Evaluation of cryopreserved ram semen following fertilization *in vitro*

by

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DEDICATION

To my mother ('Mamasilo Mohlomi), and my sisters ('Mampoeakae,'Mat'soloane, 'Malikhoa and Puleng Mohlomi) and my brothers ('Mutsi and Sello Mohlomi) for their support.

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DECLARATION

I hereby declare that this dissertation submitted by me to the University of the Free State for the degree, **Master of Science in Agriculture,** is my own independent work and has not previously been submitted by me at another University. I furthermore cede copyright of the dissertation in favour of the University of the Free State.

Masilo Henoke Mohlomi

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LIST OF ABBREVIATIONS

e.g	for example
min	minutes
hr	hour
˚C	Degree Celcius
μm	Micron
%	percentage
μl	Microliter
/	or
ml	milliliter
mg	milligram
mm	millimeter
mm^2	millimeter square
AV	Artificial Vagina
IVF	*In Vitro* Fertilization
COC’s	Cumulus Oocyte Complexes
mPBS	modified Phosphate Buffered Saline
BSA	Bovine Serum Albumin
BCB	Brilliant Cresyl Blue
FBS	Fetal Bovine Serum
SAMM	South African Mutton Merino
BO	Bracket and Oliphant
SOF	Synthetic Oviduct Fluid

kg	Kilogram
LN_2	Liquid Nitrogen
et al.	And others
SAS	Statistical Analysis System
ATP	Adenosine Triphosphate
DNA	Dioxynucliec Acid
GnRH	Gonadotropin-releasing Hormone
LH	Luteinizing Hormone
FSH	Follicle Stimulating Hormone
CO_2	Carbon dioxide
ARTs	Assisted Reproductive Technologies
pH	Potential hydrogen
OPU	Ovum pick-up
MOET	Multiple Ovulation and Embryo Transfer
IVEP	*In vitro* Embryo Production

CHAPTER 1

GENERAL INTRODUCTION

Reproductive success is a key component of economic production in ruminants, affecting both animal productivity and genetic progress (Bauersachs *et al*., 2010). The reproduction success in any animal production enterprise is then of high economical importance and this can be achieved with better applied knowledge of reproduction physiology and application of certain reproductive technologies.

When considering reproductive technologies as such, artificial insemination (AI) is probably one of the most important reproductive techniques to accelerate the genetic improvement of animals. The widespread use of AI in cattle has ultimately allowed accurate genetic evaluation and rapid dissemination of genetic merit on a national and international basis to the benefit of both the animal breeder and the consumer. It has also enabled the use of sophisticated data analysis procedures to be implemented to identify animals with superior performance.

The availability of an efficient sheep AI service would also yield similar benefits, and would greatly enhance the scope for pedigree and commercial breeders to respond positively and effectively to consumer demands. The widespread use of AI and the realization of its full potential then depend essentially on the use of frozen semen and on the availability of techniques that could result in acceptable fertility. However, the poor fertility obtained when frozen-thawed ram semen is used for cervical insemination in sheep has stimulated widespread research interest in the sheep industry. Gil *et al*., (2003) stated that the relatively low success rate of cervical AI with frozen semen in sheep has limited a wider application of the technique, calling for an improvement of insemination technique itself and/or of the survival rate of the frozen-thawed sperm. The short life span of fresh semen has on the other hand been reported to be a constraint in the use of AI in genetic improvement programs for sheep (O'Hara *et al*., 2010).

The alternative is laparoscopic AI as an effective method of insemination when using frozen-thawed semen to extend the life span, but the procedure is expensive, thus limiting its use. Welfare concerns may also limit the use of the laparoscopic AI procedure. It is generally

desirable and necessary to develop non-surgical procedures that could form the basis of making AI a practical reality in the sheep industry.

Apart from AI, facilitation of genetic improvement of animals is done through the use of certain other several assisted reproductive technologies (ART's) such as semen and embryo cryopreservation, estrous synchronization, multiple ovulation and embryo transfer (MOET), as well as *in vitro* embryo production (IVEP). The cryopreservation of bovine semen and embryos has made great progress in recent years, but little progress has been obtained in the small stock industry (Zhu *et al*., 2001). Cryopreservation of gametes is seen as an important technique for long time storage of semen and embryos for future use in the dissemination of superior genetic material. The long term conservation of sperm is especially crucial for *in vitro* fertilization (IVF) and/or AI purposes (Merlo *et al.,* 2008). Cryopreservation ultimately creates the opportunity to maintain superior genetic material at low costs, and also conserve endangered species or breeds (Gonzalez-Bulnes *et al*., 2004; Mapletoft and Helser, 2005). As well as providing some security with respect to disasters or outbreaks of disease that may seriously affect animal population survival (Kirkwood and Colenbrander, 2001). However, the cryopreservation process exposes sperm cells to physical and chemical stress and less than 50% of the sperm cells may survive, with fertilizing ability being maintained (Waterhouse *et al*., 2006).

It has further been recorded that some cryopreservation techniques, such as slow freezing are expensive, may cause physical damage to the sperm and embryos, due to crystal formation and are time consuming (Naik *et al*., 2005). The vitrification technique on the other hand may cause damage due to cryoprotectant toxicity (Naik *et al*., 2005; Sharma *et al*., 2006). Slow cooling however, seems to be the most important element used in the preservation technique in sheep, when compared to vitrification (Thuwanut, 2007).

Tests that set minimum standards for semen used for artificial insemination (Mocé and Graham, 2008) have limited value for predicting subsequent fertility of the semen sample (Mocé and Graham, 2008). However, it is documented that semen evaluation techniques such as sperm binding, oocyte penetration and *in vitro* fertilization estimates functional aspects of spermatozoa (Flowers *et al*., 2009). The assessment of functional sperm parameters under capacitating conditions has been proposed (Petrunkina *et al*., 2007). In contrast, the type of extender affects fertilization potential while it has no effect on developmental potential up to the blastocyst stage (Forouzanfar *et al*., 2010). Indeed, extender has a major effect on post-

thawed semen viability (Paulenz *et al*., 2002; Valente *et al*., 2010). In addition, semen quality is negatively affected by freezing procedures (Nur *et al*., 2010) and thus, experiments to examine the outcomes of the use of different freezing protocols have been proposed (Ramón *et al*., 2013).

In vitro embryo production (IVEP) was first performed in order to produce relatively cheap embryos on a large scale for various experimental procedures (Wani, 2002). Besides for experimental purposes, IVEP can be seen as a possible method to produce embryos in abundance to improve the reproductive efficiency of livestock (Rust and Visser, 2001). Early stage embryos are required for the production of clones, transgenics, sexed embryos and for the diagnosis of genetic defects (Wani, 2002). *In vitro* embryo production and fertilization are now important technologies for obtaining live offspring (Kikuchi *et al*., 2009). For a viable embryo to be produced, good quality sperm and oocytes are needed. The quality of oocytes is of major importance in assuring the developmental competence of embryos, which is more apparent and is determined by the oocyte's nuclear and cytoplasmic maturation attained during growth in the follicle (Sirard, 2001). Oocytes from small follicles (2-3 mm) have been shown to have a reduced developmental *in vitro* competence due to lack of pre-maturation factors that should occur during the final follicular growth phase (Cognie *et al*., 2004). A competent oocyte is generally described as the oocyte which is able to sustain embryonic development to term (Brevini-Gandolfi and Gandolfi, 2001). The number of high quality oocytes harvested from an ovary is an important consideration in the *in vitro* production of embryos. Oocytes for *in vitro* fertilization are generally collected from one of the following sources: the oviducts soon after ovulation, mature follicles shortly before ovulation or immature and atretic follicles, usually from abattoir material (Wani, 2002).

Ovaries from slaughtered animals are then the cheapest and most abundant source of primary oocytes for large scale production of embryos through IVEP (Wani, 2002). To date, some research has been done on IVEP in sheep (Wani *et al*., 2000; Galli *et al*., 2001; Wani, 2002, Rao *et al*., 2002; Cognie *et al*., 2003; Katska-Ksiazkiewicz *et al*., 2004; Locatelli *et al*., 2006; Cox and Alfaro, 2007 and Cocero *et al*., 2011). As research has also been done on breed effect on semen and cryopreservation (Mahoete, 2010; Maghaddam *et al*., 2012).

Estrous synchronization as one of the available assisted reproductive technologies, can contribute to some extent to the improvement of farm animal productivity. This involves the application of the knowledge of animal's hormonal activity to manipulate the sexual cycle.

Effective estrous synchronization can then facilitate the use of timed AI (Sa Filho *et al*., 2009). Progress in the field of estrous synchronization has been directly linked to the discovery of a wave-like pattern of follicular development (Day *et al*., 2010). Various relationships between fertility and aspects of follicular development, such as follicle size, length of the pro-estrus phase, follicular estradiol production and progesterone concentrations during follicular development to fertility have emerged and led to fundamental investigations into the mechanisms underlying these aspects (Day *et al*., 2010).

Multiple ovulation and embryo transfer (MOET) is a potential technique for increasing the efficiency of breeding program in domestic animal production. Hafez and Hafez (2000) documented that embryo transfer can be used to rapidly increase rare bloodlines, to obtain more offspring from valuable females and to accelerate genetic progress by facilitating progeny testing in females and thus reducing the generation interval.

In the sheep production industry, there is still much work to be done to improve reproductive efficiency.

The objectives of the present study were thus as follows:

A. Evaluate the effect of breed on fresh ram semen quality parameters.
B. Compare the sperm motility of ram semen from two sheep breeds following cryopreservation and thawing, based on 4 time intervals (0, 30, 60 and 120 minutes after thawing).
C. Compare the effect of two extenders on ram semen cryopreservation of two breeds of sheep.
D. Compare the effect of two ram semen freezing protocols of two sheep breeds cryopreserved using two extenders.
E. Test the fertilizing ability of the frozen-thawed ram semen following cryopreservation.

CHAPTER 2

LITERATURE REVIEW

In this chapter the different phases involved with processes of semen collection, cryopreservation and *in vitro* fertilization in sheep are reviewed in detail. Before discussing these phases a look at the male reproductive anatomy, spermatogenesis and factors affecting semen quality will be made.

2.1 Male reproductive anatomy

The semen production processes (spermatogenesis) takes place in the ram reproductive tract which has the following physiological anatomy as shown in the figure below.

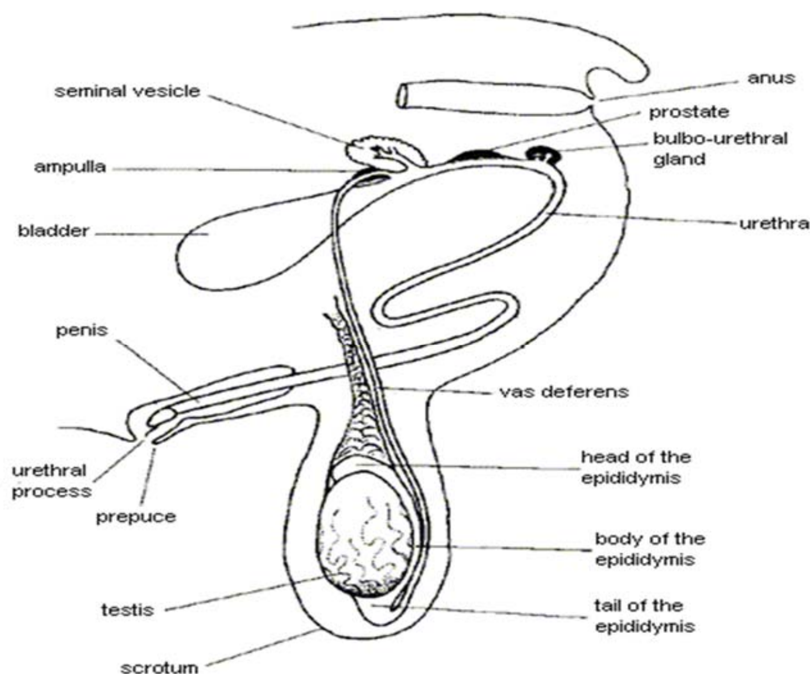

Figure 2.1 Reproductive tract of a ram (Simmons & Ekarius, 2001)

The testis is composed of coiled seminiferous tubules, in which the sperm are formed (Ganong, 2011), after which the sperm cells are then transported to the epididymis. The epididymis is

then the primary location for the maturation and storage of sperm prior to ejaculation (Costanzo, 2006; Berne and Levy, 2008). The epididymis leads into the vas deferens, which enlarges into the ampulla of the vas deferens immediately before the vas deferens enters the body of the prostate gland. The two seminal vesicles, one located on each side of the prostate gland, then empty the seminal fluid into the prostatic end of the ampulla, and the contents from both the ampulla and the seminal vesicles pass into an ejaculatory duct leading through the body of the prostate gland and then emptying into the internal urethra. Prostatic ducts also empty fluid from the prostate gland into the ejaculatory duct and from there into the prostatic urethra (Guyton and Hall, 2010; Ganong, 2011).

The urethra is the last connecting link from the testis to the exterior. The urethra being supplied with mucus derived from a large number of minute urethral glands located along its entire extent and even more so from bilateral bulbo-urethral glands (Cowper's glands), located near the origin of the urethra.

2.1.1 Spermatogenesis

During the formation of the embryo, the primordial germ cells migrate into the testes and become immature germ cells called spermatogonia which lie in two or three layers of the inner surfaces of the seminiferous tubules. The spermatogonia then begin to undergo mitotic divisions, beginning at puberty, and continually proliferate and differentiate through definite stages of development, to eventually form sperm cells (Ganong, 2011). Events and time involved during spermatogenesis are shown on the figure below.

Spermatogenesis occurs in the seminiferous tubules during the active sexual life of the male as the result of stimulation by anterior pituitary gonadotrophic hormones. In the first stage of spermatogenesis, the spermatogonia migrate between the Sertoli cells, towards the central lumen of the seminiferous tubules. The Sertoli cells are very large, with overflowing cytoplasmic envelopes that surround the developing spermatogonia all the way to the central lumen of the seminiferous tubule (Ganong, 2011). Spermatogonia that cross the barrier into the Sertoli cell layer become progressively modified and enlarged to form large primary spermatocytes. Each of these, in turn, undergoes meiotic division to form two secondary spermatocytes. After another few days, these too divide to form spermatids which are eventually modified to become spermatozoa (sperms) (Cheng and Mruk, 2010).

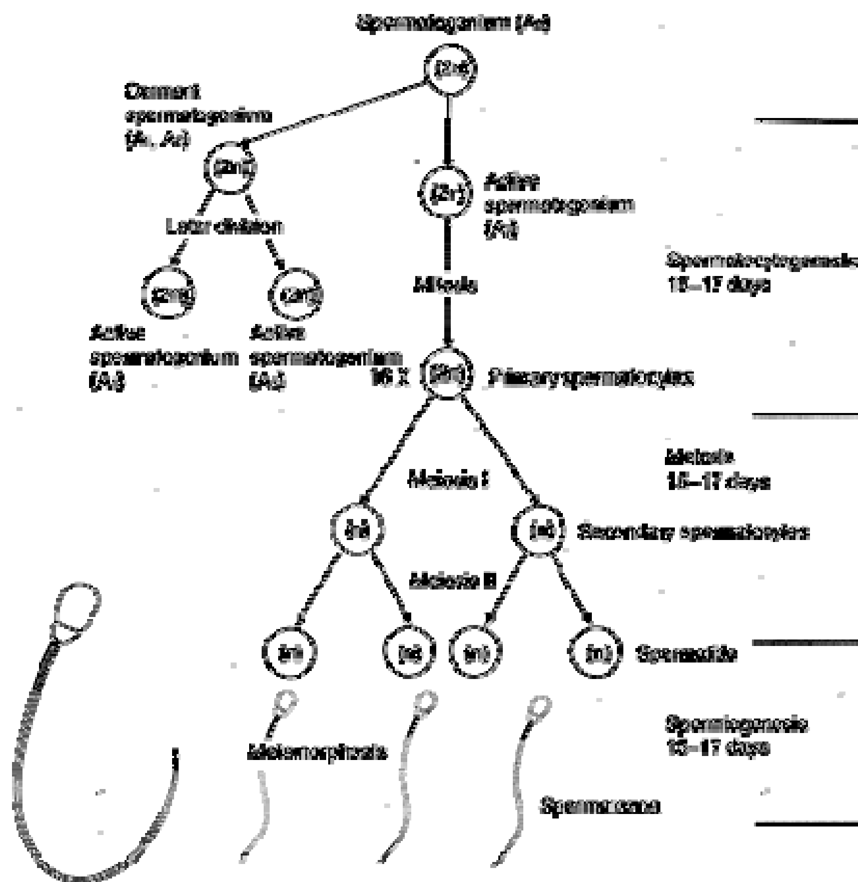

Figure 2.2 Sequence of events and time involved during spermatogenesis (Cheng and Mruk, 2010)

During the change from the spermatocyte stage to the spermatid stage, the 46 chromosomes (23 pairs of chromosomes) of the spermatocyte are divided. Thus 23 chromosomes go to one spermatid and the other 23 to the second spermatid (Ganong, 2011). In each spermatogonium, one of the 23 pairs of chromosomes carries the genetic information that determines the sex of each individual offspring. The pair being composed of one X chromosome, is called the female chromosome, and one with a Y chromosome, the male chromosome. During meiotic division, the male Y chromosome goes to one spermatid that then becomes a male sperm, and the female X chromosome goes to another spermatid that becomes a female sperm (Guyton and Hall, 2006).

2.1.2 The structure of the sperm cell

When the spermatids are first formed, they still have the usual characteristics of epithelioid cells, but soon begin to differentiate and elongate into spermatozoa. The spermatozoa are broadly composed of a head and a tail. The head is comprised of the condensed nucleus with only a thin cytoplasmic and cell membrane layer around its surface (Guyton and Hall, 2006). On the outside anterior a two thirds of the head is a thick cap, called the acrosome that is formed mainly from the Golgi apparatus. This acrosome contains a number of enzymes, similar to those found in the lysosomes of a typical cell, including hyaluronidase (which can digest proteoglycan filaments of tissues) and powerful proteolytic enzymes (which can digest proteins). These enzymes then play important roles in allowing the sperm to enter the ovum and fertilize it (Guyton and Hall, 2006).

According to Guyton and Hall (2010), the tail of the sperm, called the flagellum, has three major components mentioned below:

(1) a central skeleton constructed of 11 microtubules, collectively called the axoneme, similar in structure to that of cilia found on the surfaces of other types of cells

(2) a thin cell membrane covering the axoneme; and

(3) a collection of mitochondria surrounding the axoneme in the proximal portion of the tail (called the body of the tail).

Back and forth movement of the sperm tail (flagella movement) provides motility to the sperm cells. This movement results from a rhythmical longitudinal sliding motion between the anterior and posterior tubules that make up the axoneme. The energy for this process is supplied in the form of adenosine triphosphate (ATP), synthesized by the mitochondria in the body of the tail.

After formation in the seminiferous tubules, the sperm require several days to pass through the tubule of the epididymis. Sperm removed from the seminiferous tubules and from the early portions of the epididymis are non-motile, and they cannot fertilize an ovum. However, after the sperm have been in the epididymis for some time, they develop the capability of motility (Ganong, 2011), even though several inhibitory proteins in the epididymal fluid still prevent final motility until after ejaculation. Only a small quantity of sperms cells can be stored in the epididymis, most are stored in the vas deferens. During this time, they are kept in a deeply

suppressed inactive state by multiple inhibitory substances in the secretions of the ducts. Conversely, with a high level of sexual activity and ejaculations. During semen cryopreservation, at temperatures below -80°C, the highly concentrated, viscous solution within and outside the sperm turns into a relatively stable glassy matrix, which is basically maintained when sperm are stored at -196°C (LN_2) (Rodrequize-Martines *et al.,* 2009).

After ejaculation, the sperm cell become motile, and also becomes capable of fertilizing the ovum, a process called maturation. The Sertoli cells and the epithelium of the epididymis secrete a special nutrient fluid that is ejaculated along with the sperm. This fluid contains hormones (including both testosterone and estrogens), enzymes, and special nutrients that are essential for sperm maturation (Guyton and Hall, 2010; Ganong, 2011; Fox, 2013).

The normal motile, fertile sperm are capable of flagellated movement through the fluid medium. The activity of sperm being greatly enhanced in a neutral and slightly alkaline medium, but it is greatly depressed in a mildly acidic medium. A strong acidic medium can cause rapid death of sperm. The activity of sperm increases markedly with increasing temperature, but so does the rate of metabolism, causing the life of the sperm to be considerably shortened (Ganong, 2011).

2.1.3 The seminal vesicle

Each seminal vesicle is a tortuous, loculated tube, lined with a secretory epithelium that secretes a mucoid fluid containing an abundance of fructose, citric acid, and other nutrient substances, as well as large quantities of prostaglandins and fibrinogen (Costanzo, 2006). During the process of emission and ejaculation, each seminal vesicle empties its contents into the ejaculatory duct shortly after the vas deferens empties the sperm (Fox, 2013). This adds greatly to the volume of the ejaculated semen, and the fructose and other substances in the seminal fluid are of considerable nutrient value for the ejaculated sperm until one of the sperm fertilizes the ovum (Costanzo, 2006). According to Guyton and Hall (2010) and Costanzo (2006), prostaglandins are believed to aid fertilization in the following two ways:

(1) by reacting with the female cervical mucus to make it more receptive to sperm movement and

(2) by possibly causing backward, reverse peristaltic contractions in the uterus and Fallopian tubes to move or direct the ejaculated sperm toward the ovaries.

2.1.4 The prostate gland

The prostate gland secretes a thin, milky fluid that contains calcium, citrate ion, phosphate ion, a clotting enzyme, and a profibrinolysin (Costanzo, 2006; Guyton and Hall, 2010). During emission, the capsule of the prostate gland contracts simultaneously with the contractions of the vas deferens so that the thin, milky fluid of the prostate gland adds further to the bulk of the semen (Costanzo, 2006). The slightly alkaline characteristic of the prostatic fluid may be quite important for successful fertilization of the ovum, as the fluid of the vas deferens is relatively acidic, owing to the presence of citric acid and metabolic end products of the sperm and, subsequently, helps to inhibit sperm fertility (Ganong, 2011). The vaginal secretions of the female are also acidic. Sperm do not become optimally motile until the pH of the surrounding fluids rises to between 6.0 and 6.5. Consequently, it is probable that the slightly alkaline prostatic fluid helps to neutralize the acidity of the other seminal fluids during ejaculation, and thus enhances the motility and fertility of the sperm (Ganong, 2011).

2.1.5 Semen

Semen, which is ejaculated during mating, is composed of the seminal fluid and sperm cells. Seminal fluid arises from the sex glands, such as the seminal vesicles, the prostate gland, and the bulbourethral glands (Ganong, 2005; Costanzo, 2006; Berne and Levy, 2008). The bulk of the semen fluid is from the seminal vesicles, which is the last to be ejaculated and serves to wash the sperm through the ejaculatory duct and urethra.

The average pH of the combined semen or seminal fluids is approximately 7.5. The alkaline prostatic fluid contributing more to neutralize the mild acidity than the other components of the semen. The prostatic fluid then gives the semen a milky appearance, while the fluid from the seminal vesicles and mucous glands gives the semen a mucoid consistency (Guyton and Hall, 2010). A clotting enzyme from the prostatic fluid causes the fibrinogen of the seminal vesicle fluid to form a weak fibrin coagulum, which retains the semen in the deeper regions of the vagina, where the cervix is located (Guyton and Hall, 2006). The coagulum dissolves after a few minutes because of lysis by fibrinolysin formed from the prostatic profibrinolysin. In the early minutes after ejaculation, the sperm remain relatively immobile, possibly because of the viscosity of the coagulum. As the coagulum dissolves, the sperm simultaneously become highly motile (Guyton and Hall, 2010).

Although sperm can live for many weeks in the male genital ducts, once they are ejaculated in the semen, their maximal life span is only 24 to 48 hours at body temperature. At lowered temperatures, however, semen can be stored for several weeks, and when frozen at temperatures of -196°C, sperm has been preserved for years (Hafez and Hafez, 2000; Nur *et al*., 2010).

2.2 Factors affecting the quality of the sperm

2.2.1 Environment

Photoperiod, temperature, humidity, nutrition, diseases and parasites are some of the environmental factors that can affect animal production and reproduction (Brito *et al*., 2002). An increasing number of reports suggest that chemical and physical agents in the environment, introduced and spread by for example, human activity, may affect male fertility in humans (Jerewics *et al.,* 2009). Much has then been done on effects of environmental factors on human sperm quality and fertility (Duty *et al.,* 2003; Hauser *et al.,* 2003). So for example cattle reproduction can be affected by heat stress under high ambient temperatures and/or humidity, the body thermoregulatory mechanisms are unable to increase body heat loss and internal temperature generally increases above the physiological limits (Brito *et al.,* 2002). In the tropics, sperm production and semen quality have been shown to decrease during the hot season (Wolfenson *et al.,* 2000). The elevation of the testicular temperature generally results in an increased metabolism and oxygen demand, but testicular blood flow is limited and this increased demand cannot be supplied resulting in hypoxia, the generation of reactive oxygen species and deterioration of semen quality (Leonardo *et al.,* 2004).The maintenance of the testicular temperature at 4 to 5 °C below body temperature is essential for normal spermatogenesis in rams (Leonardo *et al.,* 2004).

2.2.2 Season of the year

Sheep are seasonal breeders (Rosa and Bryand, 2003) and suggestions by Ghalban *et al*. (2004) indicate that the optimal male performance may be obtained during the period of increasing daylight length. In spring and summer both sperm quantity and quality were recorded to be higher than that in winter or autumn in bucks. Sheep and goats thus exhibit great seasonal variation in semen quality (Leboeuf *et al*., 2000). These seasonal variations in both semen

quality and quantity are mainly due to changes in daylight length throughout the year (Ghalban *et al.,* 2004). Optimal reproductive performance of crossbred rams was recorded in late summer and the beginning autumn by Moghaddam *et al,* (2012). It was also reported that sperm mass motility increased steadily from the beginning of the mating season to the end of the mating season in rams (Ghalban *et al*., 2004). Photoperiodic signals are generally translated into stimuli on the reproduction system by changes in the pattern of secretion of melatonin from the pineal gland (Munyai, 2012). It has been documented by Zamiri *et al.* (2005), that the highest values of thyroid stimulating hormone, T4, free T4 index, testosterone, total sperm number, percentage normal sperm, percentage live sperm, sperm concentration, semen volume and scrotal circumference were recorded from early summer to winter with the lowest values being detected at the end of spring.

2.2.3 Age of the male

Increased age of the bull has been associated with decreased sperm motility and increased minor sperm defects (Brito *et al*., 2002). Mature rams then generally also have higher ejaculate volumes, sperm concentrations and total sperm per ejaculate than younger rams (Hafez and Hafez 2000; Ghalban *et al.,* 2004). According to Salhab *et al.* (2003), good quality semen can be collected from growing Awassi rams at 11 months of age. This is in agreement with the work done by Kumar *et al.* (2010) who reported that Malpura ram lambs 9-12 months of age can produce good quality semen.

2.2.4 Nutrition

It has been indicated that adequate nutritional management is crucial for successful reproduction efficiency in sheep (Smith and Akinbamijo, 2000; Fernandez *et al*., 2004; Kheradmand *et al*., 2006). Carbohydrates, protein and nucleic acid metabolism and their deficiency may for example impair spermatogenesis and libido in males (Smith and Akinbamijo, 2000; Alejandro *et al.,* 2002; Mitchell *et al.,* 2003; Kheradmand *et al*., 2006); while improved dietary intake of higher energy levels and protein supplementation in rams can improve the reproductive performance during the breeding season (Kheradmand *et al.,* 2006). The supplementation of vitamin B_{12} in semen extenders have been shown to significantly improved spermatozoa quality, viability, motility, progressive motility, normal spermatozoa and decreased morphological defects (Hamedani *et al*., 2013). Vitamin B_{12} deficiency has been reported to increase the number of abnormal sperm and decrease the motility and velocity of

sperm in male rats (Watanabe *et al*., 2003). Vitamin B_9 (folic acid) may also be vital for proper development of human sperm, as it is needed for the production of DNA (Wallock *et al*., 2001).

It has been suggested that in rams, vitamins B_1, B_6 and B_{12} play a key role in the thermoregulation of the scrotal skin and rectal temperature and help maintain libido, semen quality and fertility during heat stress (Hamedani *et al*., 2013). Azawi and Hussein (2013) also reported an increase in the viability of ram spermatozoa diluted in the Tris diluent containing vitamins C or E (stored at 5˚C, for 120 h).

2.3 The collection of the ram semen

For the collection of semen, which is the first phase, there are certain prerequisites (depending on the collection technique used) that have to be adhered to. Firstly it is essential to clean and shave the prepuce of the ram- to prevent any semen contamination during the semen collection process. Further the area where semen collection takes place must be sheltered and in a dust free environment. Thus harmful factors such as exposure to sunlight, dust and water (especially when using the artificial vagina) must be minimized during the semen collection period. Care should be taken that all equipment to be used are also clean and sterile. Generally there are two methods that are used for semen collection in rams; these are the use of the artificial vagina and/or the electrical ejaculator.

2.3.1 The artificial vagina

The artificial vagina (AV) is the most commonly used method of semen collection, as it gives an ejaculate similar to that obtained during natural mating. The clean, dry, artificial vagina is assembled and filled with hot water (45 to 50˚C), to create the required temperature and pressure. A cone with a calibrated semen collection tube is affixed to one end of the AV, while the lining at the open end is lubricated with some sterile petroleum jelly. An ewe (preferably in estrus) or a dummy ewe can be used for the semen collection. Generally sexually active rams display greater levels of investigatory olfactory behavior towards the stimulus females (Roselli and Stormshark, 2009). The temperament and libido of the ram is very important, especially during training of the rams to mount and adopt to ejaculate into the artificial vagina. To obtain good semen sample, the ram must not be allowed to mount the ewe immediately, in other words teasing the ram before mounting is important. During mounting, the ram's penis

is diverted into the artificial vagina for ejaculation. It is important to keep the semen collection tube warm (with the hand) during the collection process, so as to prevent cold shock to the ejaculate.

2.3.2 Electrical stimulation

The electro-ejaculator can be used for rams that are not trained or cannot mount an ewe due to an injury. In the case of electrical stimulation, the quality of the semen sample also depends largely on the efficient application of this technique. With electrical stimulation method, semen density is generally lower per unit volume as compared to the AV since it contains more seminal fluid or even urine. It was however documented by Hafez and Hafez. (2000) that electrical stimulation is a crude imitation of the complex natural mechanisms involved with ejaculate.

The mechanism by which the electro ejaculator operates is that a few electrical impulses (6 to 12V) applied rectally to the ram usually causes the ram to ejaculate. Since the ram exhibits strong contractions during the application of this technique, it is essential that the ram is firmly secured, thus labour intensive compared to collection using AV.

The electrode of the ejaculator is firstly lightly smeared with medicinal paraffin to facilitate the insertion of electrode into the ram's rectum and improve conductivity. The depth to which the electrode is inserted; the strength and duration of the stimuli (although varying from one individual to the next) are essential factors in other to obtain acceptable ejaculate. It is once again important to keep the semen as close as possible to the body temperature (38˚C), both during and after semen collection (Hafez and Hafez, 2000).

2.3.3 The principle involved in the use of the artificial vagina and electro-ejaculator

Ejaculation results from inherent reflex mechanisms, integrated in the sacral and lumbar regions of the spinal cord, and these mechanisms can be initiated by either psychic stimulation from the brain or actual sexual stimulation from the sex organs, but usually it is a combination of both (Guyton and Hall, 2010). Ewes in estrus however, sexually transmit stimulating olfactory cues that together with other sensory and behavioural signals, attract sexually interested rams (Roselli and Stormshak, 2009). The most important source of sensory nerve signals for initiating ejaculation is the glans penis. The glans contains a sensitive sensory end-organ that transmits impulses to the central nervous system. The action of mating on the glans

stimulates the sensory end-organs, and the sexual signals in turn pass through the pudendal nerve, through the sacral plexus to the sacral portion of the spinal cord and finally up the spinal cord to undefined areas in the brain. During natural service, the sensory nerve endings in the penile integument and the deeper penile tissues are essential for ejaculation (Hafez and Hafez, 2000). Impulses may also enter the spinal cord from areas adjacent to the penis to aid in the stimulation of ejaculation (Guyton and Hall, 2010). So for instance, stimulation of the rectal epithelium, the scrotum, and perineal structures in general may send signals into the spinal cord that add to the sexual sensation. Sexual sensations can even originate in internal structures, such as in areas of the urethra, bladder, prostate, seminal vesicles, testes, and vas deferens. Indeed, one of the causes of libido is filling of the sexual organs with secretions. The concern is that the rams should not be stressed, but rather be stimulated to provide semen that is similar to that obtained from natural mating.

Penile erection is the first sign of the male sexual stimulation, and the degree of erection is proportional to the degree of stimulation, whether psychic or physical (Guyton and Hall, 2010). This sexual stimulation then produces dilation of the arteries supplying the cavernous bodies of the penis (Fox, 2002). Stiffening and straightening of the penis in ruminants is caused by the ischiocavernosus muscle which pumps blood from the carvenous space of the crura into the rest of the corpus cavernosum of the penis (Hafez and Hafez, 2000). Erection is caused by parasympathetic impulses that pass from the sacral portion of the spinal cord through the pelvic nerves to the penis. During sexual stimulation, the parasympathetic impulses, in addition to promoting erection, cause the urethral glands and the bulbourethral glands to secrete mucus. This mucus then flows through the urethra during mating to aid in the lubrication. Most of the lubrication is provided by the female sexual organs, rather than by the male. Without satisfactory lubrication, the male ejaculation is seldom successful due to friction resulting in painful sensations that inhibit rather than excite sexual sensations (Fox, 2002).

According to Guyton and Hall (2011), when the sexual stimulus becomes extremely intense, the reflex centers of the spinal cord begin to emit sympathetic nerve impulses that leave the cord at the T-12 to L-2 vertebrae and pass to the genital organs through the hypogastric and pelvic sympathetic nerve plexuses to initiate emission, the forerunner of ejaculation. Emission begins with contraction of the vas deferens and the ampulla to cause expulsion of sperm into the internal urethra. Then contractions of the muscular coat of the prostate gland are followed by contraction of the seminal vesicles, expelling the prostatic and seminal fluid into the urethra

and forcing the sperm forward. All these fluids mix in the internal urethra with mucus already secreted by the bulbourethral glands to eventually form semen.

The filling of the internal urethra with semen elicits sensory signals that are transmitted through the pudendal nerves to the sacral regions of the spinal cord, giving the feeling of sudden fullness in the internal genital organs. Also, these sensory signals further excite rhythmical contraction of the internal genital organs and cause contraction of the ischio-cavernosus and bulbo-cavernosus muscles that compress the bases of the penile erectile tissue. These effects together cause rhythmical, wavelike increases in pressure in both the erectile tissue of the penis and the genital ducts and urethra, which then "ejaculate" the semen from the urethra to the exterior. At the same time, rhythmical contractions of the pelvic muscles and even some of the muscles of the body trunk cause thrusting movements of the pelvis and penis which also help propel the semen into the deepest recesses of the vagina and perhaps even slightly into the cervix. As documented by Hafez and Hafez (2000), ejaculation is the passage of the resultant semen along the penile urethra.

2.3.4 The hormonal principles

A major share of the control of sexual functions in both the male and the female begins with the secretion of gonadotropin-releasing hormone *(*GnRH*)* by the hypothalamus. This hormone in turn stimulates the anterior pituitary gland to secrete the gonadotropic hormones: luteinizing hormone *(*LH*)* and follicle-stimulating hormone *(*FSH*)*. LH being the primary stimulus for testosterone secretion by the testes, and FSH mainly stimulating spermatogenesis. According to Roselli and Stormshak (2009), the tonic secretion of testosterone by the testis activates the copulatory behavior in rams.

The secretion of LH by the anterior pituitary gland is also cyclic, with LH following the pulsatile release of GnRH. Conversely, FSH secretion increases and decreases only slightly with each fluctuation of GnRH secretion; instead, it changes more slowly, several hours in response to longer-term changes in GnRH. Due to the much closer relationship between GnRH secretion and LH secretion, GnRH is also widely known as LH-releasing hormone.

Testosterone is secreted by the interstitial cells of Leydig in the testes (Guyton and Hall, 2006), but only when stimulated by LH from the anterior pituitary. Furthermore, the testosterone quantity secreted increases approximately directly proportion to the amount of LH available. Testosterone secreted by the testes in response to LH has the reciprocal effect of inhibiting the

anterior pituitary secretion of LH. Most of this inhibition probably results from the effect of testosterone on the hypothalamus to decrease the secretion of GnRH. Thus, testosterone exerts a homeostatic effect on the hypothalamic-pituitary-gonadal axis through a negative feedback regulation of GnRH secretion (Scott *et al.,* 2004). Consequently, a decrease in the secretion of both LH and FSH by the anterior pituitary, and the decrease in LH will reduce testosterone secretion by the testes. Thus, whenever testosterone secretion becomes too high, this automatic negative feedback effect reduces testosterone secretion toward the desired operating circulating level. Conversely, when testosterone is too little, it allows the hypothalamus to secrete large amounts of GnRH, with a corresponding increase in anterior pituitary LH and FSH secretion and consequent increase in the testicular secretion of testosterone. FSH binds with specific FSH receptors attached to the Sertoli cells in the seminiferous tubules. This causes these cells to grow and secrete various spermatogenic substances. Similarly, the diffusing of testosterone into the seminiferous tubules from the Leydig cells in the interstitial spaces also has a strong trophic effect on spermatogenesis.

When the seminiferous tubules fail to produce sperm, secretion of FSH by the anterior pituitary gland also increases markedly. Conversely, when spermatogenesis proceeds too rapidly, pituitary secretion of FSH decrease. The cause of this negative feedback effect on the anterior pituitary is believed to be secretion of inhibin by the Sertoli cells (Guyton and Hall, 2010).

2.4 Evaluation of the semen

Sperm cells are known to be extremely sensitive to air, light, temperature fluctuations, metals, hypotonic or hypertonic liquids, soap, disinfectants, urine, perspiration and several other substances. It is therefore imperative to handle the semen as carefully as possible after collection (Hafez and Hafez, 2000). The microscopic and macroscopic examination of semen can then be done, based on the following:

2.4.1 Semen volume

Ram semen volume normally varies between 0.5 and 2.0 ml per ejaculate, depending on collection method used (Hafez and Hafez, 2000).

2.4.2 Colour and density of the semen

The normal colour varies from a thick, creamy substance to a clear watery sample, depending on the density. The colour is thus an indication of the density and also any abnormal colour indicates contamination of one kind or another. So for example, blood will cause a reddish-brown or pink colour pus cells will show up as grey or brown semen and urine as yellow or dilute semen (Hafez and Hafez, 2000).

Table 2.1 Relation of semen colour and number of sperm cells (density) per ejaculate in the ram. (Hafez and Hafez, 2000)

Colour	Number of cells ($\times 10^9$/ml)
Thick creamy (many)	5.0
Creamy	4.0
Thin creamy	3.0
Milky	2.0
Cloudy	0.7
Watery (clear)	insignificant

2.4.3 pH

The normal pH of ram semen varies between 6.4 and 6.7. Variations from these values could be an indication of an abnormality. So for example when the semen pH is too acidic – it could be due to too little prostate and seminal fluids or – if the semen is too alkaline – this is an indication of inflammation (Guyton and Hall, 2011).

2.4.4 Sperm motility and percentage live sperm

With a good semen sample, when the semen collection tube is held against the light, movement of the semen can be seen with the naked eye. For a more accurate evaluation of sperm motility, it is however necessary to examine the sample under the microscope (Hafez and Hafez, 2000). It is essential to examine the semen quality, before the semen is used. It could happen that sperm cells die because of cold shock or are negatively affected for some or other reason. This

makes it essential to examine a drop of semen under the microscope and evaluate the sperm quality.

Table 2.2 Relationship between sperm motility and the percentage live sperm in the ram (Zamiri *et al*., 2010)

5– Very strong progressive, dark waves (90% plus live cells)
4 – Strong progressive undulations (70 to 90% live cells)
3 – Weak undulations (50 to 70% live cells)
2 – Very few, weak, non-progressive undulations (25 to 50% live cells)
1 – No undulations (5 to 25% live cells)
0 – All cells dead

2.4.5 Semen smears

To carry out an examination for the percentage live/dead sperm cells and abnormalities in the semen, a smear can be made by adding a drop of eosin/nigrosin to a drop of semen on a microscope slide and examining the smear under the microscope (× 40 magnification). Dead sperm cells colour red while live sperm cells stay white and abnormalities such as loose heads, double heads, broken or curved neck and central bodies, as well as curled broken or double tails, become clearly visible. It is recommended to use only rams with less than 15% abnormal sperm. To determine the presence of bacteria or pus cells (infection), a drop of sperm can be stained using giemsa. The pus cells also stain light red (Hafez and Hafez, 2000).

2.5 Dilution of semen

Semen diluents that can be used generally to extend semen samples include pasteurized skim milk, glucose citrate or tris egg yolk. Dilution of semen is performed slowly, by gradually adding the diluents drop by drop, mixing it and keeping the mixture close to body temperature. These diluents serve as buffers, nutritional media and as protectants for the semen. Care must be taken that the semen does not undergo cold shock during dilution. Cold shock reduces the membrane permeability to water and solutes, while damaging the acrosomal membrane (Purdy,

2006). It was documented by Marco *et al.* (2005) that only semen with motility score of 4 and higher can be diluted. By using animal derived additives such as milk or egg yolk in a semen extender generally implies sanitary risks, not only through the inclusion of specific microbiological agents, but also by contaminants that may compromise the quality of semen. An alternative to egg yolk in extenders for ram semen may be soybean lecithin (Gil *et al.,* 2003; Forouzanfar *et al.*, 2010). Many possible disadvantages of using egg yolk for example bacterial contamination and variability in sperm survival have been outlined (Aires *et al*., 2003; Amirat *et al*., 2004; Fukui *et al.*, 2008). It was however documented by Munyai (2012) that skim milk and egg yolk are generally good sources of lipids which generally provide protection of the sperm membranes to temperature changes. According to Valente *et al.* (2010), egg yolk as a supplement is difficult to be replaced in ram semen extenders.

Since 50 to 60 million live sperms are required per insemination, the density of the ejaculate determines the number of ewes that can be inseminated per ejaculate and also determines the volume of the insemination dose that must be used. This volume generally varies between 0.05 and 2ml (Hafez and Hafez, 2000).

2.6 Storage of the semen

2.6.1 Liquid semen

A wide variety of diluents can be used for storing liquid semen, for example tris-fructose egg yolk, pasteurized skim milk-egg yolk, glucose citrate-egg yolk and sodium citrate. As documented by Forouzanfar *et al*. (2010), an extender should contain an energy source (glucose or fructose). Agents that comprise good extending media should be able to provide nutrients as source of energy, protect against harmful effect of rapid cooling, provide a buffer to prevent harmful shifts in pH as lactic acid is formed, maintain the proper osmotic pressure and electrolyte balance, inhibit bacterial growth and protect sperm cells during freezing (Hafez and Hafez, 2000). Antibiotics are also frequently added to the semen diluent for the purpose of destroying any possible micro-organisms, while glycerol serves as a cryoprotective agent in the semen freezing process (Munyai, 2012). The cryoprotectant prevents the crystallization of water within the sperm cells, which ultimately allows the sperm cells to be frozen rapidly (Holt, 2000; Munyai, 2012). The best results have been obtained when storing liquid semen by adding 500 to 1000 µg streptomycin to 1ml of diluted semen. Marco (2005) reported that

diluted semen can be stored successfully for up to 3 days at a temperature of 2 to 5˚C, or for 24 hrs at 18 to 20˚C.

However this method of liquid semen preservation is not very desirable as fertility is generally lower than with fresh, diluted semen. It is still a sound practice to collect semen when it is required (Hafez and Hafez, 2000).

2.6.2 Frozen semen

Cryopreservation as a technique for the long term storage of semen has many advantages, but the freezing and thawing processes generally induce detrimental effects in terms of ultra-structural, biochemical and functional sperm damage (Watson, 2000; Munyai, 2012). Resulting in a decrease of sperm motility, membrane integrity and fertilizing ability (Purdy, 2006; Munyai, 2012) Semen cryopreservation induces the formation of intracellular ice crystals, osmotic and chilling injury that gives rise to sperm damage like cytoplasmic fractures, an effect on the cytoskeleton and genome related structures (Isachenko, 2003). The membrane permeability is increased after cooling, and may be a consequence of increased membrane leakiness and specific protein channels. Cooling also affects calcium regulation and this has severe consequences on the cellular function, including cell death (Munyai, 2012). When proper cooling procedures are used, these consequences can be avoided. The use of programmable freezer have been reported with better sperm motility (Hammadeh *et al.,* 2001; Clulow *et al.,* 2008) and with less cryo-damage to the sperm cell (Petyim and Choavaratana, 2006).

There are generally two ways of preserving frozen ram semen, namely pellets or straws. In pellet preservation, the diluents tris-fructose-egg yolk- glycerol or raffi-nose-egg yolk-glycerol or lactose-egg yolk-glycerol are used. Egg yolk being the main component in the extenders for storage and cryopreservation of semen in most mammalian species, including bull, ram goat, pig and even human semen (Forouzanfar *et al.,* 2010). Egg yolk has been shown to have beneficial effects on sperm cryopreservation survival as a protector of the sperm plasma membrane and acrosome against temperature related injury, in association with other components because of the lipids that it contains (Purdy, 2006). Glycerol plays a vital role as a protectant during freezing. Gil *et al*. (2003) reported that glycerol, despite its value as a cryoprotectant, is metabolically toxic to spermatozoa and noxious to membrane integrity depending on the concentration and the temperature at which it is added. Thus calling for a compromise if optimal results are to be achieved. The toxicity of glycerol thus limits the use

of high concentrations of glycerol in the cryopreservation media (Forouzanfar *et al.,* 2010). Sodium citrate, milk based semen extenders (non-fatty milk powder and distilled water) and tris-based extenders, including egg yolk were for example used by Paulenz *et al.* (2002) and sperm viability parameters were shown to be influenced by storage time and the extender. Valente *et al.* (2010) noted that the semen extender composition has a major effect on post-thawed sperm viability. Tris-egg yolk based diluents have been reported to provide adequate cryoprotection (Salamon and Maxwell, 2000; Valente *et al.,* 2010).

In a frozen form, semen can then be stored in liquid nitrogen (- 196˚C) indefinitely. When required, it can be thawed at a temperature of 38 or 60 or 70˚C depending on the freezing method, for a period of approximately 45 seconds, where after, it is placed in the water bath at 32˚C. The dose used for AI using frozen ram semen varies between 0.1 and 0.2 ml with a density of 150 to 180 million live sperms per insemination (Hafez and Hafez, 2000).

When inseminating using frozen semen, it is desirable to keep the sperm numbers that are being inseminated as high as possible, since sperm viability decreases rapidly after thawing. At present, the conception rate achieved with frozen semen (following cervical insemination) in sheep is low and use is made of the technique of intra-uterine insemination with the aid of the laparoscopy. In this technique, semen is directly deposited in the uterine horns to significantly enhance the chances of conception, even though less sperm are inseminated with this technique. Suitable semen extenders to preserve an adequate number of spermatozoa with all the attributes needed to overcome the cervical barrier and to improve fertilization post thawing are needed to achieve these goals (Gil *et al*., 2003). This is enunciated by the recent work in that a slight decrease of post thaw ram sperm abnormalities are achieved with the use of high levels of sugars and extenders containing trehalose and raffinose (Jafaroghli *et al*., 2011). So it has been confirmed that it is possible for ram semen to be successfully cryopreserved in straws (Sabev *et al*., 2006; García-Álvarez *et al*., 2009b) and so for example lot of research has been done on ram semen preservation with good results (Gil *et al.* 2000; Holt, 2000; Salamon and Maxwell. 2000; Gil *et al*., 2003; Marco-Jiménez *et al*., 2005; Pereira *et al*., 2009; Nur *et al*., 2010; Valente *et al*., 2010; Moustacas *et al*., 2011).

2.6.2.1 Semen freezing procedures

Resent work seems to have incorporated different number of ram semen freezing protocols. The examples of protocols were as follows:

Protocol 5 ˚C (P5 ˚C) (Gil *et al*., 2003)

1. Cooling to 5˚C within 60 min in the waterbath.
2. Extend semen at 5˚C with f/2 of each extender to 0.8×10^9cells/ml. (Where f/2 refers to two fractions of glycerol)
3. Equilibration at 5˚C for 2 hr.
4. Packaging in 0.25ml mini straws at 5˚C.
5. Freezing.

Protocol 15 ˚C (P15 ˚C) (Gil *et al*., 2003)

1. Cooling to 15 ˚C within 30 min.
2. Extend semen at 15 ˚C with f/2 of each extender to 0.8×10^9 cells/ml. (Where f/2 refers to two fractions of glycerol)
3. Further cooling to 5 ˚C within 30 min in a waterbath.
4. Equilibration at 5 ˚C for 1.5 hr.
5. Packaging in 0.25ml mini-straws at 5 ˚C.
6. Freezing.

According to Gil *et al.* (2000) the freezing protocols were as follows:

Protocol 1:

Centrifugation before filling the straws to re-concentrate the diluted semen to a calculated sperm concentration of 800×10^6 sperm/ml.

1. Further dilution (22 ˚C) to a final ratio of 1+4 semen/diluents using fraction 1 of the extender (without the cryoprotectant).
2. Cooling to 5˚C within 1 hr.
3. A second dilution at 5˚C to double the volume with fraction 2 of the extender (extender with the cryoprotectant)
4. Equilibration at 5˚C for 2 hr.
5. Centrifugation at 5˚C (700g/10 min).

6. Removing supernatant to yield a calculated final sperm concentration of 0.8×10^9 sperm/ml.
7. Packaging in 0.25ml mini-straws at 5˚C.
8. Freezing.

Protocol 2:

Involved an appropriate ejaculate extension to yield 800×10^6 sperm/ml

1. Further dilution (22 ˚C) to 1.6×10^9 cells/ml with diluents fraction 1.
2. Cooling to 5˚C within 1 hr.
3. A second dilution at 5˚C to 0.8×10^9 sperm/ml with diluents fraction 2.
4. Cooling to 5˚C within 1 hr.
5. Packaging in 0.25ml mini-straws at 5 ˚C.
6. Freezing.

2.6.3 Cryoprotective agents

Cryoprotectants are used in the cryopreservation medium to reduce the physical and chemical stresses derived from cooling, freezing and thawing on the sperm cells (Purdy, 2006). Cryoprotectants can be classified as penetrating or non-penetrating agents, where the penetrating agents or intracellular cryoprotectants including glycerol, dimethyl sulphoxide, ethylene glycol and propylene glycol have low molecular weights and induce membrane lipid and protein re-arrangement, resulting in increased membrane fluidity, greater dehydration at low temperatures, reduced intracellular ice formation and an increased sperm survival rate to cryopreservation (Holt, 2000). It has been suggested that intracellular ice formation in the sperm cell is one of the major detrimental factors that reduce the viability and membrane integrity of frozen thawed sperm (Jafaroghli *et al*., 2011). Purdy, (2006) reported intracellular cryoprotectants to be solvents that normally dissolve sugars and salt in the cryopreservation medium.

Ram semen has been proven to be more difficult to cryopreserve than the semen of other farm animals (Nur *et al*., 2010). Despite advances in the cryopreservation of mammalian spermatozoa, there has been less success in ram spermatozoa cryopreservation than with bull spermatozoa (Varisli *et al*., 2008). The basis of the cryoprotective properties of glycerol is not completely understood (Aires *et al*., 2003). It has been documented by Nur *et al*. (2010) that the presence of glycerol lowers the quality and fertilizing capacity of semen. However, as

mentioned earlier, the use of cryoprotactants is obligatory for maintaining the post-thaw cryosurvival of ram semen.

2.7 *In vitro* fertilization

In the natural situations, the female reproductive tract is an excellent environment, not only for fertilization, but also for development and maturation of the fertilized ovum. For these to happen, a number of reproductive physiological activities are of vehement importance.

Reproduction begins with the development of the ova in the ovaries. In the middle of each sexual cycle, a single ovum is expelled from an ovarian follicle (ovulation) into the abdominal cavity near the open fimbriated ends of the two fallopian tubes of an ewe. This ovum then passes through one of the fallopian tubes into the uterus; if it has been fertilized by a sperm cell in the isthmic junction of infundibulum, it gets implanted in the uterus (Fox, 2002), where further development into a fetus will take place (Ganong, 2005; Costanzo, 2006).

During the fetal life, the outer surface of the ovary is covered by a germinal epithelium, which embryologically is derived from the epithelium of the germinal ridges. As the female fetus develops, primordial ova differentiate from this germinal epithelium and migrate into the substance of the ovarian cortex (Guyton and Hall, 2010). Each ovum then congregates around it a layer of spindle cells from the ovarian stroma (the supporting tissue of the ovary) and causes them to take on epithelioid characteristics, these cells are then called granulosa cells. The ovum surrounded by a single layer of granulosa cells is called a primordial follicle. The ovum itself at this stage is still immature, requiring two more cell divisions before it can be fertilized. At this time, the ovum is called a primary oocyte (Fox, 2013). During all the reproductive years of adult life, the primordial follicles develop enough to expel their ovum each sexual cycle; the remainder degenerate or become atretic. At the end of reproductive capability, only a few primordial follicles remain in the ovaries, and even these degenerate soon thereafter. All these activities are regulated hormonally (Fox, 2002).

Gonadotrophic hormones are secreted from anterior pituitary gland (Costanzo, 2006; Fox, 2013). Secretion of most of these anterior pituitary hormones is controlled by gonadotropin releasing hormones (GnRH) formed in the hypothalamus and then transported to the anterior pituitary gland by way of the hypothalamic-hypophysial portal system (Ganong, 2005; Guyton

and Hall, 2010). The gonadotrophic hormones are luteinizing hormone (LH) and follicular stimulating hormone (FSH).

Estrogen which is secreted by the follicles in small amounts has a strong effect to inhibit the production of both LH and FSH (Fox, 2013). Even when there is an availability of progesterone (secreted by ovaries), the inhibitory effect of estrogen is multiplied, even though by itself progesterone has little effect. These feedback effects seem to operate mainly on the anterior pituitary gland directly, but also operate to a lesser extent on the hypothalamus to decrease the secretion of GnRH, especially by altering the frequency of the GnRH pulses (Fox, 2002; Guyton and Hall, 2010; Fox, 2013).

In addition to the feedback effects of progesterone and estrogen, other hormones are also involved. So for example inhibin is secreted, along with the steroid sex hormones, by the granulosa cells of the ovarian graafian follicle in the same way that the Sertoli cells secrete inhibin in the male testes (Fox, 2006). Inhibin hormone has the same effect in the female as in the male, inhibiting the secretion of FSH and, to a lesser extent LH by the anterior pituitary gland (Fox, 2002; Fox, 2006; Guyton and Hall, 2010; Fox, 2013). Therefore, it is believed that inhibin may be especially important in causing the decrease in secretion of FSH and LH at the end of the female sexual cycle.

2.8 The fertilization process

Fertilization of the ovum normally takes place in the isthmic junction of the infundibulum of one of the fallopian tubes, soon after both the sperm and the ovum reach the site (Ganong, 2005; Costanzo, 2006; Guyton and Hall, 2010). However before a sperm can penetrate the ovum, it must first penetrate the multiple layers of granulosa cells attached to the outside of the ovum (the corona radiate) and then bind to and penetrate the zona pellucida surrounding the ovum proper (Fox, 2002; Ganong, 2005; Fox, 2006; Berne and Levy, 2008; Guyton and Hall, 2010; Fox, 2013).

Once a sperm has entered the ovum, the oocyte divides again to form the mature ovum plus a second polar body that is expelled. The mature ovum still carries 23 chromosomes in its nucleus.

In the meantime, the morphology of fertilizing sperm has also changed. On entering the ovum, the head swells to form a male pro-nucleus. Later, the 23 unpaired chromosomes of the male pro-nucleus and the 23 unpaired chromosomes of the female pro-nucleus align themselves to re-form a complete complement of 46 chromosomes (23 pairs) in the fertilized ovum (Guyton and Hall, 2010).

2.9 Sperm capacitation

There are certain natural changes that spermatozoa must undergo (capacitation) so as to fertilize the ovum. Although spermatozoa are said to be mature when they leave the epididymis, their activity is held in check by multiple inhibitory factors secreted by the genital duct epithelia. Therefore, when sperm are first expelled in the semen, they are unable to penetrate the ovum. However, on coming in contact with the fluids of the female genital tract, multiple changes occur that activate the sperm for the final processes of fertilization. These collective changes are called capacitation of the spermatozoa. According to Guyton and Hall (2010) some changes that are believed to occur are as follows

1. The uterine and fallopian tube fluids wash away the various inhibitory factors that suppress sperm activity in the male genital ducts.
2. While the spermatozoa remain in the fluid of the male genital ducts, they are continually exposed to many floating vesicles from the seminiferous tubules containing large amounts of cholesterol. This cholesterol is continually added to the cellular membrane covering the sperm acrosome, toughening this membrane and preventing release of its enzymes. After ejaculation, the sperm deposited in the vagina swim away from the cholesterol vesicles upward into the uterine cavity, and gradually lose much of their other excess cholesterol over the next few hours. In so doing, the membrane at the head of the sperm (the acrosome) becomes much weaker.
3. The membrane of the sperm also becomes much more permeable to calcium ions, so that calcium now enters the sperm in abundance and changes the activity of the flagellum, giving it a powerful whiplash motion in contrast to its previously weak undulating motion. In addition, the calcium ions cause changes in the cellular membrane that covers the leading edge of the acrosome, making it possible for the

acrosome to release its enzymes rapidly and easily as the sperm penetrates the granulosa cell mass surrounding the ovum and even more so as it attempts to penetrate the zona pellucida of the ovum itself. Thus, multiple changes occur during the process of capacitation. Without these, the sperm cannot make its way to the interior of the ovum and induce fertilization.

Stored in the acrosome of the sperm are large quantities of hyaluronidase and proteolytic enzymes. Hyaluronidase depolymerizes the hyaluronic acid polymers in the intercellular cement that hold the ovarian granulosa cells together (Fox, 2002; Fox, 2006; Guyton and Hall, 2010; Fox, 2013). The proteolytic enzymes digest the proteins in the structural elements of tissue cells that still adhere to the ovum. When the ovum is expelled from the ovarian follicle into the fallopian tube, it still carries with it multiple layers of granulosa cells. However, before a sperm can fertilize the ovum, it must dissolve these granulosa cell layers, and then penetrate through the thick covering of the ovum itself, the zona pellucida (Fox, 2002; Fox, 2006; Berne and Levy, 2008; Guyton and Hall, 2010; Fox, 2013). To achieve this, the stored enzymes in the acrosome begin to be released. It is believed that of these enzymes hyaluronidase is the most important in opening pathways between the granulosa cells so that the sperm can reach the ovum.

When the sperm reaches the zona pellucida of the ovum, the anterior membrane of the sperm itself binds specifically with receptor proteins in the zona pellucida (Ganong, 2005; Berne and Levy, 2008; Guyton and Hall, 2010). Then, rapidly the entire acrosome dissolves, and all the acrosomal enzymes are released (Ganong, 2005). Within minutes, these enzymes open a penetrating pathway for passage of the sperm head through the zona pellucida to the inside of the ovum. Within another few minutes, the cell membranes of the sperm head and of the oocyte fuse with each other to form a single cell (Fox, 2002; Fox, 2006; Guyton and Hall, 2010; Fox, 2013). At the same time, the genetic material of the sperm and the oocyte combine to form a completely new cell genome, containing equal numbers of chromosomes and genes from ewe and ram as discussed earlier. Thereafter the embryo begins to divide and develop.

The analysis of semen parameters cannot confirm the quality of semen with certainty, as one cannot be sure whether the sperm will fertilize or not (Mocé and Graham, 2008). Some semen evaluation techniques such as sperm binding, oocyte penetration, and *in vitro* fertilization can estimate functional aspects of the spermatozoa (Flowers, 2009). Following the work of Hafez and Hafez. (2000), classic semen evaluation techniques, as well as new automated semen

assessment technologies have yielded only estimates of the potential fertilizing capacity of spermatozoa. In addition, Petrunkina *et al*. (2007) proposed several criteria for the assessment of functional sperm parameters regarding capacitating ability. It has been suggested that the type of semen extender affects the *in vitro* fertilization potential, while it has no effect on the developmental potential up to the blastocyst stage (Forouzanfar *et al*., 2010). To date, reports arising from research has revealed the role of sperm cryopreservation for both *in vivo* and *in vitro* production of human and animal embryos (Byme *et al*., 2000; Yildiz *et al*., 2007; Forouzanfar *et al*., 2010). So for example, in sheep lot of research on sperm cryopreservation for *in vitro* embryo production has been done successfully (García-Álvarez *et al*., 2009a; García-Álvarez *et al*., 2009b; Periera *et al*., 2009; Valente *et al*., 2009; Forouzanfar *et al*., 2010; Valente *et al*., 2010).

For *in vitro* fertilization to take place, a number of processes are involved. Oocytes have to be collected washed and matured. Oocyte collection is usually performed by the isolation of follicles, dissection, aspiration or slicing of follicles or oviductal flushing (Gordon, 2003). Aspiration as such entails that all visible ovarian follicles are aspirated using a hypodermic needle, attached to a disposable syringe. The slicing technique involves the use of a surgical blade, making an incision over the entire ovarian surface into a Petri dish containing a harvesting medium (Wani *et al*., 2000; Wang *et al*., 2007). Dissection again is the method of dissecting the intact antral follicles with a subsequent carefully controlled rapture to recover morphologically acceptable oocytes with the least disruption to the surrounding cumulus cells. Laparoscopic ovum pick-up is another method of recovering the oocytes but from ovaries of live animals by aspiration through the use of laparoscope (Gordon, 2003).

The follicular oocytes are commonly recovered from ovaries of slaughtered animals (Wani, 2002) and are thereby heterogeneous in quality and developmental competence (Rodriguez-Gonzalez *et al.,* 2002; Gordon, 2003; Bhojwani *et al.,* 2007). Compact cumulus oocyte complexes (COC's) are then often recovered from slaughterhouse collected bovine ovaries (Bhojwani *et al.,* 2007). Oocytes characterized by a decreased developmental competence have a low ability to reach the embryonic stage and their development is generally disturbed (Nemcova *et al.,* 2006; Petro *et al.,* 2012).

According to Thibier (2004), with *in vitro* embryo production, one of the main biohazards concerning embryos is the repeatability in the donor female and the collection method of oocytes used, followed by maturation, fertilization and embryo development. The method of

COC recovery may influence the degree of heterogeneity of the recovered oocytes (Bhojwani *et al.,* 2007) and can also affect the efficiency of IVEP (Katska-Ksiazkiewicz *et al*., 2007). From the slicing method of oocyte collection from ovaries, COCs may be recovered not only from the antral follicles on the surface, but also from smaller antral follicles from the inside of the ovary, which may be in the earlier stages of follicular development after antrum formation (Bhojwani *et al.,* 2007) while the follicle aspiration method is a preventive sanitary measure in the *in vitro* embryo production process (Marius *et al.,* 2009). However, slicing is the most useful technique of oocyte collection, even though it does not allow for selection according to follicular diameter (Rodriguez-Gonzalez *et al.,* 2002). Following the work of Yang *et al.* (2012), a person should use caution when relying on a particular size of follicle to predict oocyte maturity. Slicing or aspiration has then generally been used for oocytes recovery in slaughtered sheep (Wani *et al*., 2000).

Higher quality oocytes percentages have been reported (80.5 ± 2.4% and 79.8 ± 1.9%) when using the aspiration method as opposed to lower quality oocytes percentages (42.7 ± 2.3% and 41.2 ± 4.8%) for the slicing method (Marius *et al.,* 2009). It should however be noted that several researchers from different countries such as Dublin, Denmark, Itally, Russia, USA, India and Spain have documented high yield of oocytes when using the slicing technique (Gordon, 2003).

Reports regarding ovum pick-up (OPU) has suggested that the technique is of high value more in India, where cow slaughter is banned for religious reasons, and there is no opportunity for researchers to obtain oocytes from the abattoir bovine ovaries, as in most other countries (Gordon, 2003). It was demonstrated by Manik *et al.* (2002), that the use of OPU is useful in recovering developmentally competent oocytes. In cattle, a great number and better quality oocytes were recovered from Gir cows (Sales *et al.,* 2010) and a high blastocyst rate obtained (Snjiders *et al.,* 2000; Blondin *et al.,* 2002; Rizos *et al.,* 2005). Lonergan (2010) suggested that one way of experimentally separating potential issues surrounding the follicle and/or oocytes for issues relating to the reproductive tract environment, is to use transvaginal ovum pick-up, coupled with IVF. However, it has been reported that certain factors such as the energy deficiency and lactation period have negative effects on oocyte quality and endocrine levels, when using OPU (Gwazdauskas *et al.,* 2000; Walters *et al.,* 2002; Leroy *et al.,* 2005).

Studies done in women demonstrated a higher live birth rate when mature oocytes were obtained at the time of the retrieval for the natural cycle of IVF/M treatment and also indicated

that there may be a higher incidence of miscarriage rate when the transferred embryos were produced from immature oocytes only (Yang *et al.,* 2012). In addition, in pigs, blastocyst development was improved by the use of oocytes from sows rather than from pre-pubertal gilts (Marchal *et al.,* 2001; Sherrer *et al.,* 2004). Embryos collected from pre-pubertal gilts, which were induced with exogenous hormones, were less likely to form blastocysts *in vitro* than embryos collected from natural pre-pubertal gilts (Peters *et al.,* 2001). Sow oocytes also recorded a higher maturation rate and a lower sperm penetration rate than gilt oocytes (Marchal *et al.,* 2001).

Studies of bovine and ovine *in vitro* produced embryos indicated limited success when pre-pubertal females provide oocytes (Sherrer *et al.,* 2004). However the staining of cumulus-oocytes complexes using brilliant cresyl blue (BCB) stain before *in vitro* maturation could be used to select developmentally competent oocytes for *in vitro* embryo production and even for nuclear transfer (Rodriguez-Gonzalez *et al.,* 2002; Bhojwani *et al.,* 2007; Piotrowaska *et al.,* 2013).

2.10 Factors affecting oocyte quality

A matter of great importance in attempting to produce animal embryos in the laboratory is the ability of oocytes to undergo fertilization and develop into an embryo and this ability must extent all the way through to the transfer and birth of a live animal. Determination of oocyte quality is generally performed by assessing the ability of oocytes to mature, to be fertilized and give rise to normal offspring (Duranthon & Renard, 2001; Hussein *et al.,* 2006; Sirard *et al.,* 2006). The utilization of the recovered oocytes must be for the production of viable embryos, without reducing their developmental competence (Wani *et al*., 2000; Shirazi *et al*., 2005; Morton *et al*., 2008). There are however several factors that affect oocyte quality such as the maturation process and the medium used, age of the animal, ovarian follicular size, stage of the estrous cycle and ovarian morphology, body condition and nutritional status, reproductive status of the donor and environmental factors (Gordon, 2003).

2.10.1 Maturation process and media used

The maturation of mammalian COC's is an important step in reaching the full developmental competence (Grupen and Armstrong, 2010; Jaskowski *et al.,* 2010). The condition of maturation *in vitro* must mimic the *in vivo* environment (Piotrowaska *et al.,* 2013). The

concentration and composition of the compounds which supplement the culture medium play a significant role in the achievement of full developmental competence by oocytes (Herrick *et al.,* 2004; Kim *et al.,* 2005; Song *et al.,* 2010; Piotrowaska *et al.,* 2013).

2.10.2 Age of the donor

The developmental competence of oocytes is determined by age of the donor. To carry out experiments in sheep, cattle, goats and pig embryo production, greater attention is given to pre-pubertal animals as the size of their oocytes have a marked influence on their ability to mature to the metaphase-II phase. A progressive decline in human fertility with advancing age is a well-known phenomenon (Gordon, 2003). In cattle, research has suggested that the big problem with oocytes from ovaries of pre-pubertal cows is associated with developmental competence (Gandolfi *et al.,* 2000; Maclellan *et al.,* 2000; Majerus *et al.,* 2000; Salamone *et al.,* 2000; Taneja *et al.,* 2000; Salamone *et al.,* 2001; Bhojwani *et al.,* 2007). A similar problem has been reported in the pig (Kikuchi *et al.,* 2009), buffalo (Yindee *et al.,* 2011), monkeys (Zheng *et al.,* 2001), as well as in sheep (Catt, 2002; Kochhar *et al.,* 2002).

2.10.3 Ovarian follicular size

Utilizing the growth phase of the first follicular wave for oocyte collection has been reported to have improved the efficiency of blastocyst production (Machatkova *et al.,* 2000). Moreover, six complementary DNA clones were identified as being differentially expressed in the dominant follicles - when suppressive subtractive hybridization was used to investigate the differential expression of the genes in dominant and subordinate follicles on day 1.3 to 2.3 of the animal's oestrous cycle (Sisco *et al*., 2002).

2.10.4 Body condition and nutritional status

Oocytes derived from the ovaries of animals with a lower body condition score (BCS) are generally of poor quality. Nutritional status has been reported to be correlated with embryo survival and is a key factor influencing efficiency in assisted reproductive technologies (Armstrong *et al.,* 2003; Webb *et al.,* 2004). The under nutrition of donor ewes in an IVEP programme, resulting in lower body weights and a lower body condition score, has a negative effect on the oocyte quality, which results in a lower cleavage rate and blastocyst formation (Borowczyk *et al.,* 2006). Nutrition thus has a significant impact on numerous reproductive functions, including hormone production, fertilization and early embryonic development (Boland *et al.,* 2001; Armstrong *et al.,* 2003; Boland and Lonergan, 2005). In sheep, conflicting

results have been reported for the effect of low or high energy diets on oocytes quality and early embryonic development (Boland *et al.,* 2001; Papadopoulos *et al.,* 2001). In cattle, increased systemic levels of ammonia and urea have been unlikely to disrupt the oviduct environment to a point where embryo survival would be impaired when the levels of ions and metabolites in bovine oviductal fluids were quantified (Kenny *et al.,* 2002). Greater competence has been documented in oocytes of dairy cows with a high body condition score, compared to those with a low score. A greater rate of follicle development was reported for cows with a more positive energy balance (Gordon, 2003). The effects of nutrition on oocyte and embryonic development may thus reflect the general energy balance, but may also be attributed to the specific nutrients in diets such as vitamins, minerals and other supplements (Wrenzycki *et al.,* 2000).

2.10.5 Morphology of the ovaries

The intra ovarian environment to which oocytes are exposed can play a major role in determining their developmental competence. The presence of a dominant follicle in either one or both ovaries has a negative effect on the competence of ovaries. Ovarian development of Greenland halibut last for more than one year (Albert *et al.,* 2001; Junquera *et al.,* 2003) as they spawn annually but successive cohorts of oocytes develop over 2 years (Kennedy *et al*., 2011).

2.10.6 Environmental factors and IVEP

It has been reported that the removal of impaired cohorts of follicles during autumn has led to the earlier emergence of healthy follicles and higher quality oocytes (Roth *et al.,* 2001). The adverse effect of elevated summer temperatures on oocyte developmental competence was also previously reported in cattle (AI-Katanani *et al.,* 2002).

2.10.7 Reproductive status of the animal and oocyte competence

The reproductive status of the ewes has been assumed to be a constant variable during the collection of ovaries at the slaughter house. It was however documented by several studies that puberty, parturition and lactational status, as well as weaning contribute and have an impact on the quality of the ovaries and as a result affect the quality of the oocytes. Follicular development is inhibited during lactation and weaning allows the recruitment and selection of follicles that will undergo pre ovulatory maturation and ovulation (Quesnel, 2009). It has been further documented by Quesnel (2009) that lactation inhibits LH secretion by inhibiting gonadotrophin

releasing hormone secretion and reducing the pituitary response to gonadotrophin releasing hormones. However as lactation progress, there is an increase in LH secretion, which allows the resumption of folliculogenesis. Severe restriction in crude protein or digestible energy has been shown to delay the post-weaning oestrus, through impairment of gonadotrophin releasing hormone and LH secretion during lactation (Yang *et al*., 2000a). Feed or protein restriction has also been shown to impair the ability of oocytes to be fertilized and develop into an embryo (Yang *et al*., 2000b). Thus the developmental stage of the follicles from ovaries of post weaned ewes would be questionable if reproductive status is not assumed to be a constant variable. However, suggestions have indicated that seasonal anoestrus involves an increased sensitivity of the hypothalamus to the oestradiol negative feedback, while high feed intake can modify the sensitivity of the hypothalamus to oestradiol, considering that ewes are seasonal breeders (Forcada and Abecia, 2006).

Before puberty, the secretion of sex hormones is very low as documented. Guyton and Hall (2010) reported that the hypothalamus does not secrete significant quantities of gonadotrophic releasing hormones. Several studies in humans have indicated problems when the sex hormones happen to be secreted abnormally (hypogonadism or hypergonadism) by the ovaries (Martini and Nath, 2009; Guyton and Hall, 2011; Martini *et al*., 2012). In hypogonadism, ovarian cycle will not occur normally (Guyton and Hall, 2011) adults cannot produce functional oocytes (Martini and Nath, 2009; Martini *et al*., 2012), while in hypergonadism, a granulosa cell tumor develops in the ovary (Guyton and Hall, 2011).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Area

All trials were conducted at the University of the Free State campus, in Bloemfontein, South Africa. The study site is located 28.57˚ south longitude, 25.89˚ east latitude and at an altitude of 1304 m above sea level. The trial was carried out from April (autumn) 2014 to April (autumn) 2015. The experimental animals were kept at the metabolic building of the university which is under the management and supervision of the Department of Animal, Wildlife and Grassland Sciences. The metabolic building was designed specifically for the handling of research animals (small stock, pigs and poultry). The *in vitro* fertilization study was conducted at the Reproduction Physiology Laboratory and the project was approved by the University of the Free State' Animal Ethical Committee.

3.2 Experimental animals

Two breeds of rams (Dorper and South African Mutton Merino) were used in the study.

3.2.1 Dorper rams

The Dorper breed originated from the successful crossing of the British Dorset ram and Black head Persian ewe in 1936 by the Meat Control Board at the Grootfontein College of Agriculture, Middelburg, South Africa. The Dorper is large, well conformed mutton sheep with a white or black head and a white body. The breed has an extended breeding season, which is strictly not seasonally limited. The ewes can produce lambs at almost any time of the year and attain a lambing rate of 150% per season under optimum grazing conditions. Dorper ewes produce large quantities of milk and have a good mothering ability. The breed is well adapted to a variety of climatic grazing conditions, although the breed was originally developed for the more arid areas of South Africa. However, today the Dorper breed is widespread throughout all provinces of South Africa and abroad. The Dorper thus performs well under various pasture and feeding conditions and also reacts most favourably under intensive feeding conditions (De

Waal and Combrinck, 2000). The plate below shows Dorper ram which was used as semen donor.

Plate 3.1 Dorper ram used as a semen donor

3.2.2 South African Mutton Merino rams

This breed was imported to South Africa from Germany in 1932 by the Department of Agriculture as part of a breeding programme. The breed was originally known as the German Mutton Merino. Through selection for a better wool quality and body conformation, the uniqueness of the South African breed was recognized in 1971 when the breed name was changed to the South African Mutton Merino (SAMM). This Mutton Merino is generally a dual purpose breed, developed to produce a slaughter lamb at an early age, as well as relatively good quality wool. The breed is renowned for its fertility, whereby lambing percentages of 150% and even higher has been obtained with mature ewes weighing 77 kg and rams weighing 127 kg (Buduram, 2004). The Mutton Merino has a renowned mothering ability and rears multiple births with high weaning weights. The SAMM also have a high milk production, enabling lambs to maintain a high growth rate and weaning weight for early maturity and hence early marketing. The breed is generally efficient feed converter and popular in feedlots. It is non-

selective in its grazing habits, and is able to utilize low quality roughage. Its grazing habits limit energy consumption and better utilization efficiency is maintained allowing for increased mutton and wool production. The South African Mutton Merino is able to perform under most climatic conditions (Buduram, 2004). The plate below shows South African Mutton Merino ram which was used as a semen donor.

Plate 3.2 South African Mutton Merino ram used as a semen donor

3.3 Management of experimental animals

Upon arrival, all eight adult rams recording mean body weight of 69.6 ± 9.2 kg and 92.5 ± 6.1 kg and mean age of 54 ± 4.7 months and 36 ± 2.1 months (Dorper and SAMM respectively) were allowed two weeks to recover and adapt before any activities were performed. Fresh water was provided *ad libitum* and fresh feed in the form of Lucerne was also provided. Vaccination was done in advance for the prevention of pulpy kidney, pasteurella, tetanus, malignant oedema or blackquarter. Plate 3.3 below shows how cleaning was done.

Plate 3.3 Cleaning process to ensure a healthy environment for the rams

Of these diseases treated for, pulpy kidney (enterotoxamia) is a disease generally caused by the toxin of Clostridium Perfringes type D (rod shaped), from the intestinal tract due to an immediate change of nutrition from a poor to a good plain of nutrition. Pasteurellosis as such is a disease caused by beta-hemolytic, gram-negative, aerobic, non-motile, non-spore forming coccobacilli in the family Pasteurellaceae, due to a combination of stressors including heat, overcrowding, exposure to inclement weather, poor ventilation, handling and transportation. While tetanus is a disease caused by a toxin of bacterium clostridium tetani, due to the gaining of entrance by bacteria into a wound or damaged tissue. Further malignant oedema is caused by the toxin Clostridium Septicum from soil and intestinal contents of the animals and normally occurs through contamination of wounds containing devitalized tissue, soil, or some other tissue debilitant or through activation of dormant spores. Finally black quarter is a disease caused by a gram-positive, spore-forming, rod-shaped, anaerobic and motile Clostridium Chauvoei that infect animals through ingestion or wounds associated with lambing, docking, castration and shearing (Senturk, 2010).

The rams were maintained in kraals where each animal was placed in a separate pen. The cleaning of the kraals was performed early every morning and late in the afternoon. Frequent hosing of the floors was done daily for proper cleaning and rubber mats were placed in each pen to provide the necessary bedding and insulation. Two South African mutton merino ewes were used as teasers for the collection of semen from the rams. The ewes were kept in close proximity of the rams. The management was routinely similar and under natural ambient temperature and under natural photoperiod throughout the trial period.

The rams were fed a maintenance diet daily to avoid animals becoming too obese or losing body weight or condition during the study period. Plate 3.4 below shows water trough that was placed in each animal pen.

Plate 3.4 Water trough placed in each pen

During the first two weeks of the adaptation period, the experimental rams were fed a maintenance pellet diet, mixed with ground Lucerne with the purpose of reducing the stress and facilitating the adaptation process, as well as keeping the rumen functional (Malejane, 2013).

After the adaptation period, the animals were regularly given 2.5 kg of pellets per day (half was given between 7:00 and 8:00 and the other half between 14:30 and 15:30). Visual examinations of all animals were done daily for any clinical sign that could indicate illness.

3.4 Preparation of the semen extenders

All the chemicals were supplied by Sigma – Aldrich, Co (St. Louis, MO, USA) unless otherwise indicated. Distilled water was obtained from the Animal Nutrition Laboratory at the University of the Free State and all the semen extenders were prepared a day before semen collection. The two semen extenders used were a Tris-egg yolk based extender and a Sodium Citrate-egg yolk based extender. Different fractions were prepared for each extender, the one without glycerol (diluent A) and the other containing glycerol (diluent B), for the two step dilution procedure during semen processing (Gil *et al*., 2003). After preparation as shown in plate 3.5 below, the semen extenders were stored at 5˚C, until utilized.

Plate 3.5 Preparation of extenders for semen cryopreservation

3.5 Semen collection and quality evaluation

Prior to semen collection, a teaser ewe was restrained (Plate 3.6) to minimize the movement and facilitate semen collection.

Plate 3.6 Restraining a teaser ewe inside the semen collection pen

The artificial vagina (AV) was then prepared, which briefly entails filling the artificial vagina with water between 50˚ C and 60˚ C (Plate 3.7). The pressure in the AV was adjusted with the aid of the amount of water used. The one end of AV was lubricated with a sterile petroleum jelly (excess lubrication of the AV being avoided as this could result in semen contamination). The temperature inside the AV was checked prior to collection using thermometer and this temperature generally ranged between 42˚C and 45˚C. Thereafter the pre-warmed (32˚C) semen collection tube was fitted to the other end of the AV and kept covered to maintain the temperature of the collection tube (Hafez and Hafez, 2000).

Plate 3.7 Preparation of the artificial vagina before semen collection

Now the ram was then allowed into the collection pen, and after mounting the teaser ewe, its penis was diverted into the AV with the necessary care not to cause any injury or contamination (Plate 3.8). When the ram's penis made contact with the warm, lubricated surface of the AV, the ram generally gave a forward thrust and ejaculated. A vigorous upward and forward thrust generally signified ejaculation. Only semen collected in the collection tube was evaluated, as semen in the surface of AV lining generally resulted in thermal damage to the sperm cells (Mitchell and Doak, 2004). This ejaculation generally occurred within 1 to 2 seconds. Semen was deposited into the collection tube and the tube then removed from the AV and semen was ready for evaluation.

Plate 3.8 Collection of semen from the rams using the artificial vagina

3.5.1 Semen evaluation

3.5.1.1 Colour of the ejaculate

The first parameter of the semen that was recorded is colour as this is an indication of the concentration of an ejaculate and also a means of checking for any contamination of the semen.

3.5.1.2 Semen volume

The volume of the ejaculate was recorded directly from the calibrated collection tube, immediately after collection. Care was taken to make sure that the tube was kept warm and in an upright position during readings.

3.5.1.3 Semen wave motion

To assess the sperm wave motion, 10 µl of semen was drawn with a pipette and placed on a pre-warmed microscope slide (32˚C) and observed microscopically under low magnification (Olympus) (x 10 magnification). The wave motion was microscopically assessed on the scale of 0-5. The assessment of the wave motion was done immediately after semen collection (Hafez and Hafez, 2000).

3.5.1.4 Sperm concentration (semen density)

Sperm concentration was determined with the aid of an improved Neubauer haemocytometer (Plate 3.9) (depth 0.100 mm and 0.0025 mm^2). 10 µl of semen was diluted in 990 µl (1: 100) distilled water, to dilute and also kill the sperm. For the sperm counting, a microscope (x100 magnification) was used. 10 µl of diluted semen was placed on the haemocytometer and covered with a cover slip. The sample was allowed for the sperm to settle for a period of 5 min. All visible sperm cells in the 5 designated (5/25) diagonal squires were counted and recorded. Sperm counting was performed 7 hrs after collection - during that period, the semen mixture was stored in the refrigerator at a temperature of 5˚C. On the haemocytometer, the 25 large squares contained 16 smaller squares, which mainly facilitated in the counting of the sperm cells. The total number of sperm cells per ml was then calculated as the number of sperm cells counted in the 5 diagonal squares $\times 5 \times 10^6$ sperm/ml (Mitchell and Doak, 2004).

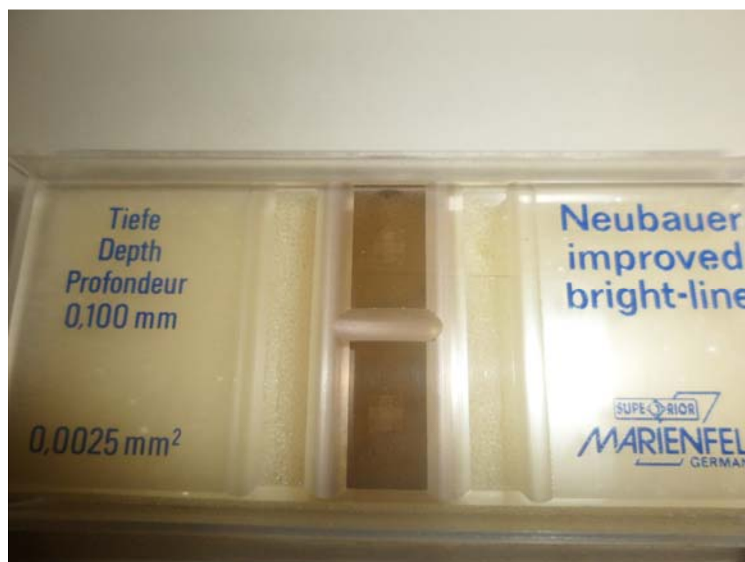

Plate 3.9 Haemocytometer used for sperm counting

3.5.1.5 Sperm viability and morphology

For the evaluation of the percentage live and abnormal sperm, smears were made using the eosin-nigrosin stain. Briefly 10 µl of semen was diluted with 100 µl of a pre-warmed (32˚C) eosin-nigrosin stain and a droplet (10 µl) of semen and stain mixture was then placed on a clean pre-warmed (32˚C) microscope slide and spread evenly with another slide to make a thin semen

smear. It is important to push, rather than to pull the edge of the second microscope slide at a 30˚ to 45˚ angle to the first slide excessive pressure is also to be avoided (Malejane, 2013). The smear was then immediately dried on the warm plate (32˚C) for less than a min. All semen smear slides were then stored in a clean tray for late determination of the percentage live and abnormal sperm.

Dead sperm coloured red and abnormalities like loose heads, double heads, broken or bent necks and mid pieces, double tails, etc, were visible and could be examined microscopically at ×1000 magnification (oil immersion). A representative sample of 100 sperm on each slide from randomly selected areas was recorded to determine the percentage live (white VS red sperm cells). The normal VS abnormal sperm were also recorded, specifying the kind of abnormality of the live sperm.

3.6 Cryopreservation of ram semen

After the collection of semen from two breeds as indicated earlier, one pooled sample of collected semen was made for each breed (Dorper and South African Mutton Merino) and both samples divided into two equal halves. Thereafter both samples were diluted with 1:1 v/v, with fraction A and then again later with 1:1 v/v with fraction B (with glycerol).

The flask (Plate 3.10) was used for the temporary storage of semen samples for the period during which samples were transported into a cold room, where temperature was decreased slowly to 5˚C.

Plate 3.10 Flask for transportation of semen from collection area to the freezing Laboratory

After 1 hr, fraction B was added at a dilution rate previously mentioned. After 2 hrs, the diluted semen samples were loaded into straws (0.25 ml, at 5 °C), sealed with polyvinyl alcohol powder of two different colours (red and yellow) and transferred to a programmable freezer as shown in Plate 3.11 (where the temperature was decreased in a stepwise manner). The starting temperature was 5 °C and semen was cooled to -20 °C at the rate of 1 °C/min. Half of the semen straws were suspended 3 - 4 cm above liquid nitrogen (Plate 3.12) for 5 min (Jafaroghli *et al*., 2011), while the other half was cooled to -80°C at the rate of 5˚C/min in the programmable freezer. All the semen straws were then plunged directly into liquid nitrogen (-196 °C) for storage. Liquid nitrogen tank is shown in Plate 3.13 below.

Plate3.11 Semen straws loaded into the programmable freezer for cryopreservation

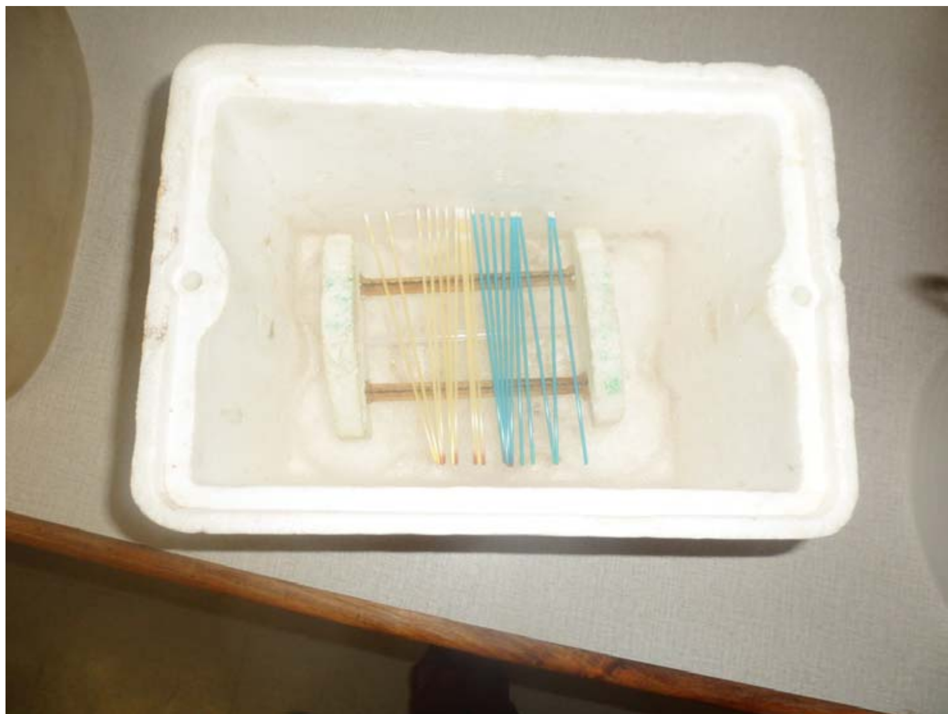

Plate 3.12 Semen straws suspended 3 – 4 cm above liquid nitrogen vapour for cryopreservation

Plate 3.13 Liquid nitrogen tank used for the storage of semen after cryopreservation

3.7 Thawing of semen for the post-thaw semen analysis

Semen straws were thawed 24 hrs after cryopreservation, by placing the straws into a flask filled with warm water (37˚C), for 30 seconds (Purdy, 2006; Jafaroghli *et al.,* 2011; Munyai, 2012). Both ends of the semen straws were cut with a pair of scissors and the semen poured into 10ml pre-warmed test tubes. The microscopic evaluation was performed at four intervals, starting immediately after thawing, 30 min, 60 min and 120 min post thawing. After every evaluation, the semen was maintained in an incubator at 37˚C.

3.8 *In vitro* fertilization

3.8.1 Ovary collection

Ovaries were collected at a local abattoir, near the Laboratory and transported to the Laboratory in sterile saline water (37˚C) in the flask within 3 hrs of slaughter. Immediately on arrival at the Laboratory, the temperature in the flask was measured and recorded. Ovaries were then washed three times in a saline (NaCl + Ultrapure water) solution.

3.8.2 Method of oocytes collection

Cumulus oocytes complexes (COC's) were recovered from the ovarian follicles by aspiration of the follicular fluid using an 18-gauge needle attached to the 10 ml syringe. The COC's were transferred into a 50 ml tube in which the complexes were allowed to settle for 10 minutes, follicular fluid was poured out and modified phosphate saline (mPBS) was added and allowed for another 10 minutes to settle, after which the oocytes were transferred to a search dish for oocyts selection under the microscope. Thereafter, oocytes were washed three times in modified phosphate saline supplemented with bovine serum albumin (BSA) and three times in M199, supplemented with fetal bovine serum (FBS). The oocytes were exposed to 26 µM of brilliant cresyl blue (BCB) diluted in mPBS for 90 minutes at 39˚C in humidified air, with 5% carbon dioxide. After exposure, the COC's were transferred into mPBS and washed three times and then the good quality oocytes according to their cytoplasm colouration (grown oocytes were those with a blue cytoplasm) were selected under microscope, the selected COC's were washed three times in M199 supplemented with FBS, before maturation (Bhojwani *et al.*, 2007).

3.8.3 Oocytes maturation *in vitro*

Four-well petri dish containing maturation medium, covered with mineral oil in each well were pre-incubated for at least 3 hrs before maturation. The maturation medium contained M199 supplemented with FBS and hormones. The COC's (50 oocytes / well) were then transferred into a pre-incubated four-well dish and incubated for 24 hrs at 38˚C (5% carbon dioxide and 90% relative humidity). Following maturation, the oocytes were then evaluated under the microscope and then processed for fertilization.

3.8.4 *In Vitro* Fertilization (IVF)

Three hrs prior to fertilization, Bracket and Oliphant oocytes fertilization medium (BO-IVF) drops were prepared (5 drops of 100 µl) for washing of oocytes and (7 drops of 50 µl) fertilization and sperm wash medium were prepared and pre-warmed. At fertilization period, a frozen semen straw was taken from the liquid nitrogen (LN_2) and thawed by dipping the straw into a flask containing warm water (37˚C), for 30 seconds. The contents of the straw was then emptied into a 15 ml falcon tube (by cutting both ends of the straw) and the tube filled to 6 ml, with warm (37˚C) Bracket and Oliphant (BO) sperm washing solution. The frozen-thawed semen suspension was then centrifuged twice at 1500 rpm for 8 min, at 38˚C. The supernatant was then carefully aspirated immediately after centrifugation without disturbing the semen pellet, using a sterile serological pipette. The semen pellet was then diluted with a pre-incubated BO-Sperm wash solution and a 5 µl aliquot of semen after dilution was placed on a microscopic slide and covered with a cover slip for the microscopic evaluation of sperm motility - which was then recorded. At the same time of washing the sperm, the oocytes were also washed in 5 drops of pre-warmed BO-IVF and transferred into 7 drops of BO-IVF for fertilization. Then 50 µl of the prepared semen was placed in the IVF aliquot containing the mature oocytes and incubated for 18 hrs at 38˚C (5% carbon dioxide and 90% relative humidity) (Bracket and Oliphant, 1975).

3.8.5 *In Vitro* Embryo Culture

After 18 hrs of oocyte-sperm incubation, presumptive zygotes were removed from the IVF aliquots into a 1.5 ml eppendorf tube containing 100 µl of pre-incubated M199 supplemented with FBS and vortexed for 1.5 min to remove the cumulus cells. After vortexing, zygotes were washed 3 times in both M199 supplemented with FBS and pre-incubated culture media (SOF-BSA) droplets (100 µl) - after which 20 - 25 zygotes were transferred into pre – incubated 50 µl droplet of SOF-BSA medium covered with mineral oil and placed in gas chamber that has three gases, thereafter the zygotes were incubated at 39˚C for 7 days in a humidified incubator (5% oxygen, 5% carbon dioxide and 90% nitrogen). During this incubation period, the culture medium was changed at 48 hrs after fertilization from SOF-BSA to SOF-FBS by aspirating medium from the aliquots and replacing the medium with the fresh pre-incubated medium with the aid of a pipette, during which the cleavage rate (2 to 8 cell percentage) was examined and recorded and embryos were then separated according to number of cell divisions. The media was changed again on day 5 of incubation. Microscopic evaluations were performed at day 6,

and day 7 for the development into the morula and blastocyst stages respectively, and results were recorded into an excel sheet for data analysis.

3.9 Data Analysis:

The statistical analysis of all collected data for fresh semen (volume, colour, concentration, motility, viability and morphology); frozen-thawed semen (motility) and performance of the frozen-thawed semen *in vitro* (cleavage - 2 to 8 cell) percentages were analyzed using the one way analysis of variance (ANOVA) procedures of SAS (2009). Tukey's Studentised Range (HSD) test (SAS, 2009) was used to determine the differences between treatment means. P values < 0.05 were considered to be significant.

CHAPTER 4

RESULTS

4.1 Effect of breed (Dorper and SAMM) on fresh semen before cryopreservation

The effect of breed (Dorper and SAMM) on fresh ram semen quality before cryopreservation is set out in Table 4.1. Semen volume and sperm motility recorded significant differences between the two breeds (P<0.05) with the South African Mutton Merino (SAMM) semen resulting in higher means than the Dorper semen for the entire observation period of the experiment. So for example 81.3 ± 0.1% versus 70.2 ± 0.2% and 1.4 ± 0.1ml versus 1.1 ± 0.1ml for sperm motility and semen volume for the SAMM and Dorper rams were recorded, respectively. There was no significant difference (P>0.05) recorded between the breeds for semen colour, concentration, percentage live sperm, percentage dead and the percentage normal sperm. So for example the general colour of the semen samples for both breeds was thin creamy.

Table 4.1 Effect of breed on fresh ram semen quality

Breed	Semen parameters (mean±SE)						
	Vol. (ml)	Colour range	Motility (%)	Conc. ($\times 10^6$/ml)	Live (%)	Dead (%)	Normal (%)
Dorper	1.1 ± 0.1^a	3.4 ± 0.4^a	70.2 ± 0.2^a	2568.3 ± 57.2^a	71.2 ± 2.5^a	28.8 ± 2.3^a	93.7 ± 2.2^a
SAMM	1.4 ± 0.1^b	3.5 ± 0.3^a	81.3 ± 0.1^b	2655.8 ± 43.1^a	75.8 ± 1.9^a	24.5 ± 1.9^a	95.2 ± 2.1^a

^{ab} Values within same column with different superscripts differ significantly (P < 0.05)

4.2 Effect of breed, different extenders and different freezing protocols on the viability of frozen ram semen for different incubation time periods after thawing

The post-thaw mean sperm motility of the Dorper and SAMM semen frozen using different extenders and freezing protocols is set out in Table 4.2 and Table 4.3, respectively. The semen frozen using the liquid nitrogen vapour protocol prior to storage, resulted in a higher sperm

motility, compared to semen frozen up to -80˚C (programmable freezer) prior to storage. The sperm viability of the frozen ram semen was inversely related to a time increase following thawing. Only the different freezing protocols and time intervals recorded a significant difference (P<0.05) throughout the entire experiment. Statistically, breeds and the different extenders recorded no significant difference (P>0.05). However, when the Tukey grouping was used (studentised range), Tris-egg yolk based extender exhibited a better sperm performance at the beginning of the experiment than the Sodium Citrate-egg yolk based extender.

Table 4.2 Mean (±SE) post-thaw sperm motility of Dorper ram semen frozen using different extenders and freezing protocols at different incubation intervals following thawing

Breed	Extender	Freezing protocol (˚C)	Incubation periods after thawing (min)	Mean±SE
Dorper	Tris-based	Vapour (-70)	0	61.4 ± 2.42^{abc1}
	Tris-based	-80	0	55.4 ± 2.93^{abd1}
	Na Citrate	Vapour (-70)	0	52.2 ± 2.350^{abc1}
	Na Citrate	-80	0	50.8 ± 3.51^{abd1}
	Tris-based	Vapour (-70)	30	52.0 ± 3.00^{abc2}
	Tris-based	-80	30	48.2 ± 3.12^{abd2}
	Na Citrate	Vapour (-70)	30	48.0 ± 3.00^{abc2}
	Na Citrate	-80	30	44.6 ± 2.34^{abd2}
	Tris-based	Vapour (-70)	60	38.4 ± 4.70^{abc3}
	Tris-based	-80	60	37.6 ± 4.50^{abd3}
	Na Citrate	Vapour (-70)	60	37.0 ± 4.36^{abc3}
	Na Citrate	-80	60	38.0 ± 3.39^{abd3}
	Tris-based	Vapour (-70)	120	32.0 ± 4.90^{abc4}
	Tris-based	-80	120	27.8 ± 4.98^{abd4}
	Na Citrate	Vapour (-70)	120	27.6 ± 5.08^{abc4}
	Na Citrate	-80	120	25.0 ± 3.87^{abd4}

$^{abcd1/2/3/4}$ Values within same column with different superscripts differ significantly (P < 0.05)

Table 4.3 Mean (±SE) post-thaw sperm motility of SAMM ram semen frozen using different extenders and freezing protocols at different incubation intervals following thawing

Breed	Extender	Freezing protocol (˚C)	Incubation periods after thawing (min)	Mean±SE
SAMM	Tris-based	Vapour (-70)	0	52.8 ± 3.22^{abc1}
	Tris-based	-80	0	54.6 ± 3.92^{abd1}
	Na Citrate	Vapour (-70)	0	52.6 ± 3.17^{abc1}
	Na Citrate	-80	0	52.6 ± 3.17^{abd1}
	Tris-based	Vapour (-70)	30	50.0 ± 3.16^{abc2}
	Tris-based	-80	30	46.6 ± 4.07^{abd2}
	Na Citrate	Vapour (-70)	30	48.0 ± 3.00^{abc2}
	Na Citrate	-80	30	46.0 ± 3.67^{abd2}
	Tris-based	Vapour (-70)	60	37.2 ± 4.40^{abc3}
	Tris-based	-80	60	29.0 ± 4.58^{abd3}
	Na Citrate	Vapour (-70)	60	37.0 ± 4.36^{abc3}
	Na Citrate	-80	60	31.0 ± 5.79^{abd3}
	Tris-based	Vapour (-70)	120	30.0 ± 4.18^{abc4}
	Tris-based	-80	120	20.0 ± 0.01^{abd4}
	Na Citrate	Vapour (-70)	120	32.0 ± 4.90^{abc4}
	Na Citrate	-80	120	22.0 ± 1.22^{abd4}

$^{abcd1/2/3/4}$ Values within same column with different superscripts differ significantly ($P < 0.05$)

4.3 Effect of breed, extender and freezing protocol on the *in vitro* performance of thawed ram semen

The *in vitro* fertilization results are summarized in Table 4.4 and in Figure 4.1. All the extenders and the freezing protocols recorded significant different ($P<0.05$) fertilization rate results throughout the entire experiment, regardless of the breed. The breed recorded no significant difference ($P>0.05$) and no interaction was found between the treatment groups. The mean cleavage percentages recorded were 33.8±0.7% when Tris-egg-yolk based extender was used with exposure to LN_2 vapour prior to storage; 30.0 ± 0.7% when Tris-egg-yolk based extender

was used with freezing up to -80˚C (programmable freezer) prior to storage; 29.9 ± 0.7% when Sodium Citrate-egg-yolk based extender was used with the aid of LN_2 vapour prior to storage and 25.7 ± 0.7% when Sodium Citrate-egg-yolk based extender was used with freezing up to -80˚C prior to storage.

Table 4.4 Mean (± SE) effect of extender and freezing protocol on *in vitro* fertilization of ram semen

Extender	Freezing Protocol (˚C)	Cleavage (%)
Tris-Egg-yolk based	Vapour (-70)	33.82 ± 0.73[a]
Tris-Egg-yolk based	- 80	30.03 ± 0.73[b]
Sodium Citrate-Egg-yolk based	Vapour (-70)	29.88 ± 0.73[c]
Sodium Citrate-Egg-yolk based	- 80	25.71 ± 0.73[d]

[abcd] Values within same column with different superscripts are significantly different (P<0.05)

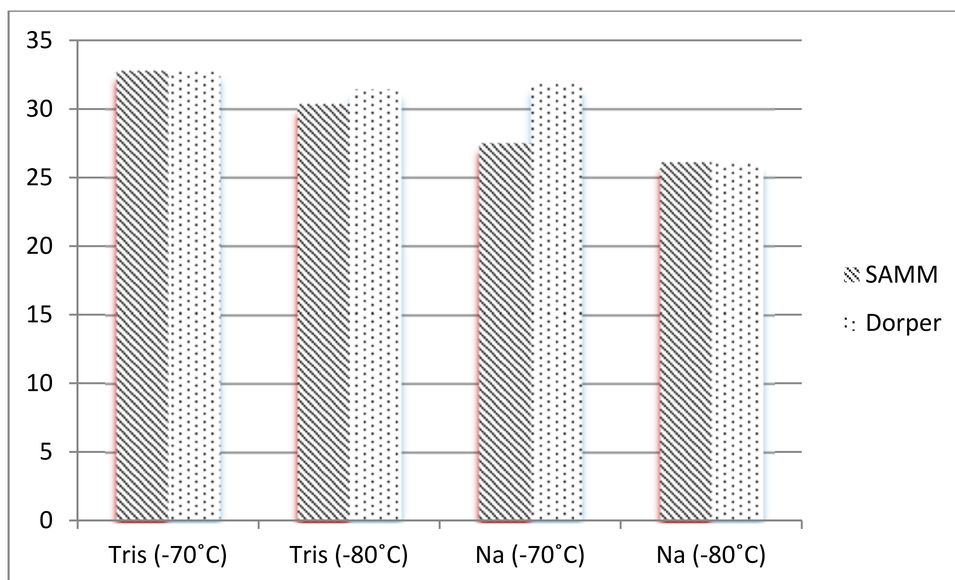

Figure 4.1 Effect of breed on *in vitro* fertilization results of frozen-thawed ram semen

Where Tris (- 70˚C) is Tris-Egg-yolk based extender with a LN_2 vapour (-70˚C) as a freezing protocol, Tris (- 80˚C) is Tris-Egg-yolk based extender with freezing up to -80˚C, Na (-70˚C) is Sodium Citrate-Egg-yolk based extender with a LN_2 vapour (-70˚C) as a freezing protocol and Na (-80˚C) is Sodium Citrate-Egg-yolk based extender with freezing up to -80˚C.

CHAPTER 5

DISCUSSION

5.1 Effect of breed (Dorper and SAMM) on fresh semen parameters prior to cryopreservation

5.1.1 Semen volume

The breed of sheep has been hypothesized to be of great importance regarding the evaluation of quality in fresh ram semen. The results of the current study are in agreement to the hypothesis. The mean semen volume (1.4±0.1and 1.1±0.1 ml) for the Mutton Merino and Dorper, respectively, suggests that the South African Mutton Merino produces significantly higher semen volumes, compared to Dorper rams. A mean ejaculate volume of 1.2 ml has been reported (Hamedani *et al.,* 2013) in Dallagh rams and 1.1±0.1ml in goats (Batisda *et al.,* 2009). Similar significance differences for sheep breeds have been reported by other researchers. So for example Mahoete (2010) reported significant differences in semen volume between Merino and Sulu rams, while (Moghaddam *et al.,* 2012) reported a significant difference between the ArkharMerino × Ghezel and Ghezel × Baluchi rams. In addition the results are also in agreement with values obtained from more recent studies. Malejane (2013) reported an average semen volume of 1.1ml in Dorper rams, while Gil *et al.* (2003), documented ejaculate volumes of 0.75 to 2 ml as being normal for rams when using artificial vagina for ram semen collection. Hafez and Hafez (2000) also indicated a range of 0.5 to 2 ml in ejaculate volume for mature rams and 0.5 to 0.7 ml in yearling rams. There are however number of factors that can affect the volume of ram semen e.g scrotal circumference (Sarder, 2005; Devkota *et al.,* 2008; Hassan *et al.,* 2009; Okere *et al.,* 2011), age of the male, nutritional status, reproductive management, method of semen collection, frequency of semen collection, season of the year and responsiveness of the ram (Hafez and Hafez, 2000; Malejane, 2013).

5.1.2 Semen colour

As set out in table 4.1, there were no significant differences in semen colour between the SAMM and Dorper sheep breeds. The general colour of the semen for both breeds was thin, creamy. These findings fall within the range as reported by Ax *et al.* (2000), who documented that fresh ram semen colour should vary from thin to thick creamy in consistency and, milky-white or pale cream in colour. This parameter generally indicates the sperm density (concentration), as well as possible contamination (Malejane, 2013). For the trial no contamination was recorded throughout the entire experiment and thus the semen and the technique of semen collection was considered to be of acceptable quality.

5.1.3 Sperm motility

Sperm motility is seen as a good indication of semen quality, fertilizing ability and animal fertility in all species. Table 4.1 illustrates a high significant difference in sperm motility ($P<0.05$) between the two breeds (81.3±0.1% and 70.2±0.2%) for the SAMM and Dorper males respectively. This finding is in agreement with Karagiannidis *et al.* (2000), who reported a range of 70 to 90% for sperm motility. Some factors have however been reported to affect sperm motility and these include age (Brito *et al.,* 2002; Salhab *et al.,* 2003; Ghalban *et al.,* 2004; Kumar *et al.,* 2010) and nutrition (Watanabe *et al.,* 2003; Hamedani *et al.,* 2013).

5.1.4 Sperm concentration

No significant differences were recorded between the two breeds. The results of this study are in agreement with the findings of Gil *et al.* (2003), who reported a sperm concentration of 2.5×10^9 sperm/ml for rams to be normal and acceptable. This was also supported by Paulenz *et al.,* (2005) and O'Hara *et al.,* (2010) who also documented acceptable sperm cell concentrations of $\geq2.5\times10^9$sperm/ml. According to the results of the present study, as set out in Table 4.1, semen concentration of SAMM was higher than the semen concentration of Dorper rams. Factors that could affect the sperm concentration include season, age of the male, degree of stimulation and nutrition (Wolfenson *et al.,* 2000; Brito *et al*., 2002; Salhab *et al.,* 2003; Zamiri *et al,* 2005; Kheradmand *et al*.,2006; Moghaddam *et al.,* 2012).

5.1.5 Sperm viability

The percentage dead or live sperm was evaluated with the aid of the eosin/nigrosin stain, whereby live sperm stain white and dead sperm stain red, as discussed in Chapter 3. This staining method indicative of the live or dead status of the sperm cell, while also presenting a good environment for the evaluation of the sperm cell morphology (Bjoerndahl *et al.,* 2003). The results of the current study regarding sperm viability are set out in Table 4.1. No significant difference (P>0.05) was recorded between the two breeds. These findings (75.8± 1.9% and 71.2± 2.5%) fall within the range of 60 to 90% that was documented by Zamiri *et al.* (2010) for the percentage live ram sperm but is higher than the findings of Schwalbach *et al.* (2006) who reported live sperm percentage mean values of 64±3% and 70±2% for Dorper rams which were managed intensively. This difference may be attributed to factors such as temperature and season of semen collection (Leboeuf *et al*., 2000; Ghalban *et al*., 2004).

5.1.6 Sperm morphology

The data of the current trial indicate no significant difference in sperm morphology or abnormalities between the SAMM and Dorper breeds (Table 4.1). These findings are in agreement with other researchers (Hafez and Hafez, 2000; Karagiannidis *et al*., 2000). The abnormalities recorded were relatively small (<10%) which is still acceptable as suggested by most researchers. Within this range of less than 10% abnormalities, the majority of sperm were seen with no tail, while some sperm were recorded with no head, no acrosome or a head with a mid-piece but without a tail. Care must always be taken to prevent injury to the sperm cell when making the smear. The relatively low percentage of abnormalities indicates that the making of semen smears was acceptable.

5.2 Effects of breed difference, different extenders and different freezing protocols on the viability of frozen-thawed ram semen

Fresh semen immediate after collection used in this research was found to be acceptable for further processing, especially for dilution and freezing as in Table 4.1. Macroscopically, the normal quality standards of fresh ram semen is 0.5 to 2.0 ml volume (Hafez and Hafez, 2000), thin to thick creamy in consistency, milky to white or pale cream in colour (Ax *et al.,* 2000). When the microscope is used, the normal concentration ranges from 1500 million sperm/ml (O'Hara *et al.,* 2010), a sperm motility of 70 to 90% (Karagiannidis *et al.,* 2000), a sperm

viability of 60 to 90% (Zamiri *et al.,* 2010) and a percentage sperm abnormalities of ≤ 10% (Karagiannidis *et al*., 2000).

The interaction of experimental factors in the current study recorded no significant differences (P>0.05). However, taking a single factor into consideration, the incubation time intervals and freezing protocols significantly affected the sperm motility (P<0.05). The negative relationship that was recorded between time intervals and sperm motility post thawing thus prohibits the use of cervical artificial insemination in ewes using frozen - thawed semen. These findings are then in agreement with the findings of Gil *et al.* (2000). The low semen storage temperatures used in the current study (-196˚C) is set to extend the fertile life span of the sperm cell, by reducing the sperm metabolic rate. It has been documented by Hafez and Hafez, (2000) that as metabolic rate increases the life span of the sperm cell decreases. This correlation has been proven in the current study at temperatures of 39˚C following thawing. In addition, reports concerning fresh semen indicated that the sperm has a short fertile life span outside the body (Morrier *et al.,* 2002) and this is due to increased cellular metabolism at higher temperatures. Even though it was suggested by Brinsko *et al.* (2000) that the freezing of semen will increase its longevity, the biggest challenge is fertility of the sperm cell after thawing. Reduction in the fertilization capacity has typically been attributed to a reduced rate in sperm motility and the freeze-thaw-induced morphological and genomic abnormalities (Morris *et al.,* 2002; Martin *et al.,* 2004). The semen frozen following the use of liquid nitrogen vapour prior to storage recorded a better sperm motility than semen frozen at -80˚C prior to storage in liquid nitrogen. It is thus suggested that in programmable semen freezing programs, semen should be exposed to liquid nitrogen vapour immediately prior to the storage into liquid nitrogen (-196˚C) and should not be frozen at -80˚C. According to the knowledge gained thus far, there is no published information regarding programmable freezing of the ram semen using temperatures similar to those used in this study. The use of the programmable freezer has however been reported to produce better sperm motility (Hammadeh *et al.,* 2001; Clulow *et al.,* 2008), with less cryodamage to the sperm cell (Petyim and Choavaratana, 2006).

The use of a Tris-egg yolk based extender showed better post thawing motility than the Na Citric-egg yolk based extender in both breeds of sheep. Similar findings have been reported where semen was stored at 5˚C and 20˚C (Paulenz *et al.,* 2002). Both extenders used in this study have been recommended by other researchers to be acceptable for ram semen cryopreservation in programs of genetic animal material improvement. In addition, Tris-egg yolk based extender has been documented to provide adequate cryoprotection (Salamon and

Maxwell, 2000). However, the difference in performance may be due to the types and concentrations of sugars used. So for example, with an increase in sugar concentrations, all post-thawed sperm characteristics were generally improved and higher sperm motility has been reported with the use of trehalose (Jafaroghli *et al.,* 2011). Furthermore, trehalose has been reported to have certain antioxidant properties (Aisen *et al.,* 2002), compared to glucose used in this study. The egg yolk has been stated to make semen evaluation problems (Moussa *et al.,* 2002), increase the risk of microbial contamination and thereby allow the production of endotoxin, hence lowering the motility of the sperm (Aires *et al.,* 2003).

5.3 Effect of breed, extender and freezing protocol on the *in vitro* performance of thawed ram semen

The sperm parameters of the frozen-thawed semen showed no significant difference ($P>0.05$) between the two breeds. The extenders and the freezing protocols were however significantly ($P< 0.05$) different throughout the entire experiment, regardless of the breed. These results are contradictory with the results of other researchers (Mahoete, 2010). The standard error of the present work (0.73) seems to be very small as compared to the standard error (3.7) of results reported by Mahoete (2010) in the Merino breed semen. This larger variation could be attributed to different factors, however, it is difficult to interpret the cause of this difference, as the materials and methods used were different. The use of a Tris-egg yolk based extender reported better cleavage results (31.91± 0.73%), compared to use of a sodium citrate-egg yolk based extender (27.8 ± 0.73%). When smaller percentages of egg yolk (10%) were included, the sodium citrate-egg yolk based extender exhibited better cleavage (Mahoete, 2010), while in this study, 20% egg yolk was used. Thus, further research is warranted on the effective levels of egg yolk inclusion in the sodium citrate extender. These *in vitro* results have confirmed the results of the current experiment for Tris-egg yolk based extender vs sodium citrate-egg yolk based extender and are in agreement with the work of Valente *et al*. (2009). Although the egg-yolk used was less (15%) (Valente *et al*., 2009) while the optimal level is allowed to be 20% (Forouzanfar *et al*., 2010), which was used in the current study. It is further documented that the freezing of ram semen in a Tris-egg yolk base with 20% egg yolk, offers a higher number of viable spermatozoa that can be used during IVF and Artificial Insemination (Forouzanfar *et al*., 2010). Furthermore, the results of the current study are in agreement with results of García-Álvarez *et al*. (2009a) who reported a range of 31% to 59% cleavage for *in vitro* results, when

vapour was used prior to storage in to liquid nitrogen. Compared to Dorado *et al*. (2007) who reported that Tris-based extender results in better IVF results, compared to other extenders.

When liquid nitrogen vapour was used in the current study, better *in vitro* results were obtained, compared to the use of -80˚C, prior to storage. No relevant literature could be found for the freezing of up to -80˚C, prior to storage in liquid nitrogen.

Variations in cleavage percentages may be due to the methods of semen quality control applied and quality assurance procedures and contributing conditions in the embryology laboratory. In the present work, the selection of oocytes for *in vitro* fertilization was performed with the aid of the brilliant cresyl blue technique, to ensure that only oocytes of good quality were used (Bhojwani *et al*., 2007). The experience of the researcher has also been reported to contribute to variation in sperm quality results (Malejane, 2013), thus affecting *in vitro* results. Different methods of semen collection may also have contributed to the variations of *in vitro* fertilization results experienced (García-Álvarez *et al.,* 2009b). However, the semen collection methods used in this study was reported to provide better results compared to other collection methods (Malejane, 2013). The reliable and repeatable ejaculates obtained are an indication of an acceptable collection technique (artificial vagina) used.

The results thus suggest that frozen-thawed ram semen can perform well in *in vitro* fertilization procedures, if a Tris-egg yolk based extender is used as the diluent in combination with exposure to liquid nitrogen vapour as a freezing protocol prior to storage. Further work is warranted to confirm these results *in vivo*.

CHAPTER 6

GENERAL CONCLUSIONS

The semen volume and sperm motility parameters showed a significant difference between the two sheep breeds ($P<0.05$) where the South African Mutton Merino (SAMM) semen performed better than the Dorper semen for the entire period of the trial. There was however no significant differences for the breeds in terms of semen colour, sperm concentration, percentage live and dead sperm and percentage normal sperm. Based on these results it could be concluded that further work must be done with a larger number of animals to increase the statistical precision and significance. This is due to the fact that few numbers of rams per breed were used in the current study and the fact that a difference was found only between two of the semen parameters.

The semen frozen using liquid nitrogen vapour prior to storage exhibited better sperm motility than semen frozen to -80˚C (programmable freezer) prior to storage. The sperm viability of the frozen ram semen was inversely proportional with the incubation time increase after thawing. Only the freezing protocols and the time intervals showed a significant difference ($P<0.05$) throughout the entire experiment. Statistically, breed difference and the different semen extenders had no significant effect ($P>0.05$). However, when Tukey's grouping was used (studentised range), the Tris-egg yolk based extender exhibited a better performance at the beginning of the experiment, compared to the Sodium Citrate-egg yolk based extender. It could thus be concluded that the Tris-egg yolk based extender should be used as a diluent for ram semen cryopreservation and freezing with the aid of liquid nitrogen vapour, prior to storage. This will lead to a better fertility if insemination is done immediately after thawing, or alternatively the laparoscopic method will remain an alternative.

The semen frozen by the use of liquid nitrogen vapour prior to storage, showed better oocyte cleavage rates post *in vitro* fertilization, compared to the semen frozen to -80˚C (programmable freezer), prior to storage. Only the freezing protocols and semen extenders exhibited a significant difference on oocyte cleavage rate post *in vitro* fertilization throughout the entire experiment. Statistically the different breeds showed no significant difference in cleavage rate post *in vitro* fertilization. Therefore, it could be concluded that in general frozen-thawed ram

semen (irrespective of the breed) can perform well in the *in vitro* fertilization procedures used if the Tris-egg-yolk based extender is used as a diluent in combination with exposure to liquid nitrogen vapour prior to storage as a freezing protocol. Further research following thawing is warranted to confirm fertility results *in vivo.* The results thus also lead to the conclusion that any of the two breeds can be used for the *in vitro* fertilization programs.

RECOMMENDATIONS

It is recommended that further research must be performed with a larger number of animals for better statistical precision or significance to determine the effect of breed on the quality of ram semen. The Tris-egg-yolk based extender should be used as diluent, in combination with exposure to liquid nitrogen vapour as a freezing protocol prior to storage. More research is also warranted to evaluate the results of these cryopreservation techniques in the field of *in vivo* fertility.

ABSTRACT

EVALUATION OF CRYOPRESERVED RAM SEMEN FOLLOWING FERTILIZATION *IN VITRO*

by

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Degree: M.Sc. (Agric.)

Three experiments were done at the University of Free State in Bloemfontain in 2014 and in 2015. Experiment 1 was carried to evaluate the effect of breed (Dorper and SAMM) on fresh ram semen parameters before cryopreservation. Experiment 2 was carried to evaluate the effects of breed difference, different extenders and different freezing protocols on the viability of frozen ram semen during different incubation periods after thawing. Experiment 3 was carried to evaluate effects of breed, extender and freezing protocol on *in vitro* fertilizing

capacity of frozen-thawed ram semen. Experiments 1 and 2 were done in 2014 during the breeding season while experiment 2 was done in 2015 from mid of January until the end of April.

For the first trial, the semen was collected with the aid of artificial vagina from two breeds of rams. The ejaculates were evaluated for the following parameters: semen volume, semen colour, sperm motility, sperm concentration, percentage live/dead sperm and sperm morphology. The parameters were then compared against the breeds to assess the breed effect. The semen volume and sperm motility showed a significant difference between the two breeds ($P<0.05$) in that South African Mutton Merino (SAMM) semen recorded better motility and higher semen volume than Dorper semen for the entire experiment. There was no significant difference between breeds for semen colour, sperm concentration, percentage live and dead sperm and sperm morphology.

For the second trial, the study to evaluate the effect of different breeds, extenders and freezing protocols on the sperm motility of ram semen including survival at different incubation periods after thawing was conducted during the natural breeding season. Semen was collected from two breeds of rams (South African Marten Merino (SAMM) and Dorper). After fresh semen evaluation and selection of good quality ejaculates, semen was pooled for each breed and divided into two then randomly allocated to Tris-egg yolk and Na-Citrate egg yolk based extenders. Semen samples were further divided into two more groups and allocated randomly to two different freezing protocols (freezing up to -20˚C using the programmable freezer and then by liquid nitrogen vapour prior to storage vs freezing to -80˚C prior to storage). Microscopic evaluation of the semen for sperm motility was performed 24 hrs following freezing. The semen frozen by the use of liquid nitrogen vapour prior to storage recorded better sperm motility than semen frozen to -80˚C prior to storage. The viability of the frozen ram semen was inversely proportional with incubation time increase after thawing. Only the freezing protocols and incubation periods exhibited a significant difference in sperm motility throughout the experiment. Statistically, the difference breeds and the different semen extenders recorded no significant difference. However, when Tukey's grouping was used (studentised range), the Tris-egg yolk based extender showed better sperm motility compared to the Na-Citrate egg yolk based extender. The results suggest that the breed does not have impact on semen freezing and the Tris-egg yolk based extender is better suited as a diluent for ram semen cryopreservation. Results also indicate that freezing with the aid of liquid nitrogen

vapour prior to storage will lead to a better fertility if insemination is done immediately after thawing or alternatively the laparoscopic method will remain an alternative.

In the last experiment, ovaries were collected during spring and at the beginning of summer at the Bloemfontein abattoir from unknown, untreated sheep and transported to the laboratory in sterile saline water (37˚C) in the flask. Cumulus oocytes complexes (COC's) were recovered from the ovarian follicles by aspiration method, washed and stained in brilliant crysel blue for selection of good quality oocytes. The COC were then washed and matured *in vitro* for 24 hrs before *in vitro* fertilization. The frozen semen (frozen by different extenders and different freezing protocols and from different breeds) was thawed and then centrifuged twice at 1500 rpm for 8minutes at 38˚C. Then 50 µl of the prepared semen was placed in the IVF aliquot containing the mature oocytes and incubated for 18 hrs at 38˚C (5% carbon dioxide and 90% relative humidity). After 18 hrs of oocyte-sperm incubation, presumptive zygotes were removed from the IVF aliquots into a 1.5 ml eppendorf tube containing 100 µl of pre-incubated M199 + FBS and vortexed for 1.5 min to strip off the cumulus cells. After vortexing, zygotes were washed 3 times in both M199 + FBS and pre-incubated culture media (SOF-BSA) droplets after which the zygotes were allocated to groups of 20 per aliquot which were covered with mineral oil and placed in gas chamber with 3 gases, then incubated at 39˚C for 7 days in humidified incubator (5% oxygen, 5% carbon dioxide and 90% nitrogen). During this incubation time, the culture medium was changed at 48 hrs after fertilization to SOF-FBS by aspirating medium from the aliquots and replacing the medium with the fresh pre-incubated medium with the aid of a pipette, during which the cleavage rate (2 to 8 cells) was examined and embryos were then separated according to number of cell division. The media was changed again during day 5. Microscopic evaluations were performed during day 6, and day 7 for the development into morula and blastocyst respectively and results were recorded into the excel sheet for data analysis.

The semen frozen by with the aid of liquid nitrogen vapour prior to storage recorded better cleavage compared to semen frozen to -80˚C prior to storage. Only the freezing protocols and semen extenders exhibited a significant difference in post *in vitro* fertilization throughout the entire *in vitro* fertilization experiment. Statistically the different breeds had no significant difference in post *in vitro* fertilization. The results suggests that, in general frozen-thawed ram semen irrespective of the breed can perform well in the *in vitro* fertilization procedures used if the Tris-egg-yolk based extender is used as diluents in combination with exposure to liquid nitrogen vapour prior to storage as a freezing protocol. Further research following thawing is

warranted to confirm fertility results *in vivo.* The results thus also indicate that the breed has no effect on the *in vitro* results of frozen-thawed ram semen.

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