3) = Evaluated 3 months, 3 hours after thawing.

The effect of breed on percentage of live sperm of frozen-thawed semen collected with an artificial vagina is presented in Table 4.12. Percentage live sperm was determined under a microscope from semen smears. Again, no significant differences were observed between the breeds.

Table 4.12. The effect of breed on percentage of live sperm (± s.e.) of frozen-thawed semen collected with an artificial vagina (microscope; counted from semen smears)

Traits	Dohne Merino	Dorper
% Live 1W(0)	37.04 ± 2.46	35.49 ± 2.60
% Live 1W(3)	33.50 ± 1.92	29.56 ± 2.03
% Live 1M(0)	37.74 ± 2.54	39.03 ± 2.69
% Live 1M(3)	34.94 ± 2.18	36.87 ± 2.31
% Live 3M(0)	39.49 ± 2.62	38.53 ± 2.77
% Live 3M(3)	34.45 ± 2.35	34.18 ± 2.49

1W(0) = Evaluated 1 week, directly after thawing; 1W(3) = Evaluated 1 week, 3 hours after thawing; 1M(0) = Evaluated 1 month, directly after thawing; 1M(3) = Evaluated 1 month, 3 hours after thawing; 3M(0) = Evaluated 3 months, directly after thawing; 3M(3) = Evaluated 3 months, 3 hours after thawing.

The trends in percentage live sperm from the fresh semen stage until three months after freezing are depicted in Figure 4.2 for the Dohne Merino and Dorper breeds for semen collected with an AV.

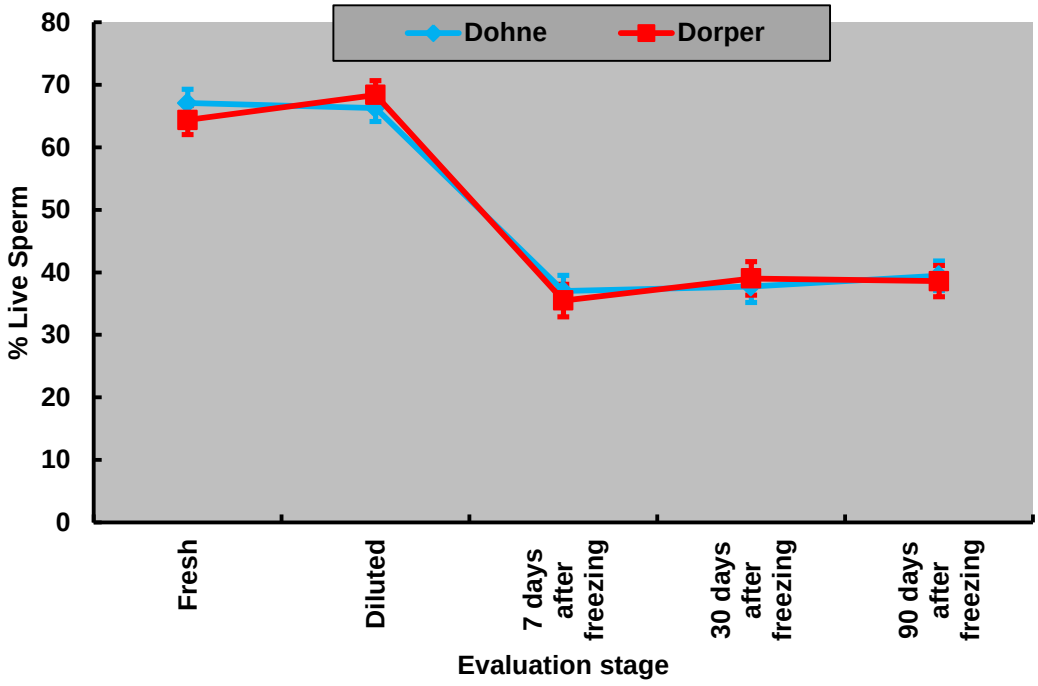

Figure 4.2. Percentage live sperm at different stages for the two breeds for semen collected with an AV

From Figure 4.2 it is evident that there were no differences between the two breeds. The percentage live sperm of both breeds dropped considerably after freezing, after which it remained constant for the remainder of the evaluation period.

4.3 Comparison of semen traits before and after freezing of semen collected with the electro-ejaculation and artificial vagina methods

The effect of collection method on semen traits measured and assessed on fresh and diluted semen is summarised in Table 4.13.

Table 4.13. The effect of collection method on semen traits (± s.e.) measured and assessed on fresh and diluted semen

Traits	Artificial Vagina	Electro-Ejaculation
Volume (ml)	1.34 ± 0.06	1.28 ± 0.52
Concentration ($x10^9$ sperm/ml)	1.20 ± 3.68	1.11 ± 3.51
Motility of fresh sample	4.76 ± 0.05	4.83 ± 0.04
Motility after dilution with Triladyl®	4.04 ± 0.07	4.19 ± 0.6
% Live sperm fresh sample	65.73^a ± 1.60	70.70^b ± 1.51
% Live sperm after dilution with Triladyl®	67.33 ± 1.58	64.17 ± 1.50

[a,b] Values with different superscripts differ significantly between methods (P <0.05).

Percentage live sperm of fresh semen collected with an AV (65.73 ± 1.60 %) was significantly lower (P <0.05), than semen collected via EE (70.70 ± 1.51 %). No differences between collection methods were observed in any of the other traits.

The distribution of semen samples collected with either the EE or AV methods over the different semen density categories is summarised in Table 4.14.

Table 4.14. Distribution semen samples from Dorper and Dohne Merino rams of the two collection methods over the different semen density categories

Method	Watery	Milky	Thin cream	Creamy	Thick cream
AV	0	3	22	22	7
EE	1	18	23	11	6

Chisq = 15.289; DF = 4; P-value = 0.004

Yates' chi-sq = 12.167; Yates' P-value = 0.016

Collection method had a significant effect on density of fresh semen samples. Density of the semen samples collected via an AV were mostly categorised as Thin Cream and Creamy. The majority of semen samples collected via EE were also categorised as Thin Cream, but more of the EE samples were categorised as Milky than those collected with an AV. The distribution of the three breeds over the different semen colour categories is summarised in Table 4.15 for semen samples collected with an artificial vagina. Colour of all semen samples collected via the AV were categorised as Creamy, while 20% of the samples collected via EE was categorised as White.

Table 4.15. Distribution of semen samples from Dorper and Dohne Merino rams of the two collection methods over the different semen colour categories

Method	White	Creamy
AV	0	54
EE	13	46

Chisq = 13.445; DF = 1; P-value = 0.000

Yates' chi-sq = 11.368; Yates' P-value = 0.001

The effect of collection method on motility of frozen-thawed semen, subjectively evaluated under a microscope, is summarised in Table 4.16. Motility of frozen-thawed semen collected with an AV was higher (P <0.05) than the corresponding values of semen collected via EE at one week, one month and three months after thawing.

Table 4.16. The effect of collection method on motility of frozen-thawed semen, subjectively evaluated under a microscope of Dorper and Dohne Merino rams

Traits	Artificial Vagina	Electro-ejaculation
Motility 1W(0)	$2.05^{a} \pm 0.11$	$1.73^{b} \pm 0.11$
Motility 1W(3)	$1.22^{a} \pm 0.09$	$0.81^{b} \pm 0.08$
Motility 1M(0)	$2.13^{a} \pm 0.11$	$1.43^{b} \pm 0.10$
Motility 1M(3)	$1.33^{a} \pm 0.08$	$0.91^{b} \pm 0.07$
Motility 3M(0)	$2.33^{a} \pm 0.11$	$1.55^{b} \pm 0.10$
Motility 3M(3)	$1.23^{a} \pm 0.08$	$0.76^{b} \pm 0.07$

[a,b] Values with different superscripts differ significantly between methods (P <0.05).

1W(0) = Evaluated 1 week, directly after thawing; 1W(3) = Evaluated 1 week, 3 hours after thawing; 1M(0) = Evaluated 1 month, directly after thawing; 1M(3) = Evaluated 1 month, 3 hours after thawing; 3M(0) = Evaluated 3 months, directly after thawing; 3M(3) = Evaluated 3 months, 3 hours after thawing.

The effect of collection method on the percentage live sperm of frozen-thawed semen is summarised in Table 4.17. Percentage live sperm was determined under a microscope using a droplet of frozen-thawed semen.

Table 4.17. The effect of collection method on the percentage live sperm of frozen-thawed semen (microscope; using a droplet of frozen-thawed semen) Dorper and Dohne Merino rams

Traits	Artificial Vagina	Electro-ejaculation
% Live 1W(0)	$28.73^{a} \pm 1.72$	$20.30^{b} \pm 1.64$
% Live 1W(3)	$21.07^{a} \pm 1.37$	$11.31^{b} \pm 1.31$
% Live 1M(0)	$25.41^{a} \pm 1.37$	$18.02^{b} \pm 1.31$
% Live 1M(3)	$17.00^{a} \pm 1.19$	$11.65^{b} \pm 1.13$
% Live 3M(0)	25.76 ± 1.36	22.81 ± 1.30
% Live 3M(3)	16.03 ± 1.16	13.80 ± 1.11

[a,b] Values with different superscripts differ significantly between methods (P <0.05).

1W(0) = Evaluated 1 week, directly after thawing; 1W(3) = Evaluated 1 week, 3 hours after thawing; 1M(0) = Evaluated 1 month, directly after thawing; 1M(3) = Evaluated 1 month, 3 hours after thawing; 3M(0) = Evaluated 3 months, directly after thawing; 3M(3) = Evaluated 3 months, 3 hours after thawing.

The percentage live sperm for frozen-thawed semen collected with an AV were higher when evaluated one week and one month after freezing. Three months after freezing there were no observed significant differences (P >0.05) between all these breeds.

The effect of collection method on the percentage live sperm of frozen-thawed semen is summarised in Table 4.18. Percentage live sperm was determined under a microscope from semen smears. Semen collected with an AV yielded frozen-thawed sampled with higher percentage live sperm (P <0.05) at all three post-freezing evaluation dates than semen from EE.

Table 4.18. The effect of collection method on the percentage live sperm of frozen-thawed semen (microscope; counted from semen smears) Dorper and Dohne Merino rams

Traits	Artificial Vagina	Electro-ejaculation
% Live 1W(0)	$36.26^{a} \pm 1.79$	$28.38^{b} \pm 1.79$
% Live 1W(3)	$31.53^{a} \pm 1.40$	$25.92^{b} \pm 1.32$
% Live 1M(0)	$38.38^{a} \pm 1.85$	$29.27^{b} \pm 1.75$
% Live 1M(3)	$35.91^{a} \pm 1.59$	$26.63^{b} \pm 1.50$
% Live 3M(0)	$39.01^{a} \pm 1.90$	$28.96^{b} \pm 1.80$
% Live 3M(3)	$34.31^{a} \pm 1.71$	$24.63^{b} \pm 1.61$

[a,b] Values with different superscripts differ significantly between methods (P <0.05).

1W(0) = Evaluated 1 week, directly after thawing; 1W(3) = Evaluated 1 week, 3 hours after thawing; 1M(0) = Evaluated 1 month, directly after thawing; 1M(3) = Evaluated 1 month, 3 hours after thawing; 3M(0) = Evaluated 3 months, directly after thawing; 3M(3) = Evaluated 3 months, 3 hours after thawing.

The trends in percentage live sperm from the fresh semen stage until three months after freezing are depicted in Figure 4.3 for the two collection methods.

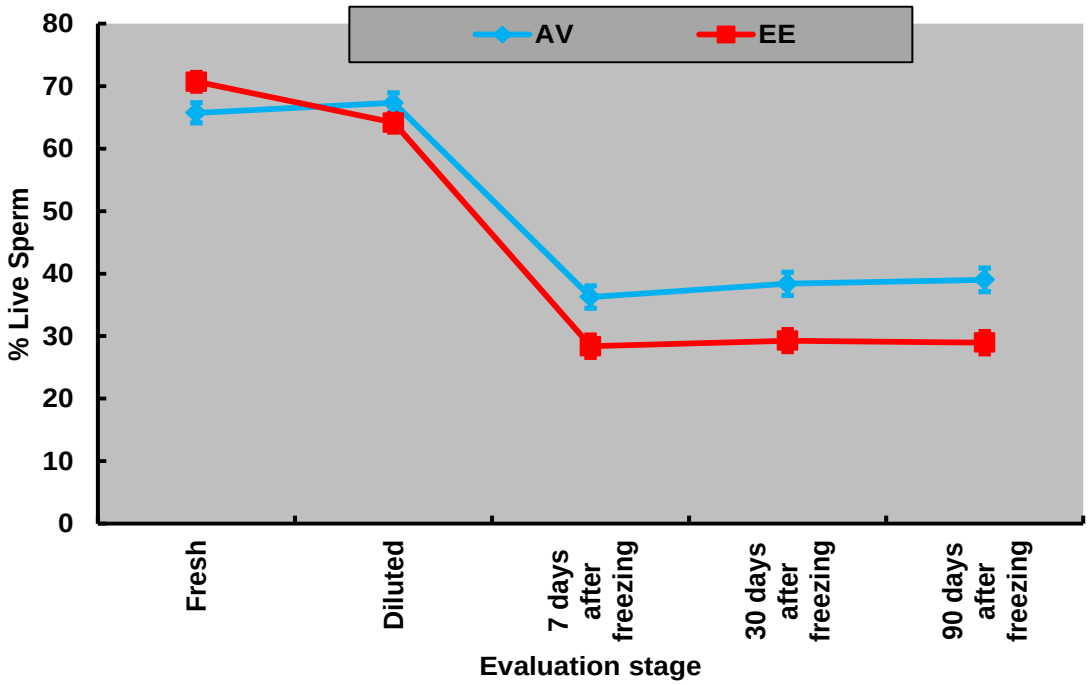

Figure 4.3. Percentage live sperm at different stages after freezing for the two semen collection methods

From Figure 4.3 it is evident that percentage live sperm of frozen thawed semen collected via the EE was lower at all evaluation stages than those of semen collected with an AV.

4.4 Interaction of breed (Dorper and Dohne Merino) x collection method (electro-ejaculation and artificial vagina)

The breed by collection method interactions are summarised for the various semen traits measured and assessed on fresh and diluted semen Table 4.19. The only significant interaction detected was for semen volume, where Dohne Merino rams produced more semen with an AV, while Dorper rams produced more semen with the EE.

Table 4.19. The breed by collection method interactions for the various semen traits measured and assessed on fresh and diluted semen

Traits	Dohne Merino		Dorper		Inter-action
	AV	EE	AV	EE	
Volume (ml)	$1.46^{a} \pm 0.08$	$1.20^{b} \pm 0.08$	1.22 ± 0.08	1.37 ± 0.08	**
Concentration ($x10^{9}$ sperm/ml)	1.21 ± 5.01	1.14 ± 5.20	1.19 ± 5.40	1.10 ± 5.29	ns
Motility of fresh sample	4.74 ± 0.07	4.85 ± 0.07	4.78 ± 0.08	4.81 ± 0.07	ns
Motility after dilution with Triladyl®	4.03 ± 0.09	4.13 ± 0.10	4.04 ± 0.10	4.24 ± 0.10	ns
% Live sperm fresh sample	67.07 ± 2.19	68.59 ± 1.94	$64.38^{c} \pm 2.32$	$72.82^{d} \pm 1.98$	ns
% Live sperm after dilution with Triladyl®	66.29 ± 2.18	63.41 ± 2.11	68.37 ± 2.30	64.92 ± 2.15	ns

[a,b,c,d] Values with different superscripts differ significantly between methods within breeds (P <0.05).

ns = interaction of breed*method not significant; ** = interaction of breed*method significant (P <0.01)

The breed by collection method interactions on motility of frozen-thawed semen, subjectively evaluated under a microscope, is summarised in Table 4.20. Motility of frozen-thawed semen collected with an AV was higher than the corresponding values of semen collected via EE in Dohne Merino rams at 7, 30 and 90 days after thawing. The same applied for Dorper rams at one week and three months after thawing of the samples. The only significant interaction detected for semen motility was at one month after freezing.

Table 4.20. The breed by collection method interactions on motility of frozen-thawed semen, subjectively evaluated under a microscope

Traits	Dohne Merino		Dorper		Inter-action
	AV	EE	AV	EE	
Motility 1W(0)	$2.09^{a} \pm 0.16$	$1.53^{b} \pm 0.15$	2.02 ± 0.17	1.93 ± 0.15	ns
Motility 1W(3)	$1.22^{a} \pm 0.12$	$0.80^{b} \pm 0.09$	$1.22^{c} \pm 0.13$	$0.81^{ad} \pm 0.09$	ns
Motility 1M(0)	$2.33^{a} \pm 0.14$	$1.32^{b} \pm 0.14$	1.94 ± 0.15	1.55 ± 0.14	*
Motility 1M(3)	$1.40^{a} \pm 0.10$	$0.78^{b} \pm 0.10$	1.26 ± 0.11	1.03 ± 0.10	ns
Motility 3M(0)	$2.43^{a} \pm 0.15$	$1.53^{b} \pm 0.15$	$2.22^{c} \pm 0.16$	$1.57^{d} \pm 0.15$	ns
Motility 3M(3)	$1.21^{a} \pm 0.11$	$0.73^{b} \pm 0.93$	$1.26^{c} \pm 0.11$	$0.79^{d} \pm 0.94$	ns

[a,b,c,d] Values with different superscripts differ significantly between methods within breeds (P <0.05).

ns = interaction of breed*method not significant; * = interaction of breed*method significant (P <0.05)

1W(0) = Evaluated 1 week, directly after thawing; 1W(3) = Evaluated 1 week, 3 hours after thawing; 1M(0) = Evaluated 1 month, directly after thawing; 1M(3) = Evaluated 1 month, 3 hours after thawing; 3M(0) = Evaluated 3 months, directly after thawing; 3M(3) = Evaluated 3 months, 3 hours after thawing.

The breed by collection method interactions on the percentage live sperm of frozen-thawed semen (microscope; using a droplet of frozen-thawed semen), is summarised in Table 4.21. The only significant interaction detected for percentage live sperm was at one month after freezing.

Table 4.21. The breed by collection method interactions on the percentage live sperm of frozen-thawed semen (microscope; using a droplet of frozen-thawed semen)

Traits	Dohne Merino		Dorper		Inter-action
	AV	EE	AV	EE	
% Live 1W(0)	$28.73^{a} \pm 1.72$	$17.86^{b} \pm 2.03$	$20.30^{c} \pm 1.64$	$22.72^{d} \pm 2.07$	ns
% Live 1W(3)	$21.07^{a} \pm 1.37$	$10.03^{b} \pm 1.61$	$11.31^{c} \pm 1.31$	$12.57^{d} \pm 1.64$	ns
% Live 1M(0)	$25.41^{a} \pm 1.37$	$15.87^{b} \pm 1.72$	18.02 ± 1.31	20.17 ± 1.75	*
% Live 1M(3)	$17.00^{a} \pm 1.19$	$9.33^{b} \pm 1.59$	11.65 ± 1.13	13.97 ± 1.61	*
% Live 3M(0)	25.76 ± 1.36	22.20 ± 2.01	22.81 ± 1.30	23.41 ± 2.05	ns
% Live 3M(3)	16.03 ± 1.16	12.73 ± 1.89	13.80 ± 1.11	14.86 ± 1.92	ns

^{a,b,c,d} Values with different superscripts differ significantly between methods within breeds (P <0.05).

ns = interaction of breed*method not significant; * = interaction of breed*method significant (P <0.05)

1W(0) = Evaluated 1 week, directly after thawing; 1W(3) = Evaluated 1 week, 3 hours after thawing; 1M(0) = Evaluated 1 month, directly after thawing; 1M(3) = Evaluated 1 month, 3 hours after thawing; 3M(0) = Evaluated 3 months, directly after thawing; 3M(3) = Evaluated 3 months, 3 hours after thawing.

The breed by collection method interactions on the percentage live sperm of frozen-thawed semen (microscope; counted from semen smears), is summarised in Table 4.22. No significant interactions were detected for percentage live sperm evaluated from semen smears.

Table 4.22. The breed by collection method interactions on the percentage live sperm of frozen-thawed semen (microscope; counted from semen smears)

Traits	Dohne Merino		Dorper		Inter-action
	AV	EE	AV	EE	
% Live 1W(0)	$37.04^{a} \pm 2.46$	$26.92^{b} \pm 2.64$	35.49 ± 2.60	29.84 ± 2.68	ns
% Live 1W(3)	$33.50^{a} \pm 1.92$	$24.07^{b} \pm 2.17$	29.56 ± 2.03	27.78 ± 2.21	ns
% Live 1M(0)	$37.74^{a} \pm 2.54$	$24.56^{b} \pm 2.70$	$39.03^{c} \pm 2.69$	$33.97^{d} \pm 2.75$	ns
% Live 1M(3)	$34.94^{a} \pm 2.18$	$23.13^{b} \pm 2.23$	$36.87^{c} \pm 2.31$	$30.13^{d} \pm 2.27$	ns
% Live 3M(0)	$39.49^{a} \pm 2.62$	$28.96^{b} \pm 2.71$	$38.53^{c} \pm 2.77$	$28.96^{d} \pm 2.76$	ns
% Live 3M(3)	$34.45^{a} \pm 2.35$	$23.75^{b} \pm 2.44$	$34.18^{c} \pm 2.49$	$25.52^{d} \pm 2.48$	ns

^{a,b,c,d} Values with different superscripts differ significantly between methods within breeds ($P < 0.05$).

ns = interaction of breed*method not significant.

1W(0) = Evaluated 1 week, directly after thawing; 1W(3) = Evaluated 1 week, 3 hours after thawing; 1M(0) = Evaluated 1 month, directly after thawing; 1M(3) = Evaluated 1 month, 3 hours after thawing; 3M(0) = Evaluated 3 months, directly after thawing; 3M(3) = Evaluated 3 months, 3 hours after thawing.

5. Discussion

Sheep breeds demonstrate differences in semen quantity (semen volume and concentration) and quality (semen motility, percentage alive and percentage of abnormal sperm) during and after the breeding seasons (Purdy, 2006; Mahoete, 2010). This difference between breeds and individual rams in semen characteristics makes it obligatory to execute semen evaluation, in order to select the best rams for breeding and thus optimising reproductive performance (Purdy, 2006; Makawi *et al*., 2007; Mahoete, 2010;).

Sperm capability to resist cold shock, usually known as freezability, is a vital requirement to maintain the plasma and outer acrosomal membranes undamaged and unharmed throughout cryopreservation and thawing. Freezability of semen can be affected by breed and collection method (Mattner & Voglmayr, 1962; Salamon & Morrant; 1963; Wulster-Radcliffe *et al*., 2001b; Marco-Jimenez *et al*., 2005; Palmer *et al*.; 2005). In the current study, breed and method of semen collection influenced survival rate of cryopreserved semen.

5.1 Semen Volume

In this study, breed had a significant effect on ejaculate volume of fresh semen collected either via electro-ejaculation or artificial vagina. Dorper rams (1.37 ± 0.08 ml) and the Dohne Merino rams (1.20 ± 0.08 ml) produced ejaculates with a higher semen volume than Namaqua Afrikaner rams (1.09 ± 0.08 ml) when using the EE. With the AV method, Dohne Merino rams (1.46 ± 0.08 ml) produces a higher ejaculate volume than Dorper rams (1.22± 0.08 ml).

The fresh semen volumes obtained in this study are higher than those obtained by Mahoete (2010) for the Merino, Pedi and Zulu breeds, which varied from 0.30 ± 0.01 to 0.60 ± 0.09 ml. The current volumes are also higher than the average ejaculate volume reported for Polish long-wool sheep rams semen of 1.1 ml, with a range of between 0.5 and 1.2 ml (Seremak *et al*., 1999). Generally the ejaculate volume of semen from mature rams varies from 0.5 ml to 2 ml (Hafez & Hafez, 2000). Chella (2015) reported semen volume varying from 0.48 ± 0.36 ml to 1.70 ± 0.76 ml for Zulu sheep at various locations and over all seasons collected via EE. Gil *et al*. (2003) reported semen volumes between 0.75 and 2 ml for semen collected with an AV in the Polish long-wool rams, which agree with the current values. Munyai

(2012) recorded semen volumes of 0.4 to 0.9 ml for Namaqua Afrikaner sheep. This was lower compared to the volume recorded in the current study of 1.09 ± 0.08 ml recorded for the Namaqua Afrikaner rams with the EE method. A nutritional effect can contribute to such differences in semen volume. Research has shown that an improvement in nutritional intake of both protein and energy during the two-month period prior to mating may increase the testicular size and subsequent sperm production by as much as 100% (Hafez 1993).

In this study, the method of collection had no effect on ejaculate volume when considering the pooled data of the three breeds. However, a significant breed by collection method interaction was observed for semen volume for the Dorper and Dohne Merino rams. Dorper rams produced more semen than Dohne Merino rams with the EE method, while Dohne Merino rams produced a higher ejaculate volume than the Doper rams with the AV method. Matthews *et al*. (2003) on the other hand reported no significant differences in semen volume of Dorper rams after using AV or EE collection methods. These current results are also in contrast with reports by Marco-Jimenez *et al*. (2005; 2008) and Guiliano *et al*. (2008) who recorded higher semen volumes in sheep and camels using electro-ejaculation compared to the artificial vagina. A higher volume of the ejaculate collected via EE could be due to the contribution of the accessory sex glands because of electrical stimuli, as suggested by Mattner and Voglmayr (1962). It is possible that the EE procedure changes the secretory function of one or more accessory glands, which could influence the amount of fluid secreted and thus the chemical composition of the seminal plasma (Jiménez-Rabadán *et al*., 2012).

Semen collection intensity is an important aspect relating to semen quality in domestic animals. In the ram, semen attributes such as ejaculate volume, sperm concentration and motility are highly correlated with the frequency of ejaculation. Semen volume will decrease with frequent collection of three or more times per day or even for an extended period of time (Hafez and Hafez, 2000). In the current study semen was collected twice a week, which should not have a major influence on semen volume, while Munyai (2012) collected on successive days (every Monday and Tuesday). The rams used in the present study received a high-protein and high-energy diet and were exercised twice weekly, exercising formed a major part of the study in preparing and maintaining the rams to be well conditioned and to try eliminate any possible accumulation of excessive fat which might have hindered the process of semen collection and ultimately the results. Combrink and Schoeman (1993) demonstrated the importance of fitness of rams during the mating season. They achieved

higher lambing percentages when rams were exercised before the mating season, more especially when rams will be kept in an intensive environment. These sentiments were echoed by Hafez, (1993) who emphasised that, rams should not be allowed to become over-fat (body condition score more than 4), as obese rams tend to be less sexually active and are more prone to heat stress.

Thus nutrition and frequency of collection had a major role in this study for recording high semen volume as compared to other studies. Hafez (1993) reported that nutrition had a direct and dramatic effect on testicular size of the rams, which again had a corresponding effect on sperm production. Louis *et al*. (1994) also reported that the volume of semen and gelatinous fraction per ejaculate was reduced when boars were fed low levels of both energy and protein compared with boars fed high levels of protein, regardless of energy intake. These results concurred with other research that confirmed an increase in sperm output with increased protein and amino acid intake (Poppe *et al*., 1974; Kim & Moon, 1990a,b). Kaya *et al*. (2002), Bester (2006) and Nel-Themaat *et al*. (2006) reported that the first ejaculate usually tends to be more than the volume in the consecutive ejaculates. When semen was collected twice per day, the average semen volume from the first ejaculate was recorded as 1.62 ml, compared to 1.06 ml from the second ejaculate. This corresponds with the fact that, where collection of semen was done once a day, 1.1 ml was obtained compared to 0.8 ml from 3 collections per day.

5.2 Sperm Motility

Sperm motility is usually believed to be one of the most important characteristics used to assess the fertilising capability of ejaculated sperm (Hashida & Abdullah, 2003). In the present study, sperm motility of the fresh semen, as assessed on a scale of 0 to 5, was not influenced by either breed or collection method.

Munyai (2012) recorded a lower total motility rate in fresh Namaqua Afrikaner semen (37.1%) collected with electro-ejaculation when compare to three other indigenous breeds (Damara - 69.6%, Pedi - 74.9% and Zulu - 56.0%) using the CASA system for sperm evaluation. The Pedi also (52.7%) recorded a significantly higher proportion of progressive motile sperm cells, compared to the Damara (36.4%), Namaqua Afrikaner (17.4%) and the Zulu (32.6%) rams. Total motility of semen after dilution and before freezing of the breeds

studied by Munyai (2012; Pedi, Damara and Zulu) ranged from 31.8% to 50.4%, while the corresponding progressive motility ranged from 21.7% to 34.9% (Munyai, 2012).

Motility of spermatozoa at thawing is considered an effective test to evaluate the quality of frozen semen as an index of view of sperm functionality (Leboeuf, 1989). It is well known that freezing and thawing impairs sperm structures and function, reducing motility as well as fertilizing capacity of sperm cells (Watson, 1995; Maxwell and Watson, 1996). Motility of fresh and diluted semen in all breeds in the current study was high (> 4), but dropped to below 2 in all breeds for the frozen-thawed semen. Percentage motility also dropped from above 70% in fresh semen to below 15% in frozen-thawed semen in a study conducted by Ak et al., (2010). Total and progressive motility also decreased in the post-thawed samples frozen in liquid nitrogen vapour to 8.8 ± 0.9% and 4.4 ± 0.7% in the indigenous breeds evaluated by Munyai (2012). Furthermore, motility 3 hours after thawing in the current study was also lower than motility assessed immediately after thawing for all breeds and collection methods. This is in accordance with findings from Kasimanickam *et al*. (2007) where deterioration in sperm motility characteristics increased with storage time.

The only recorded breed difference in motility of frozen-thawed semen in the current study is the Dorper ram semen collected via EE that had a higher motility (1.55 ± 0.14) than the Dohne Merino (1.32 ± 0.14) and Namaqua (1.03 ± 0.14) rams at the 1 month evaluation, directly after thawing.

As far as differences between EE and AV collection methods are concerned, motility of frozen-thawed semen collected with an AV and evaluated at 7, 30 and 90 days after freezing, were significantly higher than that collected via EE. These results may perhaps be ascribed to semen exposure to contaminations such as urine during semen collection with EE. These results are in accordance with results obtained by Carter et al. (1990) who compared the EE and AV collection methods and found that AV results were slightly higher than when using the EE technique, thus encourages the use of AV for semen collection, especially in cryopreservation procedures.

5.3 Semen density, concentration and colour

Hafez and Hafez (2000) stated that there is a correlation between the colour of the semen sample and sperm concentration. The viscidness which often relates to the density of the semen

sample is considered as the reflection of the number of spermatozoa present in the semen sample. Often the density (concentration) of a semen sample is reported to be correlated with the fertilisation rate (Brown & Krouse, 1973; Wolf & Inoue, 2005). The results of this current study concur with the discoveries of other studies (Bag *et al*., 2002) when assessing semen density and colour.

Considering the distribution of the semen samples collected via EE of the different breeds over the various categories, it was obvious that there were more Dorper samples that were categorised in the less dense categories. Sixty percent of the Namaqua samples were categorised as Creamy or Thick cream. These concur with the sperm concentrations, where the Dorper rams had the lowest sperm concentration ($1.10 \pm 5.29 \times 10^9$ sperm/ml) and the Namaqua rams the highest sperm concentration ($1.22 \pm 5.20 \times 10^9$ sperm/ml). These results may be attributed to the frequency of collection as the Dorper rams were also exposed to the AV method of collection. The distribution pattern and sperm concentration between semen samples of Dorper and Dohne Merino rams collected via AV were very similar.

The distribution of the semen samples collected via EE and AV over the various categories differed significantly. More of the EE samples were categorised in the less dense categories than those of the AV samples. The AV samples also had a higher sperm concentration ($1.20 \pm 3.68 \times 10^9$ sperm/ml) than the EE samples ($1.11 \pm 3.51 \times 10^9$ sperm/ml). As mentioned earlier, higher secretions by one or more accessory glands with EE could influence the amount of seminal fluid (Jiménez-Rabadán *et al*., 2012). This negatively influences the density and sperm concentration of the sample. This could explain the lower density obtained with the Dorper EE samples.

The colour of all semen samples collected via the AV were categorised as Creamy, while 20% of the samples collected via EE was categorised as White. The Dohne Merino had more White semen samples than the Dorper and Namaqua rams when semen was collected via EE. The reason for this cannot be given, as all the Dohne Merino and Dorper samples collected via AV were categorised as Creamy.

The sperm concentration in this study showed no significant differences between the three breeds in any method of collection used. These values are in accordance with those recorded by Hassan *et al*. (2009) for native Bangladesh sheep ranging from $1.03 \pm 0.61 \times 10^9$ sperm/ml

for one year old to 4.17 ± 0.58 x10^9 sperm/ml for four year old rams. Chella (2015) reported sperm concentrations varying from 1.34 ± 0.68 ml to 4.27 ± 1.38 x10^9 sperm/ml for Zulu sheep at various locations and over all seasons. The mean sperm cell concentration recorded in the present study was higher than the reported results by Fourie *et al*. (2004) with intensively managed Dorper rams (731.6 x10^6 sperm/ml), also using the electro-ejaculator as the method of semen collection. However Gil *et al*. (2003) suggested that a sperm concentration of 2.5 x10^9 sperm/ml of rams to be normal and acceptable.

The difference between this current study and the previous studies might be due to different devices that were used to determine sperm cell concentration. In this study, a haemocytometer was used whereas in the previous studies, a Spermacue® photometer was used. There are however, still several factors which might have caused differences between this current study and previous studies in terms of sperm concentration. These include breed used, nutrition, time of the year and the age of the rams (Marco-Jiménez *et al*., 2005; Bester, 2006; Yamashiro *et al*., 2006; Daramola *et al*., 2007; Sundararaman *et al*., 2007; Zarazaga *et al*., 2009).

5.4 Percentage live sperm

The effect of breed on percentage of live sperm of frozen-thawed semen collected via electro-ejaculation and counted from semen smears was variable for the three evaluation periods. However, the Namaqua samples had a lower percentage of live sperms than the Dorper at all three evaluations and the Dohne Merino at the 3 month evaluation. The percentage live sperm of all breeds dropped considerably after freezing. It remained constant for all breeds from one week until 3 months after freezing, indicating that length of storage should not have an effect on percentage live sperm for semen collected via EE. Dorper rams had the highest and Namaqua rams the lowest percentage live sperm for frozen-thawed semen. These results can directly be ascribed to a breed effect.

No differences in percentage of live sperm of frozen-thawed semen collected via AV and counted from semen smears was observed between Dorper and Dohne Merino rams. A drop in percentage of live sperm in AV samples similar to EE samples occurred in both breeds after freezing. Semen collected with an AV yielded frozen-thawed sampled with higher percentage live sperm at all three post-freezing evaluation dates than those collected via EE

and these results can be ascribed to directly to the method of collection used as well as the semen contamination associated with semen collected using EE method.

In all the breeds, the post-thaw percentage live sperm of semen collected with electro-ejaculation ranged between 15.4 and 33.9%, compared to a percentage live sperm for fresh semen that ranged between 63.0 and 72.8%. For semen collected via artificial vagina the range was from 16.0 to 39.4% for post-thaw and 64.38 to 68.37% of fresh semen. These results are lower than those obtained by Mahoete (2010) using indigenous Pedi and Zulu rams, who recorded a post-thaw percentage live sperm of 26.7 and 45.8 % as compared to 75 and 76.7% of percentage live sperm for fresh semen. These differences could most probably be ascribed to the specific freezing protocol applied. Percentage live sperm variation between rams was also observed by Garcia-Alvarez *et al*. (2009).

6. Conclusions

The aim of this study was to evaluate semen from the endangered Namaqua Afrikaner breed against that of breeds whose semen is being frozen on a commercial scale. The need for Namaqua semen to be frozen successfully is that a cryobank for this breed is being established which will include several thousand frozen semen doses.

As mentioned at the start of the thesis, it was originally planned to use the AV method to collect the semen samples, but the Namaqua rams refused to use the AV. It was therefore envisaged that assumptions could be made regarding the semen traits of Namaqua rams from AV collection with relation to the same traits of the Dorper, Dohne Merino and Namaqua rams when EE and AV methods were used.

With regard to the EE method, fresh semen from the Namaqua rams compares favourably with that of the Dorper or Dohne Merino rams, except for semen volume, but semen volume does not influence the actual quality or the fertilizing ability of the semen. However, the percentage live sperm of the frozen-thawed semen of the Namaqua rams was lower than that of the Dorper rams, indicating that the Namaqua semen collected via EE did not freeze as good as that of the Dorper semen. Neither fresh nor frozen-thawed Dorper and Dohne Merino semen collected via EE did differ significantly. Furthermore, except for semen volume, Dorper and Dohne Merino semen collected via AV did not differ significantly whether evaluated as fresh or frozen-thawed semen. However, both Dorper and Dohne Merino rams produced semen with higher motility and a higher percentage live sperm post-thaw when the semen samples were collected via an AV than via EE. From these results it can therefore be postulated that if Namaqua semen were collected via AV it could have a higher post-thaw percentage live sperm than if semen was collected via EE.

The concentration and percentage of live sperm of the fresh semen sample determine the number of straws that can be frozen from a specific semen sample. Semen samples with a motility score below 3 are not even considered for cryopreservation. If one considers the quality of the fresh semen samples of the Namaqua Afrikaner rams, and the fact that it did not differ from that of the Dorper or Dohne Merino rams, which semen is successfully being frozen on a commercial scale in South Africa, technically this implies that it should be

feasible to cryopreserve semen from Namaqua Afrikaner rams. However, the low percentage of live sperm obtained with the frozen-thawed samples for all breeds in this study, is an indication that further research is needed into more suitable freezing protocols. The post-thaw motility obtained for other indigenous breeds by Mahoete (2010) and Munyai (2012) was also unacceptably low, which further contributes to the necessity of further research into this aspect. Therefore, from the results of this study, it can be concluded that Namaqua Afrikaner semen cannot be frozen successfully for the purpose of storage in a cryobank, when using a freezing protocol based on Triladyl® as extender.

6.1 Recommendations

Advanced further studies are necessary to investigate the reason for the lower post-thaw survival rate of sperm of the Namaqua Afrikaner rams. This will be necessary as semen from this endangered breed, destined for storage in a cryobank has to be of high quality. Such resources as cryobanks are expensive and funds cannot be wasted on preserving inferior samples that could not fertilize an ovum when needed. The low percentage of live sperm obtained with the frozen-thawed samples in this, as well as other studies on indigenous breeds, is an indication that further research is needed into more suitable freezing protocols. Furthermore, any increase in post-thaw survival rate of sperm will be beneficial and it is therefore suggested that all efforts be made to solve the problem of the Namaqua Afrikaner rams that do not want to ejaculate into an artificial vagina.

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