The influence of genotype on sperm motility and sperm head morphometry of Merino (*Ovis aries*) sheep

by

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Thesis presented in fulfilment of the requirements for the degree of Master of Science in the Faculty of Agrisciences at Stellenbosch University

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April 2014

Declaration

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Summary

The application of assisted reproductive biotechnologies in sheep flocks is hampered by the susceptability of ovine sperm to cryodamage. There is still considerable scope in the improvement of cryopreservation protocols for ovine sperm to minimize the degree of damage to sperm during the cryopreservation process. Pre-cryopreservation processing has a definite effect on the survivability, motility, and fertilizing ability of sperm. Little information is however available on the potential contribution of the genetic make-up of rams, divergently selected for fecundity, on the ability of sperm to offer resistance to the damage caused by cooling, cryopreservation and thawing. The study aimed to investigate the influence of genetic selection for prolificacy (i.e. High Merino Line and Low Merino Line in terms of fecundity) on the ability of ovine sperm to offer resistance to cryodamage.

The study investigated the effect of pre-cryopreservation processing by comparing motility and morphometry traits recorded for fresh- and post-thaw Merino ejaculated and epididymal sperm samples obtained form the High and Low lines, respectively. The effect of different sperm concentrations, equilibration periods and the addition or omission of seminal plasma from cryopreserved samples on the viability and morphometrical traits were also investigated. Ejaculate samples were collected by means of the artificial vagina (AV) method from 8 High Line rams and 7 Low Line rams. Epididymal samples were collected from 6 rams of each of the High and Low lines respectively, by recovering the epididymal sperm via aspiration from the *cauda* epididymides post mortem. Ejaculate samples were subjected to macroscopic and microscopic evaluation, and epididymal samples only to microscopic evaluation, for which the Sperm Class Analyzer® program was used for the evaluation of motility and morphometric measurements. Sperm motility recordings were captured at 100 frames per second.

From findings of the study, it was concluded that genotype had no positive influence on the conception rate of the ewes mated to the High or Low Line rams, even though the rams from the two lines differed significantly in terms of their serving capacity. When sperm morphometry was evaluated for fresh ejaculate samples, the two lines differed significantly in terms of the morphometric traits elongation and ellipticity. Epididymal and ejaculated sperm obtained from Low Line rams had broader and rounder heads, compared to sperm obtained from High Line rams. When morphometry was assessed for sperm samples between the two methods of sperm recovery (collected with an AV or recovery via aspiration from the *cauda* epididymides of sacrificed rams), no morphometrical differences were observed. Significant differences were reported for the majority of the sperm motility traits (i.e. percentage motile, rapid-, medium-, slow swimming, curvilinear velocity (VCL), straight line velocity (VSL), average path velocity

(VAP), and amplitude of the lateral head displacement (ALH)) recorded for ejaculated and epididymal sperm. The motility traits ALH and beat-cross frequency (BCF) analysed for epididymal sperm differed significantly between the two lines. When epididymal sperm were evaluated post-thaw, it became evident that the sperm obtained from the High Line rams had a larger acrosome surface cover when compared to that of the Low Line ram sperm. The addition of seminal plasma to epididymal samples did not result in an improvement of the preservation of sperm motility. It is known from the literature that cryopreservation causes a decrease in sperm head size. Head width was unaffected by cryopreservation with the addition of seminal plasma in this study, indicating a potential benefit with the use of seminal plasma in the cryopreservation protocol of epididymal ram sperm.

The study compared two pre-processing techniques, i.e. the more time consuming swim-up technique (SUT) with a more time-efficient 'flush technique' (FT) to optimize the pre-processing protocol for motility assessment of sperm samples before cryopreservation of ram sperm. Comparison of the SUT and FT indicated that almost all of the motility parameters measured using the FT compared favourably with those obtained using the SUT. The results indicated that the FT can be used a more time-efficient technique to use for determining the motility of a sperm sample prior to cryopreservation.

In conclusion, line differences associated with reproduction were observed in terms of the serving capacity of the rams, with selection for fecundity influencing the morphometric traits elongation and ellipticity for sperm obtained from the two lines. Future studies should be aimed at investigating morphometric traits of ovine sperm, to correlate it with fertilizing ability of sperm post-thaw and ensure optimal cryopreservation processing.

Opsomming

Die toepassing van ondersteunende reproduksie tegnieke in skaaptroppe word bemoeilik deur die onvermoë van ram sperme om weerstand teen bevriesingskade te bied. Daar is nog baie ruimte vir die verbetering van die bevriesingsprotokolle vir skaap sperm om die omvang van bevriesingskade te verminder. Voor-bevriesing verwerking het dan 'n besliste uitwerking op die oorlewing, beweeglikheid en bevrugtingsvermoë, van skaap sperme. Min inligting is beskikbaar oor die potensiële bydrae van die genetiese samestelling van ramme wat uiteenlopend op grond van vrugbaarheid geselekteer is, op die vermoë van skaap sperme om weerstand te bied teen die skade wat deur verkoeling, diepbevriesing en ontdooiing, veroorsaak word. Die doelwit van die studie was om die invloed van genetiese seleksie vir fekunditeit (d.i. Hoë Merino Lyn en Lae Merino Lyn in terme van fekunditeit) op die vermoë van skaap sperme om weerstand teen bevriesingskade te bied, te ondersoek.

Die studie het getoets wat die bevriesing proses se effek op epididimale sperme is, deur sperm motiliteit en -morfometrie te vergelyk tussen vars gekollekteerde sperme en sperm monsters na ontdooiing. Die effek van verskillende sperm konsentrasies, ekwilibrasie tydperke en die byvoeging of uitsluiting van seminale plasma op die lewensvatbaarheid en morfometriese eienskappe van Merino ramsperme is ondersoek in die studie. Geëjakuleerde monsters is versamel met behulp van 'n kunsmatige vagina (AV) van 8 Hoë Lyn en 7 Lae Lyn ramme. Epididimale monsters is verkry van 6 ramme van elk van die Hoë en Lae Lyne, deur middel van aspirasie van die sperme uit die *cauda* epididimii nadoods. Geëjakuleerde sperm monsters is met behulp van makroskopiese en mikroskopiese metodes geëvalueer, en epididimale sperm monsters slegs mikroskopies geëvalueer, met behulp van die Sperm Class Analyzer® program wat vir die evaluasie van beweeglikheid en morfometriese afmetings gebruik is. Sperm beweeglikheids opnames is opgeneem teen 100 raampies per sekonde.

Die resultate van die studie het aangedui dat genotipe geen effek het op besetting van die ooie gepaar met die Hoë of Lae Lyn ramme gehad het nie, terwyl die dekvermoë aansienlik tussen ramme van die twee lyne verskil het. Wanneer die morfometriese eienskappe van vars geëjakuleerde sperme vergelyk was, het die lyne beduidend in terme van die morfometriese eienskappe van verlenging (*elongation)* en elliptisiteit verskil het. Die epididimale en geëjakuleerde sperme verkry vanaf die Lae Lyn ramme het ’n breër en ronder kopvorm getoon as sperme wat verkry is van die Hoë Lyn ramme. Wanneer die morfometriese eienskappe van sperme versamel met die twee verskillende metodes (d.i. kunsmatige vagina of aspirasie vanuit die *cauda* epididimides) vergelyk was, is geen morfometriese verskille waargeneem nie. Die meeste sperm beweeglikheidseienskappe (d.i. persentasie beweeglike, vinnig-, medium- en stadig-swemmende sperme, VCL, VSL, VAP en ALH) van geëjakuleerde en epididimale

sperme het verskil. Die beweeglikheidseienskappe amplitude van die laterale verplasing van die spermkop (ALH) en frekwensie waarmee sperm sy eie pad kruis (BCF), soos bepaal vir epididimale sperme, het beduidend tussen die twee lyne verskil. Met die evaluering van epididimale sperme na ontdooiing was dit duidelik dat sperme verkry van die Hoë Lyn ramme 'n groter mate van akrosoom-oppervlak gehad het, in vergelyking met sperme van die Lae Lyn ramme. Die byvoeging van seminale plasma by epididimale monsters het nie bygedra tot 'n verbetering van spermbeweeglikheid nie. Bestaande literatuur dui aan dat diepbevriesing 'n afname in die kopgrootte van sperme veroorsaak. In hierdie studie het die byvoeging van seminale plasma ’n verandering in kopgrootte voorkom, wat dui op ’n potensiële voordeel om seminale plasma in die bevriesingsprotokol van epididimale ramsperme in te sluit.

Die studie het twee beweeglikheid bepalingstegnieke vergelyk om te bepaal of die tydrowende “opswem” tegniek (SUT) vervang kan word met 'n meer tyd-doeltreffende "spoel tegniek” (FT) in die voorbevriesing verwerking protokolle van ram sperme. Vergelyking van die twee tegnieke het aangedui dat die meeste van die kinematiese eienskappe van die FT gunstig met die waardes soos verkry met die SUT, vergelyk het. Resultate het getoon dat die FT parameters goed vergelyk met die beweeglikheid parameters van die SUT, dus kan dit aangeneem word dat die FT ’n meer tyd-doeltreffende tegniek is wat vergelykbare sperm beweeglikheidsinligting oor skaap sperm monsters voor bevriesing sal verskaf.

In samevatting is verskille in terme van die dekvermoë en op morfometriese vlak, meer spesifiek die eienskappe van verlenging (*elongation)* en elliptisiteit, tussen die twee lyne waargeneem. In toekomstige studies moet die morfometriese eienskappe van skaapsperme verder bestudeer word, asook die korrelasie daarvan met die bevrugtingsvermoë na ontdooiing bepaal om sodoende die diepbevriesing protokolle van skaapsperme te optimaliseer.

Acknowledgements

Firstly, I would like to thank our heavenly Father, for He has made me what I am today.

Then, there are no proper words to convey my deep gratitude and respect for my two supervisors. Dr Helet Lambrechts, you have inspired me to become an independent researcher and you have taught me the importance of critical reasoning. Prof. Schalk Cloete, thank you for your constructive criticism which helped me to develop a broader perspective to my thesis.

My sincere thanks must also go to Prof. Gerhard van der Horst and Dr Liana Maree from the University of the Western Cape. Thank you for all your time, guidance, inputs and patience throughout my study. I truly appreciate it.

I would also like to express my greatest appreciation to Gail Jordaan for statistical analysis. Thank you so much for reminding me that the light at the end of the tunnel was indeed not a train speeding towards me.

I'd like to also thank Annelie and her team at the Elsenburg sheep section for all their help with the rams. Davie, you were a star, thank you.

I would like to thank the Meat Industry Trust Bursary Working Committee for the bursary they have awarded to me for the last two years.

Also I thank the Western Cape Agricultural Research Trust for their financial contribution towards my studies.

To all my friends at the Animal Sciences department, thank you so much for all your love and support throughout my study. For every cup of coffee, for every conversation, for every walk at the foot of the beautiful Stellenbosch mountains, for every smile and all the great times in the post-grad NARGA. It was fun, and made the journey enjoyable and exciting.

And to my Stellenbosch parents, *Oom Herman en Tannie Babie*, you kept me sane during the write-up period. Thank you so much for all your love and support.

Lastly, I would like to thank my parents. *Mamma, Pappa, baie dankie vir al die ondersteuning en liefde wat julle deur my studies aan my gegee het. Julle het nooit in my vermoëns getwyfel nie. Ek is ongelooflik lief vir julle.*

"*Ek is tot alles in staat deur Christus wat my krag gee*"
Filippense 4:13

Table of contents

Alphabetical list of abbreviations

ºC	Degrees Celsius
µL	Microliter
µm	Micrometre
ABP	Androgen Binding Protein
ACTH	Adrenocorticotrophic Hormone
AI	Artificial Insemination
ALH	Amplitude of the Lateral Head displacement
ANOVA	Analysis of Variance
AR	Acrosome Reaction
ART	Assisted Reproductive Techniques
ASMA	Automated sperm morphometry analysis
AV	Artificial Vagina
BCF	Beat/Cross Frequency
C	Concentration
CASA	Computer Aided Sperm Analysis
CNS	Central Nervous System
CRH	Corticotrophin Releasing Hormone
CV	Coefficient of Variation
DAFF	Department of Agriculture, Forestry and Fisheries
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
E	Equilibration
EE	Electro Ejaculation
ET	Embryo Transfer
FGA	Fluorogestone Acetate
FSH	Follicle Stimulating Hormone
FT	Flush Technique
G	Gravity
GH	Growth Hormone
GnRH	Gonadotrophin Releasing Hormone
GOT	Glutamic Oxaloacetic Transaminase
h	Hour
HL	High Line
HPT -axis	Hypothalamic Pituitary Testes axis
IGF	Insulin-like Growth Factor

IVC	*In Vitro* Culture
IVEP	*In Vitro* Embryo Production
IVF	*In Vitro* Fertilization
IVM	*In Vitro* Maturation
JIVET	Juvenile In Vitro Embryo Transfer
LH	Luteinizing Hormone
LIN	Linearity index
LL	Low Line
LSM	Least Square Means
Min	Minute
mL	Millilitre
MOET	Multiple Ovulation and Embryo Transfer
n	Sample size
PRL	Prolactin
PSA	*Pisum sativum* Agglutinin
ROS	Reactive Oxygen Species
PVC	Polyvinyl Chloride
s	Second
SAS	Statistical Analysis Software
SCA	Sperm Class Analyser
SEM	Standard Error of the Mean
SP	Seminal Plasma
SS	Segre-Silberberg
StAR	Steroidogenic Acute Regulatory
STR	Straightness index
SUT	Swim-Up Technique
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VSL	Straight Line Velocity
v/v	Volume / Volume

List of Figures

List of Tables

Chapter 1

General introduction

Sheep farming is practiced throughout South Africa, and allows for the utilization of semi-arid and arid regions which are otherwise unproductive with regards to other livestock species. According to a survey of the Department of Agriculture, Forestry and Fisheries, there are approximately 8000 commercial and 5800 communal sheep farms in the country. It is estimated that the national sheep population consists of about 28.8 million sheep. During the last decade the average gross production value for lamb and mutton in South Africa amounted to R 2 588 million per annum (DAFF, 2011).

Extremes in rainfall and temperatures in South Africa lead to a large variation in soil patterns, and agricultural endaveours are subject to the limitations of soil and and climate, which determine the type of production system that can be managed successfully under these extreme conditions (Smith, 2006). Most of South Africa is comprised of arid and semi-arid zones, which limit the agricultural potential of the land (Cloete & Olivier, 2010). More than 80% of the farmed land in South Africa is then only suitable for extensive livestock utilisation (Livestock Development Strategy for South Africa, 2006). Small stock farming can complement winter- or summer-grain cropping, as sheep can utilize the crop residues and most by-products of the crop farming systems (Cloete & Olivier, 2010). During periods of drought which results in low crop yields, farmers depend on sheep farming to remain economically viable. According to Cloete and Olivier (2010), combining crop production with sheep farming lends stability in a higher output, to the more risky crop enterprises throughout South Africa.

The potential of assisted reproductive techniques (ART's) to assist sheep producers to optimise the production and reproductive efficiency of their flocks has received considerable attention during the last few decades. The application of ART's such as artificial insemination (AI) and multiple ovulation and embryo transfer (MOET) allow sheep farmers to potentially produce lamb and mutton more cost-efficiently.

One of the limitations of the effective application of the respective ART's in sheep, however, is the fact that these techniques have been adapted from ART's developed for and used in the cattle industry. Factors influenced by species differences and which can determine the optimal application of ART's, include different cryopreservation protocols and thawing rates; changes in membrane lipid packing (i.e. potentially inducing temperature-dependant variation in cryoprotectants, specifically glycerol), and the concentration of cryoprotectants added (Holt, 2000).

Optimization of the ART's applicable to sheep production will enable sheep farmers to manage the reproductive performance of their animals and to optimise the number of offspring successfully weaned per ewe, and thus manage their production systems more cost-effectively to promote genetic progress within their flocks (Byrne *et al.*, 2012).

In order to optimize reproduction efficiency with the use of ART's within a production system, the quality of sperm samples used should be of high quality. There are however many factors that can affect the quality of a sperm sample, which include environmental effects (e.g. effect of season), management of the animals (e.g. nutrition and stress), and animal dependent factors (e.g. animal age, breed, health, or composition of the seminal plasma). Even with the use of superior sperm samples, the processing of these samples, that includes cooling, freezing and thawing, can adversely affect the quality of both ejaculated and epididymal sperm (Hafez & Hafez, 2008).

Despite the destructive effects of cooling, freezing and thawing, including the effect that different extenders can have on survivability, overall motility and fertilizing ability of ejaculated and epididymal sperm, very little is known about the potential contribution of the genetic make-up of rams to the ability of sperm to withstand damage caused by pre- and post-cryopreservation processing. Very little information is available on the effect of selection for specific genotypes and how it affects the quality of sperm post thaw.

Ovine cryopreservation protocols need to be improved to minimize the extent of damage caused by the cryopreservation process. The deleterious effect of cryopreservation on the viability and fertilizing ability of sperm can mostly be ascribed to alterations of the sperm acrosomal membrane which occurs mainly during the processes of cooling, freezing and thawing. The modification ofthe acrosomal membrane can be caused by the use of an ineffective cryoprotectant during equilibration, as well as too short an equilibration period to allow for the cryoprotectant to replace the water in the sperm cell. Inefficient cryoprotectants only partially replace water in the sperm cell, which contributes to the formation of ice crystals during freezing, which then acts as microscopic blades that disrupt and damage the sperm membrane. Damage to the acrosomal membrane will result in the leakage of acrosomal enzymes prior to or during thawing (Parks & Graham, 1992; Bailey *et al.*, 2000; Medeiros *et al.*, 2002).

The use of seminal plasma to decrease the amount of damage that occurs during the process of cryopreservation has been investigated by many researchers. However, there have been controversial reports of the potential effect of seminal plasma to improve the ability of epididymal sperm to withstand cryodamage, and the improvement of post-thaw viability.

The use of an optimal equilibration period is also of great importance, for if this period is too short, partial replacement of the water molecules in the sperm head will also result in altered acrosomal membranes, which do occur even with the use of an effective cryoprotectant. Several studies have reported a post-thaw sperm motility of 21% (Purdy, 2006) while others have reported higher levels in post-thaw motility of 33% (Ollero *et al.*, 1997), 41% (Pontbriand *et al.*, 1989), and 65.2% (Bag *et al.*, 2004). The large variation in results indicates that there is ample scope for improvement in the cryopreservation protocols for ovine sperm.

The aims of the study were therefore to determine the influence of genotype on quantitative and qualitative sperm traits, as assessed by means of computer-assisted sperm motility analysis. The influence of sperm concentration, equilibration period, and the addition of seminal plasma on the ability of epididymal ovine sperm to withstand cryodamage was also investigated. The potential effect of adding seminal plasma to epididymal sperm was assessed to determine whether seminal plasma needs to be included in the cryoprotective media to be used in the preservation of epididymal ovine sperm. Two motility determining techniques for ejaculated sperm were also investigated to determine whether a new and more rapid technique can replace an older, more time-consuming technique, thus indirectly streamlining the protocol for the cryopreservation of ovine sperm.

Chapter 2
Literature review

2.1 Introduction

With the human population ever-increasing, livestock industries are under pressure to increase their production. The demand for animal products is expected to increase significantly over the next few decades, with food and water security a priority for humankind in the 21st century. However, increased production will be counteracted by the effect of global warming that may result in changes in the local climate, potentially impacting agricultural pactices on a local and global scale in order to ensure that they remain sustainable and cost-effective (Nardone *et al.*, 2010).

In the livestock production sector, cost-effective and optimal production practices will allow farmers/producers to supply in the ever-increasing demand for safe food. To ensure cost-effective production practices, farmers can use different management tools to assist them in optimizing the production efficiency of their farming enterprises. Two such tools include genetic selection, and assisted reproductive technologies (ART's). Genetic breeding programs focus on the selection for traits that can contribute to improving the production and reproductive efficiency of livestock species. Traits that can be selected for include, among others, improved disease resistance (Eady *et al.*, 2003), improved feed conversion due to selection for disease resistance (Doyle *et al.*, 2011), and optimal reproductive ability (i.e. selecting for multiple rearing ability) (Cloete *et al.*, 2004; Cloete *et al.*, 2009). The selection of animals according to certain traits to specifically improve and/or optimise production and reproduction efficiency is commonly known as genetic improvement with genetic improvement being vital to the success of a sustainable agricultural food industry (Bailey *et al.*, 2000).

Assisted reproductive techniques (ART's) such as artificial insemination (AI) and *in vitro* embryo production and transfer (IVEP) can then contribute significantly, if applied properly, to optimize and ensure cost-efficient livestock production (Byrne *et al.*, 2012).

2.2 Assisted reproductive techniques

Assisted reproductive techniques (ART's) are implemented and used worldwide. One of the main aims of using ART's in livestock production is to allow high genetic merit animals to produce more offspring in a shorter period of time than would be possible naturally. Furthermore, the use of hormonal synchronization of oestrus and ovulation, in combination with certain ART techniques, allows breeders to overcome the limitation of seasonal breeding, i.e.

animals can produce offspring and milk throughout the year, especially in seasonally breeding livestock, such as sheep and goats (Baldassarre & Karatzas, 2004).

Assisted reproductive techniques have enabled breeders to optimise the production and reproduction efficiency of their systems by improving selective traits, as well as by accelerating genetic progress. Certain ART's such as AI and embryo transfer (ET) increase the selection differential, while other techniques such as juvenile *in vitro* embryo transfer (JIVET) enable producers to reduce the generation interval (Baldassarre & Karatzas, 2004). Artificial insemination and the preservation of semen are the main technologies that are used extensively in the livestock sector (Madan, 2005). Some of the most important ART's are discussed below.

2.2.1 Artificial insemination

Artificial insemination (AI) is the most widely used ART in the world, and has made a significant contribution to genetic improvement in the livestock industry (Evans & Maxwell, 1987; Baldassarre & Karatzas, 2004; Verma *et al.*, 2012).

The first successful use of AI was documented in 1780 by the Italian Spallanzani, when he inseminated a beagle bitch and obtained three puppies 62 days later (Herman *et al.*, 1994). The French (specifically the veterinarian Repiquet) started using AI in their horse breeding programme in 1890 (Herman *et al.*, 1994). Many other reports appeared in the 19th century, but it was not until the 1900's that extensive studies using farm animals were conducted in Russia, and shortly thereafter in Japan (Hafez & Hafez, 2008). Early development and implementation of AI in sheep on large scale only began in 1938 in Russia (Foote, 2002).

Artificial insemination is one of the ART's that remains most important in commercial livestock production. The popularity of this technique relies on three cornerstones, namely that its application is simple, economical and successful (Vishwanath, 2003). The use of AI can be advantageous for genetic gains, curbing the spread of sexually transmitted diseases and to exert control over management options such as the timing and spread of the lambing season. Artificial insemination also has the benefit of reducing disease transmission between animals. During natural mating, venereal diseases can be transferred from the male to the female. By screening collected sperm, problems associated with male infertility can also be avoided (Hafez & Hafez, 2008).

Genetically, AI allows for the exchange of genetic material of superior animals, it leads to improved performance and potential of livestock herds and flocks, and also accelerates the genetic gain that is possible within a herd. The amount of sperm used per insemination is much

less than is ejaculated by the male during natural mating. Thus the quality of sperm used for insemination should be of superior quality to ensure successful fertilization, even with the lower sperm concentration doses that are used in AI. The use of high quality sperm from genetically superior donors is essential to limit the transfer of unwanted traits to the next generation (Naqvi *et al.*, 2001).

With regards to management, AI promotes progeny testing under a range of environmental conditions, thus facilitating the study of genotype x environment interactions. The use of AI technology also promotes the production of large numbers of offspring, especially with the use of synchronization of oestrus in large groups of animals. The generation of fast and efficient information regarding breeding abilities and number of offspring produced is also made possible, and AI thus proves to be a beneficial research tool. The development and subsequent use of AI in the farm animal industry has led to a remarkable increase in the productivity of livestock production (Anel *et al.*, 2005).

There are however disadvantages to take into consideration before practicing AI on a large scale. Inseminators must have applicable knowledge in the field, especially to detect oestrus in females and to inseminate within the specific insemination window. Even with excellent inseminators, many females will not be impregnated at the first insemination, and thus a second round of inseminations will be required. Some farmers choose not to inseminate a second time, and rather use a bull/ram to impregnate those females which weren't impregnated using AI. This then contribute to additional costs of maintaining a bull/ram on the farm (Overton, 2005).

Another factor that needs to be considered is the stress that results from females being e.g. restrained to be inseminated. The application of AI is expensive, especially when semen cryopreserved in straws are used for insemination. The use of costly quality sperm of top ranking bulls/rams can also contribute to the costs involved using AI.

When considering either liquid stored- or frozen-thawed semen, there are several advantages and disadvantages to be considered (see Table 2.1).

Table 2.1 *Relative advantages and disadvantages of liquid and frozen-thawed semen (Hafez & Hafez, 2008)*

Liquid stored semen	**Frozen-thawed semen**
Advantages	
Low sperm numbers	Long term storage
High sire utilisation	Flexibility of use
Inexpensive storage	
Ease of use in the field	
Disadvantages	
Limited shelf life	High sperm numbers
	Expensive to store

Another ground-breaking technical advance that was made in recent years is the sexing of sperm by DNA quantification, using flow cytometry (Foote, 2002). The sexing of sperm enables a producer to increase the number of offspring of a specific gender in a closed population (Nicholas, 1996). Currently the time required to sort the billions of sperm per ejaculate is limiting its extensive commercial application, but equipment and the techniques are continuously improving, which will eventually make it a feasible ART tool. One should however take into consideration the degree of damage that is associated with these techniques (Foote, 2002).

2.2.2 Multiple ovulation and embryo transfer

Multiple ovulation and embryo transfer (MOET) is often referred to as the ART that is "to the female, what AI means to the male". The application of MOET allows for the production of more offspring from a single genetically superior female than would normally be possible, if the female was bred naturally. The use of MOET was first documented in 1975 for beef cattle, 1977 for dairy cattle, and in 1986 for sheep. The MOET reproductive tool entails the administration of exogenous hormones (FSH / progesterone) to increase the number of follicles to develop in the ovaries of the female before ovulation. The resulting ova can then be collected by means of transvaginal ultrasound-guided aspiration before ovulation, or flushing after the ova have been released during ovulation, and used in *in vitro* embryo production (IVEP) processes (Nicholas, 1996).

The *in vitro* production of embryos (IVEP) is an approach that may increase the efficiency of reproduction in animals without compromising their productive lifespan (Bousquet *et al.*, 1999). The procedure for the production of embryos *in vitro* is then applied worldwide, with different goals for a variety of livestock species, exotic, wild and endangered animals. The possibility of producing embryos by using *in vitro* technologies has captured the attention of animal scientists and entrepreneurial groups, especially when a non-invasive procedure for recovering oocytes

from the antral follicles in the live animals became a practical and effective approach (Neglia *et al.*, 2003).

In vitro maturation (IVM) of oocytes and *in vitro* fertilization (IVF) are used to produce large numbers of embryos (bovine and ovine) for transfer and micromanipulation purposes, which contributes to making the application of the ART's more economical. There are a number of factors that play a role in the success of *in vitro* embryo production, such as the source of oocytes, the source and preparation of the sperm, culture media, and culture conditions, which seem to differ for different species (Wani, 2009). The collection of oocytes by means of transvaginal ultrasound-guided follicular aspiration has routinely been performed with success in large prepuberal and adult ruminant species under various physiological and pathological conditions (Neglia *et al.*, 2003).

It is important to note that sperm do not attain their full fertilization capacity until after they are transported through the female reproductive tract to eventually reach the oviduct. There are certain physiological changes that sperm have to go through before they are able to penetrate the zona pellucida and fertilize the ovum. When the first experiments with IVF were conducted, poor results were achieved due to the fact that the sperm were not capacitated. Capacitation refers to the modification of ejaculated sperm in the female reproductive tract, thus enabling sperm to fertilize the ova. This then led to a method of treating sperm prior to IVF to capacitate sperm to achieve successful fertilization (Hafez & Hafez, 2008).

The process of capacitation allows sperm to undergo the normal acrosome reaction prior to fertilization. According to Hafez and Hafez (2008), previous experiments obtained capacitated sperm by flushing the sperm from the uterus or oviducts of a mated or artificially inseminated female. *In vitro* capacitation of sperm however differs from the *in vivo* phenomenon. The capacitation of sperm *in vitro* has been accomplished by the addition of a high ionic strength medium (e.g. calcium ionophore or caffeine), a long incubation period of 18-24 h, high pH, or washing through a Percoll® gradient however, results were not repeatable. The fact that the glyocosaminoglycan heparin capacitates bull sperm has then significantly advanced this IVF technology in farm animals. The procedure of *in vitro* embryo production (IVEP) generally involves the collection of oocytes from ovarian follicles, then completing three biologic steps: IVM; IVF; and *in vitro* culture (IVC) (Hafez & Hafez, 2008).

The first mammalian embryos were transferred by Walter Heape in 1890, when he transferred two four-cell Angora rabbit embryos into a Belgian doe. The Belgian doe then gave birth to four Belgian and two Angora young. According to Mapletoft and Hasler (2005), there have been few reports on successful mammalian embryo transfers since then, until the 1920's.

2.2.3 Economic implications of ART's in the sheep industry

Livestock farming in South Africa contributes up to 49% of agricultural output. The Department of Agriculture, Forestry and Fisheries (DAFF) of South Africa has calculated that over the past decade, the average gross production value of lamb and mutton amounted to R2588 million. From 2001 until 2009, the gross value for lamb and mutton has continuously increased. Thus the importance of reproduction efficiency and production of surplus animals are of great importance to counter the local deficit and demand. Using ART's as the basic tool, it is possible to enhance the level of production within the livestock sector (DAFF, 2011).

Sheep farming is practiced throughout South Africa and the overall improvement of the sheep industry in the country is of great importance to optimize production efficiency. By using ART's, it is possible to increase production, reproductive efficiency and the rate of genetic improvement. The use of MOET in commercial sheep flocks is a very important tool for the genetic improvement and preservation of the genetic integrity of the species. Due to the relative short history of the application of the respective ART's in sheep, the development and refinement of ovine-specific protocols to optimise production efficiency, thereby contributing to ensuring the cost-effective management of sheep flocks on a global scale (DAFF, 2011).

2.3 Reproduction in the ram (*Ovis aries*)

2.3.1 Anatomy of the male reproductive system

The reproductive system of the ram consists of a set of bilateral testes, a duct system, accessory glands, and the penis. The testes are also known as the male gonads, and are located outside the abdominal cavity, housed within the scrotum (Hafez & Hafez, 2008).

The testes of the ram descend during fetal development from the abdominal cavity through the inguinal canal to assume their position in the scrotal sack. Differences between species as far as the shape, size, and the location can occur, but the essential structure and function of these organs are the same (Frandson *et al*., 2003). The testes of the ram are usually about 10 cm long, and weigh approximately 250-300 g (Sisson & Grossman, 1975).

Each testis consists of a mass of seminiferous tubules and is surrounded by a fibrous capsule (*tunica albuginea*) (Sisson & Grossman, 1975; Frandson *et al*., 2003; Hafez & Hafez, 2008). The Leydig cells, located in the connective tissue between the seminiferous tubules, are responsible for the production and secretion of testosterone (Frandson *et al*., 2003; Hafez & Hafez, 2008). The Leydig cells secrete the male hormones into the testicular veins and lymphatic vessels (Hafez & Hafez, 2008). In seasonal breeders such as the ram, testicular size

in reproductively mature rams varies throughout the year, with the difference in size that can be attributed to the extent of spermatogenic activity in the seminiferous tubules (Hafez & Hafez, 2008).

The scrotum houses the testes, and is a cutaneous sac that conforms in size and shape to the testes (Frandson *et al*., 2003; Hafez & Hafez, 2008). The scrotum has a neck above the testes that can clearly be identified, and a septum which separates the two halves. The scrotum protects the testes, and allows for optimal thermoregulation in the testes, which will ensure that spermatogenesis takes place optimally. For optimum sperm production, the testes must be maintained at a temperature lower than the animal's core temperature. There are specialized temperature receptors within the scrotal skin that can provoke responses that tend to lower the whole body temperature (Hafez & Hafez, 2008).The *tunica dartos*, a layer of fibro-elastic tissue interspersed with smooth muscle fibers, lines the scrotum, and during colder ambient temperatures these muscles will contract and help to keep the testes closer to the abdominal wall of the animal, ensuring an optimal temperature for spermatogenesis to take place (Frandson *et al*., 2003).

2.3.1.1 The epididymis

The epididymis can be divided into three anatomic parts, namely the head (*caput*), the body (*corpus*) and the tail (*cauda*). The epididymis consists of a single narrow tube, and it performs vital functions such as transport, maturation and the storage of sperm prior to ejaculation (Hafez & Hafez, 2008).

When examined histologically, the epididymis can be distinguished into three segments, though these do not coincide with the gross anatomic regions. It is stated that the first two segments are involved with sperm maturation, whereas the third segment is responsible for sperm storage (Gatti *et al.*, 2004). The transport of sperm through the epididymis takes about 9 to 13 days in the ram. Maturation takes place as the sperm move through the epididymis; and motility tends to increase as the sperm enter the *corpus epididymis*. The specific environment within the *cauda epididymis* then provides the necessary factors that will enhance the fertilizing ability of the sperm. The *cauda* epididymis is also the main storage organ, containing about 75% of the total epididymal sperm supply (Hafez & Hafez, 2008).

2.3.1.2 Vas deferens

The vas deferens, also known as the *ductus deferens*, is a muscular tube which, at the time of ejaculation, will propel the sperm from the epididymis into the ejaculatory duct which is situated in the prostatic urethra (Frandson *et al*., 2003). Each epididymis is associated with one deferent

duct, which forms an enlargement to the end, known as the ampulla (Frandson *et al*., 2003). The ampulla serves as a storage organ for sperm.

2.3.1.3 Accessory sex glands

The accessory glands are comprised of the prostate and the bulbourethral glands. These sex glands produce fluids that are secreted into the male reproductive tract at ejaculation. The fluids then mix with the sperm and ampullary fluids to form semen. The addition of these fluids (called seminal plasma) also contributes to the transport of the sperm. Much information is available regarding the specific chemical composition of farm animal ejaculates (Hafez & Hafez, 2008).

2.3.1.4 Penis

The penis is located outside the body and fulfils two functions namely to deposit semen into the vagina of the female, while it is also required for the excretion of urine. The free extremity of the ram penis is housed within the prepuce, which is an infolding of the skin (Hafez & Hafez, 2008).

2.3.2 Spermatogenesis and sperm morphology

Spermatogenesis can be defined as the production of sperm, and it is partly controlled by the testes, but mainly initiated and regulated by the central nervous system (CNS) but more specifically the hypothalamic-pituitary-testicular (HPT) axis. The process of spermatogenesis is complex and involves mitotic cell division, meiosis and the process of spermiogenesis to produce viable sperm (De Kretzer *et al.,* 1998). Sperm primarily consist of nucleic acids, proteins, and lipids formed in the seminiferous tubules of the testes which contain a complex series of developing germ cells that will gradually mature to form the male gametes (Hafez & Hafez, 2008).

With the formation of sperm, the completed germ cells are released into the lumen of the seminiferous tubules. During this process, a proximal droplet of sperm is formed, and with the release of the sperm from the epithelium, the residual bodies need to be exposed of. Hence they are phagocytised by cells called the Sertoli cells (Hafez & Hafez, 2008).

2.3.2.1 Morphology of sperm

Mammalian sperm consist of a head, neck and a tail. The length of the sperm is species specific, and it varies in farm animals from 50µm (boar) to 90µm (bull). The spermatozoon is surrounded by the plasma membrane, which is characterized by a regional specific glycoprotein and lipid constitution (Pesch & Bergmann, 2006). The sperm head consists of the acrosome and the nucleus. The acrosome or acrosomal cap consists of a double-walled structure between

the plasmalemma and the anterior portion of the sperm head (Hafez & Hafez, 2008). The acrosome as such covers the first two thirds of the sperm head, and forms a cap-like structure around it. The specific shape and size of the acrosome is highly species specific and contains many enzymes, e.g. acrosin, hyaluronidase and many more hydrolases and esterases. These enzymes are then crucial for the lysis of the *zona pellucida* and the penetration of the corona radiata of the ovum. The enzymes are released from the acrosome during the acrosome reaction (AR) (Pesch & Bergmann, 2006).

A small subacrosomal space containing amorphous contents of low density is present between the acrosome and the nucleus. The function of this layer is to act as an adhesive layer that binds the cap of the acrosome to the underlying nucleus. The shape of the nucleus is also highly species specific. The neck of the sperm is approximately 1 µm in length, and connects the head to the flagellum (tail). The sperm necks varies in length and shape between species. Single mitochondria with small projections are detectable within the neck, and serve as an energy store for the sperm (Pesch & Bergmann, 2006).

The tail of a spermatozoon is the longest part of the sperm cell, and can be divided into three pieces, namely the midpiece, the principal piece, and the end piece. The midpiece is characterized by the mitochondrial sheath surrounding it. Mammalian sperm contain many mitochondria and have a higher survivability than sperm of aquatic vertebrates with only two mitochondria. The mitochondria are arranged end to end in helices for the purpose of high flexibility during movement of the sperm (Pesch & Bergmann, 2006).

The principal piece is the longest segment of the tail, and is enclosed by fibrous sheaths. These fibrous sheaths work as a type of scaffold for proteins in signalling pathways. It is speculated that it is involved in the regulation of sperm processes such as maturation, motility, capacitation, hyperactivation and/or the AR. The principal piece ends abruptly with the end piece (Eddy *et al.,* 2003; Pesch & Bergmann, 2006).

2.3.2.2 Blood-testis barrier

The seminiferous tubules, where spermatogenesis takes place, are not penetrated by blood or lymph vessels. The developing germ cells are sensitive and thus the seminiferous tubules need to protect these developing germ cells from chemical changes in the blood. This is accomplished by ways of a specialized permeability barrier, called the blood-testis barrier. The barrier performs a protecting function, with the help of a myoid cell layer that acts as a sealant, and the unique junctions that occur between the adjacent Sertoli cells (Hafez & Hafez, 2008).

2.3.2.3 Role of Sertoli cells

Sertoli cells are the somatic cells of the testes that are essential for the formation of the testes, and thus the process of spermatogenesis (Griswold, 1998). These cells provide a three-dimensional framework along which germ cells develop, mature, and gradually are transported towards the tubular lumen (Weinbauer & Wessels, 1999). The Sertoli cells are closely associated with the development of the spermatogonia (Hafez & Hafez, 2008). These cells facilitate the progression of the germ cells to sperm via their direct contact and the controlling of the environment setting within the seminiferous tubules. The roles of FSH and testosterone in spermatogenesis are expressed by the action of these hormones on the Sertoli cells. The number of Sertoli cells is indicative of the extent of spermatogenesis, i.e. the higher the number of cells present, the higher the concentration of sperm that will be produced (Griswold, 1998).

Sertoli cells also have the function to remove large numbers of degenerating germ cells, as the spermatogenic process is relatively inefficient, with large numbers of potential sperm degenerate before having the chance to become mature sperm (Hafez & Hafez, 2008).

2.3.3 Physiological alterations of the sperm surface

Sperm are in constant contact with their immediate environment, and depending on the specific environment, will interact or be altered by it. The plasma membrane of sperm thus acts as a two-way communication device to induce or suppress these specific changes to the sperm surface. During transit within the male reproductive system, ejaculation and entry to the female reproductive tract, sperm are exposed to many different fluids, epithelial and immune-competent cells. The epididymis, accessory sex glands, and the oviduct then create different environmental conditions that sperm are exposed to. The varied and complex protein complement of these different fluids and cells prepares the sperm surface for the process of fertilisation (Leahy & Gadella, 2011). There are different compartments and components which induce sperm membrane changes to occur, which will be discussed below.

2.3.3.1 Sperm surface modification in testis and epididymis

Sperm are transformed from round germ cells by various morphological and surface-modifying events within the testis. Part of this transformation includes the division, differentiation and meiosis of spermatogonia to form the haploid elongated spermatids during the process known as spermatogenesis. Further modification is enabled in the epididymis by exposure to a highly dynamic protein environment, which is created by the secretion and absorption of proteins across the epididymal epithelium. Within this protein-rich environment, proteins may also be

removed or modified by specific proteolytic processing, with some proteins being reported as being in an active enzymatic form in the surrounding fluid (Leahy & Gadella, 2011).

There are many potential mediators of sperm-egg communication acquired, or modified, in the epididymis. This demonstrates the significant contribution of this organ to successful fertilization. Sterols and fatty acids of the sperm membrane undergo significant alteration during the epididymal transit, which causes dramatic modifications in the membrane architecture (Leahy & Gadella, 2011). Studies conducted on rams, rats and mice sperm showed a significant reduction in the cholesterol content during the epididymal transit and an increase in the ratio of unsaturated fatty acids (Saez *et al.*, 2011). Lipid modification generally increases the membrane fluidity, and is thought to prime signalling pathways that regulate capacitation (Shadan *et al.*, 2004).

The proteins and lipids of the plasma membrane are thus remodelled in the epididymis in such a way that sperm are primed to undergo capacitation, interact with the zona pellucida and eventually fuse with the oolemma. It is also reported that sperm from the *cauda* epididymis are now ready to fertilize the ova (Leahy & Gadella, 2011).

2.3.3.2 Sperm surface and seminal plasma

At ejaculation, sperm are exposed to yet another set of surface remodelling components, e.g. the seminal plasma. The seminal plasma being a physiological secretion from multiple glands of the male reproductive tract, and a natural medium for the maturation of sperm through hormonal, enzymatic, and surface-modifying events, seminal plasma also contains a variety of biochemical components, some of which have been proven to be relatively specific in the regulation of sperm function (Barrios *et al.*, 2000).

In the oviduct, sperm undergo a last biochemical and structural modification, termed capacitation, which culminates in the acrosome reaction and fertilisation. Capacitation is defined as the series of transformations that a sperm cell normally undergoes during the migration through the female reproductive tract, to allow for the sperm to bind to the zona pellucida and undergo the acrosome reaction, and thus be able to fertilize the ovum. The main purpose/function of capacitation is to ensure that the sperm will reach the ovum at the appropriate time and in the appropriate state to ensure fertilization. This is done by regulating the rate at which these changes occur carefully, as to prime the sperm and activate all necessary mechanisms that is needed for the acrosome reaction (De Lamirande *et al.,* 1997).

In order to synchronize the onset of capacitation with the arrival of the sperm at the oocyte, there are molecules present on the surface of the sperm, which will act according to its immediate environment, and thus act on both the stimulus and the inhibition of the onset and progression of this process. When sperm capacitate too early, it is potentially due to the loss, modification and redistribution of molecules on the sperm surface (Leahy & Gadella, 2011).

There are also entities coating the sperm membrane which are called decapacitation factors. These factors are removed during the process of capacitation. These specific factors are present in the epididymal fluid and the seminal plasma and their mechanisms of action is highly complex and species dependent. These decapacitation factors of seminal plasma origin are best described in production animals (e.g. the ram, bull and boar), where they have indicated prevention or reverse capacitation of sperm under conditions of handling-induced stress (De Lamirande *et al.,* 1997; Leahy & Gadella, 2011).

2.3.4 Hormonal regulation of sperm production

The influence of hormones on sperm production is closely associated with the effect of animal age, as puberty is the main event that initiates the functionality of the male gonads. To be able to understand the effect of hormones on the quality of sperm, the integrated system of hormonal regulation of spermatogenesis needs to be examined. The sexual maturity of an animal can be judged by three factors, i.e. whether the necessary hormones are being produced, if there are enough receptors present in the target areas (i.e. the testes) for these hormones to bind to, and whether the receptors are sensitive to the stimuli of the hormonal signals regulated by the central nervous system (CNS). This integral system between the hypothalamus, the pituitary, and the testes is known as the hypothalamic-pituitary-testicular axis (HPT-axis). The functional HPT-axis consists of the typical endocrine actions of gonadotrophin, the different feedback mechanisms of steroid hormones and proteins and, most likely, a paracrine or an autocrine modulation. These mechanisms will determine the rate and efficiency of spermatogenesis (Roser, 2001).

The endocrine regulation of spermatogenesis and thus the successful formation of good quality sperm are mainly characterized by changing patterns of secretion of gonadotrophin releasing hormone (GnRH), follicle stimulating hormone (FSH), luteinizing hormone (LH), and testicular hormones, such as the androgens and inhibin (Roser, 2001).

2.3.4.1 The effect of GnRH, LH, FSH, ABP, inhibin, testosterone, and estrogen on spermatogenesis

The testes not only produce testosterone, but also a series of related steroid hormones. Androgens exert their major action on the Sertoli cells rather than directly on the germ cells. Pituitary LH stimulates the Leydig cells to secrete androgens that not only diffuses into the adjacent Sertoli cells, but are also secreted into the blood, where they feed back to the hypothalamus to decrease the release of GnRH, and thus reduce the release of stimulating LH from the anterior pituitary, as seen in Figure 2.1 below.

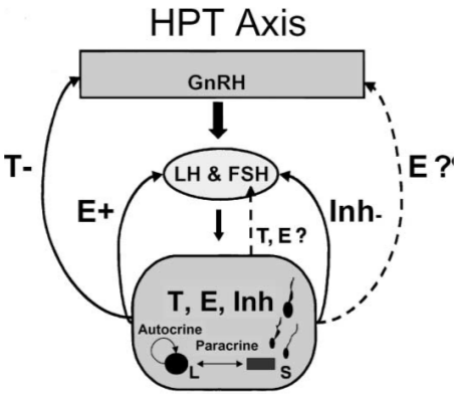

Figure 2.1 *The HPT axis of the stallion indicating the link between the hypothalamus, the pituitary and the testes, and how hormonal communication and regulation allows for the production of viable sperm (Roser, 2001)*

The secretion of inhibin and androgen-binding-protein (ABP) from the Sertoli cells is stimulated by FSH. The role of this ABP being to form a complex with the androgens so it can be transported along with the sperm into the epididymis, as these epithelial cells require a relatively high level of androgen for normal functioning. Inhibin acts as a negative feedback mechanism for the secretion of FSH, but has no significant effect on the secretion of LH (Hafez & Hafez, 2008).

A high level of testosterone is needed for the normal maturation process of sperm. Testosterone that is secreted into the seminiferous tubules is either converted into dihydrotestosterone (DHT) by the action of an enzyme called 5α-steroid reductase, or it is converted to estrogens by the enzyme aromatase (Hafez & Hafez, 2008). Estrogen modulates the GnRH modulated LH secretion through a negative feedback mechanism and estrogen also appears to be a factor in the development of the Leydig cells, and the modulation of steroidogenesis within the Leydig cells. In stallions, an abnormally high amount of estrogen is produced within the testes, thus

implying that estrogen, along with FSH and testosterone, plays a role in sperm production (Roser, 2001).

2.3.4.2 The effect of prolactin, thyroid- and growth hormone on spermatogenesis

Previous studies conducted on rats have indicated that prolactin (PRL) is an important factor in inducing the transcription of the estrogen receptor. There is also significant evidence that prolactin affects the tissues that are sensitive to the androgens. In combination with LH and GH, it also modulates the synthesis of the LH receptors, activates the synthesis of androgen, and can stimulate the process of sperm production in the rat, ram and boar (Roser, 2008). Studies conducted on horses showed that the prolactin levels of stallions are elevated during the breeding season, and lower in the non-breeding season which suggests that prolactin could be a role player in enlarging the testes during breeding season, thus elevating the production of sperm (Roser, 2001; 2008).

Thyroid hormone on the other hand is vital to the onset of mature Leydig- and Sertoli cell differentiation. The lack of thyroid hormone will lead to a decline in the steroidogenic acute regulatory (StAR) proteins, which will lead to a reduction in testosterone production, which in turn is crucial for regulating spermatogenesis (Roser, 2008).

Growth hormone (GH) exerts its effect on the function of the testis by modulating the synthesis of gonadal steroids and spermatogenesis. In the rat, studies have indicated that the presence of GH limits the amount of LH that is released by the pituitary, and it tends to increase the production and release of testicular insulin-like growth factor-1 (IGF-1). With insufficient amounts of GH, the testes size will be abnormally small, thus producing less sperm during spermatogenesis. Growth hormone affects synthesis of testosterone, thus altering spermatogenesis and sperm quality indirectly (Roser, 2008).

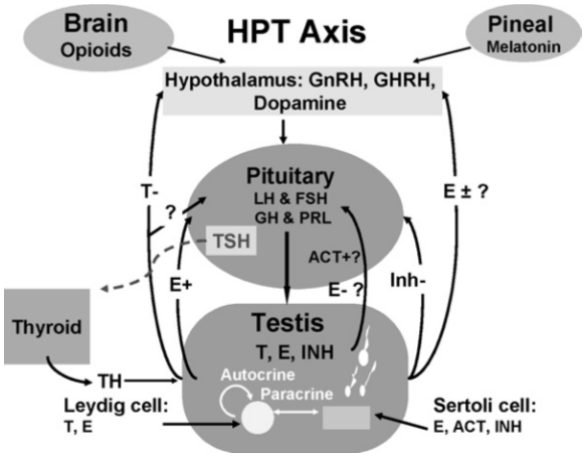

Figure 2.2 *The integrated hormonal regulation of the HPT axis; the different signals acting on the brain, pituitary and the testes for the successful maintenance of spermatogenesis (Roser, 2008)*

All the hormones (Figure 2.2) play vital roles in the formation of good quality sperm. If one should be deficient, the quality of the sperm will be influenced negatively, direct or indirectly. There are many factors that can affect the fluctuations of hormones, but mostly it is regulated by season, thus season will play an important role on the quality of sperm produced by seasonal breeders (Roser 2008).

2.3.5 Mating performance and preference of the ram

Gonyou (1984) reported that the number of ewes serviced and impregnated by an individual ram is the ultimate measure of sexual performance of rams. Thus one method of quantifying the contribution of rams to the reproduction success within a flock is by measuring mating performance. Sexual performance within populations of domestic male farm animals is however highly variable. With such a high variation in sexual performance, the selection for this trait should have a rapid response when selected for, provided that the trait selected for is heritable. A high variability in sexual performance makes it desirable for any breeder to evaluate the mating competence of individual males before inclusion in a breeding program (Chenoweth, 1981).

In a study on the effect of rams differing in their serving capacity on flock fertility, it was concluded that inadequate serving capacity is a contributing factor to insemination failure within a flock mating system. It is thus possible that the use of a high prolificacy group of rams with a high serving capacity may lead to a reduced percentage of unfertilised ewes and an improvement in the lambing percentage within a breeding flock (Kilgour & Wilkins, 1980).

Within groups of rams, dominance-subordinance relationships exist, and this social interaction between rams modify the mating preferences of the individual rams (Tilbrook *et al.*, 1987). It was also noted that rams showed a clear preference to certain ewes, and ignored others, although all ewes were in oestrus. Chenoweth (1981) also reported that the greatest single stimulus for male copulatory behaviour is the immobilization of the female. Immobilization could be due to restraints or oestrus, for the reaction of rams is the same towards both kinds of immobilization.

Groups of rams will form a social hierarchy according to dominance, and cause a difference in reproductive efficiency within the group in which dominant and subordinate/submissive rams are present. Previous studies have also indicated that when a dominant ram is viewed by an audience of rams, no change was found in the mating behaviour. However when submissive rams were viewed by an audience of dominant rams, a decrease in mounting and ejaculation frequency was recorded (Lindsay *et al.*, 1976; Gonyou, 1984).

2.4 General factors affecting sperm quality

The use of high quality viable gametes in the application of ART's is of utmost importance to ensure the success of these techniques to lower input costs, and to ensure the production of viable offspring.

There are certain factors that should be considered when evaluating the quality of sperm. Some of the parameters indicative of sperm quality include overall motility, total volume, concentration, % dead/live, % normal/abnormal, and the integrity of the acrosome, to name a few. There are however certain factors that can influence these traits and thus reduce fertility, impacting negatively on the reproductive efficiency of males. Some of these factors are discussed below, and how they affect the quality of sperm.

2.4.1 Animal age

For the age of the male to have an effect on sperm quality, one should keep in mind that an animal needs to reach sexual maturity before viable sperm is produced. Puberty is reached when an animal is able to produce gametes and also manifest complete sexual behavioural sequences. This is when the circulating concentrations of gonadotrophin increase, as a result of sex steroids and a possible influence from the responsiveness to gonadotrophin releasing hormone (GnRH), is produced by the hypothalamus and ultimately regulates the release of gonadotrophins. Once the gonadotrophins are produced and released to reach their target sites,

it is possible for the male to initiate the process of spermatogenesis. However, the onset of puberty does not indicate sexual maturity, as the hypothalamic pituitary testis axis (HPT-axis) should be fully functional for the testes to produce sperm capable of fertilizing the ovum. The HPT-axis will be fully functional when all three components are integrated. Basically sexual maturity is the gradual adjustment between the increased release of gonadotrophin and the ability of the gonads to assume steroidogenesis and gametogenesis at the same time (Hafez & Hafez, 2008). In the ram, spermatogenic maturity is usually reached at an age between 18-20 months, depending on the breed (Mandiki *et al.*, 1998).

Previous studies have shown that the age of the male can influence the production of sperm, as it affects the quality and viability of the sperm. Studies conducted on aging rodents showed histological changes occurring in the testes with age, and these changes decrease the quality of sperm produced. The latter study also concluded that vacuoles were found to decrease within the germ cells, and thus result in a thinner seminiferous epithelium. This then results in a reduction in the amount of spermatids produced and the process of spermatogenesis then eventually disappearing in mice by 33 months of age. A decrease was also recorded in the semen volume produced, as the males aged (Kidd *et al.,* 2001). The effect of age on sperm concentration has been studied, and it seems there are some contradictions in the literature regarding this relationship. Some studies found a decrease in sperm concentration (Haidl *et al.,* 1996), while others concluded an increase in sperm concentration with increasing age (Rolf *et al.,* 1996; Brito *et al.,* 2002). Results reported by Kidd *et al. (*2001) do not support the fact that sperm concentration decreases with increasing age.

It has however been found that in bulls, the scrotal circumference and testicular dimensions showed a rapid increase in young bulls, a more gradual increase in mature bulls, and it could even decrease in older bulls (Brito *et al.,* 2002). This is relevant, as the scrotal circumference indicates the level of epididymal duct development. This indicates the possible level of spermatogenesis and also the concentration of sperm that can be expected per ejaculate. When adult rams are compared to pubertal rams, there is an increased level of spermatogenesis efficiency in the adult rams. This higher efficiency can be attributed to the fact that the HPT axis has fully matured and thus there is an increase in the number of round spermatids from which the sperm are originating, and also that there is an increase in the capacity of the epididymal maturation that occurs (Mandiki *et al.*, 1998). A positive correlation between the circumference of the scrotum and the total amount of sperm being produced, thus a larger circumference will ultimately mean more sperm (Almquist *et al.*, 1976).

In bulls, it was found that the semen quality, in terms of normality and concentration, was higher in bulls the of age group 3-7 years than those in the age group of younger than 3 years or older

than 7 years. It should be noted, however, that there are early and late maturing breeds, that could affect the specific age that sperm quality was tested on (Dowsett & Knott, 1996). Studies conducted on pubertal rams showed that these males produced ejaculates with an increased number of immature cells, coupled with a poor progressive motility, when compared to adult rams (Mandiki *et al.*, 1998).

Human studies have indicated that in general aging is associated with a lower percentage of normal sperm. Several studies showed a direct correlation between age and sperm morphology, although some contradictions do exist in the literature. Other studies show no direct relationship between the age of the animal and sperm morphology (Kidd *et al.,* 2001).

In terms of sperm motility, studies done on humans tend to show a negative linear relationship between animal age and the motility of sperm, although there are a small percentage of studies that show no relationship between age and sperm motility. With this in mind, the majority of the studies done provide strong and consistent evidence that sperm motility tends to decrease with increased age (Kidd *et al.,* 2001). The results of these studies on aging men could be as a result of increasing age, which decreases the number of Leydig cells in the testis. Thus the amount of testosterone produced is decreased, which has a direct effect on spermatogenesis and thus the production of normal, viable sperm (Plas *et al.*, 2000).

Simitzis *et al.* (2006) concluded that ram age influenced the expression of sexual interest. The study indicated that young rams not previously exposed to ewes, often failed even at short distances, to identify the state of the ewe's sexual receptivity. These rams also exhibited reduced sexual performance when oestrus ewes were identified. The low sexual performance was accompanied by low expression of the Flehmen reflex and higher rates of nudging, when compared to the mature rams.

It should be remembered to consider the effect of nutrition, for if the animal's diet is deficient in energy, the onset of puberty will be delayed. After sexual maturity has been attained, spermatogenesis occurs throughout the life of a male. In order to use the best sperm for ART's, the animals used to collect semen from need to be selected carefully. The best quality sperm comes from strong healthy males, which are fed correctly formulated diets and managed properly. If one of these should be insufficient, the quality of the sperm could be compromised (Hafez & Hafez, 2008).

2.4.2 Nutrition

Females are generally more susceptible to reduced fertility, caused by a nutritional restriction than males. The effect of nutritional deficiencies is then noticeable in the delay of the onset of puberty, and also affects characteristics of the semen. The negative effects of nutritional deficiencies are more severe in young or growing animals, compared to mature adult males. It is known that nutritional deficiencies affect the endocrine system, rather than the spermatogenic function of the testes, thus the effect of nutrition on the quality of sperm is indirect via the endocrine functions. There are however a few ways in which nutrition can affect male fertility some of which are protein, caloric, as well as vitamin deficiencies. However there are also certain factors like toxic agents and minerals that could play a vital role in balancing a diet for optimal production (Hafez & Hafez, 2008).

2.4.2.1 Effect of under- and overfeeding

When a mature male is fed at low levels, it will still be able to maintain the same sperm production and the necessary testosterone to support spermatogenesis. This, however, is not true for young males that are still investing energy and protein resources into growth, for a low level of nutrition will lead to retarded growth and delayed sexual development, as well as a delay in the onset of puberty. This is due to the inhibition of the endocrine activity within the testes which in turn will slow down growth and the secretory function of the male reproduction organs (Hafez & Hafez, 2008).

Studies have showed that when rams, bulls, and boars were fed a low energy diet for extended periods, the libido and the testosterone production are affected long before it can be seen in the quality of the semen. The effect of underfeeding can be corrected for in mature males, although it is not always successful when done with young males, due to the extent of harm done to the germinal epithelial cells of the testis (Hafez & Hafez, 2008).

Previous studies have indicated that the dietary energy level fed to bull calves post-weaning affected the process of spermatogenesis profoundly. Bulls fed a high energy diet (80% grain, 20% forage) showed a decrease in progressively motile sperm, compared to those fed a moderate-energy diet (100% forage). The percentage of morphologically normal sperm was also affected by the diet. Previous studies have also indicated that males with a better body condition showed a decrease in seminal plasma quality compared to those with a weaker body condition. Further Coulter *et al.* (1997) stated that the reason for this could be ascribed to the excess subcutaneous fat that accumulates around the scrotum when males are fed a high energy diet. This then inhibits the testis from being temperature regulated at a lower

temperature than the internal body temperature of the animal, thus affecting the production of sperm.

When young males are fed a protein deficient diet, the libido and semen quality is affected markedly. The deficiency of protein then affects young males much more than mature bulls, boars or rams. The ram is however special in the sense that a high protein diet is not essential for the optimal production of sperm (Hafez & Hafez, 2008).

2.4.2.2 Vitamin deficiencies

The deficiency of specific vitamins leads to a decrease in the reproductive ability of most farm animals. The deficiency of dietary vitamin A leads to the degeneration of the testes, most probably due to the indirect effect on the pituitary to inhibit the release of gonadotrophin into the blood. When such a deficiency occurs, the process of spermatogenesis can be restored and corrected by a gonadotrophin hormone- or vitamin A injection, unless the damage done to the testes is too severe to repair (Hafez & Hafez, 2008).

Another important vitamin is vitamin E, which plays an important role in the process of normal reproduction, although the specific role in the success of fertilizing ability is unclear (Hafez & Hafez, 2008). Vitamin E does however, play an important role as an antioxidant, scavenging for free radicals to protect sperm against the damage that can be caused by reactive oxygen species (ROS). The oral supplementation of vitamin E and organic selenium during a 6 month trial on humans, showed a statistically significant increase in the semen, seminal plasma, as well as the blood vitamin E levels (Keskes-Ammar *et al.*, 2003).

There has however, been some controversy in the literature on the specific effect on seminal plasma and the blood vitamin E levels. In a study by Keskes-Ammar *et al.*, (2003) there was a significant improvement in the sperm motility when the vitamin E-Se treatment was applied. It was also stated that the oral administration of vitamin E was advantageous to the fecundity rate of ewes inseminated with the sperm of supplemented rams. It also improved the fertilization rate *in vitro*.

2.4.2.3 Mineral deficiencies and possible toxic agents

Information on the specific deficiencies of minerals on male reproduction is generally scarce. An iodine deficiency in bulls has been said to cause a decrease in the libido and semen characteristics. Other minerals that have been found to show improvement of sperm production and fertility when supplemented are copper, cobalt, manganese and zinc (Hafez & Hafez, 2008).

Zinc is one of the important role players in male reproduction, as it is involved extensively in nucleic acid and protein metabolism, and is thus essential to the process of cell differentiation and replication. Zinc is also essential for the synthesis of reproductive hormones, such as testosterone and gonadotrophin releasing hormone (GnRH) while it is also required for the attachment of the sperm head to the tail, as well as the production of an antibacterial substance, which is produced in the prostate gland and released into the seminal plasma. Supplementing zinc increases sperm production per day while it also plays a role in reducing the proportion of abnormal sperm (Kendal *et al*., 2000).

There are many natural chemicals, ionizing radiations, and earth salts that may impair the male reproductive system development and function, called endocrine disrupting chemicals (EDC) (Knez, 2013). Exposure to these types of chemicals during the early stages of development can disrupt normal development, and also alter disease susceptibility later in the animals' life (Schug *et al.*, 2011). A possible inhibitor of spermatogenesis is the presence of plant estrogens in forages. Although substantial research efforts have been made, there is still no final answer to the problem of endocrine-disrupting chemicals and their effect on fertility (Hafez & Hafez, 2008).

2.4.3 Season

Most species in the temperate climates, including sheep, show an annual cycle of reproduction which can be divided into a breeding season and a non-breeding season (i.e. a period of near infertility) (Langford *et al.,* 1987). The breeding season can generally be considered to be determined by the gestation length of specific species, so to ensure the parturition at the time of year that will ensure the optimal survival percentage of the young (Langford *et al.,* 1987).

The initiation of the breeding period is determined by photoperiod, and since sheep are short-day breeders, the start of the breeding season will be indicated with the change of season when the days become shorter (i.e. autumn). The ram is sensitive to changes in the photoperiod, thus the signals sent from the eyes to the hypothalamus changes, and this initiates the production and release of hormones by both the hypothalamus and the testes. This entails stimulating the secretion of FSH and LH, which in turn will induce the secretion of testosterone.

With the winter solstice during spring, testicular regression of the ram is induced by inhibiting the secretion of gonadotrophin and, hence, testosterone production. During the non-breeding season the production of sperm is thus depressed (Langford *et al.,* 1987).

In studies performed with the use of photoperiod treatments (melatonin) on rams, it was found that with continuous exposure to short days, the overall FSH and testosterone levels remained above the basal levels. Prolactin levels were however depressed, the size of the scrotum remained near the maximum, and the production of elevated numbers of motile sperm was continued during this exposure to short days (Langford *et al.,* 1987).

A study on boars showed that the quality of the semen varied between seasons, and that there is a significantly higher level of sperm produced in autumn, compared to the low production recorded during the summer. In the same study, it was concluded that sperm quality parameters showed clear differences with seasonal changes. These fluctuations are likely mediated by hormonal mechanisms and controlled by photoperiod. Thus one can assume that even in the domesticated swine breeds, there is a part of the primitive nature of wild pig still being responsible for coordinating seasonal breeding (Ciereszko *et al.,* 2000). However, a study conducted on rams showed that contrary to the correlation between the changes in testicular size and sperm production, one of the characteristics of quality sperm, namely motility, did not vary greatly over seasons (Mandiki *et al.*, 1998).

A study performed on British goats also indicated a significant effect of season on all the parameters of semen quality. It was clear that there are differences in sperm quality between different species, but within species and between breeds there might be a significant difference (Ahmad & Noakes, 1996).

The effect of season was investigated on ram ejaculates, and it was concluded that season had an important effect on sperm morphometric traits. The morphometric traits include specific sperm head size and dimensions measured to give a more representative idea of how different treatments affect sperm shape and ultimately fertility. This study also indicated that different sperm sub-populations were observed in different seasons, indicating that sperm reserves are depleted during specific seasons (mating season), when compared to other seasons (the non-breeding season) (Martí *et al.*, 2012).

2.4.4 Breed

Current farming systems rely on different breeds of sheep, bred for the production of a specific product like wool, meat, and milk. Some sheep farmers are also exploring the beneficial influence of crossbreeding on production (Simitzis *et al.*, 2006).

A study conducted on Suffolk, Texel and Dorset Horn rams showed that when compared, breed had an effect on the wave motion of the semen. It was found that the Dorset Horn produced

semen which had an overall higher wave motion scoring. The same study concluded that breed had a significant effect on the density of the semen. This study showed that all the other characteristics of semen quality were not affected (Boland *et al.*, 1985). However, in another study performed on various ram breeds (i.e. Najdi, Naemi, Merino, Somalian, and Sudanese sheep) it was found that the overall mean values of sperm count, percentage of motile sperm, and percentage of live sperm were significantly affected by breed. The sperm concentrations and percentage live sperm being higher for the Najdi, Naemi and Merino rams, when the breeds were compared (Abdel-Rahman *et al.*, 2000). Total progressive sperm motility was also significantly influenced by breed (Kasimanckam *et al.*, 2007).

When motility was studied in different cattle breeds, it was concluded that the sperm motility and concentration was significantly different among breeds. Overall, breed differences were evident in the study, as sperm motility and concentration were considerably lower for the Brahman and Santa Gertrudis bulls when compared to Angus and Hereford bulls. It should however be noted that the Santa Gertrudis breed is a late maturing breed, and that this could explain the low sperm output in the age group of 16-20 months. Angus and Herefords are again early to medium maturing breeds thus their optimal sperm production will be earlier than the later maturing bulls (Fields *et al.,* 1979).

Studies conducted on bulls in Brazil to compare *Bos indicus* with *Bos taurus* cattle, the study concluded that sperm production of *B. indicus* bulls was higher than that of *B. taurus* bulls (Brito *et al.*, 2002). One should however take into consideration that many other factors play a role in spermatogenesis. Spermatogenesis is dependant on temperature variations, and thus it is suspected that animals with a more thermo-tolerant response to heat stress (*B.iIndicus*) will have a better production of sperm overall compared to animals not so thermo-tolerant (*B. taurus*). The same study indicated that sperm defects were more prominent in *B. Taurus* bulls (Koivisto *et al.*, 2009).

The fact that breed has a significant effect on the semen quality was also confirmed by studies conducted on boars. There were however no differences when the acrosin activity of the boar sperm were tested between breeds (Ciereszko *et al.,* 2000). Similar studies conducted on the effect of breed on the sexual behaviour of rams showed that different sheep breeds exhibited different levels of sexual interest per ewe (Chios rams vs. Karagouniki rams) (Simitzis *et al.*, 2006).

2.4.5 Selection

Selection for improved reproduction traits were never considered to be practical, due to the low heritability estimates. However, experiments conducted on mice and sheep for genetic response to selection made researchers reconsider (Burfening *et al.*, 1993).

The reproduction potential of an animal is generally governed by the interactions between its genes and the environment. Thus the genetic makeup of an animal determines the reproductive physiology and behaviour of that animal within its environment (Krasnow & Steiner, 2006). Selection often focusses on the phenotype of animals and the selection for specific traits, without considering that correlated responses could potentially impact reproduction success negatively. A study by Bench *et al.* (2001) indicated that if there is sufficient genetic variation within a sheep population, it is possible to obtain a significant response by selecting for ram sexual performance in male offspring within a single generation.

Moore (1981) hypothesized that there are two ways in which rams from high and low prolificacy flocks can contribute to the variation in the number of lambs born per ewe joined. One of which is a difference between genotypes, in terms of the fertilizing ability of their sperm. The other being in differences in the ability of sperm obtained from high and low prolificacy rams to fertilize ova from multiple- and single-ovulating ewes. However, studies were unable to conclude that the variation in number of offspring produced per joining was due to genotype differences (Moore & Whyman, 1980; Moore, 1981).

The use of serving capacity tests generally enable sheep producers to select higher performing rams to include in breeding programmes. Bench *et al.* (2001) suggested that it is possible that sire serving capacity is related to the fecundity of the female offspring. This was illustrated in the study by Wilkins and Kilgour (1978) as their study concluded that lambing success was significantly greater for the female offspring of high-performing rams.

Studies conducted on two lines of Merino sheep which were divergently selected for reproductive traits (for lamb survival in relation to lambing and neonatal behaviour between the lines) concluded that progeny from the higher line rams had improved birth weights and higher overall survival, when compared to the progeny of the lower reproductive line rams (Cloete & Scholtz, 1998). When assessed according to the number of lambs weaned per ewe joined, the two genetically diverse lines differed appreciably in offspring survival. The breeding of animals at a younger age can shorten the generation interval for genetic selection and possibly increase the number of offspring produced throughout their reproductive lifespan (Cloete *et al*., 2009).

2.4.6 Stress

There are many types of stress, some of which can be immobilization, electro-shock and physiological stress (Tohei *et al.*, 1996). Different organisms react differently to stress, as it is not only influenced by the type, intensity and duration of the stressor, but it also depends on the level of circulating sexual steroid hormones present in the organism at that time (Janett *et al.*, 2006).

There exists many environmental and nutritional stressors an animal can be exposed to, and include factors such as housing- or handling stress. The suppression of the reproductive capacity of mammals due to stress is a fact. When an animal is stressed, the hypothalamic-pituitary-adrenal (HPA) axis secretes a range of hormones. These hormones include vasopressin and corticotrophin releasing hormone (CRH) and these can affect all three levels of the HPT axis (Janett *et al.*, 2006). Stress is thus accompanied by both an increase in the activity of the HPA axis, and a reduction in the activity of the HPT axis (Tohei *et al.*, 1996). The activity of CRH together with β-endorphin will directly inhibit the release of GnRH from the hypothalamus, thus inhibiting the hormonal cycle that regulates the production of sperm. With the CRH-mediated increase of ACTH secretions, an increase in circulating glucocorticoids follows. Glucocorticoids have been proven to inhibit both the hypothalamus and the pituitary gland to secrete hormones thus indirectly inhibiting the process of spermatogenesis (Janett *et al.*, 2006). In later studies it was concluded that glucocorticoids show a direct inhibitory effect on testosterone production. Corticosterone is a glucocorticoid and a high concentration of serum corticosterone has been reported to increase the sensitivity to the negative feedback effects of testosterone, and thus also inhibit the testicular function of the animal (Tohei *et al.*, 1996).

There are several factors that may increase the percentage of abnormal sperm being produced, one of which is heat stress. If high ambient temperatures are combined with a high humidity, a male can be rendered sterile for up to six weeks. Better management practices could minimize this effect of heat stress (Hafez & Hafez, 2008).

There is, however, a difference between the effects of acute vs. chronic stress on the production of testosterone. Studies have shown that chronic stress will decrease the production of testosterone, whereas the effects of acute stress on testosterone production are much more unpredictable. Different researchers have reported no changes, decreases and even increases in the level of testosterone production when chronic stress was studied. Stress then influences the quality of semen, as it lowers the motility and the density of sperm, and there even seems to be an increase in the amount of morphologically abnormal sperm (Janett *et al.*, 2006).

2.4.7 Disease

There are many specific and non specific infections that have an impact on the reproductive performance of animals (Salisbury *et al.*, 1978). Infections of bacterial or viral origin within the genital tract or the male may be important causes for male infertility. The process of infection within the animal body may then lead to a decrease of the process of spermatogenesis, destruction of sperm function and/or the obstruction of the seminal tract. Brucellosis and mycotoxins are two factors that can affect the reproductive success of animals (Keck *et al.*, 1998).

2.4.7.1 Effect of brucellosis

This disease is caused by an organism called *Brucella abortus,* and is commonly known as contagious abortion (Salisbury *et al.*, 1978). When studies were performed on rams, it was found that the semen quality was reduced remarkably in affected animals. This decrease in sperm quality has been manifested through poor motility, low total sperm output, and a high proportion of the sperm being morphological abnormal (Cameron & Lauerman, 1976). A large number of leukocytes are usually also present and the epididymis enlarged, although atrophy of the testes is common (Kimberling, 1988). The variation found between and within rams seems to be related to the advancement of the disease within the epididymis, and also the allocation and the severity of the lesions in the reproductive tract (Cameron & Lauerman, 1976).

2.4.7.2 Effect of mycotoxins in feed

Aflatoxins are food-borne, secondary toxic fungal metabolites that form from *Aspergillus flavus* and *Aspergillus parasiticus*. The consumption of aflatoxin-contaminated feed stuffs has been shown to affect the quality of sperm. Aflatoxins are hepatotoxic, hepatocarcinogenic, and mutagenic (Mathuria & Verma, 2008). The results of the latter study are tabulated in Table 2.2

Table 2.2 *Effect of aflatoxin induced changes (mean ± SEM) on the sperm parameters of mice (adapted from Mathuria & Verma, 2008)*

Parameter	Untreated control	Low dose Aflatoxin	High dose Aflatoxin
Total sperm count (million/mL)	45.41^{a} ± 0.14	35.51^{b} ± 0.06	27.54^{c} ± 0.04
Sperm viability (%)	90.43^{a} ± 0.05	60.57^{b} ± 0.06	45.43^{c} ± 0.08
Sperm motility (%)	85.43^{a} ± 0.05	50.37^{b} ± 0.04	35.55^{c} ± 0.05
Sperm morphological abnormalities (%)	10.59^{a} ± 0.06	60.50^{b} ± 0.07	80.60^{c} ± 0.06

The results in Table 2.2 clearly indicate that the total sperm count, sperm viability, motility, and morphological abnormalities were all affected by the aflatoxins in the feed. The number of normal sperm was markedly lower when high concentrations of aflatoxins were present in the feed possibly due to a loss in membrane integrity of the sperm, while the sperm motility also

decreased appreciably. The reason for the decrease in motility can possibly be ascribed to the effect of aflatoxin on the membrane permeability. The quantity of abnormal sperm also increased as the level of aflatoxin in the feed increased (Mathuria & Verma, 2008). Fumonisin B_1 is also a mycotoxin and occurs naturally in maize and maize-based feeds. Studies done on pubertal boars showed a significant influence of the toxin on sperm production and reserves (Gbore & Egbunike, 2008).

2.4.7.3 Fever as a symptom of disease

The mammalian testes functions optimally at a temperature that is lower than that of body temperature. Thus the regulation of this specific temperature is of importance for the process of spermatogenesis. Elevated temperatures are generally a symptom of many diseases, as fever, which impacts negatively on sperm production and integrity (Hafez & Hafez, 2008). Too high temperatures can decrease spermatogenesis, and also result in the proteins in the epididymis to undergo degradation. The epididymal proteins are also important for the process of sperm maturation, motility, and fertilization (Mathuria & Verma, 2008).

2.4.8 Seminal plasma

It is generally considered that the presence of seminal plasma during cryopreservation protects the membrane of the sperm, but this protective effect is evident in all species. The investigation of seminal plasma as an additive to mediate the damaging effects of cryopreservation was considered appropriate, due to the stabilising effect it has on the sperm membrane (Leahy & Gadella, 2011).

There are contradictory findings concerning the addition of seminal plasma to sperm samples to minimize cryopreservation damage, and the correlation with post-thaw fertilizing ability of the sperm. Most studies concluded that seminal plasma added to post-thaw samples was beneficial in terms of sperm membrane stability (Ollero *et al.,* 1997; Barrios *et al.,* 2000; Martínez-Pastor *et al.,* 2006; Maxwell *et al.,* 2007; Bernardini *et al.,* 2011), sperm motility (Graham, 1994; Ollero *et al.,* 1997; Azerêdo *et al.,* 2001; Bernardini *et al.,* 2011), and the improved overall post-thaw survivability and viability of sperm (Troedsson *et al.,* 2005; Leahy *et al.,* 2010). However, several studies reported no effect (i.e. no improvement in conception rates) when seminal plasma was added to post-thaw samples (O'Meara *et al.,* 2007; Leahy *et al.,* 2010).

There are many factors that may play a role in whether seminal plasma will improve the motility/survivability/viability/structure stability of sperm. Some of these factors include the specific male that the seminal plasma was recovered from, the specific fraction that was utilised, and the type of processing that the seminal plasma was subjected to (i.e. fractionation of

seminal plasma). The season during which the seminal plasma was collected, is also important. Studies have indicated that seminal plasma recovered from males during the breeding season had a more favourable effect, than samples collected during the non-breeding season. Variation in the processing protocols also play a big role, for example the point at which the seminal plasma was added to the sample (pre- or post-cryopreservation), and the precise concentration of the sperm sample as the ratio of number of sperm to seminal plasma may affect the outcome. All of the above factors contribute to the controversial effect that seminal plasma tends to have on field fertility (Domínguez *et al.,* 2008; Leahy *et al.,* 2010).

It is known that seminal plasma proteins protect ram sperm from cold shock or cryodamage caused during the routine freezing protocol. Cold shock can generally occur when sperm are cooled or frozen in the absence of any cryoprotectants. The specific protective component in seminal plasma has been identified as a 14 kDa BSP protein, which has been indicated to prevent or reverse the damage done to the sperm cell with cold shock. Bull, but not ram sperm membranes are generally destabilised by the protective proteins. The reason for this is not as yet clear, although it could be related to the fact that there are differences in stimulation for capacitation (Leahy & Gadella, 2011).

2.4.9 Other physiological related factors

There are several abnormalities of the male reproductive system that may occur due to a genetic defect or disease although only some of these are truly characterized as genetic anomalies. Some examples would be cryptorchidism and testicular hypoplasia, which is common in all farm species. There has also been a high incidence of abnormal sperm observed when abnormal spermiogenesis occurs. This leads to sperm defects, which in turn will affect fertility (Hafez & Hafez, 2008). A summary of the effects of abnormal spermiogenesis are set out in Table 2.3.

Table 2.3 *Summary of some hereditary sperm defects which affect fertility (Hafez & Hafez, 2008)*

Sperm defect	Description of the defect	Fertility status
Knobbed sperm	Acentric thickening of the acrosome	Sterility
Decapitated	The head and the tail are separated at the neck region	Sterility
Dag defect	Folding of tail over the midpiece, giving the impression of a swollen midpiece	Infertility
Pseudo-droplet	Rounded or elongated thickening of the midpiece	Sterility
Corkscrew	Tail defect	Infertility
Diadem effect	Nuclear pouch formation	Infertility
Sterilizing tail stump	Tail defect	Sterility

2.4.9.1 Cryptorchidism

Sometimes one or both testes fail to move down from the abdominal cavity into the scrotum, this phenomenon is known as cryptorchidism. It is known to be more common in swine and horses compared to any other farm animal. Since the testes is then located within the abdominal cavity (mostly in the inguinal canal), the testes are not able to regulate the temperature at levels cooler than body temperature, thus causing unfavourable conditions for spermatogenesis to occur. Thus animals that are bilaterally cryptorchid will be sterile, in contrast to unilateral cryptorchid animals, which are able to produce fertile sperm, in reduced numbers due to the condition (Hafez & Hafez, 2008).

When steroidogenic function is considered, there seems to be controversy regarding the ability of cryptorchid testis to produce testosterone. It seems as if the cryptorchid testes are less sensitive to exogenous gonadotrophin than normal scrotal testes in the bull and the ram. However, *in vitro* studies have indicated that steroid production of unilateral cryptorchid stallions and boars, by Leydig cells, was the same for both the contralateral and the abdominal testes (Hafez & Hafez, 2008).

2.4.9.2 Testicular hypoplasia

All farm animals are potential candidates for testicular hypoplasia. This is a congenital defect in which the potential for development of the epithelium (where spermatogenesis occurs) is lacking. This defect is also likely only to be suspected at puberty or later, as only then will the animal show reduced fertility or even sterility. Hypoplasia can occur in only one or both testes. In the case of a sterile bull, the semen will be of poor quality as it will be watery and contain little or no sperm. If the case of hypoplasia is not that severe the semen, libido and the animals' ability to mate are not affected, even though reduced sperm numbers may be expected (or even abnormal sperm in the case of the ram). The size of a hypoplastic testis is generally much smaller than that of a normal testis (Hafez & Hafez, 2008).

2.4.9.3 Wolfian duct aplasia

There are several structures that originate from the Wolffian duct like segments of the epididymis, the ductus deferens, etc. that are not fully developed and which will result in abnormal spermatogenesis. These malfunctions can be either unilateral or bilateral, even if the gonads were fully developed. There are other defects too, e.g. epididymal stenosis, which results in the spermatocoele (i.e. cysts containing sperm). This usually occurs when the sperm accumulate in the ductus efferentes, and thus lead to the formation of a spermatocoele, found in the epididymal head of post-pubertal males (Hafez & Hafez, 2008).

Apart from genetic defects, reproductive processes can malfunction without being an inherited disease or defect, for there are diseases caused by environmental factors and stress that cause a wide spectrum of reproduction problems (Hafez & Hafez, 2008).

2.5 Semen collection

To ensure the successful application of artificial insemination, one needs to collect high quality sperm from superior rams. Semen can be collected from rams via one of two methods, namely electro-ejaculation (EE) or the use of an artificial vagina (AV).

2.5.1 AV vs. EE

The EE method of collection is performed by inserting a bipolar electrical probe into the ram's rectum. Then low voltage electrical stimulation is given for two to four seconds, at about ten to twenty second intervals, until the ram ejaculates. There are however several problems when using the EE, some of which is the high variability in both the sperm concentrations and the ejaculate volume. Urine contamination of the sample is also quite common when using EE. Another negative aspect of EE is the fact that it is stressful to the ram as the ram has to be restrained and the process is highly uncomfortable. The collection method of EE should only be used in extreme situations (Hafez & Hafez, 2008).

The use of the AV is ethically a more acceptable methodh to collect semen from a ram. With EE, no training of the ram or preparation of ewes is required prior to collection. The AV method requires the training of the rams, with most rams if docile enough, that can be trained within a week (personal observation). The AV consists of a polyvinylchloride (PVC) pipe (i.e. T-junction shape) of about 20-25cm long, with a softer latex inner lining. Studies conducted on rams showed that the length of the AV affects the percentage sperm loss, with a shorter AV having the least, compared to a longer AV (12.9% vs. 50.8%) (Romano & Christians, 2009).

A glass collection tube is placed at the one end of the AV. Care should be taken to warm the collection tube prior to collection (37˚C), so as to reduce the cold shock the sperm may experience, when ejaculation occurs. The AV is filled with warm water (approximately 48˚C) between the outer and inner layers to create a warm pressure, mimicking the female reproductive tract. The inside of the AV will then be at a temperature of approximately 42˚C. A ewe is restrained (dummy), while the ram is brought to her. As the ram mounts the ewe, his penis is gently diverted into the AV. After ejaculation, the collection tube containing the sample is placed in a waterbath at 37˚C and processed for insemination or cryopreservation (Hafez & Hafez, 2008).

Studies have indicated that when the two methods are compared, EE resulted in lower recovery efficiency, due to contamination with urine or the lack of response to electrical stimulation. When the quality of fresh semen samples are compared for the two methods of semen collection, no significant differences was recorded. One study did however indicate a higher number of stable and functional sperm in frozen-thawed sperm for samples collected with EE, when compared to the AV method (Marco-Jiménez *et al.,* 2005).

Although the EE method is faster and more convenient than using the AV, semen quality is often reduced and semen characteristics altered with the use of EE (Wulster-Radcliffe *et al.,* 2001; Marco-Jiménez *et al.,* 2005).

2.5.2 Collection of epididymal sperm

In domestic animals, AI relies almost exclusively on the semen that is obtained by means of EE. In sheep, however both the EE and AV are the main methods to obtain sperm for AI purposes. There is however also a need for a post-mortem sperm collection method for in the tragic event that a genetically superior male animal dies, its sperm can be collected and preserved, thus letting the genetics of that animal live on long after its death (Anel *et al.,* 2003).

Ideally epididymal sperm should be collected, extended and cryopreserved immediately or used directly for insemination (Tamayo-Canul *et al.,* 2011). Often this is not possible, and thus the testes may need to be kept on ice for a while, before any processing can take place. A study showed that stored ram epididymides at 5˚C, still contained viabled sperm up to 48h post-mortem, although fertility was found to decrease with time (Kaabi *et al.,* 2003).

Epdidymal sperm collection involves the recovery of the testes of the animal, and removing the connective tissue around the testicles. The epididymis is then separated from the testicle, and the *cauda* epididymis is then separated from the entire epididymis. After transfer of the segment of the *cauda* epididymus to a Petri dish, incisions are made at the distal end of the *cauda* to expose the sperm to the outer environment. The sperm are then allowed to swim out into a sodium citrate buffer (Lone *et al.,* 2011). Care should however be taken to minimise or prevent microbial contamination, which can cause sperm agglutination, reduced sperm motility and viability, and the inhibition of the acrosome reaction (Santiago-Moreno *et al.,* 2009). Care should also be taken not to cut blood vessels when incisions are made into the *cauda* epididymis, as blood will contaminate the sperm sample. Relevance to this, if one has dissected the *cauda* free of the connective tissue, there should be minimal blood vessels present, i.e. the maount of blood in them so little that it will be diluted with the collection medium (Kaabi *et al.,* 2003).

When epididymal sperm and ejaculated sperm are compared, it has been stated that there were significant differences in the fertilizing ability of the sperm when used to inseminate females after being cooled. After collection of epididymal sperm, the protocols for evaluation, preparation and cryopreservation are generally the same as for ejaculated sperm samples (Alapati *et al.*, 2009).

2.6 Evaluation of sperm quality

The successful application of assisted reproductive techniques relies on both the female and the male components to maximize fertilization success. The sperm used for insemination or embryo production need to be selected carefully to ascertain the contributions by the selected males. Male infertility can rather be identified as a multi-factorial syndrome which encompasses a wide variety of specific disorders (Tsakmakidis *et al.*, 2010). The impact of male infertility on the reproductive efficiency in an animal production system is high, for a single male can serve multiple females, by either natural mating or by means of artificial insemination. Semen evaluation is done complementary to the clinical examination, and has been found to be useful and accurate (Ganter, 2008).

There are certain factors that have to be considered when semen samples are to be evaluated. The examination should focus on the overall appearance of the sample, motility, total volume, concentration, % live, % morphologically abnormal sperm, and the integrity of the acrosome (Salisbury *et al.*, 1978). One should however, remember that although these routine semen evaluation tests are useful indicators, they cannot predict the exact fertility of a ram, since the process of fertilization is complex. There is no single test available that can accurately predict the fertility of a semen sample. Standard macroscopic evaluation procedures for semen analysis are considered as relatively poor indicators of the fertilising ability of sperm (Tsakmakidis *et al.*, 2010).

There have been guidelines set up by the Society for Theriogenology that state the minimal acceptable thresholds for semen characteristics that must be met for a ram to pass a breeding soundness evaluation. Some of these include a physical examination by a veterinarian and perhaps a vaccination/health certification (Hafez & Hafez, 2008). There are minimal standards for males, and according to Hafez & Hafez (2008) for bull semen the standards include the following requirements:

- More than 500 million sperm/mL present;
- More than 50% of motile sperm with progressive forward movement;
- More than 80% of sperm with normal morphology.

In general sperm evaluation should be a rapid and effective process to ensure that carefully collected samples can be processed and preserved for further use as soon as possible. When the evaluation of semen samples indicates no motile sperm present, and the reproductive system of the animal has been fully examined for disease, it can be concluded that the animal is sterile (Hafez & Hafez, 2008).

Both ejaculate- and epididymal samples are subjected to macroscopic and microscopic evaluation procedures.

2.6.1 Macroscopic evaluation

When farmers make use of their own rams to inseminate ewes, macroscopic semen evaluation is often the only method used. It focuses on the appearance of a sample in terms of volume, colour, and mass motility. The evaluation of these parameters are called macroscopic evaluation, for it can be evaluated with the naked eye, without the use of a microscope (Evans & Maxwell, 1987).

2.6.1.2 Semen volume

The semen volume produced differs amongst farm animals (Table 2.4). Younger animals tend to produce less sperm than older animals, and the smaller size species will also produce a smaller ejaculate, compared to larger species. The average volume of semen produced by farm animals is presented in Table 2.4.

Table 2.4 *Semen volume recorded for different farm animals (adapted from Gordon, 2004)*

Species	Semen volume produced by single ejaculation (mL)
Stallion	50-100
Bull	5.0
Ram	0.5-2.0
Boar	200-250

The total amount of semen produced are affected by age, environment, health status, semen collection procedure, season of the year, frequency of collection, as well as breed and individual animal differences (Hafez & Hafez, 2008).

2.6.1.2 Colour

The colour appearance of ram semen is a milky-white or a pale creamy colour indicating a high sperm density in the sample. Colour scoring is generally a subjective way to evaluate semen. Colour is assessed on a numeric scale, ranging from 0 – 5 (0 being almost clear and 5 being thick creamy white).

In the bull and ram, the ejaculate colour should be relatively uniform, with a dense appearance indicative of a high sperm cell concentration. The sample should also be free from any hair, dirt or any other contaminants. It is possible for bulls/rams to sometimes produce a yellowy semen sample, which could be due to the harmless presence of riboflavin in the feed. This yellow colour should not be confused with a sample contaminated by urine as urine contamination will generally be accompanied by a strong urine odour. If a semen sample appears to have a pinkish colour, it could be an indicator of the presence of blood, likely due to injury of the penis during collection (Hafez & Hafez, 2008).

2.6.1.3 Mass motility

Sperm motility is determined either with the naked eye, or when undiluted semen is placed on a microscope slide without a coverslip under a light microscope. It is usually referred to as the mass cloud movement of sperm in undiluted semen. Normal viable ram semen exhibits prominent wave-like motion that is characterised by, continuous moving and swirling motions created by the motile sperm. The appearance of mass motility depends on the sperm concentration, the percentage of immotile sperm, and the degree of activity expressed by the rest of the sperm in the sample. Abnormal sperm can also influence the amount of mass movement observed, for their swimming pattern will be impaired and in most cases, non-progressive in nature (Evans & Maxwell, 1987).

Mass sperm motility is scored on a scale of 0-5, with 5 being the highest % motile sperm (80-100%) and 0 indicating no motile sperm. The score is based on vigorous movement and the formation of these waves (Table 2.5).

Table 2.5 *Score sheet used to determine the amount of vigour present in the mass wave motion of a sperm sample (Adapted from Hafez & Hafez, 2008)*

Score	Aspects of wave motion
0	Total immobility (All sperm static)
1	Individual movement (about 10% motile)
2	Very slow movement (no wave formation, 20-40% motile)
3	General wave movement, slow amplitude of waves (45-65% motile)
4	Vigorous wave movement, no eddies (70-85% motile)
5	Rapid, dense wave motion, eddies present (90% + motile)

2.6.2 Microscopic evaluation of sperm

Microscopic evaluation of sperm entails the analysis of sperm samples to determine the percentage live sperm, motility, concentration, general morphology, and the integrity of the acrosome. Microscopic evaluation is much more accurate than only macroscopic evaluation, and usually accompanies macroscopic evaluation. The microscopic evaluation of sperm at a magnification factor of 200 or 400 will be adequate, by measuring a few parameters (Hafez & Hafez, 2008):

- % sperm motile (normal being 70-90% motile),
- % sperm which are progressively motile,
- Sperm velocity (on a scale of 1 being stationary to 4 being rapid),
- Longevity of sperm motility in raw semen, and in extended semen.

2.6.2.1 Percentage live sperm

Several staining procedures can be used to estimate the percentage of live sperm in a sample. One example is of the combination of nigrosin-eosin, which indicates the proportion of live sperm in contrast to the proportion dead. When the staining material is added to an aliquot of a sperm sample, and a smear made, live sperm will not absorb any stain, while dead sperm will stain red against the dark nigrosin background. There are however other staining materials that include for example Spermac®, SpermBlue®, etc. that are equally popular for live/dead staining (Hafez & Hafez, 2008).

2.6.2.2 Sperm motility

Motility is one of the most important pre-requisites for sperm to realize their fertilizing ability within the female reproductive tract (Lambrechts *et al.*, 2000). To assess the motility of sperm, the subjective estimation of the viability of sperm is involved, and also the quality of the movement. Motility can be measured in raw and extended sperm samples. The motility of sperm is also highly susceptible to environmental factors and thus it is necessary to protect semen from harmful agents or conditions prior to analysis. When raw semen is evaluated, it is an indicator of sperm performance in its own accessory gland fluid (Hafez & Hafez, 2008).

There are many patterns of motility, although general patterns in diluted semen appear in a long semi-arc pattern. When sperm are found to swim in circles, it could be a result of cold shock. An oscillatory motion also indicates aged or drying cells. Any abnormal motility patterns are associated with infertility or sub-fertility in males (Hafez & Hafez, 2008).

Sperm motility is strongly related to the ability of the sperm cell to migrate through the female genital tract and to find, interact, and fertilize the oocyte (Suarez & Pacey, 2006). Computer-assisted-semen-analysis (CASA) supports the objective assessment of many sperm characteristics, such as motion, motility and morphology. The use of CASA instruments has demonstrated high levels of precision and reliability (Tsakmakidis *et al.*, 2010). The system can be programmed to use a different number of frames per second settings to track the sperm successfully and to include the analysis of the precise motion immediately. This makes it a popular method used to evaluate sperm motility. The use of flow cytometry is also often used to detect viable sperm based on binding of fluorescent dyes. The time lapse photomicrography permits visualization of sperm tracks, but these automated systems are expensive, although very accurate (Hafez & Hafez, 2008).

Studies done have indicated that the motility of sperm declines dramatically after a process of freeze-thawing, although this effect is less pronounced in diluted and chilled semen (Gundogan *et al.*, 2010). The motility of sperm has been found to be preserved by the addition of sugars (glucose and mannose) to the diluents when freezing semen (Salamon & Maxwell, 2000).

2.6.2.3 Sperm concentration

Accurately determining the number of sperm in 1 mL of a sperm sample is an important part of microscopic evaluation as this number will determine the number of inseminations that can be carried out with a single ejaculate. The average sperm concentration of ram ejaculates range between 3.5×10^9 to 6.0×10^9 sperm/mL. There are many ways in which the concentration of a sperm sample can be measured some of which include the use of a hemacytometer, colorimeter, or a spectrophotometer (Hafez & Hafez, 2008).

The hemacytometer is time consuming, although very accurate. This method of determining the concentration of a sperm sample is done on a microscope slide which has precisely scored chambers on it. The slide is then placed under the microscope and the sperm per chamber are counted manually. The use of colorimeters or spectrophotometers that are calibrated with the hemacytometer is less time consuming and alow precise (Evans & Maxwell, 1987; Hafez & Hafez, 2008).

A computer automated semen analysis (CASA) system can also be used to accurately determine the concentration of a sample. The Sperm Class Analyser (SCA®) is a CASA system that accurately determines the concentration of a sample using a sample of known dilution, a positive displacement pipette, and Leja® slides. Computerized systems such as the SCA® system are programmed and calibrated initially for a species with the aid of a hemacytometer.

There is however debate regarding the accuracy of the CASA system, when compared to the manually counted chambers of the hemacytometer. Kuster (2005) concluded that the differences between the use of CASA and hemacytometers can be explained by the Segre-Silberberg (SS) effect, which implies that the depth of the slide chambers used for concentration determination causes Poiseuille flow. The SS effect can then cause errors in the estimation of particle concentration. It has been debated that hemacytometers are 100 µm in depth, and thus the SS effect does not apply, whereas the commercially used capillary-loaded disposable slides are mostly all at a depth of 20 µm, and are thus prone to the SS effect (Kuster, 2005).

As with volume, the concentration of sperm also differs between animals (Table 2.6).

Table 2.6 *Sperm concentrations of different farm animals (adapted from Hafez & Hafez, 2008)*

Animal	**Sperm concentration (sperm/mL)**
Bull	$2.0x10^8$ (young bulls); $1.8x10^9$ (mature bulls)
Boar	6.0 to $10.0x10^8$
Stallion	100.0 to $150.0x10^6$
Ram	3.5 to $6.0x10^8$

Another way of assessing concentration of ram semen is by using consistency to estimate the average concentration (Evans & Maxwell, 1987). Table 2.7 illustrates how the evaluation system can be used to assess the concentration from the consistency of the sample.

Table 2.7 *Consistency scoring of sperm concentration (Hafez & Hafez, 2008)*

		Number of sperm (x 10^9)	
Score	**Consistency**	**Mean**	**Range**
5	Thick creamy	5.0	4.5-6.0
4	Creamy	4.0	3.5-4.5
3	Thin creamy	3.0	2.5-3.5
2	Milky	2.0	1.0-2.5
1	Cloudy	0.7	0.3-1.0
0	Clear (watery)	Insignificant	Insignificant

2.6.2.4 Sperm morphology

Every sperm sample contains some abnormal cells. Morphologically abnormal sperm (Figure 2.3) of males have been associated with lower levels of fertility and even sterility for many years (Saacke, 2008). When the number of morphologically abnormal sperm exceeds 20%, fertility will generally decline. There are typically three types of abnormalities namely primary, secondary or tertiary abnormalities. Primary abnormalities are associated with the head of the

sperm and the acrosome, whereas secondary abnormalities refer to the presence of a droplet on the midpiece of the tail; and tertiary abnormalities refering to all other tail defects found (Hafez & Hafez, 2008).

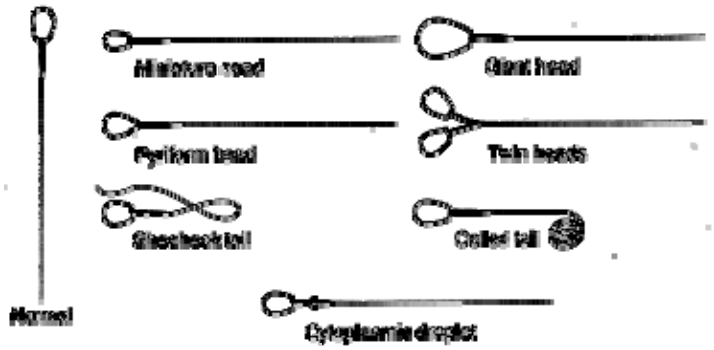

Figure 2.3 *Morphological sperm abnormalities (Sources from www.nongae.gsnu.ac.kr)*

If one takes into consideration the very small, intricate privileged specific paths offered by the cervix and mucus of females for species having intravaginal semen deposition (like cattle and sheep), flagellar pattern is considered crucial for sustainable transport of sperm, which will result in the removal or even degradation of cells with abnormalities of the tail and also protoplasmic droplets (also affecting the swimming pattern). With this, it can be concluded that impaired or abnormal sperm motility may be the direct underlying basis for sperm exclusion, based upon the morphology of the sperm head as well (Saacke, 2008).

Several staining procedures are used for the evaluation of sperm morphology. Stallion sperm morphology is generally examined with air-dried semen smears, and by using a light microscope at a 1000x magnification. There exists a strong positive correlation between the percentage morphologically normal sperm and motility. Thus, if the sperm motility is low and the percentage of morphologically normal sperm is high, it could indicate that laboratory errors may have occurred, thus resulting in a decrease of sperm motility. If an abnormal morphology count of more than 15% is found, it should preferable not to use the sample for AI purposes (Hafez & Hafez, 2008).

In rams, abnormal sperm percentages vary with season, with higher numbers of abnormal sperm occurring during spring and decreasing as the breeding season advances. Sperm morphology can then be assessed by using a range of staining procedures, of which the use of eosin-nigrosin stain or SpermBlue® are but a few.

With this method, at least 150 sperm needs to be evaluated to be representative of a sample, with sperm being categorized into one or more of the following five categories (Hafez & Hafez, 2008):

- Tailless,
- Abnormal heads,
- Abnormal tail formations,
- Abnormal tail formations with a proximal cytoplasmic droplet,
- And abnormal tail formations with a distal droplet.

2.6.2.5 Acrosome integrity

It is very important for sperm to have an intact acrosomal membrane, for it is regarded as a good indicator of high sperm quality. Studies have shown that an intact functional sperm membrane is important to achieve fertilization and DNA integrity is essential for embryonic development. Acrosome integrity can then be evaluated with appropriate interference or phase contrast microscopy, or with special staining preparations (Saacke, 2008; Gundogan *et al.*, 2010).

There are, however, a number of methods that can be used to measure acrosomal integrity. The most commonly used method is using a plant lectin, labelled by a fluorescent probe. There are also many other lectins available for the assessment of acrosomal integrity, some of which are toxic (like *Ricinus communis* agglutinin). *Pisum sativum* agglutinin (PSA) is a lectin from pea plants, and since they cannot penetrate an intact acrosomal membrane, only acrosomes with damage will stain. There are, however, other lectins that bind only to the outer acrosomal membranes of fixed sperm, which then indicate acrosome-intact cells (Gillan *et al.,* 2005).

2.6.2.6 Morphometry

According to Vicente-Fiel *et al.* (2013), sperm are the most morphologically diverse cell types in the animal kingdom. The traditional routine evaluation of semen for use in assisted reproductive techniques includes the assessment of motility, morphology and acrosome integrity. Although well documented guidelines are used for the assessment of morphology, the standard manual evaluations result in an increase of variation due to subjective results recorded by different technicians and laboratories (Hidalgo *et al.*, 2007). The lack of objectivity when assessing morphology has provided the incentives for the developing of software to accurately obtain sperm dimensions and size (De Paz *et al.*, 2011).

The use of computer-assisted sperm morphometry analysis systems provides a series of objective parameters that have made it possible to standardize sperm morphometric evaluation. The sperm head morphometric parameters measured include head length, -width, -area, -perimeter, ellipticity, elongation, regularity, roughness, and the percentage of acrosome coverage (Vicente-Fiel *et al.*, 2013).

The high precision of using computer assisted sperm morphometry analysis (CASMA) has made it possible to detect the slightest differences in sperm head dimensions between fertile and subfertile males (Casey *et al.*, 1997). As technologies in sperm morphometry research develop, it has become clear that extensive heterogeneity exists within ejaculate samples between, and within species (indicating the existence of sperm sub-populations). Ram sperm head morphometry has been characterized using the Sperm-Class Analyzer (SCA®) (Maroto-Morales *et al.*, 2010).

The existence of well-defined sperm sub-populations within an ejaculate has been acknowledged and accepted by the scientific community (Martí *et al.*, 2012). Sperm sub-populations are then likely to represent sperm in different physiological states (Abaigar *et al*. 2001). Harvested epididymal samples are generally a heterogeneous accumulation of various sperm sub-populations, which is the product of different spermatogenic waves maturing along the epididymides and prior to sampling, were stored in the *cauda* epididymis as different cell cohorts (Rodríguez-Martínez, 2006). Previous studies have indicated that heterogeneity among sperm sub-populations may have functional significance (Holt, 1996; Thurston *et al.*, 1999). The specific physiological role of these sub-populations is poorly understood, although studies have indicated that the ability of sperm to undergo capacitation and sperm fertilizing ability may vary according to the sub-population which is under consideration (Harrison, 1996; Holt, 1996). Other studies have focussed on specific cut-off values for swimming speed to identify and quantify species specific sperm sub-populations (Maree & van der Horst, 2013).

The effect of season on morphometric traits was investigated, and a study by Martí *et al.* (2012) confirmed that there was an important seasonal effect on sperm morphometric traits. The latter study also concluded that the distribution of sub-populations seemed to be related to the season and the quality of the ejaculate sample, which could be beneficial to use as an indicator of sperm function.

2.7 Cryopreservation of semen

The ability of sperm to be preserved either short or long-term is of great importance in the livestock industry, especially when ART's are used. To fertilize a large number of ewes with semen from an outstanding ram sometimes requires transport of the sperm from the collection site to the farm. Thus ways of preserving semen was developed, being either via liquid storage or frozen storage (known as cryopreservation). Using these storage techniques, the metabolism of the sperm is slowed so that the lifespan of sperm is extended. Cryopreservation is used for the long-term storage of both ejaculated semen and epididymal collected sperm (Salamon & Maxwell, 2000).

2.7.1 Principles of cryobiology

Cryopreservation of sperm entails the complete arrest in the developmental and metabolic processes within the sperm. Sperm are thus held in a state of 'suspended animation' until they are required for fertilization (Pesch & Bergmann, 2006). Gametes can easily be damaged by cryopreservation and /or thawing either by the formation of large intracellular ice crystals or by the increased intracellular concentration of solutes and the changes that accompanies these phenomena. These changes are a result from basic dehydration of cells during the process of cryopreservation. The process of rapid freezing minimizes this degree of damage due to the solution effect, although it causes the formation of large ice crystals that can cause severe mechanical damage. The process of slow freezing prevents the formation of the large ice crystals, although it then may lead to an increased degree of damage from the solution effects (Hafez & Hafez, 2008).

Stress induced by ice crystal formation is mainly associated with accompanying osmotic pressure changes in the unfrozen fraction. Glycerol is added to extenders as a cryoprotectant, as it is indispensably necessary for the deep-freezing of sperm. Glycerol acts primarily by reducing the quantity of ice being formed at any sub-zero temperature (Pesch & Bergmann, 2006), by lowering the temperature at which freezing occurs (Hammerstedt *et al.,* 1990; Hafez & Hafez, 2008). Care should however be taken with the concentration of glycerol added to the extender, for it can cause osmotic and toxic damage if inclusion concentrations are too high (Leahy & Gadella, 2011). With this in mind, it is obvious that the optimal freezing rate for a specific tissue will depend on the relative tolerance it has to damage from ice crystals and the toxicity from the solution (Hafez & Hafez, 2008).

There are two critical ranges of temperature of which low rates are necessary for the optimal survival of the gametes; these are from -4˚C to -60˚C during the cooling process and then when the temperature ranges between -70˚C to -20˚C with the rewarming of the sperm. Liquid

nitrogen (-196˚C) satisfies the environment of gametes to be preserved for longer periods within this state of suspended animation, with no biologic activity occurring (Hafez & Hafez, 2008).

2.7.2 Effect of storage on sperm quality

When discussing the preservation of sperm the term survivability arises, as the technique may impair sperm to fulfil their function of transport in the female reproductive system, hyper-activating, capacitating and fertilizing the ovum/ova. Survivability of sperm is essential for usage in ART's. There are mainly two types of storage, one being short term and the other being long term storage.

2.7.2.1 Short-term storage

Short-term storage involves the storing of semen samples in a liquid state at low temperatures of 0-5ºC or 10-15ºC (Salamon & Maxwell, 2000). Short-term storage is used when the distance from the collecting station to the farm is not that far, thus insemination with the collected sperm occurs shortly after collection (Gundogan *et al.*, 2010). It is also used when semen is stored for up to two days in the fridge, until needed for insemination. The use of cooled ram semen affects sperm motility, morphology and is also known to result in a lower survivability in the reproductive tract of the female when compared to fresh ram semen (Gundogan *et al.*, 2010). When sperm need to be stored for longer periods of time, freezing (cryopreservation) is used.

2.7.2.2 Long-term storage

The use of liquid nitrogen to freeze semen is the other alternative to liquid storage. It is a fast and effective way to store semen for longer periods, though there is a significant difference among species when long-term storage is used. There are a fairly large proportion (40-60%) of ram sperm that have the ability to protect their motility after the process of freeze-thawing, but only a small proportion of about 20-30% of them will remain biologically unharmed (Salamon & Maxwell, 2000). Some of the sperm may be motile, but damaged, and may thus not have the ability to successfully fertilize the ovum (Salamon & Maxwell, 2000).

Cryopreservation exerts another effect by 'helping the maturation process along' to such an extent that some of the sperm will have already become capacitated, and thus their lifespan within the female tract to get to the ovum for fertilization when inseminated is shortened markedly (Salamon & Maxwell, 2000). If the prematurely capacitated sperm do not reach the ovum in time, they will die in the lower part of the female reproductive tract (Salamon & Maxwell, 2000).

In the unlikely event that damaged, but still mobile sperm, reach the ovum and succeeds in fertilizing it, chances are that the developing embryo will suffer early embryonic death (Salamon & Maxwell, 2000).

If sperm samples are stored for extended periods, irrespective of the type of diluent used, the specific storage temperature, dilution rate, or the conditions in which the sperm are stored, they deteriorate as the period of storage is increased (Salamon & Maxwell, 2000). This is mainly due to the accumulation of toxic products formed due to the metabolism of the sperm, largely of the reactive oxygen species (ROS) formed through the peroxidation of lipids in the sperm membranes (Salamon & Maxwell, 2000).

Another factor that could potentially reduce the fertilization success is when semen is inseminated at too late a stage relative to oestrus. Transport and survival of sperm is negatively impacted during the phase of late oestrus, and there is also a tendency for shorter periods of oestrus to have a lower fertilization rate overall. To ensure maximal chances of fertilization success in sheep AI, the optimum time to inseminate ewes has been set as 48-52 hours after sponge withdrawal (Salamon & Maxwell, 2000).

2.7.3 Sperm surface alteration during cryopreservation and specific cryodamage

In spite of the scope of research that has already been done on the subject, the process of cryopreservation remains a highly damaging process to the sperm cell. Only in certain species (e.g. bull) cryopreservation is routinely and successfully applied. The reason for this is based on both the biochemical and the practical differences between species (Leahy & Gadella, 2011). One of the reasons for the ineffectiveness of cryopreservation in all species is the damage that is done to the sperm in terms of motility post thaw. To overcome this, sperm needs to be deposited closer to the site of fertilisation. Some species show a greater susceptibility to freeze-thaw damage than others (Leahy & Gadella, 2011).

Another factor that needs to be considered is between-male variation in terms of sperm obtained from different males to differ in their ability to withstand cryodamage. Variation in terms of sperm to offer resistance to cryodamage is also observed between and within ejaculates of an individual. The process of cryopreservation dramatically alters the composition of the sperm membrane and its extracellular matrix. With this kept in mind, some changes are reversible, while other changes are fatal (Leahy & Gadella, 2011).

The sperm membrane is the primary protector against injury to sperm. Freezing results in protein changes on the sperm surface, and it also has distinct effects on the lipid composition

and integrity of the sperm membrane (Buhr *et al.,* 1994). Fluctuations in temperature and cell dehydration induce specific changes in the lateral-phase separation of lipids, and thus a re-ordering of the membrane components and the loss of polyunsaturated fatty acids and cholesterol. This re-ordering alters the permeability of the sperm surface to water, ions and cryoprotectants (Drobnis *et al.,* 1993).

There is however an advantage to cryopreservation, as it permits an indefinite interruption of sperm development. However, sperm are altered by the process of cryopreservation and thus when revived by warming, they emerge in an advanced state, which is typically characterised by the need for capacitation (Leahy & Gadella, 2011).

2.7.3.1 Cryopreservation damage

There are three basic types of cryogenic damage that can affect sperm. These are ultrastructural (i.e. physical damage), biochemical, and functional in nature. Ultrastructural damage occurs to the plasma and the acrosome membranes, the mitochondrial sheath, the acrosome and the axoneme of the sperm head, and the extent of such damage is generally more severe in ovine than bovine sperm. The outer membrane is the more vulnerable part, when compared to the inner part of the internal membrane (Salamon & Maxwell, 2000).

Biochemical changes are known to accompany ultrastructural damage during freeze-thawing, and usually result in the loss of important cell contents. Substances that can be lost during biochemical damage include glutamic oxaloacetic transaminase (GOT), amino acids, lipoproteins, etc. and the denaturation of the DNA portion of the spermatozoon can also occur, as changes in the chromatin structure of various other mammalian sperm have been observed (Salamon & Maxwell, 2000).

The effects of ultrastructural and biochemical cryogenic changes to sperm is thought to play a role in the decrease of the functional integrity, survival *in vivo*, and their fertilizing ability (Salamon & Maxwell, 2000). During the cryopreservation process, at least 50% of the sperm in a sample can be killed or rendered static. Previous studies have indicated that the high percentage of static sperm can be decreased with the use of a high pre-freezing dilution rate (Leahy *et al.,* 2010b).

2.7.3.2 Effect of cold shock

With the processing of sperm, the term ‘cold shock’ always seems to feature. Mammalian sperm are sensitive to cooling from body temperature to near the freezing point of water (0˚C). Cold shock can be defined as the damage to sperm during cooling which is observed as an

irreversible loss of motility of individual sperm upon rewarming (Medeiros *et al.,* 2002). Cold shock is believed to impair the function of the membrane proteins that are necessary for the maintenance of structural integrity or ion metabolism (Watson, 2000). Cold shock also affects other cellular functions, such as the loss of selectivity in the permeability of the cell membrane (Simpson & White, 1986; Medeiros *et al.,* 2002).

Phospholipids and cholesterol, two components of the sperm membrane, are very important as cold shock causes the loss of lipids from the sperm. It has been reported that the susceptibility of sperm to cold shock can be directly linked with the high ratio of unsaturated: saturated fatty acids in the phospholipids, and relatively low cholesterol concentration (White, 1993; Ollero *et al.,* 1997). Specifically the high concentration of unsaturated fatty acids in the sperm membranes makes them susceptible to peroxidation damage, which is known to adversely affect motility, metabolism, ultrastructure and overall fertility of the sperm. With a decrease in ambient temperature, the calcium uptake of sperm increases, and this effect is intensified if the cooling rate is rapid (White, 1993). Calcium regulates many functions in sperm, as it has been proven to modulate adenyl cyclase activity and motility of ram sperm (PGoh & White, 1988), as well as initiate the acrosome reaction and hyperactivation in sperm (Ho *et al.*, 2002).

Sperm of the ram are more sensitive to cold shock stress than those of other species such as the rabbit, bull, or human. The difference in response to cold shock stress is mainly due to the differences in the membrane lipid composition (Ollero *et al.,* 1997).

Preventative measures should be thus taken to minimize the occurrence of cold shock, and include amongst other control of the rate of cooling, and the addition of protective agents such as lecithin, proteins, lipoproteins, milk and egg yolk to semen extenders (White, 1993; Medeiros *et al.,* 2002; Andrabi, 2009).

2.7.4 Standard cryopreservation protocols

The fertility of cryopreserved sperm largely depends on the care that was initially taken with the extension of the sample prior to freezing, as well as during the thawing of extended sperm samples.

Processing of sperm samples for cryopreservation purposes include the dilution of the sample with an extender, equilibration of the sample, and step-by-step cryopreservation of the sample to eventually be stored in liquid nitrogen.

2.7.4.1 Dilution

Semen samples are diluted for technical and biological reasons. With natural mating, fertile rams deposit several million sperm into the ewes' vagina during a single mating. With artificial insemination, sperm from genetically superior males can be used to inseminate a large number of females, when compared to the number of ewes that can be served during natural mating (Vera-Munoz *et al.,* 2009).

A study conducted on bulls in Venezuela indicated that the motility and the membrane functionality following cryopreservation at high sperm dilution rates decreased (Vera-Munoz *et al.,* 2009). Many other researchers have reported similar results (Garner *et al.,* 2001; Prathalingam *et al.,* 2006; Ballester *et al.,* 2007). These results may be due to the lower concentration of seminal plasma at the higher dilutions, for as previously mentioned, seminal plasma plays an important role in protecting the sperm membranes during the process of cooling, cryopreserving and thawing. Leahy *et al.* (2010a) stated that ram sperm can be extended at high rates prior to freezing, but preferably not post-thaw. It was also found that dilution prior to freezing improved the motility, viability and the acrosome integrity, assessed over a 6 hour incubation period (Leahy *et al.,* 2010b).

There are many extenders commercially available to be used in cryopreservation protocols. There are specific functions that a good extending media need to fulfil, which include:

- Providing nutrients as a source of energy,
- Protecting against harmful effects of rapid cooling,
- Provide buffering effect to prevent harmful shifts in pH as lactic acid is formed,
- Maintaining the proper osmotic pressure,
- Inhibits any form of bacterial growth,
- Increasing the volume of the semen, to use it for multiple inseminations, and
- Protecting the sperm membranes during the process of freezing.

Dilution should be done as soon as possible after collection and examination. Care should always be taken to warm the cryodiluent to 37˚C prior to adding it to a sperm sample to prevent cold shock (Hafez & Hafez, 2008).

2.7.4.2 Equilibration

Equilibration refers to the total time during which the sperm remains in contact with the cryodiluent, before the process of freezing commences. During this period, the cryoprotective agent is allowed to penetrate into the sperm cell to establish a balanced intracellular and extracellular concentration. It should be kept in mind that it is not only the concentration of

cryoprotective agent that plays a role in equilibration, but also of the other osmotically active extender components. During slow cooling, the dehydration of sperm can proceed to the point of osmotic equilibrium between the intracellular and the extracellular compartment. An increase in the cooling rate may lead to dehydration of the cells at a rate not fast enough, leading to the formation of intracellular ice crystals within the cell. When optimum cooling and/or freezing rates are used, sperm will remain exposed to the unfavourable conditions for a much shorter period of time (Andrabi, 2009).

EXTENDERS FOR EQUILIBRATION

There is a wide range of additives that can be used in the long-term storage of semen to extend the lifespan of frozen-thawed semen by slowing their metabolism, and protecting them during the process of cooling and freezing. The use of egg yolk in media used for the short- and long-term storage provides protection against the potential temperature shock of cooling, and it has been proven to minimize the loss of the acrosomal enzymes and prevent degenerative changes which occur in the acrosome during short-term storage. The use of milk as part of an extender for storing ram semen in liquid is common, as the high protein fraction serves as a type of buffer against pH fluctuations, and it also serves as a chelating agent to protect sperm from any heavy metals that could be present, thus maintaining the quality of semen (Salamon & Maxwell, 2000).

Many compounds have been tested for their efficacy as sperm cryoprotectants, but the one that is most often used in cryopreservation protocols is glycerol. Glycerol itself is known to be metabolized by bull, ram, boar and goat sperm (Holt, 2000). The addition of high concentrations of glycerol is however, limited by its toxicity. The level of toxicity should be considered together with the rate of cooling and freezing, the method of adding glycerol and the composition of the diluents (Salamon & Maxwell, 2000).

Fructose is a simple carbohydrate present in the semen of the ram, and the fact that it is readily metabolized by sperm, results in this sugar being included in cryodiluents. There is a range of other sugars such as glucose and mannose that are also included in storage diluents, and which has been proven to preserve the motility of sperm, thus preserving its quality (Salamon & Maxwell, 2000).

2.7.4.3 Cryopreservation protocols

When it comes to the packaging for frozen storage, the most common packaging for ram semen is pellets and polyvinyl chloride (PVC) straws, with the latter available in either 0.25 or 0.5 mL straws (Maxwell *et al.,* 1995). The straw-method consists of filling the straws with the cryodiluted semen, and then sealing them with a PVC powder. The freezing of the straws can be done by

either hanging the straws in the vapour above the liquid nitrogen, or using a controlled-rate freezing machine (Salamon & Maxwell, 2000). The pelleting method consists of dropping semen droplets into holes which are engraved on the surface of a block of dry ice. For rams and other smaller ruminants, the 0.25 mL straw is the preferred cryopreservation method, as the straws are compatable with laparoscopic insemination pipettes, especially as less sperm is required for this technique of insemination (Holt, 2000).

Generally the pelleting of diluted ram semen on dry ice has been found to be superior to the method of freezing in straws when straws are in the liquid nitrogen vapour. In contrast, better fertility results have also been reported when the straw method was used (Graham *et al.,* 1978).

2.7.4.4 Thawing protocols

Sperm samples are kept at low temperatures (-196ºC) until needed. In the freeze-thaw procedure, the warming of the sample is just as important as the freezing phase for sperm survival. The thawing protocol used depends on the specific rate of cooling used, and if it has been sufficiently high to induce the intracellular freezing, or low enough to produce cell dehydration. In the first case, it is proposed to thaw fast, to prevent recrystallization of any intracellular ice which may be present in the sperm (Andrabi, 2008).

Thawing of straws is usually performed by immersing them in a pre-heated water bath. The thawing time should be controlled carefully to avoid killing the sperm cells by overheating (Hafez & Hafez, 2008). Generally it is considered that fast thawing rates are required in order to obtain the best sperm recovery rates. The standard protocol used for the thatwing of cryopreserved bull sperm involves immersion of the straw in 35˚C water, for a period of 30 seconds. There are however many other combinations of temperature and duration of immersion when thawing straws; some indicate that 37-39˚C for 30 seconds is preferred (Graham, 1994; Ollero *et al.,* 1997). Other studies have found satisfactory results using higher temperatures (65˚C) for a shorter immersion period (6 seconds) (Martínez-Pastor *et al.,* 2006). Care should be taken to not leave straws that are being thawed at too high temperatures for extended periods, as this may result in pH fluctuations and potentially contribute to the degradation of the proteins (Andrabi, 2008).

Thawing of pellets are best in a liquid medium at about 40˚C, but to make it practical under field conditions, an ice-water thawing bath can be easier to maintain and may result in satisfactory outcomes in terms of post thaw sperm quality (Hafez & Hafez, 2008).

2.8 Aims of the study

The potential of assisted reproductive techniques (ART's) to assist sheep producers to optimise the production and reproductive efficiency of their flocks has received considerable attention during the last few decades. Artificial insemination (AI), and multiple ovulation and embryo transfer are ART tools, and effective use can enable farmers to produce lamb and mutton more cost-effectively. One of the major limitations of using ART's, however, is that the existing techniques have been adapted from the respective ART's developed for the cattle industry.

Optimization of the respective ART's for the sheep industry are however important, as it will enable sheep farmers to better manage the reproductive ability of their animals and thus run their farming systems more cost-effectively while at the same time promoting genetic progress within a flock. Previous studies have investigated the use of ART's to increase ovine production and reproductive efficiency. However, the effect of genetic selection for reproduction in sheep, and specifically the contribution of the ram, is poorly understood.

This study aimed to determine the contribution of the ram to reproductive efficiency in terms of mating dexterity and semen quality. The effect of divergent genotypes was also investigated on epididymal sperm, and the possible effect it might have on the cryopreservation status of sperm. Generally it is known that ovine sperm membranes are damaged during the process of cryopreservation, and thus the use of seminal plasma to optimize the cryopreservation protocol for sheep was investigated.

Lastly, it was noted that the standard techniques for evaluation of ovine semen samples, specifically the evaluation of sperm motility, was a time consuming factor which contributes to the decrease in overall sperm quality of the sample prior to processing for cryopreservation, and thus also the viability and survivability of sperm post-thaw. To address this problem, a new technique (flush technique) was compared with the conventional swim-up technique to evaluate whether the flush technique can be used as a better alternative, when used for semen evaluation prior to processing protocols for cryopreservation purposes.

Chapter 3

Materials and methods

3.1 Experimental location

The study was conducted on the Elsenburg Research Farm (33°51'S, 18°50'E) outside Stellenbosch in the Western Cape, South Africa. The climate of the region is classified as Mediterranean, and the annual precipitation during the winter months (April to September) is on average approximately 600mm.

3.2 Experimental animals

Ethical clearance was obtained from the Departmental Ethics Committee for Research on Animals (DECRA) of the Department of Agriculture, Western Cape (DECRA reference number R11/45). All animal care and procedures used in the study were performed in such a manner that it adhered to the guidelines stipulated in the South African National Standards document 10386:2008.

A total of 27 Merino rams (*Ovis aries*) aged 2 to 5 years were used in the study. The rams are part of a research flock established and maintained at the Elsenburg research farm. The rams were maintained under uniform nutritional conditions, and to avoid energy partitioning towards wool growth between individual rams, all animals were shorn approximately 8 weeks prior to the onset of semen collection. Throughout the mating season (January to February) and during the lambing season in winter (June-July), irrigated kikuyu grass (*Pennisetum clandestinum*) paddocks were utilised as pasture.

Prior to the training of the rams to ejaculate into an artificial vagina (AV), a total of six ewes were synchronized using 40mg fluorogestone acetate (FGA) intra-vaginal progestagen sponges (Ovakron™; Ramsem, Bloemfontein, SA). A general antibacterial cream was applied to the intra-vaginal sponges, and inserted using a sponge applicator (Ramsem, Bloemfontein, South Africa). After 14 days, the intravaginal sponges were removed and the ewes came into oestrus 36h later.

The synchronized ewes were used to train the rams to mount and ejaculate into the AV. After training the rams, a single Merino ewe (not in heat) was restrained, and used as a dummy female for semen collection (Figure A1; Appendix A). Rams were successfully trained to ejaculate into the AV within a period of two weeks (Figure A1; Appendix A).

3.2.1 Flock history and selection for the multiple rearing trait

The rams and ewes used in the study were sourced from a base population that was established in 1986 on the Tygerhoek Research Farm, near Riviersonderend in the Southern Cape. The experimental layout consisted of two genetically diverse lines of Merino sheep, where selection was based on the maternal ranking values for the number of lambs that were reared per joining opportunity. The entire genetic resource flock was transferred to Elsenburg during 1992 for detailed behavioural studies on the parturient and early postnatal behaviour of ewes and lambs (Cloete & Scholtz, 1998). Replacements for the High Line (HL) were selected from the progeny of ewes that reared more lambs, than the number of lambing opportunities offered. Replacements for the Low Line (LL) in contrast, were offspring from ewes that reared fewer lambs than they had lambing opportunities (i.e. ewes that were either infertile or that had lost all progeny born at least once) (Lambrechts *et al.*, 2000). Selection was based on ranking values according to maternal lamb-rearing ability, augmented by estimated breeding values for the number of lambs weaned from the progeny group of 2002 (Cloete *et al*., 2004; 2009). These breeding values were estimated from a single trait repeatability model fitted to repeated ewe reproduction records.

3.3 Behavioural study of mating performance of rams from the HL and LL

For this part of the study, 54 HL and 23 LL rams, aged approximately 1.5 years were used to conduct serving capacity tests. Merino ewes were synchronised using Ovakron intra-vaginal sponges (Ramsem, Bloemfontein, South Africa) for approximately 14 days so to induce oestrus 36h after sponge withdrawal. Rams were then individually placed with 5-8 synchronized ewes in a testing pen. The latter number was more than the number of ewes used in similar studies by Kilgour (1985) of 3-5 ewes. No feed or water was made available to the animals in the observation pens during the observation sessions. Ram behaviour was recorded for a period of 20 minutes, which differed from the two 1h observation periods of Kilgour (1985).

Behavioural data recorded during each observation session included:

- Time to the first mount (measured in seconds): This is the time that elapsed from introduction to the ewes until the first mounting occurred. A terminal value of 1200 seconds was given to rams that failed to mount during the 20-minute observation period.
- Time to the first serve (measured in seconds): This is the amount of time that elapsed from introduction to the ewes until the first successful serve which was characterized by a distinct thrust of the pelvis and a sudden backward jerk of the head. This is usually followed by a period during which the ram expressed little sexual interest. A terminal value of 1200 seconds was given to rams that failed to mount during the 20 minute observation period.

- Number of mounts: This was the number of mounts observed within the 20 minute observation session.
- Number of serves: This was the number of serves observed within the 20 minute observation session.

3.4 Collection of sperm and seminal plasma samples

Sperm samples used in the study consisted of ejaculated and epididymal samples obtained from HL and LL rams.

3.4.1 Collection and processing of ejaculated samples

Semen was obtained from 15 mature Merino rams (8 HL; 7 LL), aged between 2 to 5 years, using an artificial vagina (AV) (Brady & Gildow, 1939; Hafez & Hafez, 2008). Rams were trained to ejaculate into an AV by using 6 synchronized ewes, as described in Section 3.2. Subsequent collections were facilitated by one Merino ewe that was restrained and used as a dummy/teaser (Section 3.2).

The HL and LL rams were randomly allocated to three groups of five each, and semen was collected three days per week over a period of four weeks from each of these three groups. Semen was thus collected from each ram, once a week, with a total of three collections per ram for the period of the study. On collection days, the rams were brought in from the pasture, and placed in individual indoor pens (2m X 1m), until used for semen collection. Semen collection was performed in a separate pen, where a ewe was restrained as the dummy/teaser for collection. The AV was prepared beforehand to ensure that the collection conditions optimised the viability of the semen samples. The temperature in the AV ranged between 42-45^{O}C, and was monitored between each sample collection procedure.

Prior to collection, care was taken to pre-warm all equipment (collection tube, AV inner, Ham's F10 medium, microscope slides and pipette tips) to 37ºC to avoid cold-shock damage to the sperm during collection and processing. During collection, care was taken to prevent contamination of the samples.

3.4.2 Collection of epididymal samples

Rams for this part of the study were transported to an approved European Union export abattoir (Roelcor), located in Malmesbury in the Western Cape. The testes of each ram were removed immediately post mortem, placed in pre-marked plastic bags and transported on ice to the laboratory for further processing. Upon arrival at the laboratory, the epididymides of each testis

was dissected free, and moistened with pre-warmed Ham's F10 solution (37ºC) (Sigma-Aldrich, South Africa) to prevent dehydration of the tissue (Figure A2; Appendix A).

To obtain the epididymal sperm samples, each *cauda* epididymis was processed as follows:

- The *cauda* epididymides were dissected free from all connective tissue and blood vessels by using ophthalmic scissors and a pincette to expose the individual tubules (Figure A2; Appendix A). Upon exposure, a segment of each *cauda* epididymis was then removed and transferred to a small Petri dish (Lasec, South Africa) containing Ham's F10 solution.
- Each segment of epididymal tubules was then sliced by using a standard sized scalpel blade to release the epididymal sperm (Varisli *et al.*, 2009) into the Ham's F10 solution.
- The sperm - Ham's F10 mixture is then transferred to an Eppendorf tube and placed in a portable incubator (37˚C) to allow sperm to stabilize.

3.4.3 Preparation of seminal plasma

Seminal plasma was collected from the same 15 mature Merino rams that were trained to donate the ejaculate samples. After collection of the ejaculates, each sample was centrifuged (Beckman Coulter, Allegra™ X-22R Centrifuge) (2000 x g, 15 min at 4°C), and the supernatants from all the samples were pooled (i.e. equal parts from all rams) and stored at -20°C until used (Bernardini *et al*, 2011). Before use, the seminal plasma was thawed at room temperature. According to Domínguez *et al.* (2008), ram seminal plasma can be stored at either -18°C or -196°C, without affecting the ability of the seminal plasma protein binding onto the sperm surfaces or alteration of the protein composition.

3.5 Evaluation of collected samples

After collection the samples were subject to macroscopic (only ejaculate samples) and microscopic evaluation (ejaculate and epididymal samples).

3.5.1 Macroscopic evaluation

Post-collection macroscopic evaluation included samples being assessed for volume, mass motility and colour. Volume being determined by using the calibrations on the collection tube, as seen in Figure 3.1.

The colour and viscosity of the semen samples were subjectively evaluated by the same technician during the trial. The colour was graded according to a scale ranging from 0 – 5 with 0 being almost clear and 5 being a thick creamy white sample (Hafez & Hafez, 2008).

Figure 3.1 *Calibrated glass tube (left) used for ejaculate collections with an artificial vagina (AV) (right)*

Ram sperm generally exhibits a wavelike motion which can be seen if viewed in reflected light. The same effect can be seen by placing a 50µL drop of a sample on a pre-heated (37ºC) microscope slide and assessed by using a light microscope. After evaluation, mass motility score was determined according to a scale of 0-5, as presented in Table 3.1 below (Hafez & Hafez, 2008).

Table 3.1 *Classification of the mass motility exhibited by sperm for amount of vigour present in the wave motion of semen (Hafez & Hafez, 2008)*

Classification of wave motion	**Score**
Total immobility	0
Individual movement	1
Very slow movement	2
General wave movement, slow amplitude of waves	3
Rapid wave motion, no eddies	4
Rapid wave motion, eddies present	5

3.5.2 Microscopic evaluation

Microscopic evaluation of sperm samples included the accurate determination of the sperm concentration, the recording of specific motility parameters of sperm trajectory and the evaluation of morphometric parameters of the sperm head by using the Sperm Class Analyser® system (SCA®, Microptic, Spain). The SCA® technology provides fast, accurate and objective results, with a high repeatability that would not be possible to attain when using standard, subjective methods.

The SCA® system is comprised of three components, i.e. a bright field microscope fitted with a heated stage to visualize the sample, a digital camera for image capturing, and a computer on which the SCA® software has to be uploaded and installed. The motility and concentration module of the SCA® software automatically identifies and measures the motility parameters of the individual sperm. In this study, images for analysis were recorded at a frame rate of a 100 images/second. A literature survey failed to identify studies using a similar capture rate in previous ovine studies.

3.5.2.1 Sperm concentration

The accurate determination of sperm concentration is required to calculate the exact amount of extender to be added to a specific sperm sample for dilution purposes. Precise measurements are key, and thus a positive displacement pipette was used to pipet 5µl of the undiluted sample into 495µl Ham's F10 solution (Sigma-Aldrich, South Africa), thus diluting the sample 100 times. After dilution with the pre-warmed (37°C) Ham's F10 solution, the mixture was gently mixed to ensure an even distribution of medium in the sperm sample. A Leja® slide chamber was then filled with the diluted sample to determine the sperm concentration of the sample, which was then recalculated by multiplying with 100 to accurately determine the initial sperm concentration.

3.5.2.2 Analysis of motility

The SCA® was used to obtain accurate concentration and motility parameters throughout the study. Leja® slides (20µm depth, 5µl volume; Delfran, South Africa) were used throughout the study for all motility and concentration determinations. Motility was determined by using one of two techniques, namely the swim-up or flush technique, by loading an aliquot of each sperm sample into a Leja® chamber, and subsequently analysed with the SCA® motility and concentration module.

The SCA® provides automatic, immediate and objective results of motility parameters in a complete report. The SCA® software detects motile (type a, b and c) and static (type d) sperm

automatically (Figure 3.2). The measured motility parameters and their descriptions are presented in Table 3.2.

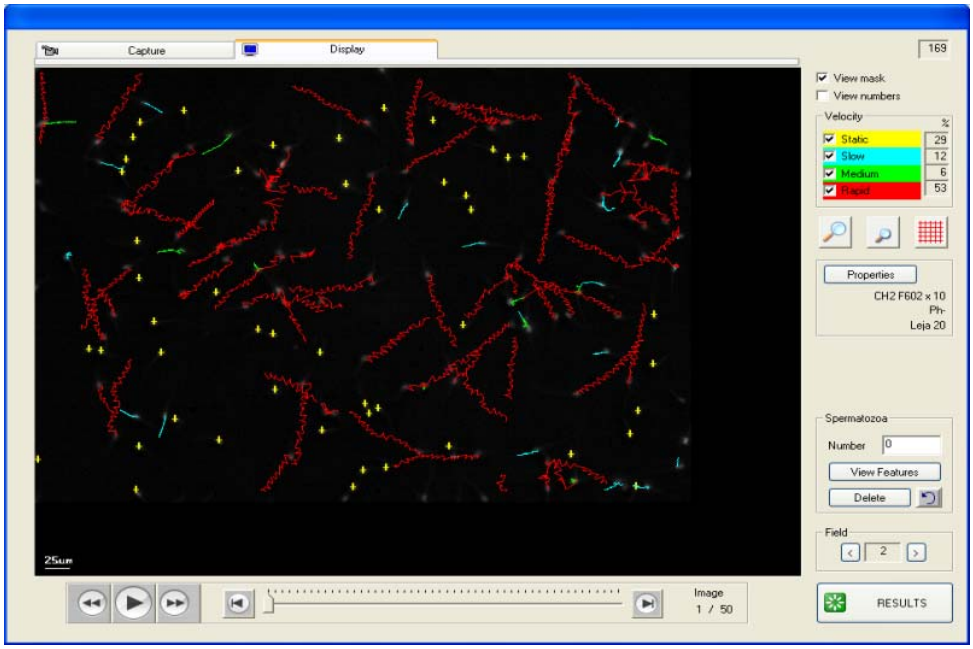

Figure 3.2 *This screenshot visualizes the analysis of sperm motility and concentration by using the SCA® module for concentration and motility. Sperm paths are classified as either type a (red), type b (green), type c (blue) or type d (yellow).*

Execution of motility determining techniques

The potential of two motility evaluation techniques to obtain an indication of the motility of a sperm samples were evaluated as part of this study, with the techniques being the swim-up technique (SUT) and the flush technique (FT). The swim-up technique is a well-documented method for the separation of highly motile sperm. However, one drawback of the SUT is that a considerable period of time elapses from sample collection, until the motility recordings can be made (minimum of 10 minutes). A flush technique (FT), developed by Prof G. van der Horst (gvdhorst@uwc.ac.za), may potentially allow for a more rapid assessment of sperm motility. This may then contribute to optimizing the pre-insemination or cryopreservation integrity and viability of ram sperm. The two techniques are detailed below:

THE SWIM-UP TECHNIQUE (SUT)

Prior to motility recording, 0.5 mL pre-warmed Ham's F10 (37ºC) (Sigma-Aldrich, South Africa) was transferred to an Eppendorf tube. An aliquot (10 µL) of the fresh sperm sample was then carefully placed at the bottom of the Eppendorf tube. The sperm - Ham's F10 mixture was then incubated at a temperature of 37ºC for 10 minutes, which allowed for the most motile and viable sperm to swim upwards towards the overlaying Ham's F10 layer.

After the 10 minute waiting period, a cloud became visible within the sample. Sampling for motility recording was then performed by collecting an aliquot from just above the 'cloud', and this sample is then transferred to a chamber of a pre-warmed Leja® slide (Delfran, South Africa). Motility recordings were captured in two to three fields per samples, at 100 frames per second using the SCA® system.

THE FLUSH TECHNIQUE (FT)

Prior to recording of sperm motility, a Leja® slide (Delfran, South Africa) was pre-warmed on the heated stage, at 37ºC. An aliquot of approximately 2µL of each fresh sperm sample was placed at the entrance of a Leja slide chamber, after which Ham's F10 medium was added to the chamber contents at the same point of entry, thus 'flushing' the sperm so that the sample distributed evenly throughout the chamber. Each slide was then placed on the heating stage for 7 to 10 seconds to allow for the sample to stabilize (i.e. no currents observed within the chamber), and for the sperm to reach maximum motility within the chamber environment.

The SCA® system was then used to capture the motility of each sample. For analysis purposes, two to three different fields of each sample were captured, at a capturing rate of 100 frames per second.

Table 3.2 *Description of motility parameters measured in this study (http://www.micropticsl.com)*

Motility parameter	**Description**
Motility	Percentage of sperm on field showing motility, classified into fast, medium, or slow motility, and thus also the amount of static/immotile sperm.
Progressive motility	Percentage of sperm moving progressively forward.
Curvilinear velocity (VCL)	Measured in µm/s. It is the time/average velocity of a sperm head along its actual curvilinear trajectory.
Average path velocity (VAP)	Measured in µm/s. It is the time/average of a sperm head along the spatial average trajectory of sperm.
Straight/line velocity (VSL)	Measured in µm/s. It is the time/average velocity of a sperm head along the straight line between the first detected position and the last detected position.
Amplitude of the lateral head displacement (ALH)	Measured in µm. It is the magnitude of the lateral displacement of a sperm head about its spatial average trajectory. It can be expressed as either a maximum, or an average of these displacements.
Straightness (STR)	This measures the linearity of the spatial average path, and is calculated by dividing the VSL with the VAP.
Linearity (LIN)	It measures the linearity of the curvilinear trajectory, calculated as the VSL divided by the VCL.
Wobble (WOB)	This measures the oscillation of the actual trajectory.
Beat/cross frequency (BCF)	Measured as beats/s. This is the time average-average rate at which the curvilinear sperm trajectory crosses its average path trajectory.

All the above parameters are measured automatically by the SCA® system, and the data are then captured in Excel sheets for statistical analysis.

Equipment used for the capture and analysis of motility recordings

Sperm motility was assessed using the Motility and Concentration module of the SCA® software, version 5.1 (Microptic S.L., Barcelona, Spain). The capture of the data was done using a Basler A602fc digital camera (Microptic S.L., Barcelona Spain), which was mounted (C-mount) on an Olympus CH2 microscope (Wirsam, Cape Town, South Africa), equipped with phase contrast optics and a heated stage. The camera was connected to an Apple MacBook using a firewire cable. The software settings are summarized in Table 3.3 below.

Table 3.3 *Software settings (capturing- and analytical properties) for the SCA® system used in the study*

CAPTURING PROPERTIES		
Number of images	50	
Images/second	100	
Optics	PH- (negative phase contrast)	
Chamber	Leja 20	
Automatic analysis	YES	
ANALYTICAL PROPERTIES		
	Min	Max
Particle area	3	100
Slow	50	80
Medium	80	180
Rapid	180	-
Progressive (%STR)	80	-
Circular (%LIN)	-	50
Connectivity	14	
VAP Points @100 images/second	11	

3.5.3 Analysis of morphometric parameters

SpermBlue® (Microptic S.L., Barcelona, Spain) was developed for the evaluation of human and animal sperm morphology (Van der Horst & Maree, 2009). SpermBlue® fixative and staining material was then used throughout this study for morphometric analyses (for details on SpermBlue® staining method, please refer to Appendix A).

The SCA® morphology module was used for all analyses, as this software assesses detailed data for each individual sperm such as size (length, width, area) and head shape (ellipticity, elongation, roughness, and regularity), percentage acrosome coverage, specific size of the midpiece, and the angle of insertion into the sperm head. For the purpose of this study, only head- and acrosome-related morphometric parameters were considered (see screenshot of morphometric head parameters in Figure 3.3).

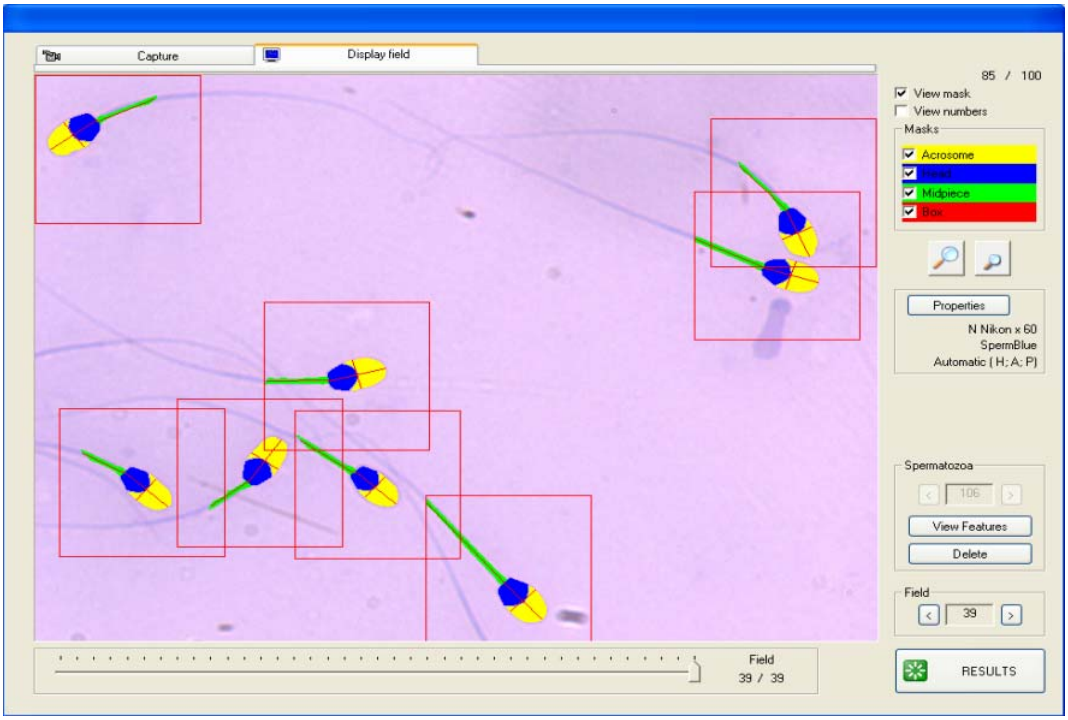

Figure 3.3 *Screenshot of visualisation following analysis of sperm morphometry using the SCA® software. The SCA® system indicates and identifies the acrosome (yellow), head (blue) and midpiece (green)*

The evaluation of the morphometric parameters was performed in systematically selected areas of the slide. Sperm were excluded if the head of sperm was lying on the edge, and if sperm overlapped, since it creates a physical barrier that the SCA® system cannot analyse. On each slide 100 sperm were analysed (and averaged) to complete the evaluation. The settings for

contrast and brightness remained the same throughout the study, although the light setting was adjusted accordingly to capture the best possible images on the slides.

Sperm analysis was performed at random on the different regions of the slide. After analysis of sperm, each image and corresponding analysis mask were compared manually. If obvious incorrect analyses were performed, these sperm were deleted from the field. The acrosome, head and midpiece regions are represented by yellow; blue and green respectively (Figure 3.4). Morphometric analysis excluded overlapping sperm and sperm positioned with heads on the edge of the captured field, due to assessment problems.

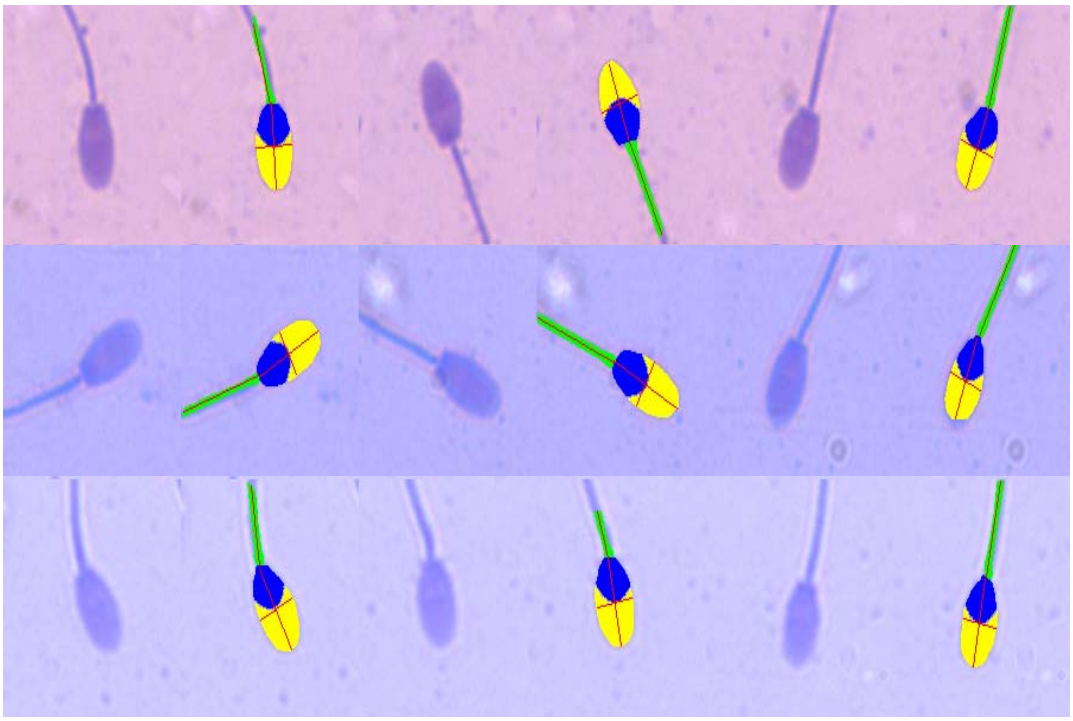

Figure 3.4 *The above images depict the morphometric analysis of sperm stained with SpermBlue®. The different sections are indicated in different colours: acrosome (yellow), head (blue) and midpiece (green). Each individual stained sperm cell is shown on the left, with the analysed sperm to its immediate right*

The morphometric parameters measured are summarized in Table 3.4. There were four software-calculated indexes, namely ellipticity, elongation, roughness and the regularity, all of which consider the standard morphometric measurements (head-length, -width, -area, etc.). It also gives an indication of the sperm head shape. The ellipticity parameter indicates whether sperm heads are tapered or thin i.e. where the value for ellipticity is high and the heads of the sperm are thin.

Elongation on the other hand indicates the roundness of the sperm heads. Values closer to zero suggest a rounder head. The roughness index indicates whether sperm heads are amorphous or irregular (indicated with low values), and the regularity index indicates whether sperm heads are pyriform (McAlister, 2010).

Table 3.4 *Formulas used to compute morphometric sperm head parameters, measured by the SCA® (L: length; W: width; A: area; P: perimeter) (Maree et al., 2010)*

Morphometric parameter	Symbol/formula
Head length (µm)	L
Head width (µm)	W
Head area (μm^2)	A
Head perimeter (µm)	P
Head ellipticity	L/W
Head elongation	(L-W)(L+W)
Head roughness	4π(A/P2)
Head regularity	π(L*W/4*A)

Equipment used for capturing images and analysis thereof for morphometric properties

A Basler A312fc digital camera (Microptic S.L., Barcelona, Spain) was mounted (C-mount) on a Nikon Eclipse 50i (IMP, Cape Town, South Africa) with bright field optics. The camera was connected by a six pin firewire cable to a Belkin firewire card of a Lenovo desktop computer. Fields of sperm observed with a 100x objective were digitally analysed using the Morphology module of the SCA® system (Van der Horst & Maree, 2009). The software settings are summarized in Table 3.5.

Table 3.5 *Software settings (analytical properties) for the SCA® system used in the study for morphometric analysis*

ANALYTICAL PROPERTIES		
Setting: RAM		
	Min	Max
Particle area	2	80
Sperm to analyse	100	
Size box	150	
Detection	AUTOMATIC	
Stain	SPERMBLUE	
Acrosome inversion	NO	

3.6 Sample processing

The use of the SCA® made it possible to evaluate samples at both the motility and morphometric level. Prior to evaluation of samples, a sperm processing protocol was used, leading to analysis and cryopreservation.

After the sperm samples were collected and placed in the incubator, a series of steps followed. These steps included the dilution, equilibration, loading of the straws and the process of cryopreservation.

3.6.1 Sample dilution

No dilution of ejaculate samples were performed in this study. After the accurate concentration was obtained for epididymal samples, each sample was diluted to three concentrations (C1: 100-, C2: 150-, and C3: 200 $x10^6$/mL) by using a commercial cryodiluent (Ramsem, Bloemfontein, South Africa, see Appendix A for details).

3.6.2 Equilibration time

Equilibration refers to the total time during which sperm remain in contact with the cryoprotectant before the process of freezing commences. During this period, the cryoprotectant was allowed to penetrate into the sperm cell to establish a balanced intracellular and extracellular concentration to prevent the formation of ice crystals within the sperm cells and causing damage to the sperm membranes.

After dilution, samples are equilibrated in a refrigerator (4˚C), according to the protocols presented in Table 3.6.

Table 3.6 *Experimental design used to demonstrate the effects of concentration and equilibration on sperm traits (E: equilibration period, at 1, 2 or 3 hours; C: sperm concentration, at 100-, 150- or 200 $x10^6$/mL)*

Sperm concentration	**Equilibration period**		
	1 hour (E1)	**2 hours (E2)**	**3 hours (E3)**
C1 (100 $x10^6$/mL)	E1C1	E2C1	E3C1
C2 (150 $x10^6$/mL)	E1C2	E2C2	E3C2
C3 (200 $x10^6$/mL)	E1C3	E2C3	E3C3

After each hour of equilibration smears were prepared on microscope slides of each concentration, and fixed using SpermBlue® fixative, to be stained (Figure A4; Appendix A) and

mounted for morphometric analysis, according to Section 3.5.3. For a more detailed report on how smears were prepared, please refer to Appendix A.

3.6.3 Loading of straws

After each equilibration period, two straws were made per treatment group. For this study, French Mini straws (0.25cc, Minitüb) were used. Straws were labelled with a waterproof permanent marker prior to loading. Information recorded on each straw included the date of collection, ram number, and the specific experiment number. Thus if a sample was collected on 3 July, from a ram with identification number 0.029, diluted to 100 million sperm/mL (C1) and equilibrated for two hours (E2), the identification would be: *0.029 3Jul C1E2*.

Straws were placed in the refrigerator to cool to 4-5ºC, beforehand. After loading, the straws were then sealed with a coloured PVC sealing powder to facilitate the easy identification of each treatment group (Taurus®, George, South Africa). The PVC powder transforms into a gel-like substance when coming into contact with any liquid, thus sealing the sample within the straw and preventing contamination of the sample.

3.6.4 Cryopreservation protocol

After loading the straws, cryopreservation processing commenced. The protocol for cryopreservation included:

- Liquid nitrogen transferred from a liquid nitrogen tank to a small Styrofoam cooler box, and half filled. After the straws were sealed with the PVC powder, straws were placed 3 cm above the level of the liquid nitrogen (vapour). This distance between the straws and the liquid nitrogen allow the straws to be suspended in the liquid nitrogen vapour, which is estimated to be at a temperature of approximately -80˚C (Visser, 1974). The straws were then allowed to rapidly cool in the vapour for a period of 15 minutes (Lambrechts, 1996).

- After being suspended in the liquid nitrogen vapour for 15 minutes, the straws were immediately plunged into the liquid nitrogen (-196˚C) for a period of 5 minutes.

- Straws were then transferred from the Styrofoam cooler box to a canister within a storage liquid nitrogen tank. Care was taken to prevent the straws being subjected to temperature fluctuations.

3.6.5 Storage and thawing procedure

Cryopreserved samples were stored in a commercial 25L liquid nitrogen storage tank (Schorn Cryogenics, South Africa), and the level of the liquid nitrogen was monitored frequently to ensure that an adequate liquid nitrogen level was maintained in the tank at all times.

Straws were thawed individually by transferring the straw directly from the liquid nitrogen into a prepared water bath at 35˚C for 30 seconds (37ºC for 30 seconds - Ollero *et al.*, 1997; 35ºC for 20 seconds - Azerêdo *et al.*, 2001). Each straw was then dried with a piece of paper towel, cut open with clean scissors, and the contents of the straw emptied into a 1.5 mL Eppendorf tube. The tube was then placed in a portable incubator at 37ºC for a period of one minute to allow stabilization of sperm after thawing. After the waiting period, the sample was removed from the incubator and evaluated for all motility parameters. Smears of the thawed samples were prepared, fixed, stained and mounted for morphometric analysis.

3.6.5.1 Protocol for seminal plasma addition post thaw

The possible beneficial effect of seminal plasma on post-thaw epididymal samples was evaluated by thawing individual straws with a concentration of 200 million sperm/mL, and equilibrated for 3h (standard), as described in Section 3.6.2. After transferring the straw contents to a 1.5 mL Eppendorf tube (Lasec, South Africa), 20% whole seminal plasma was added (details of seminal plasma collection discussed in Section 3.4.3). The contents of the straw and seminal plasma mixture were gently mixed, and placed in a portable incubator at 37ºC for 15 minutes (Bernardini *et al*., 2011). After the period of post-thaw incubation, each sample was removed from the incubator and evaluated for all motility parameters. Smears of seminal plasma treated samples were prepared, fixed, stained and mounted for morphometric analysis according to Section 3.5.3.

3.7 Post-thaw evaluation of samples

After the post-thaw incubation period, samples required dilution to accurately assess motility parameters with the SCA® system. Samples were diluted at a ratio of 1:4 (thus diluted 5 times) with Ham's F10 solution (Sigma-Aldrich, South Africa). An aliquot of the diluted sample was then pipetted into a pre-warmed four chamber Leja® slide, and allowed to stabilize for 30 seconds on the heated microscope stage. Motility recordings were captured and analysed according to capturing- and analytical pre-set properties (as described in 3.5.2.2). For each of the thawed samples, a "no analyse" recording of two seconds were captured from the undiluted sperm sample. These recordings were captured to confirm the total percentage motile sperm

determined by the SCA® system. The percentage motility was also determined by manually counting the number of motile sperm and by dividing this number by the determined concentration within the captured fields.

3.8 Statistical analysis

All data were analysed by generalized linear models, using PROC GLM of SAS Enterprise Guide 5.1 software, Version 9.3 of the SAS System for Windows. The procedure generates least square means (LSM), adjusted for multiple comparisons using the Bonferroni t-test. For the comparative analyses, the one-way analysis of variance (ANOVA) approach was used. Each collected sample was evaluated and assessed as individual samples. All results were expressed as the mean ± standard error of the mean.

All tables and graphs were prepared in Microsoft Excel (2010). All data collected with the SCA® were converted from *.mot file format to the Excel file format prior to analysis. All results were expressed as the mean ± standard error of the mean. Findings were considered statistically significant when $p<0.05$.

Chapter 4

The influence of mating dexterity and genotype on the semen quality of two genetically diverse Merino lines

Abstract

The goal of any breeding system is to improve the reproduction efficiency within the flock. The contribution of rams to the reproductive performance of ewes is still unclear. A research flock selected for two genetically divergent lines (i.e. the high line (HL) and the low line (LL)), based on maternal ranking values, established in 1986 was used. Selection for the rearing of multiple lambs can contribute to the overall optimal management of sheep flocks, and thus ensure cost-effective management of production systems. This study investigated whether the genetic make-up of rams contribute to the reproduction efficiency of a flock, in terms of conception rates and sperm quality (as qualified by motility and morphometry traits). Rams from the HL and LL were assessed in terms of mating performance in twenty minute mating tests. Matings, conception rates and lamb birth weights were recorded. Semen samples were obtained by means of the artificial vagina method from 15 Merino rams (8 HL; 7 LL), and epididymal sperm samples were obtained from 12 sacrificed Merino rams (6 HL; 6 LL) selected from the same base population. Sperm motility and morphometry recordings were made and analysed using the SCA® system, with motility recordings captured at 100 frames/second. Rams from the HL significantly outperformed the LL rams in terms of the number of services during the mating performance tests. On average, conception rates were higher for ewes mated with HL rams. Lambing data showed no significant differences for birth weights of lambs sired by HL or LL rams. No significant differences were also found for motility parameters measured between ejaculated sperm from the HL and LL rams. However, the morphometrical analysis indicated differences in elongation and ellipticity index values. When ejaculated sperm was compared to the epididymal sperm, significant differences were obtained for most of the motility parameters, although morphometrically no significant differences were found between the ejaculated and epididymal sperm, independent of the genetic line. In conclusion no noteworthy differences were obtained between sperm obtained from the HL or the LL, and thus further research should be focussed on the ewe contribution to the overall reproduction performance of a flock.

4.1 Introduction

The potential of assisted reproductive techniques (ART's) to assist sheep producers to optimise the production and reproductive efficiency of their flocks has received considerable attention during the last few decades. The reproduction potential of an animal is governed by the interactions between its genes and the environment. The genetic makeup of an animal determines the reproductive physiology and behaviour of that animal. Selection often focusses on the phenotype of animals, and selection for specific traits without considering correlated responses, could potentially impact reproduction success negatively (Krasnow & Steiner, 2006).

Rams and ewes both contribute to the reproduction success of a breeding flock. Certain studies have investigated the contribution of the ram in terms of correlations with certain phenotypic traits. These studies reported a positive correlation between reproductive characteristics such

as testicular diameter and sperm quality when sheep of high and low prolificacy were evaluated (Land, 1974; Knight, 1984; Rege *et al.*, 2000). Bradford (1970) proposed the idea of rams contributing to the variation in conception rate of ewes through a difference in the fertilizing ability of their sperm. Moore (1981) hypothesized that there are two ways in which rams from high and low prolificacy flocks can contribute to the variation in the number of lambs born per ewe mated. One of these is the difference between genotypes in terms of the fertilizing ability of their sperm, and the other differences in the ability of sperm obtained from high and low prolificacy rams to fertilize ova from multiple- and single-ovulating ewes. However, studies were unable to conclude that the variation in number of offspring produced per mating was due to genotype differences (Moore & Whyman, 1980; Moore, 1981).

One method of quantifying the contribution of rams to the reproduction success within a flock is by measuring the mating performance. Sexual performance within populations of domestic male farm animals is generally highly variable (Chenoweth, 1981). With such a high variation in sexual performance, the selection for this trait should have a rapid response when selected for. Dunnington and Siegel (1985) reported an eighty-fold (80x) increase in successful matings by the 20th generation measured in the higher line, when selection was based on the sexual performance of roosters. This high variability in sexual performance makes it desirable for any breeder to evaluate the mating competence of individual males before inclusion in a breeding program.

A study on the effect of rams differing in their serving capacity on flock fertility concluded that inadequate serving capacity is a contributing factor to insemination failure within a flock mating system (Kilgour & Wilkins, 1980). It is thus possible that the use of a high prolificacy group of rams with high serving capacity may lead to a reduced percentage of unfertilised ewes and an increase in the lambing percentage within a breeding flock.

A study by Bench *et al.* (2001) indicated that if there is sufficient genetic variation within a sheep population, it is possible to obtain a significant response by selecting for ram sexual performance in both male and female offspring within a single generation.

In 1986 two genetically diverse lines of Merino sheep were established by selection criteria according to maternal ranking values of the ewes. Details with regard to the selection procedure of replacement animals for two divergent lines can be found (Cloete & Durand, 1994; Cloete *et al.*, 2009). In brief, the progeny of ewes that reared >1 lamb per mating constituted the replacement animals for the high line (HL), with progeny of ewes rearing <1 lamb per mating were used as replacements animals for the low line (LL). Lamb survival rate in relation to lambing and neonatal behaviour between the lines has been studied and it was concluded that

the HL lambs showed improved birth weights and overall survival, when compared to the LL lambs. When assessed according to the number of lambs weaned per ewe mated, the two lines differed appreciably in their reproductive potential (Cloete & Scholtz, 1998).

When assessed according to the number of lambs weaned per ewe joined, the two lines differed considerably in terms of their reproductive potential. The study therefore aimed to investigate the influence of genetic selection on the prolificacy of the flock (for ejaculated- and epididymal recovered sperm) and the contribution of the respective rams in this regard. Special attention was given to motility and morphometric parameters that can potentially provide an explanation for the reported differences in siring ability of the two lines.

4.2 Materials and methods

4.2.1 Experimental location

The experiments were conducted at the Elsenburg research farm (33°51'S, 18°50'E), outside Stellenbosch in the Western Cape, South Africa.

4.2.2 Experimental animals

The animals used in this study originated from the same base population (aged between 2 and 5 years), and were allocated to 3 treatment groups; i.e. mating dexterity-, ejaculate- and epididymal group. The first group (mating dexterity group) consisted of 54 high line (HL) and 23 low line (LL) rams and were used to determine the effect of genotype on mating ability. The second group (ejaculate group) consisted of 8 HL and 7 LL rams, and they were trained for collection of semen with an artificial vagina (AV). The last group (epididymal group) consisted of 6 HL and 6 LL rams, which were sacrificed to obtain epididymal sperm for comparison with ejaculate samples.

All rams were maintained according to ethically approved husbandry practices, and under uniform nutritional conditions. Rams in the ejaculate group were all shorn 8 weeks prior to the commencement of semen collection, to avoid variability in energy partitioning towards wool growth.

Ethical clearance was obtained from the Departmental Ethics Committee for Research on Animals (DECRA; number R11/45). All animal care and procedures used in the study were performed in such a manner that it adhered to South African National Standards 10386:2008.

4.2.3 Observation of the reproductive performance

The mating dexterity group of rams were used to compare mating performance between the HL and LL genotypes. All rams were tested for mating performance at an age of approximately 1.5 years, and data was pooled for rams born in the years 2007 to 2010 (therefore stemming from the same contemporary groups as those rams aged 2 to 5 years at the time the study was conducted).

Merino ewes were synchronised with the use of oestrogen (intravaginal sponges), so that they came into oestrus 36h after the removal of the sponges. Rams from both the HL and the LL were individually placed with 5-8 synchronized ewes in a testing pen. Ram behaviour was then observed for a period of 20 minutes. These tests were based on comparable tests as described by Kilgour and Whale (1980). However these researchers used an observation period of 20 minutes pen testing, followed by two 1h periods of pen testing conducted. The data recorded during the twenty minute observation periods included:

- Time to first mount (seconds) (TMOUNT)
- Time to first serve (seconds) (TSERVE)
- Number of mounts during the 20 minute period (NMOUNT)
- Number of matings during the 20 minute period (NSERVE)

A successful mating was characterized by the thrust of the pelvis, and a sudden backward jerk of the head, followed by a period in which the ram expressed little sexual activity.

4.2.4 Semen collection

Rams were trained to ejaculate into an AV prior to commencement of semen collection (for more detailed information on the training of the rams and the AV collection technique, please refer to Chapter 3).

Semen samples were collected from 5 rams per day, at three intervals per week, for a duration of three weeks (n=45). Care was taken to ensure all equipment and instruments were pre-warmed (37ºC) to avoid cold shock to sperm. After collection, samples were subjected to macroscopic evaluation, which include volume, colour and macroscopic motility evaluation. Please refer to Chapter 3 for a more detailed description of macroscopic evaluation criteria and protocols. After macroscopic evaluation, semen samples were transferred to Eppendorf tubes (Lasec, South Africa), and placed in a portable incubator at 37ºC, until further processing.

4.2.5 Collection and processing of epididymal samples

Immediately after slaughter, the testes of each ram were collected, and placed in sample bags that were clearly marked with each ram's information. The testes were then transported on ice to the laboratory, with the time that elapsed from collection to processing ranging from 1.5 to 4 hours. Epididymal sperm were collected by means of aspiration into a Ham's F10 solution (Sigma-Aldrich, South Africa), transferred to Eppendorf tubes (Lasec, South Africa) and placed in an incubator until further processing (for protocol and details, please refer to Chapter 3). Care was taken to ensure all equipment and instruments used were pre-warmed to 37°C, to avoid potential cold shock to the epididymal sperm.

4.2.6 Motility and morphometry recordings and analyses

All motility recordings were captured using the swim-up technique as evaluation technique. The swim-up technique entails the transfer of 0.5 mL pre-warmed Ham's F10 (37ºC) to an Eppendorf tube, after which an aliquot (10 µL) of the fresh sperm sample is placed at the bottom of the Eppendorf tube. The tube is then incubated at a temperature of 37ºC for a period of 10 minutes, which allowed for the most motile and viable sperm to swim up towards the overlaying Ham's F10 layer.

After a 10 minute waiting period, a cloud was visible within the sample. Sampling for motility recording was then performed by collecting an aliquot from just above the 'cloud', and the sample was then transferred to a chamber of a pre-warmed Leja® slide (Delfran, South Africa). Two to three fields were used to capture motility per sample at 100 frames per second using the SCA® system.

Specific motility and morphometric parameters measured with the SCA® system included progressive motility, non-progressive motility, total motile, static, rapid, medium, slow, VCL, VSL, VAP, LIN, STR, ALH, and BCF (refer to Chapter 3 for a more detailed description of the parameters, and the determinations).

Smears were prepared for each individual ram, using the SpermBlue® (Microptic, S.L., Barcelona, Spain) staining kit (Van der Horst & Maree, 2009). SpermBlue® allows for the morphometric analysis of sperm samples. The morphometric parameters measured in this study include head length, -width, -perimeter, -area, ellipticity, elongation, roughness, regularity and the percentage of acrosome coverage. Refer to Chapter 3 for a more detailed description of the parameters, and the calculations.

4.2.7 Statistical analysis

The data were analysed with generalized linear models, using PROC GLM of SAS Enterprise Guide 5.1 software, Version 9.3 of the SAS System for Windows. The procedure generates least square means (LSM) and was adjusted for multiple comparisons with the Bonferroni t-test. For the comparative analyses, the one-way analysis of variance (ANOVA) approach was used. All results are expressed as the mean ± standard error of the mean. For conception rate comparisons, Wald's Chi square comparison test was used in a 2 x 2 contingency table by using coded data (1=conception, 0=no conception). Findings were considered statistically significant at $p<0.05$.

4.3 Results and discussion

4.3.1 Ram behaviour and conception rates of ewes served by HL and LL rams

The reproductive potential between rams from the two genetically diverse lines were compared by assessing the conception rates of the ewes that have been served by the HL and LL rams for the lambing year of 2012. Results were compared by using the Chi-square test in a 2 x 2 contingency table. The results are summarized in Table 4.1.

Table 4.1 *The conception rates of ewes served by rams of the High Line (HL) and Low Line (LL), respectively, for the 2012 lambing season*

Line	Number of ewes joined	Number of ewes lambed	% Ewes lambed
HL	177	163	90.80
LL	116	102	85.29

Results following comparison of conception rates between the HL and the LL were not significant ($p=0.19$). The averages observed in this study compare well to that of Lambrechts (1996), where the conception rates of ewes were documented over a period of three consecutive years (1993-1995). The overall averages of ewes mated by HL and LL rams were consistent with that found in the present study (HL: 93.4%; LL: 84.8% respectively).

The results from this study however differ from that found by Winfield and Cahill (1987), as conception rates between Corriedale ewes joined with rams with high mating competency (mount to serve ratio) and low mating competency indicated 51.1% and 55.7% conception rates, respectively. Differences may be due to breed differences, or because of varying environments.

Prior to mating the rams with the ewes for the breeding season, the mounting and serving behaviour of the 1.5 year old replacement HL and LL rams were observed and documented

annually to determine if the two lines differed in terms of their serving capacity. Results are presented and discussed below.

Table 4.2 *Least square means (±SEM) depicting the mounting and serving behaviour of rams from the High Line (HL) and Low Line (LL) within a 20 minute testing period (pooled data for rams born 2007-2010).*

	HL		LL	
	(n=54)		(n=23)	
	Mean ± SEM	**Range (CV)**	**Mean ± SEM**	**Range (CV)**
TMOUNT (s)	122.89 ± 30.73	964.00 - 1200 (183.77)	66.52 ± 26.93	553.00 - 1200 (194.17)
NMOUNT	8.00 ± 1.10	0.00 - 36.00 (100.97)	5.78 ± 2.07	0.00 - 38.00 (171.59)
TSERVE (s)	198.81 ± 39.59	1108 - 1200 (146.33)	145.65 ± 55.95	879.00 - 1200 (184.22)
NSERVE	2.17[c] ± 0.28	0.00 - 7.00 (93.50)	0.74[d] ± 0.20	0.00 - 3.00 (130.39)

[c, d] Columns with different superscripts differ significantly (p<0.01).

Data presented includes the time till first mount (TMOUNT), the number of mounts (NMOUNT), the time till first serve (TSERVE) and the number of matings (TSERVE).

From these results it is evident that there were no significant differences obtained for the time untill the first mount, the number of mounts, or the time until the first mating. However, a significant difference was obtained between the rams from the HL and the LL when the number of successful matings (NSERVE) were compared (p=0.002; Table 4.2). The HL rams were capable of more matings in the 20 minute session, compared to the LL rams (2.17 ± 0.28 and 0.74 ± 0.20 respectively).

The results obtained in this study are consistent with those reported by Lambrechts *et al.* (2000), as the number of matings for rams of the HL group differed significantly (p<0.01) from the number of serves of rams from the LL (2.64 ± 0.20 and 1.64 ± 0.24 respectively). Results in a study by Bench *et al.* (2001) are also consistent with results obtained in the current study. However the latter study indicated a higher number of matings for ram lambs born from the high performance sires (3.3 ± 1.2) and the low performance sires (2.8 ± 0.8) per testing session. The higher number of serves could be explained by the length of the test period, for rams in the study of Bench *et al.* (2001) were observed over a longer period of 30 minutes compared to the 20 minute observation periods used in the current study.

The average for the number of mounts combined with the higher (p<0.01) number of matings for the HL rams when compared to the LL rams, suggest that the overall mating dexterity of the HL being higher than that of the LL rams. It can be speculated that the rams from the LL are subordinate in social ranking within the flock, and it is possible that the presence of dominant rams in nearby pens could affect the mounting and ejaculation of these subordinate rams

(Lindsay *et al.*, 1976; Tilbrook *et al.*, 1987). The social interaction between rams should however not be underestimated, for this could possibly contribute to the difference in the number of matings in the HL and LL rams. A study by Malo *et al.* (2006) on red deer, reported variable fertility rates between males, and suggested that a male's ability to successfully fertilize ova may contribute substantially to differences observed in male reproductive success.

The mating season for this flock lasted 46 days. The cumulative lambing rate of the ewes that were joined with the HL- and LL rams for the period of the mating season was compared, to determine if there was any difference in lambing rate of the two groups. Results for the cumulative lambing rate are presented in Figure 4.1. The number of lambs born as singles or as twins is also set out for the ewes that were served by HL and LL rams respectively (Table 4.3).

Table 4.3 *The number of lambs born as either multiples (twins/ triplets) or singles when compared for ewes served by High Line (HL) or Low Line (LL) rams*

Lamb born status	Number of lambs	Total lambs per line	Lambing %
HL lambs (multiple)	84	183	45.90
HL lambs (single)	95	183	51.91
LL lambs (multiple)	44	102	43.14
LL lambs (single)	57	102	55.88

The results obtained indicated that there was no difference in the lamb yield for the HL and LL groups. The average percentage of lambs born as singles (HL rams; 51.9% vs. LL rams; 55.9%) and as multiples (HL rams; 45.9% vs. LL rams; 43.1%) did not differ between the HL- and LL- matings. From Figure 4.1 it is evident that most of the lambs from both the HL and the LL matings were born in the first half of the mating season. This is consistent with the results obtained by Lambrechts (1996).

Although on average no differences were found between the percentage of lambs born as singles or multiples, the results indicate that the percentage of lambs born from matings of the HL was 87.4% and the lambs born from LL matings was 75.5% on day 23 of the lambing season.

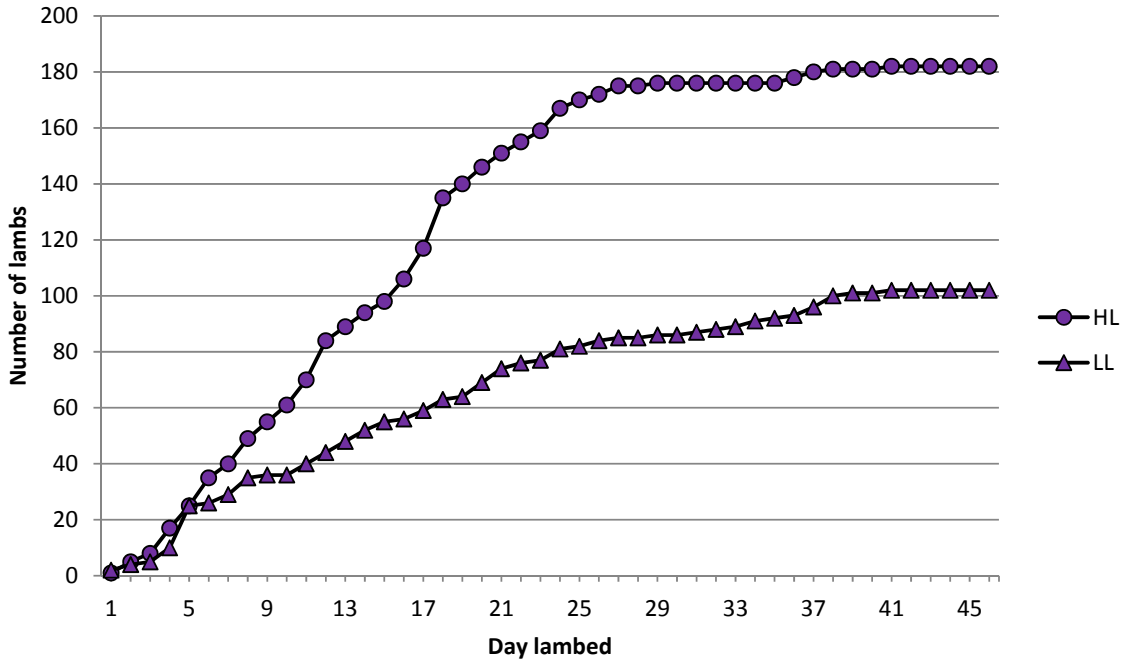

Figure 4.1 *The cumulative lambing rates of ewes served by the High Line (HL) rams and the Low Line (LL) rams, during the 2011-2012 mating season*

With no difference between single- and multiple lambs born between the HL and the LL, it was attempted to determine if there was any difference in birth weights of the lambs of the progeny born to the HL and LL rams respectively. The results for lamb birth-weights are presented in Table 4.4.

Table 4.4 *A comparison of the birth weights (mean ± SEM) obtained for lambs born from ewes mated to High Line (HL) and Low Line (LL) rams, respectively (range of values, as well as the CV is included)*

	HL	**LL**
	(n=179)	**(n=101)**
Birth weight (kg)	3.96 ± 0.07	3.95 ± 0.10
Min	2.3	1.9
Max	6.5	6.5
CV	17.08	18.71

After comparing conception rates, mating performance and lamb birth weights between HL and LL mated animals, differences were only observed for conception rates and mating performance (rams) between the lines. Further investigation into sperm quality parameters was conducted to possibly explain the difference between the lines regarding the number of offspring produced.

4.3.2 Macroscopic evaluation

EJACULATE SAMPLES

All semen samples collected from the 15 HL (n=8) and LL (n=7) rams were evaluated for macroscopic motility, and the results are presented in Table 4.5. No significant differences were found between the semen samples of the HL and the LL rams for macroscopic motility, sperm concentration or ejaculate volume (Table 4.5).

Table 4.5 *Macroscopic and microscopic sperm traits (mean ± SEM) of ejaculated samples obtained from Merino rams from the High Line (HL) and Low Line (LL)*

	HL		LL	
	(n=23)		(n=23)	
	Range (CV)	mean ± SEM	Range (CV)	mean ± SEM
Concentration (x10^6/mL)	1550 – 8580 (42.91)	3647.29 ± 319.45	1600 – 6760 (45.28)	3502.27 ± 338.08
Volume (mL)	0.5 – 1.5 (32.95)	0.88 ± 0.06	0.5 – 1.25 (31.38)	0.84 ± 0.06
Macroscopic motility	3 – 5 (17.08)	4.04 ± 0.14	3 – 5 (18.38)	3.86 ± 0.15

No significant differences were obtained for macroscopic motility, sperm concentration or ejaculate volume between samples obtained from HL and LL rams. This may suggest that the difference in reproductive performance observed between the lines could be due to underlying parameters such as morphometric dimensions and sperm head size or the influence of sub-populations within ejaculate samples between individual rams.

The macroscopic results obtained in this study are in contrast to those obtained by Lambrechts (1996), who found that ejaculated sperm obtained from 4 HL rams exhibited on average a lower motility, when compared to that of 4 LL rams. However, collections followed a 6 week mating season in the study of Lambrechts, (1996) while the rams in the current study were not exposed to ewes prior to semen collections. Depletion of sperm reserves after the 6 week mating season in the study of Lambrechts (1996) could potentially have contributed the lower motility due to depletion in fully competent and motile sperm reserves in the HL rams.

4.3.3 Microscopic evaluation

4.3.3.1 The effect of genotype on sperm motility parameters of fresh ejaculated semen obtained from HL- and LL rams

Results obtained by microscopic evaluation for sperm motility traits (i.e. total motile, non-progressive motile, progressive motile, static, rapid, medium, slow, VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF) of fresh ejaculated sperm are presented in Table 4.6 (see Chapter 3 for

more detailed information on motility parameter calculation and units of measure). The recorded motility traits showed a considerable range in both lines and are presented in Table 4.6.

Table 4.6 *A comparison of the range of deviation within least square means (mean ± SEM) observed between the motility parameters for sperm obtained from fresh ejaculates of High Line (HL) and Low Line (LL) rams*

Motility parameter	HL (n=23)		LL (n=23)	
	Range (CV)	Mean ± SEM	Range (CV)	Mean ± SEM
Total Motile (%)	73.02 – 97.24 (8.16)	85.78 ± 2.08	48.25 – 95.38 (15.59)	34.95 ± 3.83
Non-progressive motile (%)	17.12 – 81.89 (41.12)	37.58 ± 3.67	11.34 – 75.52 (58.29)	47.18 ± 3.80
Progressive motile (%)	7.82 – 73.53 (29.65)	48.20 ± 3.63	5.23 – 79.83 (44.49)	82.14 ± 2.17
Static (%)	2.76 – 26.98 (49.23)	14.22 ± 2.08	4.62 – 51.75 (71.70)	59.92 ± 3.58
Rapid (%)	40.70 – 82.38 (17.70)	64.92 ± 3.43	15.86 – 82.35 (35.25)	16.07 ± 2.21
Medium (%)	4.41 – 27.75 (43.24)	15.47 ± 2.12	4.15 – 55.84 (82.64)	6.15 ± 0.63
Slow (%)	1.26 – 10.73 (46.09)	5.39 ± 0.61	1.84 – 16.33 (55.57)	17.86 ± 2.17
VCL (µm/s)	185.88 – 389.61 (17.31)	270.98 ±10.42	141.83 – 342.65 (21.47)	257.25 ± 10.88
VSL (µm/s)	85.29 – 169.65 (17.44)	120.81 ± 6.03	45.48 – 186.90 (30.57)	119.73 ± 6.29
VAP (µm/s)	120.24 – 205.05 (11.93)	156.57 ± 5.09	93.15 – 205.45 (19.95)	152.16 ± 5.32
LIN (%)	25.76 – 55.28 (13.56)	45.00 ± 1.65	23.74 – 59.00 (21.21)	46.26 ± 1.72
STR (%)	50.09 – 85.97 (10.43)	77.00 ± 2.15	47.15 – 90.97 (16.46)	77.14 ± 2.24
WOB (%)	51.43 – 66.80 (7.12)	58.34 ± 0.94	49.63 – 68.25 (8.55)	59.57 ± 0.99
ALH (µm)	2.50 – 4.78 (17.67)	3.34 ± 0.12	2.26 – 4.37 (17.48)	3.128 ± 0.12
BCF (Hz)	38.96 – 54.55 (8.42)	45.71 ± 1.49	18.08 – 59.69 (21.45)	45.43 ± 1.55

There were no significant differences ($p>0.05$) for the motility traits of total motile, non-progressive motile, progressive motile, percentage static, VCL, VSL, VAP, rapid, medium, slow,

LIN, STR, WOB, ALH or BCF between sperm obtained from ejaculates of HL and LL rams (Table 4.6).

Research on motility traits of genetically divergent lines of rams is limited, however these results are similar to that of Lambrechts *et al.* (2000). No significant differences were obtained between any of the motility parameters obtained for ejaculates from HL or LL rams, as measured with the earlier version of the SCA® program (the Sperm Motility Quantification (SMQ) programme).

4.3.3.2 The effect of genotype on sperm morphometric traits of fresh ejaculated sperm obtained from HL- and LL rams

The results obtained by microscopic evaluation for sperm morphometric traits (i.e. head-length, -width, -area, -perimeter, ellipticity, elongation, roughness, regularity, and acrosome coverage) of fresh ejaculated sperm are presented in Table 4.7. The morphometric parameters obtained throughout the study showed considerable variation (the magnitude of the variation for each trait being included).

Table 4.7 *A comparison of the range of deviation within least square means (LSM) observed between the morphometric parameters for sperm obtained from fresh ejaculates of High Line (HL) and Low Line (LL) rams respectively*

Morphometric parameter	HL		LL	
	(n=13)		(n=13)	
	Range (CV)	Mean ± SEM	Range (CV)	Mean ± SEM
Length (µm)	7.92 – 8.70 (2.89)	8.36 ± 0.07	7.73 – 8.70 (3.03)	8.40 ± 0.07
Width (µm)	4.39 – 4.94 (3.26)	4.61 ± 0.04	4.34 – 4.93 (3.50)	4.74 ± 0.05
Area (µm^2)	36.48 – 43.35 (4.50)	39.44 ± 0.49	34.33 – 43.63 (6.00)	40.65 ± 0.68
Perimeter (µm)	17.93 – 19.79 (2.85)	18.84 ± 0.15	17.54 – 19.82 (3.22)	19.10 ± 0.17
Ellipticity	1.75 – 1.88 (2.27)	1.81^c ± 0.01	1.75 – 1.80 (0.78)	1.78^d ± 0.00
Elongation	0.27 – 0.31 (3.60)	0.29^c ± 0.00	0.27 – 0.28 (1.26)	0.28^d ± 0.00
Roughness	1.35 – 1.47 (2.44)	1.40 ± 0.01	1.38 – 1.43 (1.12)	1.40 ± 0.00
Regularity	0.73 – 0.79 (2.05)	0.77 ± 0.00	0.76 – 0.79 (1.04)	0.77 ± 0.00
Acrosome coverage (%)	54.00 – 55.21 (0.75)	54.51 ± 0.11	53.49 – 55.17 (0.94)	54.20 ± 0.14

$^{c, d}$ Columns with different superscripts differ significantly ($p<0.01$)

Significant differences were observed for the sperm morphometric traits ellipticity and elongation ($p=0.0035$ and $p=0.0032$, respectively; Figure 4.2 – 4.3).The higher ellipticity and elongation values of ejaculated sperm obtained from the HL rams indicated that they had more

elongated and less tapered shape than sperm obtained from the LL. Malo *et al.* (2006) reported that sperm with more elongated heads swim faster although this was not evident in the current study, as no differences were observed for sperm motility parameters between the HL and LL rams.

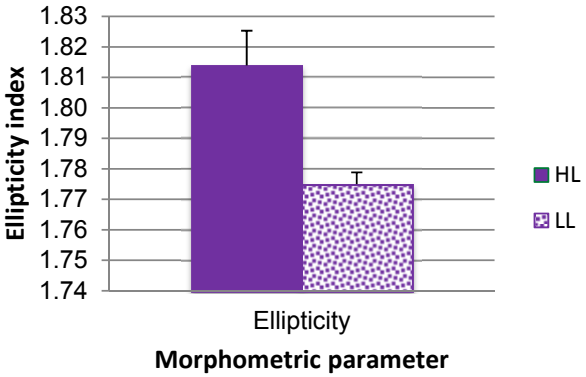

Figure 4.2 *Sperm morphometric parameter head ellipticity, as determined for ejaculated samples obtained from High Line (HL) and Low Line (LL) Merino rams, respectively*

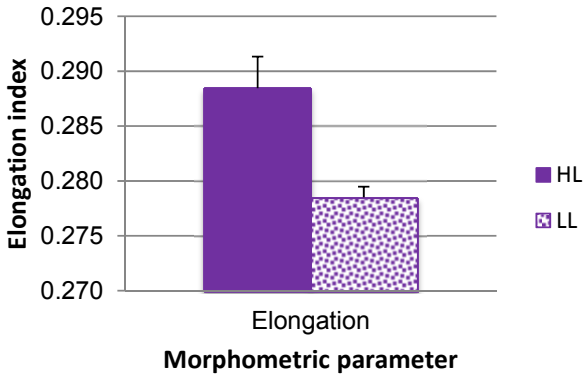

Figure 4.3 *The sperm morphometric trait head elongation, as determined for ejaculated samples obtained from High Line (HL) and Low Line (LL) Merino rams, respectively*

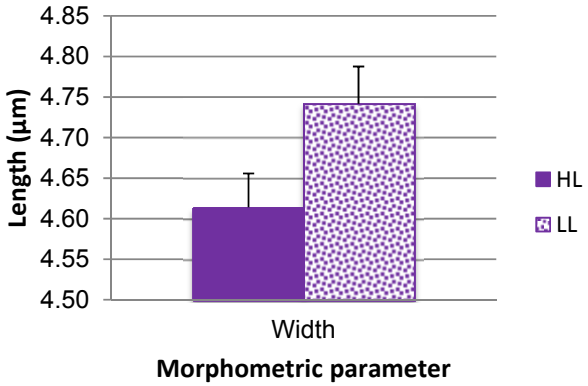

Figure 4.4 *The sperm morphometric trait head width, as determined for ejaculated samples obtained from High Line (HL) and Low Line (LL) Merino rams, respectively*

The difference in head width was also very close to the level of significance between sperm obtained from the two lines (p=0.051). Thus it can be stated that there is a tendency for LL ram sperm to be broader than that of HL ram sperm.

A study by Casey *et al.* (1997) on morphometric differences in sperm head dimensions of fertile and sub-fertile stallions, reported that the width of sperm heads obtained from stallions in the sub-fertile group tended to be larger than those obtained from stallions in the fertile group (p<0.08). These results supported a contention that differences in the dimensions of sperm heads between fertile and sub-fertile males may exist. In another study, on abalone, it was proposed that the specific sperm head dimensions/shape may influence the penetration ability of sperm through the zona pellucida (Grubert *et al.*, 2005). The cumulative evidence reinforces an argument that sperm head morphology may contribute to the differences observed in the number of offspring produced by ewes mated to rams from the two genetically diverse lines.

Understanding the adaptive significance of sperm form and function has always been a challenge to biologists due to the fact that sperm are highly specialized cells operating at a microscopic level, in a complex environment (Gage, 1998). Overall, the morphometric parameters measured on fresh ejaculated ram sperm in this study are consistent with that found by several other researchers for head length, -area, -perimeter, ellipticity, elongation, roughness, and regularity (Martí *et al.*, 2011b; Maroto-Morales *et al.*, 2012; Yániz *et al.*, 2012).

4.3.3.3 The comparison of motility and morphometry between ejaculated and epididymal sperm obtained from sexually mature Merino rams

The results for the motility recordings of fresh collected ejaculated and epididymal sperm samples are presented in Table 4.8. The motility parameters obtained throughout the study showed appreciable variation, as is evident from Table 4.8.

Table 4.8 *A comparison of the range of deviation observed between the motility parameters for sperm obtained via ejaculation or recovered from the cauda epididymis of Merino rams, aged 2-5 years*

Motility parameter	Ejaculate sample		Epididymal sample	
	(n=45)		(n=12)	
	Range (CV)	Mean ± SEM	Range (CV)	Mean ± SEM
Total motile (%)	48.25 – 97.24 (12.19)	84.04[a] ± 1.47	69.30 – 99.52 (9.49)	92.39[b] ± 3.01
Non progressive motility (%)	11.34 – 81.89 (49.06)	36.33 ± 2.82	15.69 – 85.07 (54.58)	44.41 ± 5.77
Progressive motility (%)	5.23 – 79.83 (36.92)	47.71 ± 2.72	8.96 – 80.77 (45.34)	47.97 ± 5.56
Static (%)	2.76 – 51.75 (64.19)	15.96[a] ± 1.47	0.48 – 30.70 (115.23)	7.61[b] ± 3.01
Rapid (%)	15.86 – 82.38 (26.86)	62.53[c] ± 2.34	61.40 – 96.15 (12.76)	85.62[d] ± 4.79
Medium (%)	4.15 – 55.84 (65.10)	15.76[c] ± 1.38	1.28 – 8.33 (55.27)	4.01[d] ± 2.81
Slow (%)	1.26 – 16.33 (51.43)	5.75[c] ± 0.41	0.61 – 5.95 (67.26)	2.76[d] ± 0.84
VCL (µm/s)	141.83 – 389.61 (19.27)	264.41[c] ± 7.29	436.19 – 578.03 (8.82)	473.71[d] ± 14.90
VSL (µm/s)	45.48 – 186.90 (24.90)	120.29[c] ± 5.56	86.38 – 295.98 (31.65)	199.52[d] ± 11.37
VAP (µm/s)	93.15 – 205.46 (16.03)	154.46[c] ± 4.04	201.72 – 348.28 (13.94)	266.09[d] ± 8.27
LIN	23.74 – 59.00 (17.59)	45.60 ± 1.36	14.94 – 58.09 (31.19)	42.55 ± 2.77
STR	47.15 – 90.97 (13.50)	77.07 ± 1.75	33.78 – 90.67 (22.83)	73.97 ± 3.57
WOB	49.63 – 68.25 (7.84)	58.93 ± 0.76	44.23 – 68.35 (12.72)	56.31 ± 1.56
ALH (µm)	2.26 – 4.78 (17.71)	3.24[c] ± 0.08	4.54 – 5.21 (4.72)	4.78[d] ± 0.16
BCF (Hz)	18.08 – 59.69 (15.81)	45.57 ± 1.03	39.89 – 57.07 (11.87)	49.55 ± 2.11

[a, b] Columns with different superscripts differ significantly ($p<0.05$)

[c, d] Columns with different superscripts differ significantly ($p<0.01$)

Statistical analysis revealed significant differences for the traits total motile ($p=0.0158$), rapid ($p<0.0001$), medium ($p=0.0004$), slow ($p=0.0023$), static ($p=00.0158$), VCL ($p<0.0001$), VSL ($p<0.0001$), VAP ($p<0.0001$), and ALH ($p<0.0001$) between sperm recovered from the *cauda* epididymides of the experimental animals, and those obtained from ejaculated samples via the AV (Table 4.8). Epididymal sperm showed higher values for the percentage motile, when compared to that observed for ejaculate sperm (92.4% ± 3.0% vs. 84.0% ± 1.5%, respectively).

These results are consistent with that found in the literature for bulls (Graham, 1994; Alapati *et al.*, 2009), and boars (Matás *et al.*, 2010) that agree on a higher percentage of motile sperm for epididymal sperm, when compared to ejaculated sperm directly after collection. However a study by Monteiro *et al.* (2011) on stallions concluded that the motility of epididymal sperm directly after recovery was lower than that for fresh ejaculated sperm (29.4% vs. 79.6%). However the latter study indicated that after dilution the motility of epididymal sperm increased to such an extent that motility observed for epididymal sperm was considerably higher than that of ejaculated sperm after dilution (84.0% vs. 77.1%).

The results in this study shows that epididymal sperm had higher values for the motility traits rapid swimming speed, VCL, VSL, VAP, and ALH, when compared to ejaculated motility parameters. Since the samples recovered from the *cauda* epididymides in the current study were suspended in Ham's F10 solution after collection, it is possible that the components on the Ham's F10 solution potentially contribute to the initiation of sperm motility and metabolism of sperm from the inactive/dormant state in which sperm occur within the epididymis. Exposure to the Ham's F10 can be considered as a form of chemical shock that has no detrimental effect on sperm viability (Turner & Reich, 1985).

Morphometric traits were also investigated to determine whether there are differences between sperm obtained via ejaculation and sperm recovered from the *cauda* epididymis of Merino rams. The results for the morphometric trait analysis of epididymal recovered sperm samples are presented in Table 4.9. The morphometric traits obtained throughout the study showed considerable variation, and are also summarised in Table 4.9.

Table 4.9 *A comparison of morphometric parameters (mean ± SEM) of ram sperm, obtained by ejaculation and recovery from the cauda epididymides of Merino rams, respectively*

Morphometric parameter	Ejaculate sample (n=15)		Epididymal sample (n=12)	
	Range (CV)	Mean ± SEM	Range (CV)	Mean ± SEM
Length (µm)	7.92 – 8.70 (2.65)	8.45 ± 0.06	7.73 - 8.7 (2.96)	8.28 ± 0.07
Width (µm)	4.39 – 4.94 (3.77)	4.71 ± 0.05	4.34 - 4.91 (3.22)	4.63 ± 0.04
Area (µm^2)	36.48 – 43.35 (4.90)	40.56 ± 0.51	34.33 - 43.63 (5.92)	39.35 ± 0.70
Perimeter (µm)	17.93 – 19.79 (2.87)	19.12 ± 0.14	17.54 - 19.82 (3.09)	18.75 ± 0.17
Ellipticity	1.75 – 1.88 (2.48)	1.80 ± 0.01	1.75 - 1.83 (1.18)	1.79 ± 0.01
Elongation	0.27 – 0.31 (3.97)	0.28 ± 0.00	0.27 - 0.29 (1.9)	0.28 ± 0.00
Roughness	1.35 – 1.47 (2.00)	1.4 ± 0.01	1.37 - 1.45 (1.65)	1.41 ± 0.01
Regularity	0.73 – 0.79 (1.77)	0.77 ± 0.00	0.76 - 0.78 (1.3)	0.77 ± 0.00
Acrosome coverage (%)	53.49 – 55.17 (0.99)	54.26 ± 0.14	54.04 - 55.21 (0.66)	54.50 ± 0.11

Results showed no significant differences in any of the morphometric traits measured when sperm samples obtained by ejaculation or recovered from the *cauda* epididymis were compared ($p>0.05$). There was however a tendency approaching significance for head length ($p=0.07$) indicating that sperm obtained via ejaculation tended to have longer heads, when compared to epididymal sperm.

4.4 Conclusions

The current study suggested that there exists a considerable difference between the HL and LL in terms of the mating performance (amount of serves) for the respective lines. However, no differences were observed for conception rates and lamb birth weights between ewes served by the HL and LL rams, respectively.

Microscopic evaluation showed that most motility parameters did not differ between sperm obtained from HL- and LL ram ejaculates. Morphometric analysis indicated significant differences between the lines for the index parameters ellipticity and elongation. Sperm obtained via ejaculation or recovered from the epididymis differed for most motility parameters evaluated, although no morphometric differences were found.

Further research is suggested to clarify the relationship between sperm quality and the reproductive performance of rams genetically selected for prolificacy. Aspects that warrant investigation include the effect of sperm sub-populations and hyperactivation status, and the effect of these traits on motility and morphometric traits of rams of genetically diverse backgrounds. Another possible area of research deserving further study is the possible contribution of the female component to the overall reproduction efficiency of a breeding flock.

Chapter 5

The influence of genotype, sperm concentration and equilibration period on sperm traits and the ability of epididymal sperm to withstand cryodamage

Abstract

The multiple rearing ability of an animal can be defined as the ability of the animal to produce a large number of weaned offspring within its reproductive lifespan. In sheep, selection for this trait can contribute to the income derived and the economic viability of commercial and small-scale production systems. This study investigated whether genotype (i.e. selection for ability of ewes to rear multiple lambs), sperm concentration, and period of equilibration influenced the ability of epididymal sperm, obtained from two genetically diverse lines, to offer resistance against cryodamage. Selection of the lines was based on maternal ranking values of the ewes for rearing multiple lambs. Twelve Merino rams, aged between 2 and 5 years, were sacrificed and the epididymides collected. Epididymal sperm were obtained via aspiration from the *cauda* epididymis, and motility recordings were captured at 100 frames per second using the Sperm Class Analyzer (SCA®) system. Motility and morphometry was assessed directly after collection and post-thaw. Sperm samples were exposed to nine different protocols of equilibration period and sperm concentration combinations, to optimise the protocol for epididymal sperm cryopreservation. Results showed that genotype had a significant effect on the motility parameters ALH ($p=0.016$) and BCF ($p=0.008$), and fresh morphometric parameters head width, ellipticity and elongation ($p<0.05$). Post-thaw evaluation indicated that morphometric parameters head area and percentage acrosome coverage differed significantly between genotypes. When motility and morphometry for equilibration period was evaluated regardless of sperm concentration, no significant differences were observed between 1 and 3 hour equilibration periods. No significant differences were obtained for the different pre-equilibration sperm concentrations regarding motility or morphometric parameters. Further research on equilibration periods is however warranted to elucidate the findings of this study.

5.1 Introduction

In South Africa, more than 80% of the farmed land is suitable for extensive livestock farming only (Livestock Development Strategy for South Africa, 2006), and precision livestock farming contributes up to 49% of the agricultural output (DAFF, 2011). Very little is however known regarding the potential contribution of the genetic make-up of rams on the ability of sperm to offer resistance to the damage caused by cooling, cryopreservation and thawing.

Selection for specific reproductive traits such as number of lambs weaned per ewe mated can contribute to income derived from the sale of surplus animals, and enhance genetic progress within the livestock sector (Cloete *et al.*, 2009). The heritability of reproduction traits for mammals are however considered to be low ($h^2=0.10$), although a study by Lavara *et al.* (2013) indicated that the heritability of rabbit sperm head dimensions ranged between 0.2 and 0.29.

Selection based on sperm traits may thus also be a viable option to increase the reproduction efficiency of rams.

Selection for improved reproduction traits has been an on-going process. A study on the effect of genotype on sperm quality by Lambrechts (1996) indicated differences in conception rates when selection was based on the ability of ewes to rear multiple offspring. Burfening *et al.* (1993) conducted a study on the selection of sheep for reproductive rate and the estimated genetic change in reproductive rate, and concluded that when the two lines with different reproductive rates were compared, the high line showed a greater response to selection. The latter study also indicated that the reproductive index responded well to selection.

Two genetically diverse lines of Merino sheep were established by selection of potential replacement animals for and against maternal ranking values of the ewes. Details with regard to the selection procedure of replacement animals for the two divergent lines are set out by Cloete and Durand (1994) and Cloete *et al*. (2009). Cloete *et al.* (2009) indicated that selection for multiple lambs and conception rate was higher for the HL ram matings than that for the LL matings without a reduction in lamb survival. The two lines differed in their reproductive potential, as reflected by number of lambs weaned per lambing opportunity (Cloete & Scholtz, 1998; Cloete *et al.*, 2009).

Lambrechts *et al.* (2000) reported on the differences in sperm traits between HL and LL rams, and concluded that there were no significant differences for any of the sperm parameters between the lines. This study focussed mainly on motility and morphology of sperm, with no analysis of sperm morphometric traits being performed. Morphometric analysis using CASA systems is considered as an objective and accurate way of measuring sperm dimension and size.

Processing of sperm, which include cooling, freezing and thawing, adversely affect the behavioural and functional capacity of sperm. Several researchers reported that cryopreservation affects sperm morphometry in several species, including rams (Martí *et al.*, 2011a; Yániz *et al.*, 2012), goats (Gravance *et al.*, 1997; Marco-Jiménez *et al.*, 2006; Hidalgo *et al.*, 2007), boars (Hirai *et al.*, 2001), and gilthead sea breams (Gallego *et al.*, 2012). However, the latter studies were conducted on ejaculated samples. No studies on the effect of cryopreservation on epididymal sperm morphometry were found in the literature.

A number of authors reported acceptable survival rates of epididymal sperm after cryopreservation and thawing, among others in red deer (Martinez-Pastor *et al.*, 2006), dogs (Hewitt *et al.*, 2001), buffalo (Lambrechts *et al.*, 2000), rams (Kaabi *et al.*, 2003), bulls

(Martins *et al.*, 2007), and boars (Suzuki & Nagai, 2003). However, there is room for improvement in the protocol for cryopreservation, as there are several contradictory results in the literature on the optimum sperm concentration, as well as the equilibration period to be used to minimize the extent of cryodamage to sperm.

The aim of this study was to investigate the effect of genotype, sperm concentration and equilibration period on sperm motility and sperm head morphometry of epididymal sperm samples freshly collected and post-thaw.

5.2 Materials and methods

5.2.1 Experimental location Elsenburg

The experiments were conducted at the research farm (33°51'S, 18°50'E), outside Stellenbosch in the Western Cape, South Africa.

5.2.2 Experimental animals

Twelve mature Merino rams (*Ovis aries*) (aged between 2 and 5 years) were sacrificed in the summer for this study. The animals were part of a base population of rams that were divergently selected for and against the ability of the ewes to rear multiples. In brief, progeny of ewes that reared >1 lambs per joining constituted the replacement animals for the high line (HL), with progeny of ewes rearing <1 lamb per joining were used as replacements animals for the low line (LL). Lam survival in relation to lambing and neonatal behaviour between the lines had been previously studied and it concluded that the HL lambs showed improved birth weights and overall survival rates, when compared to the LL lambs for selection (Cloete & Scholtz, 1998). Six rams were obtained from the high line (HL), and six rams were obtained from the Low line (LL). For more information on the selection strategy and flock history, please refer to Chapter 3.

Prior to slaughter, all animals were maintained according to ethically approved husbandry practices and under uniform nutritional conditions. Ethical clearance was approved by the Departmental Ethics Committee for Research on Animals (DECRA; number R11/45). All animal care and procedures used in this study were consistent with the South African National Standards 10386:2008.

5.2.3 Collection of epididymal samples

The testes of each ram were removed immediately post mortem and placed in pre-marked plastic bags and transported on ice to the laboratory. The epididymides of each testis was then dissected free, and moistened with Ham's F10 solution (Sigma-Aldrich, South Africa) to prevent

dehydration. The *cauda* epididymides were dissected free, transferred to Petri dishes and sliced with a scalpel blade to release the epididymal sperm into pre-warmed Ham's F10 solution (37˚C). Samples were transferred to a portable incubator (37˚C) to allow stabilization of sperm (for more details on protocol used, please refer to Chapter 3).

5.2.4 Experimental design

After collection, a positive displacement pipette was used to determine the concentration of each sample. After the accurate concentration was obtained, each sample was diluted to three concentrations (C1: 100-, C2: 150-, and C3: 200 $x10^6$ sperm/mL) by using Ramsem® cryodiluent (Ramsem, Bloemfontein, South Africa, see Appendix A, subsection A.4 for details). These diluted samples were then equilibrated for one, two, or three hours at 4ºC in a refrigerator. There were thus 9 treatments, i.e. C1E1, C1E2, C1E3, C2E1, C2E2, C2E3, C3E1, C3E2, and C3E3. The layout of the experimental design for the 9 treatments is set out in Table 5.1. This protocol was carried out for both HL and LL epididymal sperm samples. The statistical design thus reflected a 2 (selection line) x 3 (dilution concentration) x 3 (equilibration period) factorial designed experiment.

Table 5.1 *Experimental design used to demonstrate effect of concentration and equilibration on sperm parameters (E: equilibration period, at 1, 2 or 3 hours; C: sperm concentration, at 100-, 150- or 200 $x10^6$/mL)*

Concentration	**Equilibration period**		
	1 hour (E1)	**2 hours (E2)**	**3 hours (E3)**
C1 (100 $x10^6$/mL)	E1C1	E2C1	E3C1
C2 (150 $x10^6$/mL)	E1C2	E2C2	E3C2
C3 (200 $x10^6$/mL)	E1C3	E2C3	E3C3

After each equilibration period, two straws were loaded of each treatment (for details of loading, refer to Chapter 3). For morphometric analysis, smears were made, fixed, stained and mounted (SpermBlue®; Appendix A, subsection A.1-A.3) to be evaluated for each of the 9 treatments. Straws were then cryopreserved according to the technique described in Chapter 3, and thawed after 210 days. Post-thaw motility was recorded with the use of the SCA®, and smears were made, fixed, stained and mounted to evaluate post thaw morphometry for each treatment.

5.2.5 Analysis of data using the SCA®

Motility was recorded directly after the recovery of epididymal sperm, and post thaw. Throughout the study the Sperm Class Analyser (SCA®) software was used to obtain accurate, objective measurements of motility and morphometric parameters. Leja® slides (20µm depth,

5µl volume; Delfran, South Africa) were used for motility recordings. Motility was determined by loading an aliquot of the sperm sample into a pre-warmed (37ºC) Leja® chamber, and subsequent analysis with the SCA® motility and concentration module.

The parameters measured for motility included total motility, progressive motility, non-progressive motility, static, rapid-, medium-, slow motility, VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF. For details on motility recording, specific traits considered, equipment used and software settings, please refer to Chapter 3.

Morphometric analysis focussed on head shape and dimensions, while the midpiece and flagella of sperm was not evaluated for the purpose of this study. Morphometric data were obtained by analysing pre-stained (SpermBlue®) and mounted sperm on slides using SCA® version 5.1. The software automatically measured sperm head length, -width, -area, -perimeter, -elongation, -ellipticity, -roughness, -regularity, and the percentage of acrosome coverage. For more details on the protocol, trait definitions, equipment used and software settings please refer to Chapter 3.

5.2.6 Statistical analysis

The data were analysed by generalized linear models, using PROC GLM of SAS Enterprise Guide 5.1 software, Version 9.3 of the SAS System for Windows. The procedure generated the least square means (LSM) and adjusted derived significance levels for multiple comparisons with the Bonferroni t-test. For the comparative analyses between the high line (HL) and low line (LL) parameters, the one-way analysis of variance (ANOVA) approach was used. All results were expressed as the mean ± standard error of the mean (SEM). Factorial analysis was performed on data, with the main effects reported and the results of interactions presented in Appendix C, Table C1-C5. Findings were considered statistically significant when $p<0.05$.

5.3 Results and discussion

More than 20 300 individual sperm representative if 12 rams (aged 2 to 5 years), were analysed in the current study in an attempt to quantify the effect of genotype, sperm concentration, and equilibration period on post-thaw epididymal sperm morphometric parameters. A total of 108 straws were prepared, cryopreserved, thawed and evaluated for motility and morphometry to assess the ability of sperm to withstand cryodamage following the evaluated treatments. Parameters of significance ($p<0.05$), are additionally illustrated as graphs.

5.3.1 Evaluation of sperm motility

5.3.1.1 The effect of genotype on fresh collected sperm motility of epididymal sperm

Results obtained by microscopic evaluation for sperm motility traits (i.e. percentage motile, non-progressive motile, progressive motile, static, rapid, medium, slow, VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF) of fresh epididymal sperm are set out in Tables 5.2 and Table 5.3 (for more detailed information on motility trait calculation and units of measure, see Chapter 3).

Table 5.2 *The range between the minimum and the maximum values of the High Line (HL) and the Low Line (LL) Merino rams for all measured motility traits*

Motility parameters	HL	LL
	Range (CV)	Range (CV)
	(n=12)	(n=12)
Total motility (%)	85.98 - 99.52 (5.25)	69.3 - 99.39 (12.58)
Non progressive motile (%)	23.56 - 85.07 (41.37)	15.69 - 58.36 (57.91)
Progressive motility (%)	8.96 - 69.25 (63.25)	41.03 - 80.77 (26.95)
Static (%)	0.48 - 14.02 (75.53)	0.61 - 30.70 (134.65)
Rapid (%)	72.35 - 96.15 (10.19)	61.4 - 96.15 (15.59)
Medium (%)	1.72 - 8.33 (61.38)	1.28 - 6.76 (50.95)
Slow (%)	0.86 - 5.3 (72.57)	0.61 - 5.95 (68.97)
VCL (µm/s)	436.19 - 578.03 (11.76)	437.99 - 494.07 (4.06)
VSL (µm/s)	86.38 - 295.98 (46.40)	172.94 - 281.81 (17.52)
VAP (µm/s)	201.72 - 348.28 (20.56)	258.8 - 310.80 (7.38)
LIN	14.94 - 58.09 (45.82)	37.52 - 57.04 (15.24)
STR	33.78 - 84.98 (30.24)	66.82 - 90.67 (12.53)
WOB	44.23 - 68.36 (18.74)	56.14 - 62.91 (4.43)
ALH (µm)	4.59 - 5.21 (4.55)	4.54 - 4.81 (2.28)
BCF (Hz)	39.89 - 51.23 (9.21)	46.08 - 57.07 (7.60)

Table 5.3 *The motility traits of freshly recovered epididymal sperm (mean ± SEM) obtained from High Line (HL) and Low Line (LL) Merino rams, aged 2-5 years*

	HL	LL
	(n=12)	(n=12)
Total motility (%)	93.51 ± 4.10	91.46 ± 3.75
Non progressive motile (%)	58.19 ± 9.59	32.94 ± 8.75
Progressive motility (%)	35.32 ± 8.52	58.52 ± 7.77
Static (%)	6.49 ± 4.10	8.54 ± 3.75
Rapid (%)	86.46 ± 5.14	84.93 ± 4.69
Medium (%)	4.47 ± 1.02	3.62 ± 0.93
Slow (%)	2.57 ± 0.87	2.91 ± 0.79
VCL (µm/s)	489.94 ± 18.27	460.19 ± 16.68
VSL (µm/s)	172.52 ± 27.16	222.02 ± 24.79
VAP (µm/s)	260.73 ± 17.31	270.56 ± 15.80
LIN	35.82 ± 5.47	48.15 ± 4.99
STR	64.63 ± 6.75	81.75 ± 6.16
WOB	53.37 ± 3.11	58.76 ± 2.83
ALH (µm)	4.95^{a} ± 0.08	4.64^{b} ± 0.07
BCF (Hz)	44.93^{c} ± 1.83	53.40^{d} ± 1.67

[a, b] Columns with different superscripts differ significantly ($p<0.05$)

[c, d] Columns with different superscripts differ significantly ($p<0.01$)

Most motility parameters showed no significant difference between the epididymal sperm obtained from HL and LL rams (Table 5.3). Non-progressive motility (p=0.084), progressive motility (p=0.075) and index parameter straightness (STR) (p=0.09), tended to differ between the different ram lines. The percentage non-progressive motile sperm tended to be higher for epididymal samples obtained from the HL compared to the LL (58.2% ± 9.6% and 32.9% ± 8.8%, respectively). To the contrary the percentage progressive motile sperm tended to be higher for the LL epididymal sperm samples, compared to HL rams (35.3% ± 8.5% and 58.5% ± 7.8%, respectively). The index trait STR tended (p=0.094) to be higher for LL epididymal sperm when compared to the HL (81.8 ± 6.2 compared to 64.6 ± 6.8, respectively).

The ALH between the HL and LL differed significantly (p=0.016; Figure 5.1), where the HL showed a higher displacement value for ALH (4.95µm ± 0.08 µm) when compared to the ALH of the LL sperm (4.64 µm ± 0.07 µm). These results indicate that sperm from the HL may have had a more vigorous swimming motion than that of the sperm obtained from the LL rams. These findings are in contrast with those reported by Lambrechts (1996). The current study indicated that when based on average values, the LL rams showed a higher ALH than that of the HL rams. This can potentially be attributed to time of slaughtering, as the rams were slaughtered prior to the mating season thus little reproductive activity would encourage a homogenous population of epididymal sperm leading to a higher ALH, as sperm would hyperactivate more readily due to the higher percentage of mature sperm observed within the epididymal samples.

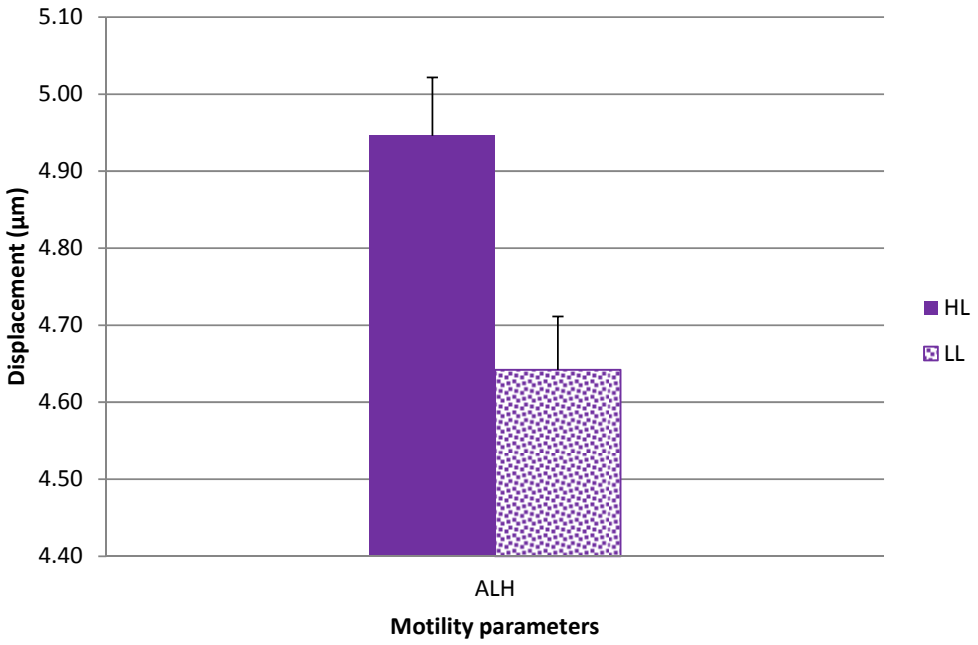

Figure 5.1 *The sperm motility trait amplitude of the lateral head displacement (ALH) for fresh collected epididymal sperm obtained from High Line (HL) and Low Line (LL) Merino rams respectively*

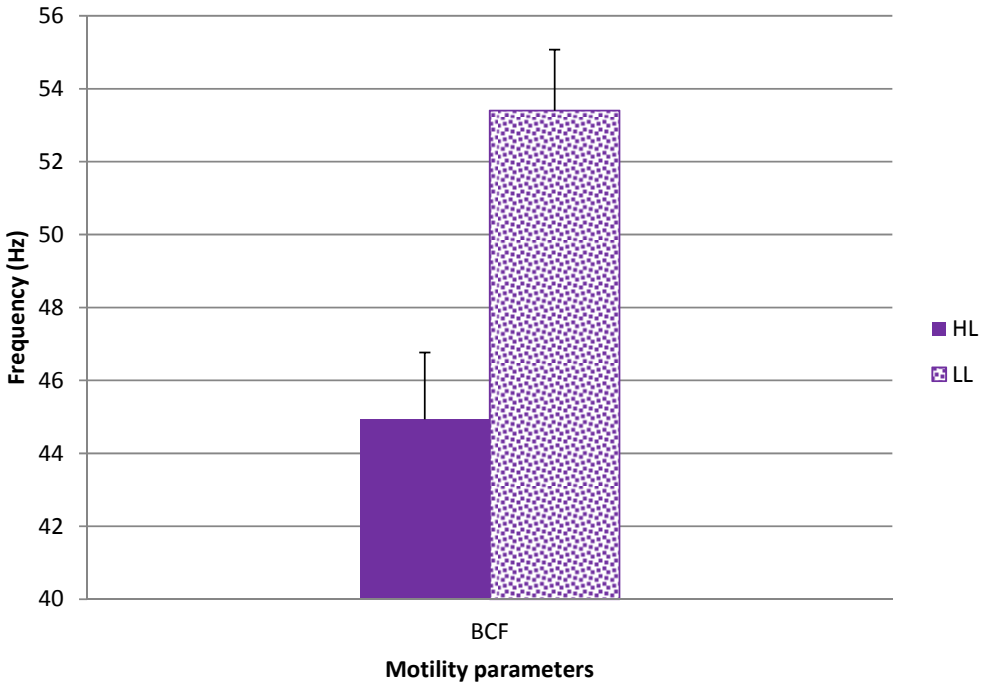

Figure 5.2 *The sperm motility trait beat/cross frequency (BCF) for fresh epididymal sperm samples obtained from High Line (HL) and Low Line (LL) Merino rams, respectively*

Sperm obtained from the LL rams reflected higher (p=0.008) values for beat/cross frequency (BCF) (53.40Hz ± 1.67Hz) than sperm obtained from the HL rams (44.93Hz ± 1.83Hz; Figure 5.2). It can thus be speculated that sperm from the LL rams are in a more mature stable state and thus more sensitive to chemical changes that is required for and initiate hyperactivation and capacitation. The differences between the BCF values of the lines could potentially be attributed to the presence of different sub-populations of sperm within the epididymal samples. Sperm sub-populations are likely to represent sperm in different physiological states of readiness to be hyperactivated (Abaigar *et al*. 2001).

The harvested epididymal samples may be considered as a heterogeneous accumulation of various sperm sub-populations, which is the product of different spermatogenic waves maturing along the epididymides and prior to recovery, were stored in the *cauda* epididymis as different cell cohorts (Rodríguez-Martínez, 2006). Previous studies have indicated that heterogeneity among sperm sub-populations may have a functional significance (Holt, 1996; Thurston *et al.*, 1999). The specific physiological role of these sub-populations is poorly understood, although studies have indicated that the ability of sperm to undergo capacitation and sperm fertility may vary depending on the sub-population which is under consideration (Harrison, 1996; Holt, 1996). Other studies have focussed on specific cut-off values for swimming speed to identify and quantify species specific sperm sub-populations (Maree & van der Horst, 2013), although this was not applied to the current study.

5.3.1.2 The effect of genotype on post thaw sperm motility

Results obtained by microscopic evaluation for sperm motility in post thaw epididymal sperm is presented in Table 5.4. The range and coefficient of variance (CV) values are also indicated.

Table 5.4 *Post thaw motility results (mean ± SEM) of epididymal sperm obtained from High Line (HL) and Low Line (LL) Merino rams aged 2-5 years*

	HL		LL	
	n=51		n=49	
	Mean ± SEM	**Range (CV)**	**Mean ± SEM**	**Range (CV)**
% Motile sperm	1.92 ± 0.50	0.45 - 8.72 (185.27)	2.17 ± 0.70	0.43 - 8.95 (225.42)

Post thaw motility recordings throughout the study indicated a low percentage of motile sperm, when compared to other studies (33% - Ollero *et al.*, 1998; 65.2% - Bag *et al.*, 2004; 21% - Purdy, 2006; 36.67% - García-Álvarez *et al.*, 2009). It should however be noted that the motile sperm within these low percentage motile samples had vigorous swimming velocities.

Results showed that when the percentage motile sperm was compared, no significant differences were found between post-thaw epididymal sperm obtained from HL or LL rams. Lambrechts *et al.* (2000) found that the average percentage motile sperm was higher for ejaculated sperm obtained from the LL rams when compared to sperm from the HL rams. This was not the case in this study, and thus it indicated that epididymal sperm from the HL and LL are equally susceptible to cryodamage.

5.3.1.3 The effect of sperm concentration and equilibration period on post-thaw sperm motility

Results obtained for microscopic sperm motility traits of post-thaw epididymal sperm are presented Table 5.5 (see Chapter 3 for more detailed information on the motility trait calculations and units of measurement). The motility traits showed a considerable variation in both lines.

No significant differences were observed for the interactions between the concentration and equilibration period treatments for total motile sperm post-thaw (see Appendix C, Table C1 for tabulated interactions).

Table 5.5 *The post-thaw percentage motile sperm observed for the three different sperm concentrations, equilibrated at 1, 2, and 3 hours, respectively*

Post-Thaw Motility	Equilibration period	Range	Mean ± SEM
C1 Concentration 100x10^6/mL (n=36)	1 hour	0.97 - 3.61	1.88 ± 0.27
	2 hours	0.52 - 3.04	1.54 ± 0.24
	3 hours	0.76 - 3.37	1.96 ± 0.28
C2 Concentration 150x10^6/mL (n=36)	1 hour	0.43 - 5.78	1.91 ± 0.51
	2 hours	0.43 - 4.59	2.05 ± 0.40
	3 hours	0.74 - 8.72	2.78 ± 0.64
C3 Concentration 200x10^6/mL (n=36)	1 hour	0.68 - 7.83	2.01 ± 0.63
	2 hours	0.44 - 3.77	1.65 ± 0.26
	3 hours	0.72 - 8.95	2.60 ± 0.74

When the effect of sperm concentration was considered, regardless of equilibration period, no significant differences were found for parameter total sperm motility between the C1, C2 and C3 sperm concentrations. There exists controversy in the literature regarding the optimum sperm concentration and equilibration period required during the cryopreservation of sperm to minimise cryodamage. Alvarez *et al.* (2012) suggested that higher sperm concentrations (800 x10^6/mL) lead to much lower sperm motility post-thaw. A study conducted on ram lambs concluded that when sperm were stored at 4ºC, better results for all the motility traits were

obtained with a sperm concentration of 200 x10^6/mL, compared to a concentration of 50 x10^6/mL (Kasimanickam *et al.*, 2007). Similarly Gundogan *et al.* (2010) found that a sperm concentration of 100 x10^6/mL resulted in higher motility, a higher percentage normal morphology and an increase in membrane integrity when compared to a concentration of 25 x10^6/mL, using liquid storage. A contradictory study by Leahy *et al.* (2010b) suggested that high pre-freezing dilution (20 x10^6/mL) improved the post-thaw function of ram sperm.

The industry standard for equilibrating a sperm sample with a cryodiluent is a minimum of 3 hours, although when equilibration periods were compared regardless of dilution concentration, no significant differences were observed between one, two, or three hours of equilibration (E1, E2, or E3). The present results suggest that the equilibration period can be reduced to one hour, resulting in the same post-thaw motility results. However, the very low percentage of motile sperm in this study warrants further studies in this regard.

5.3.2 Evaluation of sperm morphometry

Morphometric evaluation is the preferred choice of assessing the effect of cooling, freezing and thawing, as it uses the specific head dimension and shape to evaluate the status of sperm (De Paz *et al.*, 2011; Lavara *et al.*, 2013).

5.3.2.1 The effect of genotype on morphometric parameters of the sperm head of fresh collected epididymal sperm

Results obtained regarding the microscopic sperm morphometry traits of fresh collected epididymal sperm are presented in Table 5.6.

Table 5.6 *The morphometric traits of freshly collected epididymal sperm (mean ± SEM), obtained from High Line (HL) and Low Line (LL) rams, respectively*

Morphometric parameter	HL (n=54)		LL (n=48)	
	Range (CV)	Mean ± SEM	Range (CV)	Mean ± SEM
Length (µm)	7.60 – 8.49 (2.47)	8.05 ± 0.03	7.63 – 8.42 (2.43)	8.02 ± 0.03
Width (µm)	4.14 – 4.64 (2.57)	4.41[c] ± 0.02	4.30 – 4.72 (2.08)	4.49[d] ± 0.01
Area (µm^2)	34.01 – 40.64 (4.04)	37.08 ± 0.20	34.66 – 40.94 (3.87)	37.46 ± 0.21
Perimeter (µm)	16.95 – 19.09 (2.71)	18.09 ± 0.07	17.19 – 19.13 (2.55)	18.10 ± 0.07
Ellipticity	1.75 – 1.88 (1.73)	1.83[c] ± 0.00	1.72 – 1.87 (1.65)	1.79[d] ± 0.00
Elongation	0.27 – 0.31 (2.70)	0.29[c] ± 0.00	7.63 – 8.42 (2.43)	0.28[d] ± 0.00
Roughness	1.35 – 1.55 (3.17)	1.43 ± 0.01	4.30 – 4.72 (2.08)	1.44 ± 0.01
Regularity	0.72 – 0.80 (2.13)	0.76 ± 0.00	34.66 – 40.94 (3.87)	0.76 ± 0.00
Acrosome coverage (%)	50.28 – 55.07 (1.26)	54.29 ± 0.09	17.19 – 19.13 (2.55)	54.21 ± 0.06

[c, d] Columns with different superscripts differ significantly (p<0.01)

There were no significant differences between sperm obtained from HL or LL rams for the morphometric traits head length, -area, -perimeter, -roughness, -regularity, or acrosome coverage (Table 5.6). In contrast, there were significant differences found between sperm from the HL rams and LL rams regarding the morphometric traits head width (p=0.0003), ellipticity (p<0.0001), and elongation (p<0.0001). The difference in head width results suggest that sperm from the LL rams had broader heads than sperm obtained from the HL rams (4.49µm ± 0.01µm vs. 4.41µm ± 0.02 µm respectively, Figure 5.3).

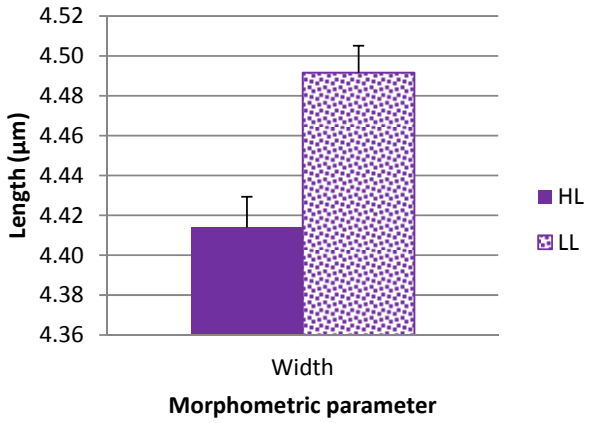

Figure 5.3 *The sperm morphometric trait head width, as determined for fresh epididymal samples obtained from High Line (HL) and Low Line (LL) Merino rams, respectively*

The morphometric index traits ellipticity and elongation are directly correlated, as they are calculated using the same values in different formulas (see Chapter 3). The differences in ellipticity index values suggest that the HL sperm (1.83 ± 0.00) were thinner and more tapered than LL sperm (1.79 ± 0.00, Figure 5.5). The index value for elongation confirms that the LL sperm are slightly more round in shape (0.28 ± 0.00) than that of the HL rams (0.29 ± 0.00, Figure 5.4).

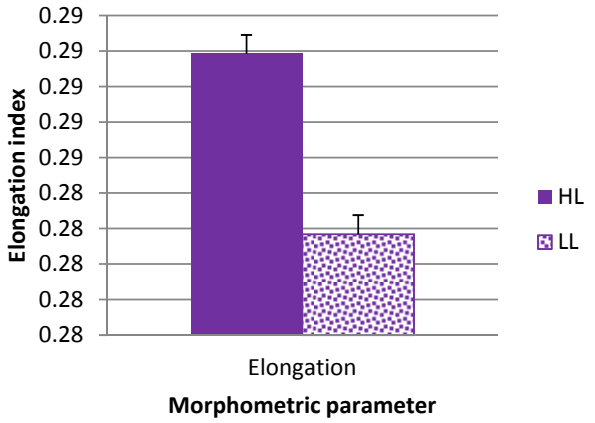

Figure 5.4 *The sperm morphometric trait head elongation, as determined for fresh epididymal samples obtained from High Line (HL) and Low Line (LL) Merino rams, respectively*

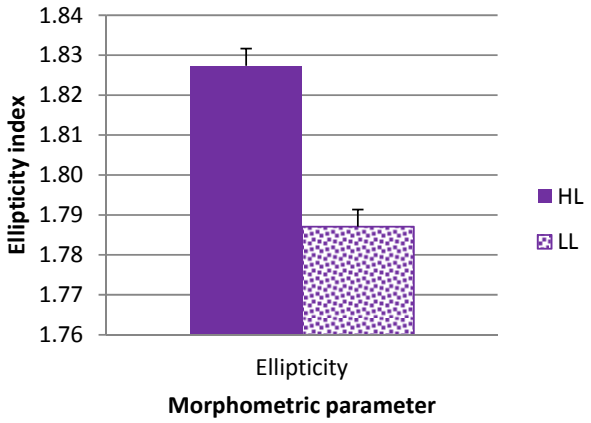

Figure 5.5 *The sperm morphometric trait head ellipticity, as determined for fresh epididymal samples obtained from High Line (HL) and Low Line (LL) Merino rams, respectively*

The values measured for ellipticity and elongation of ovine sperm were similar to that found by Maroto-Morales *et al.* (2009) for the characterization of morphometric parameters for fresh ram sperm heads. Snook (2005) stated that longer sperm may increase the competitive potential of a sperm sample, for sperm with long, thin and tapered heads may have higher swimming velocity, live longer, and perhaps suggest a higher quality sperm sample. This can possibly explain the differences observed in the number of offspring produced between the ewes mated to the HL and LL rams.

5.3.2.2 The effect of genotype on sperm head morphometry of post-thaw epididymal sperm

The post-thaw sperm morphometry results are presented in Table 5.7.

Table 5.7 *The morphometric parameters of post-thaw epididymal sperm (mean ± SEM), obtained from High Line (HL) and Low Line (LL) rams, respectively (range and CV values are also included)*

Morphometric parameter	HL (n=54)		LL (n=48)	
	Range (CV)	Mean ± SEM	Range (CV)	Mean ± SEM
Length (µm)	7.66 – 8.65 (2.58)	8.19 ± 0.03	7.81 – 8.51 (2.50)	8.16 ± 0.03
Width (µm)	4.25 – 4.87 (3.22)	4.58^{c} ± 0.02	4.34 – 4.96 (2.50)	4.67^{d} ± 0.02
Area (µm^2)	36.19 – 44.10 (3.99)	39.66^{a} ± 0.22	36.81 – 43.41 (3.47)	40.28^{b} ± 0.20
Perimeter (µm)	17.20 – 19.61 (2.86)	18.48 ± 0.07	17.75 – 19.51 (2.61)	18.56 ± 0.07
Ellipticity	1.70 – 1.87(2.17)	1.79^{c} ± 0.01	1.67 – 1.82 (1.75)	1.75^{d} ± 0.00
Elongation	0.26 – 0.30(3.50)	0.28^{c} ± 0.00	0.25 – 0.29 (2.93)	0.27^{d} ± 0.00
Roughness	1.37 – 1.54 (2.73)	1.46 ± 0.01	1.41 – 1.54 (2.40)	1.47 ± 0.01
Regularity	0.71 – 0.78 (2.13)	0.75 ± 0.00	0.71 – 0.77 (1.54)	0.75 ± 0.00
Acrosome coverage (%)	52.98 – 54.69 (0.74)	53.88^{c} ± 0.06	52.63 – 54.27 (0.65)	53.64^{d} ± 0.05

[a, b] Columns with different superscripts differ significantly ($p<0.05$)

[c, d] Columns with different superscripts differ significantly ($p<0.01$)

Significant differences were observed in almost all the morphometric traits measured for epididymal sperm obtained from the two genetically diverse lines. From the results it is evident that there were significant differences between the sperm of the HL and LL rams for the morphometric parameters head width ($p=0.001$), -area ($p=0.04$), ellipticity ($p<0.0001$), elongation ($p<0.0001$) and acrosome coverage ($p=0.0019$) obtained from post-thaw samples (Table 5.7).

The thawed sperm from the LL rams showed higher values for sperm head width than those from HL rams (4.67μm ± 0.02 μm vs. 4.58 μm ± 0.02 μm; Figure 5.6). The LL sperm also had larger surface area than those of the HL sperm (40.28μm ± 0.20μm vs. 39.66μm ± 0.22μm respectively; Figure 5.7).

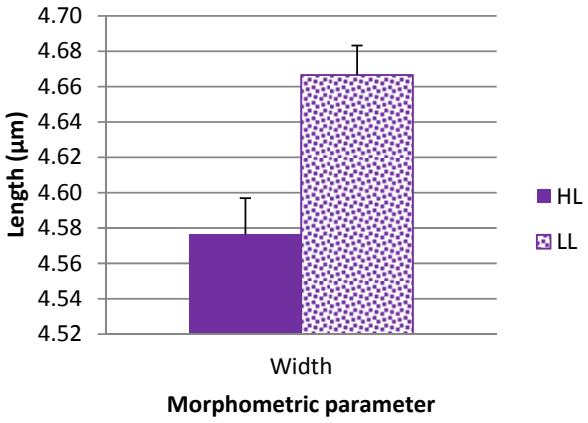

Figure 5.6 *The sperm morphometric trait head width, as determined post-thaw for epididymal samples obtained from High Line (HL) and Low Line (LL) Merino rams, respectively*

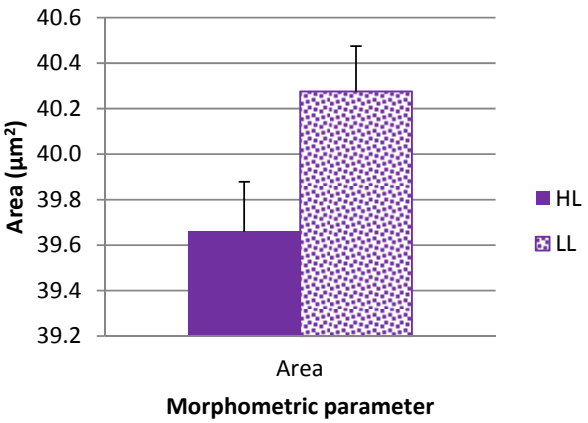

Figure 5.7 *The sperm morphometric trait head area, as determined post-thaw for epididymal samples obtained from High Line (HL) and Low Line (LL) Merino rams, respectively*

The ellipticity and elongation index values indicated that the HL sperm tend to be thinner, more tapered, while LL sperm tend to be more round in shape, when compared post-thaw (Figure 5.8 and Figure 5.9). A study by Snook (2005) stated that longer sperm may increase the competitive potential of a sperm sample. Sperm with long, thin and tapered heads may have a higher swimming velocity, live longer, and perhaps indicate a higher quality sperm sample. This difference could possibly be involved in the difference between ram lines in terms of number of offspring produced by ewes mated to HL or LL rams.

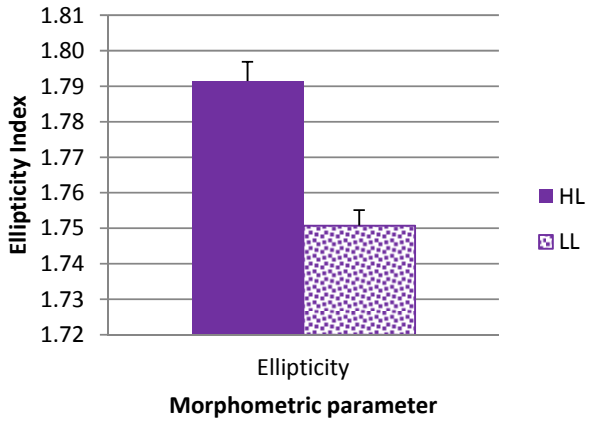

Figure 5.8 *The sperm morphometric index parameter head ellipticity, as determined post-thaw for epididymal samples obtained from High Line (HL) and Low Line (LL) Merino rams, respectively*

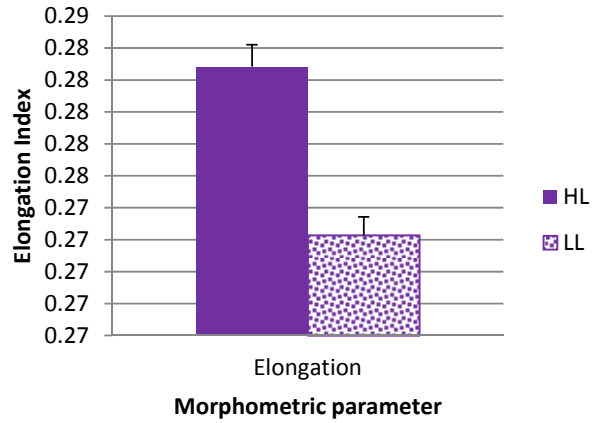

Figure 5.9 *The sperm morphometric index parameter head elongation, as determined post-thaw for epididymal samples obtained from High Line (HL) and Low Line (LL) Merino rams, respectively*

The area of the sperm covered with the acrosomal cap was significantly larger in the HL sperm, when compared to that of the LL sperm (Figure 5.10).The specific shape and size of the acrosome is reported to be highly species specific, with the acrosome as such containing many enzymes, including acrosin, hyaluronidase and many more hydrolases and esterase. These enzymes are then essential for lysis of the *zona pellucida* and the penetration of the corona radiate of the oocyte. These enzymes are then only the released from the acrosome during the acrosome reaction (AR). The acrosome covers the first two thirds of the sperm head and forms a cap-like structure around it (Pesch & Bergmann, 2006). Taking this into consideration, it can be postulated that the larger percentage acrosome coverage observed in post-thaw epididymal HL sperm, will result in a larger amount of enzymes present in the acrosomal cap. Since the acrosomal enzymes are responsible for penetration of the *zona pellucida* and thus fertilization, this result is a possible indicator that semen from HL- rams may have a higher fertilization success rate than that of LL rams.

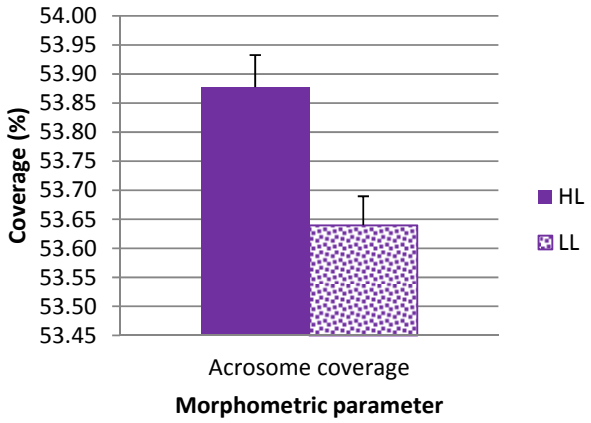

Figure 5.10 *The sperm morphometric trait percentage acrosome coverage, as determined post-thaw for epididymal samples obtained from High Line (HL) and Low Line (LL) Merino rams, respectively*

5.3.2.3 The effect of sperm concentration and equilibration period on sperm head morphometry of freshly collected epididymal sperm

Results obtained for the effect of sperm concentration and equilibration period (main effects) on the morphometry of fresh collected epididymal sperm are presented in Table 5.8. Investigation of interaction effects (i.e. C1E1, C1E2, C1E3, C2E1, C2E2, C2E3, C3E1, C3E2, and C3E3) indicated no significant differences (see Appendix C, Table C2 and Table C3).

Table 5.8 *The effect of sperm concentration and equilibration time on the morphometric measurements (mean ± SEM) of fresh epididymal sperm obtained from adult Merino rams*

Fresh Morphometry		Equilibration 1 hour E1		Equilibration 2 hours E2		Equilibration 3hours E3	
		Mean ± SEM	Range	Mean ± SEM	Range	Mean ± SEM	Range
Concentration 100x10^6/mL C1 (n=108)	Length	8.10 ± 0.06	0.67	8.07 ± 0.08	0.89	8.06 ± 0.05	0.54
	Width	4.50 ± 0.03	0.34	4.45 ± 0.04	0.47	4.45 ± 0.03	0.32
	Area	37.90 ± 0.45	5.20	37.30 ± 0.59	6.16	37.22 ± 0.40	4.32
	Perimeter	18.27 ± 0.14	1.65	18.15 ± 0.19	2.14	18.12 ± 0.11	1.26
	Ellipticity	1.80 ± 0.01	0.15	1.81 ± 0.01	0.10	1.81 ± 0.01	0.10
	Elongation	0.29 ± 0.00	0.04	0.29 ± 0.00	0.03	0.29 ± 0.00	0.03
	Roughness	1.43 ± 0.01	0.13	1.43 ± 0.01	0.11	1.43 ± 0.01	0.08
	Regularity	0.76 ± 0.00	0.04	0.76 ± 0.00	0.06	0.76 ± 0.00	0.02
	Acrosome coverage	54.29 ± 0.13	1.40	54.12 ± 0.13	1.43	54.17 ± 0.11	1.33
Concentration 150x10^6/mL C2 (n=108)	Length	8.01 ± 0.05	0.61	8.02 ± 0.07	0.61	8.00 ± 0.05	0.45
	Width	4.44 ± 0.03	0.31	4.43 ± 0.04	0.43	4.45 ± 0.03	0.36
	Area	37.16 ± 0.39	4.57	37.06 ± 0.52	6.13	37.01 ± 0.35	4.24
	Perimeter	18.06 ± 0.12	1.41	18.07 ± 0.16	1.35	17.97 ± 0.13	1.54
	Ellipticity	1.81 ± 0.01	0.12	1.81 ± 0.01	0.13	1.80 ± 0.01	0.11
	Elongation	0.29 ± 0.00	0.03	0.29 ± 0.00	0.03	0.29 ± 0.00	0.03
	Roughness	1.44 ± 0.01	0.15	1.43 ± 0.01	0.14	1.45 ± 0.01	0.13
	Regularity	0.76 ± 0.00	0.04	0.76 ± 0.00	0.05	0.76 ± 0.00	0.03
	Acrosome coverage	54.50 ± 0.12	1.22	53.84 ± 0.39	4.79	54.17 ± 0.09	0.93
Concentration 200x10^6/mL C3 (n=108)	Length	8.08 ± 0.05	0.56	8.02 ± 0.05	0.66	7.97 ± 0.05	0.58
	Width	4.47 ± 0.03	0.30	4.45 ± 0.04	0.44	4.41 ± 0.03	0.41
	Area	37.55 ± 0.39	4.71	37.28 ± 0.43	5.37	36.79 ± 0.44	4.65
	Perimeter	18.15 ± 0.12	1.21	18.09 ± 0.14	1.76	17.93 ± 0.14	1.58
	Ellipticity	1.81 ± 0.01	0.13	1.81 ± 0.01	0.13	1.81 ± 0.01	0.10
	Elongation	0.29 ± 0.00	0.03	0.29 ± 0.00	0.03	0.29 ± 0.00	0.02
	Roughness	1.44 ± 0.01	0.15	1.44 ± 0.02	0.17	1.44 ± 0.01	0.15
	Regularity	0.76 ± 0.00	0.05	0.76 ± 0.01	0.06	0.75 ± 0.00	0.05
	Acrosome coverage	54.48 ± 0.08	0.80	54.33 ± 0.17	2.25	54.37 ± 0.12	1.28

No significant differences were observed between the sperm concentrations (C1, C2, and C3), or equilibration periods (E1, E2, and E3). There was however a tendency for the acrosome coverage to differ between equilibration periods (p=0.0721). The percentage acrosome coverage was lowest for the 2h equilibration (54.33% ± 0.17%) and highest for the one hour equilibration (54.48% ± 0.08%). This was unexpected, as one would expect the results would

follow a linear trend according to the amount of time used to equilibrate the sperm samples. When interactions were investigated, no significant differences were found between treatments for the different sperm concentrations and equilibration periods.

The findings in previous studies were inconclusive pertaining to the optimum sperm concentration and equilibration period needed to minimize the extent of cryodamage to sperm. Based on lambing rates, Alvarez *et al.* (2012) suggested that the optimal sperm concentration is between 200 x10^6/mL and 400 x10^6/mL. In research done on ram-lambs it was concluded that better results for all motility parameters could be obtained with a sperm concentration of 200 x10^6/mL compared to a lower sperm concentration of 50 x10^6/mL, when stored at 4ºC, (Kasimanickam *et al.*, 2007). Similarly Gundogan *et al.* (2010) found that a sperm concentration of 100 x10^6/mL resulted in higher motility, better morphology and an increased membrane integrity, when compared to a concentration of 25 x10^6/mL when using liquid storage. This evidence supports the fact that sperm concentrations above 100x10^6/mL and below 400x10^6/mL results in better sperm motility and viability. The range of concentrations in the present study did not allow inferences regarding sperm concentrations below 100x10^6/mL.

The standard equilibration with a cryodiluent used in industry requires a minimum of three hours. However, the results in this study suggested no differences for morphometric head traits between equilibration periods of 1 and 3 hours (E1 and E3). According to these results, it is possible to reduce the equilibration period to 1 hour, while still maintaining the same morphometric trait values as for 3 hours.

5.3.2.4 The effect of sperm concentration and equilibration period on post thaw morphometric parameters of the sperm head

Results obtained for the effect of sperm concentration and equilibration period (main effects) on sperm morphometry of post-thaw epididymal sperm are presented in Table 5.9. Interactions were investigated, although not presented here (Appendix C, Table C4 and Table C5).

The results showed no significant differences between the treatment combinations for sperm concentration and equilibration periods (i.e. C1E1, C1E2, C1E3, C2E1, C2E2, C2E3, C3E1, C3E2, and C3E3).

Table 5.9 *The effect of sperm concentration and equilibration on the morphometric measurements (mean ± SEM) of post-thaw epididymal sperm obtained from adult Merino rams*

Post-Thaw Morphometry		Equilibration 1 hour E1		Equilibration 2 hours E2		Equilibration 3hours E3	
		Mean ± SEM	Range	Mean ± SEM	Range	Mean ± SEM	Range
Concentration 100x10^6/mL C1 (n=108)	Length	8.20 ± 0.05	0.61	8.28 ± 0.07	0.74	8.24 ± 0.05	0.53
	Width	4.64 ± 0.03	0.38	4.70 ± 0.04	0.49	4.66 ± 0.04	0.46
	Area	40.14 ± 0.31	3.46	40.82 ± 0.42	4.35	40.36 ± 0.41	4.28
	Perimeter	18.60 ± 0.12	1.30	18.79 ± 0.15	1.56	18.66 ± 0.14	1.57
	Ellipticity	1.77 ± 0.01	0.14	1.76 ± 0.02	0.16	1.77 ± 0.01	0.11
	Elongation	0.28 ± 0.00	0.04	0.28 ± 0.00	0.04	0.28 ± 0.00	0.03
	Roughness	1.46 ± 0.01	0.11	1.46 ± 0.01	0.12	1.46 ± 0.01	0.16
	Regularity	0.75 ± 0.00	0.04	0.75 ± 0.00	0.04	0.75 ± 0.00	0.06
	Acrosome coverage	53.77 ± 0.09	1.00	53.68 ± 0.12	1.46	53.90 ± 0.07	0.89
Concentration 150x10^6/mL C2 (n=108)	Length	8.19 ± 0.07	0.67	8.04 ± 0.04	0.36	8.18 ± 0.05	0.58
	Width	4.62 ± 0.04	0.38	4.54 ± 0.05	0.54	4.61 ± 0.03	0.48
	Area	40.03 ± 0.48	5.33	39.03 ± 0.48	4.76	39.80 ± 0.39	4.61
	Perimeter	18.55 ± 0.16	1.69	18.18 ± 0.12	1.30	18.52 ± 0.13	1.46
	Ellipticity	1.77 ± 0.01	0.12	1.77 ± 0.02	0.15	1.78 ± 0.01	0.15
	Elongation	0.28 ± 0.00	0.03	0.28 ± 0.00	0.04	0.28 ± 0.00	0.04
	Roughness	1.47 ± 0.01	0.10	1.49 ± 0.01	0.11	1.46 ± 0.01	0.09
	Regularity	0.75 ± 0.00	0.05	0.74 ± 0.00	0.05	0.75 ± 0.00	0.04
	Acrosome coverage	53.51 ± 0.12	1.17	53.48 ± 0.12	1.26	53.92 ± 0.09	1.21
Concentration 200x10^6/mL C3 (n=108)	Length	8.14 ± 0.09	0.98	8.16 ± 0.06	0.69	8.11 ± 0.05	0.50
	Width	4.58 ± 0.06	0.60	4.64 ± 0.04	0.47	4.58 ± 0.03	0.35
	Area	39.81 ± 0.72	7.91	40.10 ± 0.45	4.44	39.43 ± 0.26	2.70
	Perimeter	18.43 ± 0.23	2.41	18.51 ± 0.16	1.69	18.38 ± 0.10	1.08
	Ellipticity	1.78 ± 0.01	0.16	1.76 ± 0.01	0.13	1.77 ± 0.01	0.15
	Elongation	0.28 ± 0.00	0.04	0.28 ± 0.00	0.03	0.28 ± 0.00	0.04
	Roughness	1.48 ± 0.02	0.13	1.48 ± 0.01	0.14	1.47 ± 0.01	0.10
	Regularity	0.74^a ± 0.0057	0.05	0.74^{ab} ± 0.0042	0.05	0.74^b ± 0.0034	0.03
	Acrosome coverage	53.78 ± 0.14	1.28	53.83 ± 0.11	1.34	53.90 ± 0.14	1.47

$^{a, b}$ Columns with different superscripts differ significantly ($p<0.05$)

When dilution concentrations were compared, regardless of the equilibration period, the only trait that showed a significant difference was the morphometric parameter regularity (p=0.039). The regularity index was highest for C1 (100 x10^6 /mL), when compared to the lowest index value C3 (200 x10^6 /mL). There were however parameters that showed a tendency to differ between the different concentrations, which include head length (p=0.0585), head width (p=0.054), head area (p=0.073), head perimeter (p=0.066).

When the main effects of equilibration periods were considered, a significant difference was obtained for the morphometric parameter acrosome coverage (p=0.014). The 2h equilibration period (E2; 53.67%) differed significantly from the 3h equilibration (E3; 53.91%) period. These results were unexpected, as one would assume differences for the one hour equilibration or the 3h equilibration, but not for the 2h equilibration period.

5.4 Conclusions

This study investigated the effect of genotype, sperm concentration and equilibration period on motility- and morphometric parameters of epididymal sperm obtained from 12 Merino rams. No significant differences were observed between the HL and LL rams for the percentage of total motile sperm in either the fresh or post-thaw evaluated samples. Significant differences were however observed between the two lines for the motility parameters ALH and BCF in the freshly collected epididymal sperm.

The morphometric analyses showed that sperm from the LL rams had wider, more rounded heads than those of the HL rams when compared to the sperm from the HL that were thinner and more tapered in both fresh and post-thaw samples. Cryopreservation resulted in sperm from the LL rams to have smaller acrosome coverage, when compared to that of the HL rams.

The number of sperm cryopreserved per 0.25cc straw, and duration of equilibration period did not result in significantly different effects regarding sperm motility and morphometric integrity for fresh epididymal sperm. Post-thaw assessment of the morphometric trait regularity differed significantly between the 2 lines, when sperm concentration was considered as one of the main effects.

Future research should focus on morphometric head parameters of epididymal sperm. The effect of using different pre-cryopreservation sperm concentrations and equilibration periods on sperm morphometric traits deserves more attention, so as to optimize the cryopreservation protocol for ovine sperm samples. Another field that needs more attention is the relationship of sperm dimensions with ewe fertility. Specific sperm shape and size within species could elucidate the differences observed in the number of offspring produced between genetically diverse lines of Merino. Incorporating lambing success by using insemination with sperm from genetically diverse lines of Merino rams could possibly shed light on the aspects of difference observed within the genetically diverse lines.

Chapter 6

The potential of seminal plasma to minimize the extent of cryodamage in epididymal samples

Abstract

Little is known about the effect of seminal plasma on the morphometric traits of epididymal ovine sperm. The specific role of seminal plasma in ovine sperm function and viability remains unclear due to the contradictory inhibiting and stimulatory effects that have been reported in post-thaw sperm samples. Previous studies on the effect of seminal plasma focussed on post-thaw motility, and did not investigate the effect of cryopreservation on the morphometric traits of epididymal sperm. Morphometric information may potentially provide a more accurate indication of cryodamage, and thus the viability of sperm post-thaw. Epididymal sperm samples were obtained by sacrificing 12 adult Merino (*Ovis aries*) rams, and the harvested sperm being subjected to cryopreservation to assess the potential stabilising and protective potential of seminal plasma on sperm viability and motility. Semen samples were collected by means of the artificial vagina method from 15 adult Merino rams, and processed to obtain the seminal plasma fraction. Seminal plasma was then pooled and stored at -20ºC until used. Three treatment groups were assessed. The first two treatments involved the addition of 20% (v/v) whole seminal plasma either after recovery from the *cauda* epididymis, or to post thaw samples. These two treatments were then compared to epididymal samples from the same individuals, to which no seminal plasma was added. Smears were prepared after equilibration with an extender, and of post-thaw samples for each treatment, and morphometric analysis was performed using SCA® software. No differences were reported between treatments for motility parameters, although a significant difference was found for the morphometric parameter head width ($p=0.016$) indicating that the addition of seminal plasma supresses head deformation during cryopreservation and post-thaw, and thus potentially minimizing the extent of cryodamage in epididymal sperm. Further research is warranted to elucidate the findings of the current study.

6.1 Introduction

The South African sheep industry has been under pressure during recent years to farm more cost-effectively, and to produce optimally in every aspect to satisfy the ever-increasing demand for safe food. To ensure cost-efficient production practices, farmers can make use of different management strategies to lower the input costs. Assisted reproductive biotechnologies which include artificial insemination and embryo transfer among others are some of the management tools that may assist to optimise the reproductive potential of breeding flocks.

The main difference between ejaculated samples and samples recovered via aspiration of the *cauda* epididymis is the presence of seminal fluid in ejaculated samples. Cryopreservation results obtained by Varisli *et al.* (2009) reported that ejaculated ram sperm was more sensitive to various cryobiologically relevant stress conditions, compared to epididymal sperm.

Epididymal sperm undergo considerable cellular, biochemical and osmotic changes during the process of epididymal maturation and post addition of seminal fluids. These changes are necessary to prepare sperm to participate in the action of normal fertilization (Yeung *et al.*, 2006).

Epididymal sperm, when compared to ejaculated sperm, are more susceptible to the detrimental effect of cryopreservation, with damage that can occur as early as the equilibration stage of pre-cryopreservation processing (Varisli *et al.*, 2009). Pre-cryopreservation processing, cryopreservation, and post-thaw processing can all affect the behavioural and functional capacity of sperm, which can result in a marked reduction in overall motility (O'Meara *et al.*, 2007). These changes that occur can be attributed to thermal, mechanical, chemical, and osmotic stress (Holt, 2000; Medeiros *et al.*, 2002; Leahy & Gadella, 2011).

A potential approach to improve the ability of epididymal sperm to withstand cryodamage is the addition of seminal plasma during the pre- and/or post-thaw phase (Graham, 1994; Ollero *et al.*, 1997; Martínez-Pastor *et al.*, 2006). Seminal plasma is a physiological secretion that originates from the respective accessory reproductive glands of the male (Hafez & Hafez, 2008), and plays an important role as a maturation medium for ejaculated sperm through hormonal, enzymatic and surface-modifying events upon entering the female reproductive tract. The organic component of seminal plasma is also responsible for sperm metabolism, pH, osmolarity and overall sperm function (Barrios *et al.*, 2000; Troedsson *et al.*, 2005; Hafez & Hafez, 2008; Muiño-Blanco *et al.*, 2008).

There are however, controversial reports of the potential effect of seminal plasma to improve the ability of epididymal sperm to withstand cryodamage, and to improve the post-thaw viability of epididymal samples. Previous studies have reported a beneficial effect in treating epididymal sperm with seminal plasma. Sperm traits that were positively influenced by seminal plasma treatment (ejaculated- and epididymal sperm), include sperm motility and viability (Graham, 1994; Azerêdo *et al.*, 2001; Troedsson *et al.*, 2005; Domínguez *et al.*, 2008; Leahy *et al.*, 2010a; Bernardini *et al.*, 2011), sperm ultra-structure and the stability of the head (Ollero *et al.*, 1997; Maxwell *et al.*, 2007), as well as an improved resistance to cold shock damage (Barrios *et al.*, 2000).

Other studies have however indicated no beneficial effect of treating epididymal sperm with seminal plasma on sperm motility, or conception rates (O'Meara *et al.*, 2007; Leahy *et al.*, 2010). Azerêdo *et al.* (2001) found that the plasma membrane integrity of epididymal sperm was significantly reduced by the process of freezing, whether or not seminal plasma was added to the sperm samples.

Standard microscopic evaluation of semen samples includes the assessment of motility, morphology and acrosome integrity to evaluate the quality of a sperm sample. Although well documented guidelines are used for the assessment of these evaluations, the standard manual evaluation causes an increase of variation due to subjective results recorded by different technicians and laboratories. There is thus a need to obtain objective and accurate analyses of sperm morphology, and this need has given rise to automated sperm morphometry analysis (ASMA) systems. More and more studies rely on specific head dimension and shape parameters (morphometry) to determine the effect of cooling, freezing and thawing on sperm (Hidalgo *et al.*, 2007).

Studies focussing on the effect of seminal plasma on post thaw samples are primarily evaluated on motility, and little to no attention is paid to sperm morphometry. Evaluating morphometric data can prove to be of more value for *in vitro* reproductive technologies, as functional sperm (with the optimum head length, width, area etc.) can potentially be more important than primarily assessing post-thaw sperm motility.

The aim of this study was therefore to determine the potential cryoprotective and stabilising effect of seminal plasma, when added to epididymal samples prior to cryopreservation, and post-thaw, on sperm head morphometry and motility.

6.2 Materials and methods

6.2.1 Experimental location

The experiments were conducted at the Elsenburg research farm (33°51'S, 18°50'E), outside Stellenbosch in the Western Cape, South Africa.

6.2.2 Experimental animals

Twelve mature Merino rams (*Ovis aries*), aged between 2 and 5 years, were sacrificed for purposes of this trial. Prior to slaughtering, the rams were maintained according to ethically approved husbandry practices, and under uniform nutritional conditions. Another group of rams (15 rams) from the same base population were used to collect ejaculates by means of the artificial vagina (AV) technique, from which the seminal plasma fraction was collected and used in this trial. Ethical clearance was approved by the Departmental Ethics Committee for Research on Animals (DECRA; number R11/45). All animal care and procedures used in the study were performed in such a manner that it reflected South African National Standards 10386:2008.

6.2.3 Collection and processing of epididymal samples

Immediately after slaughter, the testes of each ram were collected, and placed in sample bags that were clearly marked with each ram's information for easy identification. The testes were transported on ice to the laboratory, with the time elapsing from collection to processing ranging from 1.5 to 4 hours. Epididymal sperm were collected by means of aspiration (for more details on aspiration technique, please refer to Chapter 3). The sperm samples (epididymal sperm in Ham's F10 solution as described in Chapter 3) were then transferred to 1.5 mL Eppendorf tubes (Lasec, South Africa), and placed in an incubator (37°C), until further processing. Care was taken to ensure all equipment and instruments used were pre-warmed to 37°C to avoid potential cold shock to epididymal sperm.

The concentration of the recovered samples were determined using a positive displacement pipette to transfer an aliquot of sperm to a predetermined amount of Ham's F10 solution in order to determine the concentration when using the SCA® programme. Positive displacement pipettes ensure high accuracy, as it forces the entire sample out of the pipette tip.

After concentration determination, samples were diluted with pre-warmed Ramsem® cryodiluent mixture (Ramsem®, Bloemfontein, South Africa) to obtain an end concentration of 200 $x10^6$ sperm/mL, and equilibrated for 3 hours. After equilibration, the samples were loaded into labelled 0.25 cc straws (Taurus®, George, South Africa), and sealed with a PVC powder (Taurus®, George, South Africa). After loading of the straws, the samples were then cryopreserved according to the method used by Lambrechts (1996). For a more in detail description of the respective processes, please refer to Chapter 3.

For morphometric measurements, smears were made on Starfrost® slides of each sample after equilibration and post thaw. These smears were then fixed and stained using SpermBlue® and mounted with DPX mounting medium (Merck, South Africa). All pre-mounted slides were analysed for morphometry using the SCA® morphology module.

6.2.4 Preparation of seminal plasma

A different group of rams from the same resource flock were trained to ejaculate into an artificial vagina (AV) to obtain semen samples. After collection of the ejaculates, the samples were centrifuged (2000 x G, 15 min at 4°C) and the supernatants from all the rams were pooled (equal parts from all rams) to obtain a representative sample for the addition of seminal plasma in the experiment. The collected seminal plasma was then stored at -20°C, until used (Bernardini *et al*, 2011). Before use, seminal plasma was thawed at room temperature. Prior to

adding seminal plasma to epididymal samples, the seminal plasma was pre-heated to 37°C in an incubator.

6.2.5 Experimental design

The experimental design to determine the potential beneficial effect of seminal plasma on the viability and survivability of epididymal sperm is presented in Table 6.1.

Table 6.1 *Experimental design encompassing three treatments to determine the potential beneficial effect of whole seminal plasma on the viability and survivability of epididymal ram sperm*

Treatment group	Description of protocol
Control group (industry protocol)	Epididymal sperm were obtained by means of aspiration, and diluted with a commercial cryoprotectant (Ramsem®) to obtain a concentration of 200 million sperm/mL, and equilibrated for 3h at 4ºC. After equilibration, the samples were cryopreserved according to the technique in Chapter 3, and thawed after 210 days. Post-thaw motility was recorded with the use of the SCA®, and smears were made, fixed, stained and mounted to evaluate post thaw morphometry.
SP added prior to equilibration	Epididymal spermatozoa were obtained by means of aspiration, and diluted with a commercial cryoprotectant (Ramsem®) to obtain a concentration of 200 million sperm/mL. Whole seminal plasma (37ºC) was added at 20%($^{v}/_{v}$) to the pre-equilibration samples. Epididymal sperm-seminal plasma samples were then allowed to equilibrate for 3 h at 4ºC, before they were cryopreserved according to standard protocols. Straws were thawed after 210 days. Post-thaw motility was recorded with the use of the SCA®, and smears were made, fixed, stained and mounted to evaluate post thaw morphometry.
SP added post-thaw	Epididymal spermatozoa were obtained by means of aspiration, diluted with a commercial cryoprotectant (Ramsem®) to obtain a concentration of 200 million sperm/mL, and equilibrated for 3h at 4ºC. After equilibration, the samples were cryopreserved according to the technique described in Chapter 3, and thawed after 210 days. Straws were thawed individually (i.e. for 30 seconds at 35ºC), and the contents of each straw was then transferred to individual 1.5mL Eppendorf tubes (Lasec, South Africa). An aliquot (50µL) of seminal plasma (37ºC) was then slowly added to each sample, gently mixed, and allowed to incubate for 15 minutes at 37ºC in a portable incubator (Bernardini *et al*, 2011) prior to further analysis. Post-thaw motility was then recorded with the use of the SCA®, and smears were made, fixed, stained and mounted to evaluate post thaw morphometry.

6.2.6 Analysis of data using the SCA®

Motility recordings were made after collection and post-thaw, however, since the effect of seminal plasma addition will only be determined on post-thaw motility results, the fresh sperm motility recordings are not presented here.

Throughout the study the Sperm Class Analyser (SCA®) was used to obtain accurate, objective measurements of motility and morphometric parameters. Leja® slides (20µm depth, and 5µl volume; Delfran, South Africa) were used for motility recordings. Motility was determined by loading an aliquot of the sperm sample into a Leja® chamber, and subsequently analysed with the SCA® motility and concentration module of the programme.

For details regarding the motility recordings, analysis, equipment used and software settings, please refer to Chapter 3. Once motility recordings were captured, specific motility parameters were measured by the SCA®. The measured motility parameters included progressive motility, non-progressive motility, total motile, static, rapid, medium, slow, VCL, VSL, VAP, LIN, STR, ALH, and BCF. Analysis of the above motility parameters were automatically performed by the SCA® system. For more details, and the calculation of parameters, please refer to Chapter 3.

Morphometric data was obtained by analysing pre-stained (SpermBlue®) and mounted sperm on slides using SCA® version 5.1. The SCA® automatically measures sperm head length, -width, -area, -perimeter, -elongation, -ellipticity, -roughness, -regularity, and the percentage of acrosome coverage. For more details on protocol, parameter estimation, equipment used and software settings please refer to Chapter 3.

6.2.7 Statistical analysis

The data were analysed by the generalized linear models, using PROC GLM of SAS Enterprise Guide 5.1 software, Version 9.3 of the SAS System for Windows. The procedure generates least square means (LSM) and adjusted for multiple comparisons with the Bonferroni t-test. For the comparative analyses, the one-way analysis of variance (ANOVA) approach was used. All results are expressed as the mean ± standard error of the mean. Findings were considered statistically significant at $p<0.05$.

6.3 Results and discussion

6.3.1 Sperm motility parameters

The motility parameters obtained throughout the study showed considerable variation and are indicated in Table 6.2. Motility results for epididymal sperm samples obtained from 12 rams, treated without seminal plasma, seminal plasma addition prior to equilibration and addition of seminal plasma post thaw are set out in Table 6.2. It should be noted that due to defective straws, some samples were lost during the thawing procedure when slides were placed in water, pre-heated to 37ºC.

Table 6.2 *The effect of addition of seminal plasma (No SP, no seminal plasma added; SP Pre Equil, seminal plasma added prior to equilibration; SP Post Thaw, seminal plasma added to post thaw epididymal samples) (mean ± SEM) on the total percentage motile sperm of post thaw epididymal samples obtained from Merino rams, aged 2 to 5 years*

	No SP		**SP Pre Equil.**		**SP Post Thaw**	
	(n=11)		**(n=8)**		**(n=9)**	
	Mean ± SEM	**Range (CV)**	**Mean ± SEM**	**Range (CV)**	**Mean ± SEM**	**Range (CV)**
Motility (%)	2.64 ± 0.85	0.72 – 8.95 (107.20)	2.85 ± 1.00	0.84 – 9.50 (98.96)	3.26 ± 0.94	0.72 – 10.68 (86.71)

Post-thaw motility of samples where seminal plasma was added prior to equilibration or post-thaw, did not differ ($p>0.05$) in both instances from motility of samples where no seminal plasma was added (Table 6.2). These findings are consistent with other studies that reported on the inability of seminal plasma to improve epididymal sperm motility post-thaw (Graham, 1994; Salamon, 1973).

Similarly, some studies have indicated that the addition of seminal plasma to have detrimental effects on sperm motility of the ram, bull and boar (de Lamirande & Gagnon, 1984; Graham, 1994) contradictory to other studies reporting a beneficial effect on sperm motility and viability post-thaw with the addition of seminal plasma for rams (Ghaoui *et al.*, 2007; Domínguez *et al.*, 2008; Leahy *et al.*, 2010a), goats (Azerêdo *et al.*, 2001), stallions (Troedsson *et al.*, 2005), Iberian red deer (Martínez-Pastor *et al.*, 2006), boars (de Graaf *et al.*, 2008) and bulls (Garner *et al.*, 2001).

6.3.2 Sperm head morphometry

In the current study, more than 2800 individual sperm obtained from 12 rams aged 2 to 5 years, were analysed to quantify the effect of seminal plasma on the post-thaw epididymal sperm morphometric parameters.

Large variation was observed with the analysis of the morphometric parameters when seminal plasma was added to epididymal sperm samples, prior to equilibration and post thaw, when compared to no addition of seminal plasma. The variation observed between the morphometric parameters measured in the study for the three treatment groups (including coefficient of variation values (CV)) are summarized and tabulated in Table 6.3. The effect of the seminal plasma treatments on epididymal samples are set out in Table 6.4 (indicated as mean ± SEM for all morphometric parameters).

Table 6.3 *Morphometric sperm head parameters as obtained for post-thaw epididymal sperm treated with seminal plasma prior to equilibration (SP Pre. Equil) or post-thaw (SP Post. Thaw.) and compared to sperm without seminal plasma treatment (NO SP)*

	NO SP		SP Pre Equil		SP Post Thaw	
	(n=11)		(n=8)		(n=9)	
Morphometry parameter	**Range**	**CV**	**Range**	**CV**	**Range**	**CV**
Length (µm)	7.89 - 8.38	2.01	8.01 – 8.52	2.00	8.01 – 8.68	2.58
Width (µm)	4.36 - 4.71	2.10	4.50 – 4.83	2.38	4.58 – 4.89	1.93
Area (μm^2)	37.82 - 40.52	2.18	38.22 – 42.66	3.58	38.31 – 43.77	3.68
Perimeter	17.88 - 18.96	1.78	18.26 – 19.25	2.17	18.05 – 19.76	2.81
Ellipticity (µm)	1.72 - 1.87	2.55	1.71 – 1.83	2.17	1.71 – 1.83	2.14
Elongation	0.26 - 0.30	4.15	0.26 – 0.29	3.58	0.26 – 0.29	3.55
Roughness	1.42 - 1.52	2.28	1.41 – 1.51	2.39	1.41 – 4.53	2.69
Regularity	0.73 - 0.76	1.52	0.74 – 0.76	1.14	0.72 – 0.77	1.95
Acrosome coverage (%)	53.13 - 54.60	0.87	53.15 – 54.49	0.78	53.11 – 54.85	0.97

Table 6.4 *A comparison of all morphometric head parameters on the effect of adding seminal plasma (SP Pre Equil, seminal plasma added prior to equilibration; SP Post Thaw), and no seminal plasma (No SP added) to post thaw epididymal sperm samples (mean ± SEM), obtained from Merino rams, aged 2 to 5 years*

	No SP added	SP added Pre Equil.	SP added Post Thaw
	(n=11)	(n=8)	(n=9)
Length (µm)	8.11 ± 0.05	8.25 ± 0.06	8.21 ± 0.07
Width (µm)	4.58^{a} ± 0.03	4.71^{b} ± 0.04	4.71^{b} ± 0.03
Area (μm^2)	39.43 ± 0.26	40.76 ± 0.52	40.56 ± 0.50
Perimeter	18.38 ± 0.10	18.75 ± 0.14	18.61 ± 0.17
Ellipticity (µm)	1.77 ± 0.01	1.75 ± 0.01	1.76 ± 0.01
Elongation	0.28 ± 0.00	0.27 ± 0.00	0.27 ± 0.00
Roughness	1.47 ± 0.01	1.46 ± 0.01	1.47 ± 0.01
Regularity	0.74 ± 0.00	0.75 ± 0.00	0.75 ± 0.00
Acrosome coverage (%)	53.90 ± 0.14	53.58 ± 0.15	53.76 ± 0.17

[a, b] Columns with different superscripts differ significantly ($p<0.05$)

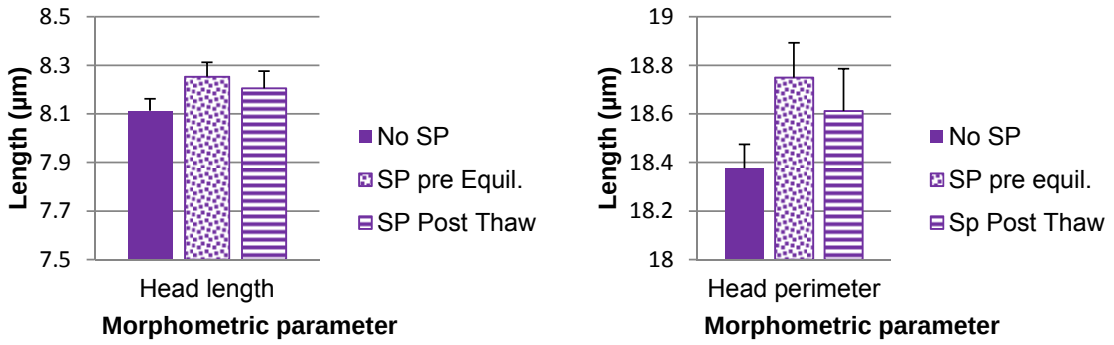

Figure 6.1 *A comparison of morphometric parameters head length and -perimeter (mean ± SEM) post-thaw, for the effect of seminal plasma addition (prior to equilibration or post thaw) or no addition to epididymal sperm*

Results showed that when head length and head perimeter was assessed, no significant differences ($p>0.05$) were found when seminal plasma was added to epididymal sperm (Table 6.3; Figure 6.1).

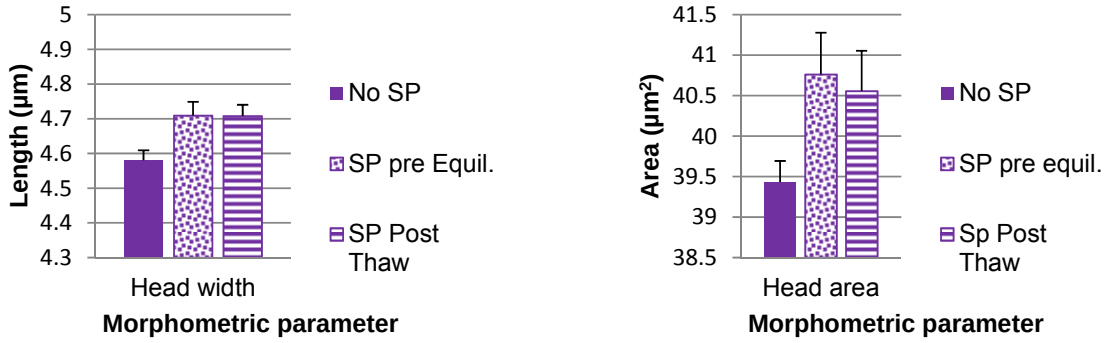

Figure 6.2 *A comparison of morphometric parameters head width and -area (mean ± SEM) post-thaw, for the effect of seminal plasma addition (prior to equilibration or post thaw) or no addition to epididymal sperm obtained via aspiration of the cauda epididymis of Merino rams, aged 2 to 5 years*

Significant differences were obtained for the morphometric head parameter width when epididymal sperm with no addition of seminal plasma were compared to epididymal sperm to which seminal plasma has been added prior cryopreservation or post-thaw ($p=0.0016$; Table 6.3). The head width measured between slides prepared from samples where seminal plasma was added prior to equilibration did not differ from samples where seminal plasma was added post thaw (4.71µm ± 0.04µm vs. 4.71µm ± 0.03µm respectively). The sperm head width of

samples of the control group (no SP added; 4.58µm ± 0.03µm) indicated decreased head widths, thus it would seem that with no seminal plasma added to samples, cryopreservation induced head shrinkage. A study by Hidalgo *et al.* (2007) on goat sperm, indicated that freeze-thawing provoked a reduction in the sperm head dimensions of cryopreserved samples, which corresponds well to that found in this study. It can thus be assumed that the addition of seminal plasma to the epididymal sperm samples maintained normal sperm head width.

Comparison of the sperm head area tended to differ between epididymal sperm treated with seminal plasma when compared epididymal sperm not treated with seminal plasma (p=0.071; Table 6.3). Results indicate that sperm not treated with seminal plasma (39.43µm^2 ± 0.26µm^2) tended to measure smaller surface area values than that of sperm treated with seminal plasma prior to equilibration (40.76µm^2 ± 0.52µm^2) and post thaw (40.56µm^2 ± 0.50µm^2) (see Figure 6.2). Other studies have indicated that the addition of seminal plasma to ejaculated sperm samples improve overall survival, and fertilizing ability of sperm (Ollero *et al.*, 1997; Maxwell *et al.*, 2007). A larger sperm head area could indicate that the epididymal sperm treated with seminal plasma stabilizes the sperm head membranes in order to prevent loss of genetic material and thus improve fertilizing ability.

The comparison of morphometric index parameters was also assessed, and is set out in Figure 6.3 and Figure 6.4.

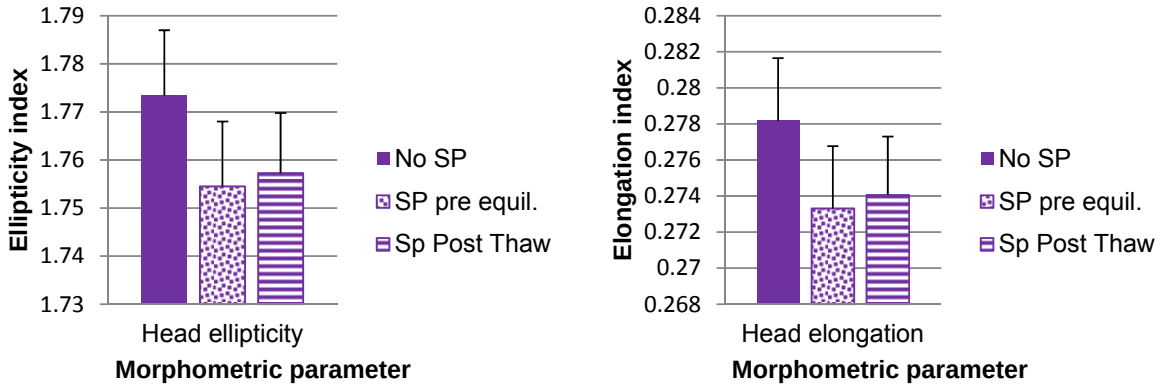

Figure 6.3 *A comparison of morphometric index parameters head ellipticity and -elongation (mean ± SEM) post thaw, for the effect of seminal plasma addition (prior to equilibration or post thaw) or no addition to epididymal sperm*

Results indicated that when the effect of seminal plasma addition (prior equilibrium and post thaw) was evaluated for head ellipticity, -elongation, -roughness and -regularity, for epididymal sperm measured post-thaw, no significant differences ($p>0.05$) were found (Table 6.3). The acrosome coverage percentage was also evaluated for the effect of seminal plasma addition on morphometric epididymal sperm parameters, and no significant differences were found (Table 6.3).

Despite the many studies done on the effect of seminal plasma to minimise cryopreservation damage by stabilizing the sperm acrosomal membrane, there is still an on-going debate regarding the specific advantages and disadvantages of using seminal plasma to optimize sperm quality post thaw. Some studies have indicated that the addition of seminal plasma to ejaculated sperm samples had a beneficial effect, although others have concluded that the addition of seminal plasma had little or no effect on the motility sperm traits of rams.

These contradictory reports imply that seminal plasma is a complex biological fluid that contains a wide variety of components that may affect the function and integrity of sperm. The contradictions which are evident from the use of seminal plasma could possibly be related to the inherent variability that exists in the composition of seminal plasma between males or ejaculates and/or the source of the seminal plasma used (Muiño-Blanco *et al.*, 2008). Artificial insemination (AI) results indicated that the addition of seminal plasma did not increase pregnancy rates when used for AI (O'Meara *et al.*, 2007).

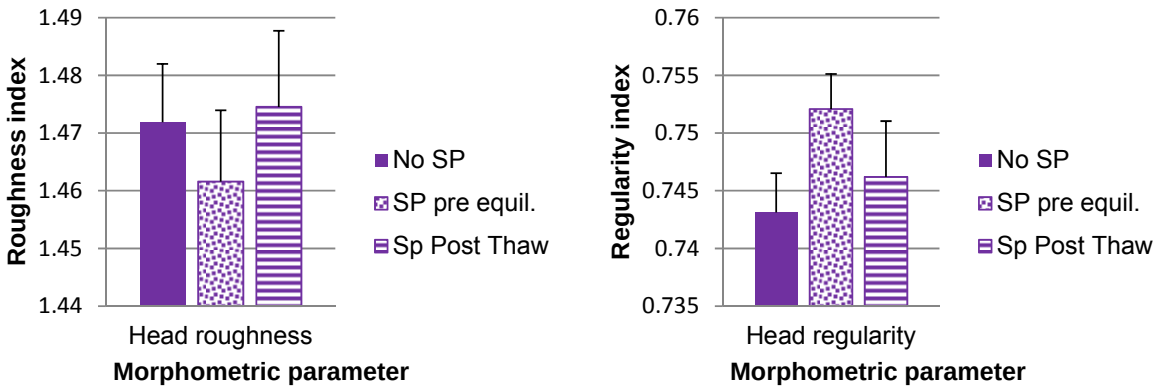

Figure 6.4 *A comparison of morphometric parameters head width and -area (mean ± SEM) post-thaw, for the effect of seminal plasma addition (prior to equilibration or post thaw) or no addition to epididymal sperm*

6.4 Conclusions

The addition of seminal plasma to epididymal samples had no discernable effect on post-thaw sperm motility as measured by the SCA® system. However, differences were obtained for the morphometric parameter head-width, which could have a possible effect on the fertilizing ability of the sperm. However small, differences between the treatment groups indicate that there is a possibility that the addition of seminal plasma minimizes the extent of cryodamage in epididymal samples.

This study reports the first information the effect of seminal plasma on the morphometric traits of post-thaw epididymal sperm. Further research is warranted to verify the results obtained in this study. Studies to establish the potential correlation between sperm morphometric traits with conception rate of ewes should also be explored, as this can promote the use of morphometry in evaluation of sperm samples for assisted reproductive techniques (ART's). It can also be advantageous to optimize a protocol which includes the addition of seminal plasma, for epididymal sperm cryopreservation for use in ART's on a commercial basis.

Chapter 7

A comparison of the swim-up and flush technique to assess motility traits of Merino semen samples

Abstract

Sperm motility is one of a set of criteria that is used to quantify and qualify the fertilizing ability of sperm. The use of the swim-up technique (SUT) in assisted reproductive procedures is well documented, with the technique being particularly effective in the identification of highly motile sperm for e.g. IVF purposes. The use of the SUT involves a period of 10 minutes of exposure of sperm to the medium before the motility of a sample can be recorded by using the Sperm Class Analyzer® (SCA®) system. The flush technique (FT) enables the capture of sperm motility shortly after collection (i.e. within a minute). This study compared the techniques in terms of motility traits recorded for freshly ejaculated ram sperm. Motility evaluations were performed using 45 semen samples obtained from 15 Merino rams (*Ovis aries*) at weekly intervals. Motility recordings were captured at 100 frames per second. The overall sperm motility of samples subjected to the FT was 89.2%, which was significantly higher than the motility of sperm subjected to the SUT (83.9%; $p<0.006$). Ram sperm are particularly susceptible to cryodamage, and shortening the interval from collection to cryostorage may possibly minimize the negative effects of cryopreservation, thus potentially improving the viability of ram sperm, post-thaw. The results indicate that the FT can be used to assess the motility of ram sperm, as results obtained for most of the SCA® parameters compares favourably to that obtained with the SUT.

7.1 Introduction

Many intensive livestock production systems rely on the use of assisted reproductive techniques (ART's) to improve production traits, by increasing the number of progeny from desired animals and by the dissemination of germplasm nationally and internationally. ART's also has an important role to play in the conservation of the genetic make-up of livestock. Incorporating ART's into management and breeding of livestock production systems can thus contribute to the overall cost-efficiency of such systems, and contribute to accelerating genetic progress.

A prerequisite of the successful application of the ART's, is that good quality gametes are used. This implies that sperm samples conserved should have adequate motility (if used for AI), normal morphology and intact acrosomes. Sperm motility is one of several sperm traits that provide an indication of the overall viability of a sample. In the animal, sperm motility is important for the transport of sperm through the cervix (with natural service), and the utero-tubal junction to reach, interact with and fertilize the oocyte (Suarez & Pacey, 2006). It is also stated that sperm motility facilitates the penetration of the *zona pellucida* of the ovum during fertilization (Herman *et al.*, 1994).

Objective measurement of motility parameters is facilitated by using computer assisted sperm analysis (CASA) instruments. Measurements obtained with CASA provide more data with a degree of objectivity and detail which could possibly quantify small differences in sperm motility not identifiable with the naked eye (Rodriquez-Martinez *et al.*, 2008). The Sperm Class Analyser (SCA®), a CASA system, digitise microscopic images of sperm trajectories (both velocities and lateral movement), which describes the patterns of motility and other re-calculated kinematic variables (Rodriquez-Martinez *et al.*, 2008).

Macroscopic evaluation of semen samples as soon as possible after collection comprises the recording of semen volume and density, followed by the subjective assessment of the sperm concentration and motility according to a numeric scale (see Chapter 3) (Rodriquez-Martinez *et al.*, 2008). Motility analysis of semen should also be performed as soon as possible after collection to ensure that a reliable estimate of the sample's viability is obtained, and that a sample can be processed as soon as possible for either insemination or cryopreservation purposes. Ovine spermatozoa, when compared to those of bovine species, are particularly susceptible to degeneration and a reduction in viability after collection. Therefore it is important to minimize the period of time used for pre-cryopreservation processes.

The use of the swim-up technique (SUT) as a separation technique for isolating populations of highly motile sperm for use in ART's like *in vitro* fertilization (IVF) is well documented (Arny & Quagliarello, 1987; Parrish & Foote, 1987; Ing *et al.*, 1991; Shamsuddin & Rodriquez-Martinez, 1994; García-López *et al.*, 1996; Jameel, 2008; Mehmood *et al.*, 2009). In addition to the SUT, there are other separation techniques that can be used for the isolation of highly motile sperm, some of which include the Percoll gradient, mini-Percoll, Sperm Select (sodium hyaluronate) and Sperm Prep (sephadex) (Ng *et al.*, 1992; Buzby *et al.*, 1993; Parrish *et al.*, 1995). Percoll and mini-percoll gradients are generally used for isolation of sperm by density centrifugation. Percoll consists of silica beads coated with polyvinylpyrolidone, which 'filters' sperm by separating them on the base of their density (Parrish *et al.*, 1995). The Sperm Select technique consists of the migration of sperm through a sodium hyaluronate solution, after which it is incubated and the top layer subsequently removed and evaluated (Buzby *et al.*, 1993). Sperm Prep on the other hand, makes use of a filtration column through which sperm are filtered and collected (Buzby *et al.*, 1993).

A drawback of the SUT is that considerable time elapses from when sample collection takes place, until motility recordings are made which in turn may impact negatively on the viability of a sperm sample. A flush technique (FT), developed by G. van der Horst (gvdhorst@uwc.ac.za), may potentially allow for a more rapid assessment of sperm motility. Motility analyses by using

samples subjected to the FT can potentially contribute to shortening the time lapse from collection to the recording of sperm motility. This can then potentially contribute to optimizing the pre-insemination or cryopreservation integrity and viability of ram sperm.

The aim of this study was to therefore to compare the FT and the SUT in terms of the motility parameters recorded, and to determine the potential of the FT to substitute the SUT as a method to obtain a reliable and rapid indication of ovine sperm motility.

7.2 Materials and methods

7.2.1 Experimental location

The experiments were conducted at the Elsenburg research farm (33°51'S, 18°50'E), outside Stellenbosch in the Western Cape, South Africa.

7.2.2 Experimental animals

Fifteen sexually mature Merino rams (aged between 2 and 5 years) were used in this study. The rams were maintained according to ethically approved husbandry practices, and under uniform nutritional conditions. Ethical clearance was provided by the Departmental Ethics Committee for Research on Animals (DECRA; number R11/45). All animal care and procedures used in the study were performed in such a manner that it was in line with South African National Standards 10386:2008.

Rams were trained to ejaculate into an artificial vagina (AV) prior to commencement of the semen collection. For more detailed information regarding the training of the rams and the AV collection technique, please refer to Chapter 3.

Semen samples (n=45) were collected from 5 rams per day, at three intervals per week, over a period of 3 weeks (thus three ejaculates per ram). Care was taken to ensure all equipment and instruments were pre-warmed (37ºC) to avoid the effect of cold shock (Chapter 3). After collection of ejaculates, samples were transferred to Eppendorf tubes (Lasec, South Africa), and prepared for motility determining techniques.

7.2.3 Recording of sperm motility

7.2.3.1 Execution of the swim-up technique (SUT)

Prior to motility recording, 0.5 mL pre-warmed Ham's F10 (37ºC) (Sigma-Aldrich, South Africa) was transferred to a 1.5 mL Eppendorf tube. An aliquot (10 µL) of the fresh sperm sample was

then placed on the bottom of the Eppendorf tube. The tube was then incubated at 37ºC for a period of 10 minutes, which allowed for the most motile and viable sperm to swim up towards the overlaying Ham's F10 layer.

After the 10 minute waiting period, a cloud became visible within the sample. Sampling for sperm motility recording was then performed by collecting an aliquot from just above the 'cloud', and the sample was transferred to a chamber of a pre-warmed Leja® slide (Delfran, South Africa). Two to three fields were used to capture motility per sample at 100 frames per second, using the SCA® system.

7.2.3.2 Execution of the flush technique (FT)

Prior to the recording of sperm motility, a Leja® slide (Delfran, South Africa) was pre-warmed on the heated stage (37ºC) prior to use. An aliquot (± 2µL) of each fresh sperm sample was placed at the entrance of a Leja slide chamber, after which Ham's F10 medium was added to the chamber contents at the same point of entry, thus 'flushing' the sperm so that the sample distribute evenly throughout the chamber. Each slide was then placed on the heating stage for 7 to 10 seconds to allow for the sample to stabilize (i.e. no currents observed within the chamber), and the sperm to reach maximum motility within the chamber environment. The SCA® computerised system (version 5.1) was then used to capture the motility of each sample. For analysis purposes two to three different fields were captured for each sample, at a frame speed of 100 frames per second.

7.2.4 Motility analysis and evaluation

Once motility recordings were captured, specific motility parameters were measured by the SCA®. The measured motility parameters included progressive motility, non-progressive motility, total motile, static, rapid, medium, slow, VCL, VSL, VAP, LIN, STR, ALH, and BCF. Analysis of the above motility traits were analysed automatically by the SCA® system. For more details, and the calculation of traits, refer to Chapter 3. Each analysis was saved separately and pooled for statistical analysis.

7.2.5 Statistical analysis

The results were generated using PROC GLM of SAS Enterprise Guide 5.1 software, Version 9.3 of the SAS System for Windows. The generalized linear model (GLM) procedure was used to estimate least square means (LSM). Obtained means were adjusted for multiple comparisons with the Bonferroni t-test. For the comparative analyses, the one-way analysis of variance (ANOVA) approach was used. Each ejaculate sample was analysed individually to obtain the

respective motility trait values, and saved as separate excel files. The values obtained from all the ejaculate samples were pooled, and statistical analysis was performed on the pooled data. All results were expressed as the mean ± standard error of the mean (SEM). Findings were considered statistically significant at $p<0.05$.

7.3 Results and discussion

The motility parameters obtained throughout the study showed a considerable range as presented in Table 7.1. Averaged motility results for semen samples obtained from the 15 rams, processed by using either the flush (FT) or swim-up (SUT) technique are set out in Table 7.1 (raw data is available in Appendix D, Table D1).

Table 7.1 A comparison of motility parameters (mean ± SEM) of ejaculated ram spermatozoa, as prepared for motility analyses by using either the flush (FT) or the swim-up (SUT) technique

	FT		SUT	
	(n=45)		(n=45)	
	Range (CV)	Mean ± SEM	Range (CV)	Mean ± SEM
Motile sperm (%)	50.10 - 98.78 (10.07)	89.23^{c} ± 1.32	48.25 - 97.24 (10.66)	83.97^{d} ± 1.32
Non-progressive motile (%)	21.98 - 78.18 (35.11)	44.41^{a} ± 2.30	11.34 - 81.89 (42.41)	36.63^{b} ± 2.29
Progressive motile (%)	9.39 - 71.78 (34.23)	44.8 ± 2.26	5.23 - 79.83 (32.29)	47.34 ± 2.25
Static (%)	1.22 - 49.90 (83.44)	10.77^{c} ± 1.32	2.76 - 51.75 (55.83)	16.03^{d} ± 1.32
Rapid (%)	7.44 - 86.46 (21.15)	61.66 ± 1.92	15.86 - 82.38 (20.86)	62.28 ± 1.92
Medium (%)	6.50 - 57.26 (36.01)	22.58^{c} ± 1.20	4.15 - 55.84 (50.95)	15.90^{d} ± 1.19
Slow (%)	0.42 - 17.29 (54.46)	4.99 ± 0.40	1.26 - 16.33 (46.76)	5.79 ± 0.40
VCL (µm/s)	123.27 - 329.41 (16.35)	244.32^{a} ± 5.89	141.83 - 389.61 (15.08)	263.96^{b} ± 5.87
VSL (µm/s)	44.00 - 168.14 (20.88)	111.55 ± 3.43	45.48 - 186.90 (19.39)	119.65 ± 3.42
VAP (µm/s)	67.31 - 201.20 (14.00)	148.55 ± 3.07	93.15 - 205.46 (13.45)	154.01 ± 3.05
LIN (%)	26.09 - 60.02 (16.69)	45.44 ± 1.12	23.74 - 59.00 (16.62)	45.45 ± 1.11
STR (%)	48.66 - 84.93 (11.92)	74.13 ± 1.30	47.15 - 90.97 (11.45)	76.89 ± 1.30
WOB (%)	48.88 - 70.93 (7.62)	60.85^{a} ± 0.68	49.63 - 68.25 (7.85)	58.87^{b} ± 0.68
ALH (µm)	2.09 - 4.16 (14.64)	2.88^{c} ± 0.06	2.26 - 4.78 (12.97)	3.24^{d} ± 0.06
BCF (Hz)	27.15 - 56.98 (12.06)	44.26 ± 0.79	18.08 - 59.69 (11.70)	45.44 ± 0.78

$^{a, b}$ Columns with different superscripts differ significantly ($p<0.05$)

$^{c, d}$ Columns with different superscripts differ significantly ($p<0.01$)

7.3.1 Results obtained for the percentage of total motile, non-progressive motile, progressive motile and static sperm

Ram sperm are particularly susceptible to cryodamage, and thus it is important to shorten the interval between collection and cryopreservation (dilution, equilibration with an extender/ cryoprotectant, loading of straws etc.) to improve the viability of sperm and to minimize the negative effects of cryopreservation post thaw.

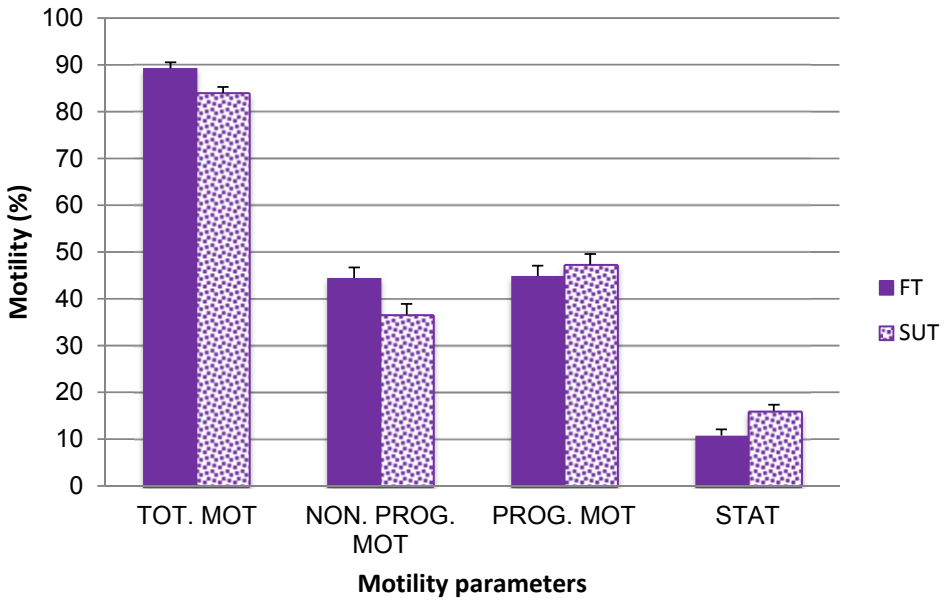

Figure 7.1 *Comparison of the FT with the SUT for ejaculate samples obtained from fifteen Merino rams for the motility parameters total motile (Tot. Mot.), non-progressive motile (Non. Prog. Mot.), progressive motile (Prog. Mot.) and static (Stat.)*

7.3.1.1 Total motile sperm

When the overall motility of the samples was compared, the FT and the SUT differed significantly (p=0.006; Table 7.1). The FT resulted in higher values for sperm motility, when compared to the SUT (89.23% ± 1.32% and 83.97% ± 1.32% respectively). Although the FT showed a higher percentage of total motile sperm, it would not be appropriate to state that the accuracy for one technique is higher, compared to the other.

There are many factors that can influence the motility of a sample, including sampling area and the ratio of Ham's F10 solution, to the concentration of sperm. The motility values found in this study for the SUT are consistent with values reported by McAlister (2010). The SUT was compared to the use of the PureSperm® density gradient as means of sperm processing

techniques in the latter study, with the results indicating that there were no significant differences between the total percentage motile sperm when using either the SUT or the PureSperm® density gradient as separation technique (82.97% ± 5.14% vs. 91.34% ± 2.35%). Thus the results obtained with the FT method for total motile were consistent with values derived when the PureSperm® density gradient was used as a separation technique.

7.3.1.2 Non-progressive motile sperm

Significant differences were observed between the FT and the SUT when the non-progressive motile sperm were evaluated (p=0.019, Table 7.1). The FT resulted in higher values for the percentage non-progressive motile sperm, when compared to the SUT (44.41%± 2.30% vs. 36.63%± 2.29% respectively). This difference could be attributed to the pre-selection of sperm populations when using the SUT, as most sperm obtained with the SUT will yield progressive motility sperm within the 'selection cloud'. The FT incorporated the whole sample, thus possibly yielding a more accurate analysis of the sample in its entirety.

7.3.1.2 Progressive motile sperm

No significant differences were found between the percentage progressive motile sperm, when the FT was compared to the SUT (Table 7.1).

7.3.1.2 Static sperm

Since the total motile percentage is directly linked (inverse) to the percentage of static sperm, it is expected that the % static will also differ significantly between the results obtained with the use of the FT, compared to that of the SUT (p=0.006; Table 7.1). The percentage of static sperm obtained by the use of the SUT was higher than the percentage of immotile sperm obtained with the FT (16.03%± 1.32% vs. 10.77% ± 1.32%, respectively). As mentioned previously, this difference in trait values should not be accepted as irrevocable proof of one technique being superior compared to the other.

7.3.2 Results obtained for rapid-, medium- and slow-swimming sperm

7.3.2.1 Rapid swimming sperm

The percentage rapid-swimming sperm did not differ between the FT and the SUT (61.66% ± 1.92% vs. 62.28% ± 1.92%, respectively; Table 7.1).

7.3.2.2 Medium swimming sperm

The comparison of the FT and the SUT showed that there is a significant difference between the two techniques. The FT resulted in a higher percentage of medium velocity sperm,

compared to the SUT (22.58% ± 1.20% vs. 15.90% ± 1.19%, respectively; Table 7.1). This difference is apparent in Figure 7.1. The percentage of sperm with medium swimming speed is consistent with that found for human sperm with the SUT (McAlister, 2010).

7.3.2.3 Slow swimming sperm

The results showed no significant differences for the percentage of slow-swimming sperm between the FT and the SUT (4.99% ± 0.40% vs. 5.79% ± 0.40%, respectively; Table 7.1).

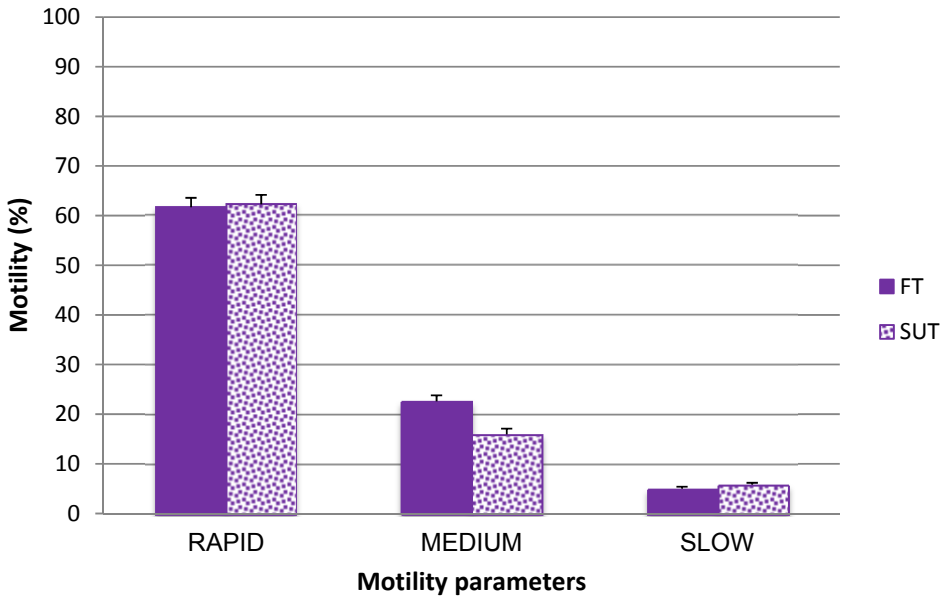

Figure 7.2 *A comparison of motility parameters rapid, medium and slow of ejaculated ram sperm, as prepared for motility analyses by using the flush (FT) and swim-up (SUT) techniques, respectively*

7.3.3 Results obtained for VCL, VSL and VAP

7.3.3.1 Curvilinear velocity (VCL)

Semen samples evaluated by the SUT had significantly higher mean values for VCL (p=0.0206) than the same samples assessed with the FT (263.96 µm/s ± 5.87 µm/s and 244.32 µm/s ± 5.89 µm/s respectively; Table 7.1, Figure 7.3). It is possible that sperm measured with the SUT could be in a more hyperactivated mode due to the amount of activity exhibited during the separation technique in the Ham's F10 solution, when compared to the FT.

7.3.3.2 Straight/line velocity (VSL)

The results indicate that there were no significant difference between the SUT and the FT for VSL. However, VSL did show a tendency to differ (p=0.098; Table 7.1). The VSL results obtained by using the FT (111.55µm/s ± 3.43µm/s) and the SUT (119.65µm/s ± 3.42µm/s) indicated that sperm measured with the SUT tended to have a higher straight line velocity. This could be due to chemical shock, as sperm was exposed to the Ham's F10 solution for a period of ten minutes prior to motility measurement. The FT only exposed sperm to Ham's F10 for a fraction of the time, and thus one can assume sperm had not yet reacted to the difference in medium, and thus have resulted in lower values for VSL when compared to the SUT.

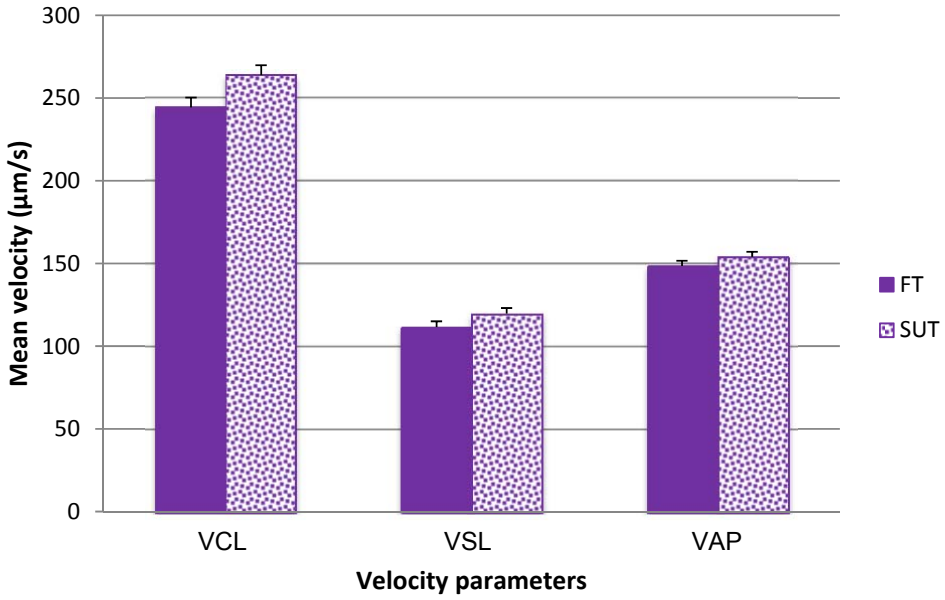

Figure 7.3 *A comparison of motility parameters curvilinear velocity (VCL), straight line velocity (VSL) and average path velocity (VAP) of ejaculated ram sperm obtained from 15 Merino rams, for motility analyses by using the flush (FT) and swim-up (SUT) techniques, respectively*

7.3.3.3 Average path velocity (VAP)

When VAP was compared between the FT and the SUT, the results indicate that there was no significant difference between the two techniques (148.55µm/s ± 3.07µm/s vs. 154.01µm/s ± 3.05µm/s respectively; Table 7.1).

7.3.4 Results obtained for linearity, straightness, wobble, amplitude of the lateral head displacement and beat/cross frequency

7.3.4.1 Linearity index (LIN)

No significant differences were obtained with the comparison of the motility determining techniques for the trait linearity.

7.3.4.2 Straightness index (STR)

The straightness index did not differ significantly when the FT and the SUT was compared (Table 7.1; Figure 7.4).

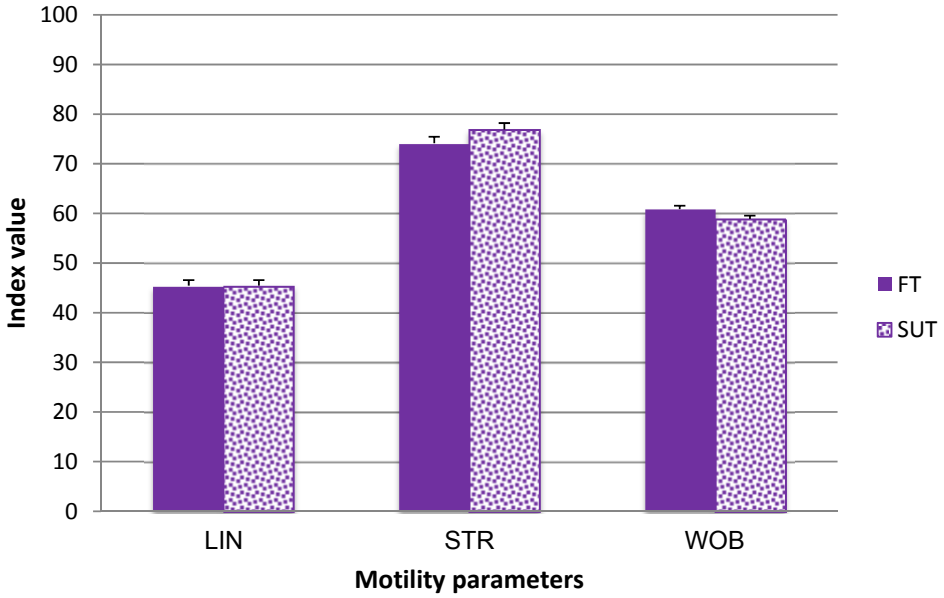

Figure 7.4 *A comparison of motility index parameters linearity (LIN), straightness (STR) and wobble (WOB) of ejaculated ram sperm obtained from 15 Merino rams, as prepared for motility analyses by using the flush (FT) and swim-up (SUT) techniques, respectively*

7.3.3.4 Wobble index (WOB)

The results showed that when the techniques were compared, the wobble (or oscillation index) differed significantly between the FT and the SUT (p=0.0429; Table 7.1). The FT showed a marginally higher oscillation index value (60.85 ± 0.68) when compared to the SUT (58.87 ± 0.68) (Figure 7.4). This difference could have resulted from sperm obtained with the SUT being in a more hyperactivated state, compared to sperm measured with the FT. With a higher wobble index value it can be assumed that sperm measured with the SUT may have possibly been exposed to chemical shock (in Ham's F10 solution), leading to a changed state of the sperm

membranes, possibly changing towards the capacitation and hyperactivation mode. Sperm measured with the FT are only exposed to the chemical shock for about a minute, when compared to the 10 minute waiting period for the SUT.

7.3.3.5 Amplitude of the lateral had displacement (ALH)

It is evident from Table 7.1 and Figure 7.5 that the FT differs significantly from the SUT when mean ALH distances were compared. The SUT showed a higher value for lateral head displacement (3.24µm ± 0.06µm) in comparison with the FT (2.88µm ± 0.06µm).

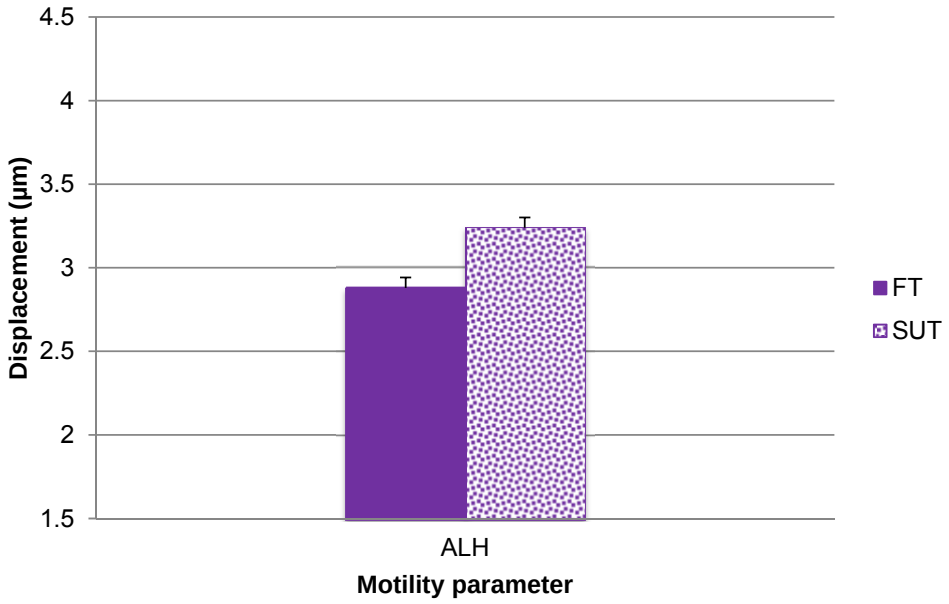

Figure 7.5 *A comparison of the motility parameter amplitude of the lateral head displacement (ALH) of ejaculated ram sperm obtained from 15 Merino rams, as prepared for motility analyses by using the flush (FT) and swim-up (SUT) techniques, respectively*

7.3.3.6 Beat/cross frequency (BCF)

BCF did not differ between the samples subjected to the SUT or the FT for motility evaluation (Table 7.1; Figure 7.6).

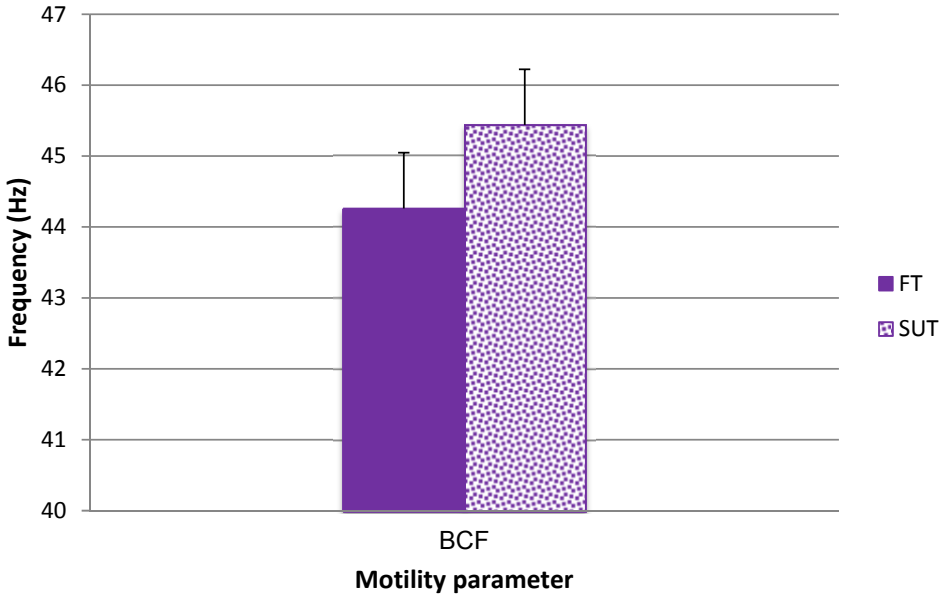

Figure 7.6 *A comparison of the motility parameter beat/cross frequency (BCF) of ejaculated ram sperm obtained from 15 Merino rams, as prepared for motility analyses by using the flush (FT) and swim-up (SUT) techniques, respectively*

7.4 Conclusions

When compared to the standard more time consuming swim-up technique, most of the kinematic parameters obtained with the FT compared favourably with those obtained with the SUT. The FT can be thus be used as an alternative to the more time consuming SUT in the preparation of sperm samples for motility analysis with SCA®.

Further research is required to verify the potential contribution of sperm sub-populations to the motility traits found in this study to verify the use of the FT as a substitute for the more time-consuming SUT.

Chapter 8

General conclusions & recommendations

The potential of assisted reproductive techniques (ART's) to assist sheep producers to optimise the production and reproductive efficiency of their flocks has received considerable attention during the last few decades. The demand for safe food increases with the increase in the human population, and therefore it is important to investigate the potential of genetic selection, and the use of assisted reproductive techniques (ART's) to optimize reproduction efficiency of a sheep population in such a way that it will satisfy this need on a global scale.

Artificial insemination (AI), and multiple ovulation and embryo transfer are ART tools, and effective use can enable farmers to produce lamb and mutton more cost-efficiently. One of the major limitations of using ART's, however, is that the existing techniques have been adapted from the respective ART's developed for the cattle industry.

Optimization of the respective ART's for the sheep industry is important, as it will enable sheep farmers to manage the reproductive ability of their animals to optimise the number of offspring successfully weaned per ewe, and thus manage their production systems more cost-effectively, and promote genetic progress within a flock at the same time. Previous studies have investigated the use of ART's to increase ovine production and reproductive efficiency. However, protocols are not optimised to the same extent as it is in the cattle industry. The effect of genetic selection for reproduction in sheep should be studied to investigate whether such selection would result in genetic resources more suitable for the successful application of ART's in the sheep industry.

This study was aimed at determining the contribution of the ram to the overall reproductive efficiency of a flock, as assessed in terms of mating dexterity and semen quality. The effect of divergent genotypes was also investigated on epididymal sperm motility and mophometry, and the possible effect it may have on the cryopreservation status of sperm. Generally it is known that ovine sperm membrane integrity is compromised during the process of cryopreservation, and thus the use of seminal plasma to optimize the cryopreservation protocol to minimize the degree of damage to the sperm membranes were investigated. Lastly, it was noted that the standard techniques for evaluation of sperm samples, specifically the evaluation of motility, was a time consuming factor, which contributes to the decrease in overall sperm quality of the sample prior to processing for cryopreservation, and thus also the viability and survivability of sperm post-thaw. To address the time consuming factor, a new technique (flush technique) was compared with the conventional swim-up technique to see whether the flush technique can be

used as a faster alternative when used for semen evaluation prior to cryopreservation processing.

Influence of mating dexterity and genotype on the sperm quality of two genetically diverse Merino lines

The aim of the study was to investigate the influence of genetic selection (i.e. HL; LL) on the prolificacy of sheep flocks, and sperm quality (motility and morphometry) of two genetically diverse lines of Merino rams (i.e. the High Line (HL) and the Low Line (LL)) selected for and against the number of lambs weaned per lambing opportunity. The current study suggested that there was a considerable difference between the HL and LL in terms of the mating performance of the rams. No differences were however observed for conception rates between ewes served by the HL and LL rams, respectively. The HL rams served more frequently during the 20 minute serve capacity test period, when compared to the LL rams. Lamb birth weight did not differ between lambs that resulted from HL and LL matings.

When assessed by means of computer-assisted sperm motility analysis, no significant differences were found between the sperm motility parameters obtained for the HL and LL rams. Morphometric analysis indicated significant differences between HL and LL sperm in terms of the two index parameters ellipticity and elongation. Method of sperm recovery (ejaculated in an artificial vagina vs. collection from the epididymis of slaughtered rams) was also evaluated, and results indicated differences for most motility parameters, although no morphometric differences were found.

The lack of significant differences indicated that other factors may affect the reproduction efficiency difference observed between the two lines. Further research is suggested to clarify the relationship between sperm quality of rams with a high mating performance, when compared to rams with a low mating performance. A possible area of research deserving further study is the potential contribution of the female component to the overall reproduction efficiency of a breeding flock in terms of ova quality and physiological state of the ewe during the mating season. Future studies should also include the effect of sperm sub-populations and hyperactivation status, and the effect of these on motility and morphometric traits of rams of genetically diverse sires.

The influence of genotype, sperm concentration and equilibration period on sperm traits and the ability of epididymal sperm to withstand cryodamage

This part of the study investigated the effect of genotype, sperm concentration and equilibration period on motility and morphometric parameters of epididymal sperm obtained from 12 Merino rams, aged 2 to 5 years.

No significant differences were found for the percentage total motile sperm between the HL and the LL rams for either fresh or post-thaw evaluated sperm samples. Significant line differences were however observed between the motility parameters ALH and BCF recorded during the SCA® analysis of fresh epididymal samples.

When the morphometric parameters of fresh and post-thaw epididymal sperm were considered, sperm from LL rams showed wider (broader) and more rounded heads, compared to sperm obtained from the HL rams. It was also evident that fresh and post-thaw epididymal sperm from the HL were more elongated and tapered, when compared to that of the LL. Post-thaw morphometric analysis also indicated that sperm from the HL rams had larger acrosome coverage of the sperm head, when compared to sperm obtained from the LL rams.

When the potential interaction of sperm concentration and equilibration period was evaluated and compared, no significant differences were observed between any of the nine treatment combinations. Post-thaw morphometric evaluation of epididymal sperm did however indicate significant differences when the main effect of sperm concentration was evaluated across equilibration periods for the morphometric trait regularity. The regularity index indicated whether the sperm were pyriform in shape, or not (pear shaped).

Very little research is available on the morphometric traits of epididymal sperm, thus it is justified to encourage further research in this field. The two genetically diverse lines of Merino sheep show differences in terms of their overall reproduction success and with the differences observed in head shape and size, the correlation between morphometric traits and fertilizing ability of sperm should be investigated. Future research concentrating on sperm competition, and possible discrimination by the ewe reproductive system should also be considered. Combining lambing success by using insemination with sperm from genetically diverse lines of Merino rams with morphometric measurements, could possibly shed light on the underlying causes of differences observed within and between the genetically diverse lines.

The potential of seminal plasma to minimize the extent of cryodamage in epididymal sperm samples

The aim of the study was to determine whether seminal plasma added to sperm samples prior to equilibration or post-thaw will limit the extent of cryodamage experienced by epididymal sperm.

The addition of seminal plasma to epididymal samples had no beneficial effect on post-thaw motility, as assessed by the SCA® system. However, differences were obtained for the morphometric parameter head width, where no seminal plasma was added to epididymal sperm prior to equilibration or post-thaw. Assuming that cryodamage causes a reduction in all measured morphometric parameters, it is possible that the addition of seminal plasma could be beneficial to minimize cryodamage in epididymal sperm.

This study presents the first information on the influence of seminal plasma on morphometric traits of epididymal sperm post-thaw. Further research is warranted to verify the results, and increase the available information on the morphometric traits of sheep sperm.

The results obtained for this part of the overall study emphasizes further research to correlate morphometric traits of sheep sperm with conception rate of ewes to determine whether morphometric traits need to be included in sperm quality tests for ART's. The development of a short and long-term storage protocol that includes the addition of seminal plasma for the cryopreservation of epididymal sperm for use in ART's on a commercial scale could also benefit the sheep industry.

A comparison of the swim-up and flush technique to assess sperm motility traits of Merino semen samples

Ovine sperm are highly susceptible to cryodamage, and thus the time that elapses between collection and cryopreservation should be minimized to improve post-thaw viability. Standard sperm motility determining techniques (swim-up technique (SUT)) are time consuming, and sperm processing must be postponed, until motility is determined. This study investigated the potential of the flush technique (FT) as an alternative method to assess the motility of fresh semen samples obtained from rams as a substitute to the SUT.

When the FT is compared to the standard more time consuming SUT, most of the kinematic parameters obtained with the FT compared favourably with those obtained using the SUT.

Some parameters (total motile sperm; wobble) indicated higher values when using the FT and other parameters (VCL; ALH) indicated higher values when using the SUT. The inclusion of the possible effect that sperm sub populations may have on the specific motility traits may be important to clarify some of the results that were obtained. The FT can thus be used as an alternative to the more time consuming SUT in the preparation of sperm samples for motility analysis with SCA®.

References

Abaigar, T., Cano, M., Pickard, A.R., Holt, W.V., 2001. Use of computer-assisted sperm motility assessment and multivariate pattern analysis to characterize ejaculate quality in Mohor gazelles (*Gazella dama mhorr*): effects of body weight, electroejaculation technique and short-term semen storage. Reprod. 122, 265-273.

Abdel-Rahman, H.A., El-Belely, M.S., Al-Qarawi, A.A., and El-Mougy, S.A., 2000. The relationship between semen quality and mineral composition of semen in various ram breeds. Small Rumin. Res. 38(1), 45-49.

Ahmad, N. and Noakes, D.E., 1996. Seasonal variations in the semen quality of young British goats. Br. Vet. J. 152(2), 225-236.

Alapati, R., Stout, M., Saenz, J., Gentry, G., Godke, R., and Devireddy, R., 2009. Comparison of the permeability properties and post-thaw motility of ejaculated and epididymal bovine spermatozoa. Cryobiol. 59(2), 164-170.

Almquist, J.O., Branas, R.J., and Barber, K.A., 1976. Postpuberal changes in semen production of Charolais bulls ejaculated at high frequency and the relation between testicular measurements and sperm output. J. Anim. Sci. 42, 670-676.

Alvarez, M., Tamayo-Canul, J., Anel, E., Boixo, J.C., Mata-Campuzano, M., Martinez-Pastor, F., Anel, L., and de Paz, P., 2012. Sperm concentration at freezing affects post-thaw quality and fertility of ram semen. Theriogenology. 77, 1111-1118.

Andrabi, S., 2009. Factors affecting the quality of cryopreserved buffalo (bubalus bubalis) bull spermatozoa. Reprod. Domest. Anim. 44(3), 552-569.

Anel, L., De Paz, P., Alvarez, M., Chamorro, C., Boixo, J., Manso, A., Gonzalez, M., Kaabi, M., and Anel, E., 2003. Field and in vitro assay of three methods for freezing ram semen. Theriogenology. 60(7), 1293-1308.

Anel, L., Kaabi, M., Abroug, B., Alvarez, M., Anel, E., Boixo, J., Fuente, L.F., and De Paz, P., 2005. Factors influencing the success of vaginal and laparoscopic artificial insemination in churra ewes: A field assay. Theriogenology. 63(4), 1235-1247.

Arny, M., and Quagliarello, J., 1987. Semen quality before and after processing by a swim-up method: Relationship to outcome of intrauterine insemination. Fertil. Steril. 48(4), 643-648.

Azerêdo, G., Esper, C., and Resende, K., 2001. Evaluation of plasma membrane integrity of frozen-thawed goat spermatozoa with or without seminal plasma. Small Rumin. Res. 41(3), 257-263.

Bag, S., Joshi, A., Naqvi, S.M.K., and Mittal, J.P., 2004. Effect of post-thaw incubation on sperm kinematics and acrosomal integrity of ram spermatozoa cryopreserved in medium-sized French straws. Theriogenology. 62, 415-424.

Bailey, J.L., Bilodeau, J., and Cormier, N., 2000. Semen cryopreservation in domestic animals: A damaging and capacitating phenomenon. J. Androl. 21(1), 1-7.

Baldassarre, H., and Karatzas, C., 2004. Advanced assisted reproduction technologies (ART) in goats. Anim. Reprod. Sci. 82: 255-266.

Ballester, J., Johannisson, A., Saravia, F., Håård, M., Gustafsson, H., Bajramovic, D., and Rodriguez-Martinez, H., 2007. Post-thaw viability of bull AI-doses with low-sperm numbers. Theriogenology. 68(6), 934-943.

Barrios, B., Pérez-Pé, R., Gallego, M., Tato, A., Osada, J., Muiño-Blanco, T., and Cebrián-Pérez, J. A., 2000. Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol. Reprod. 63(5), 1531-1537.

Bench, C.J., Price, E.O., Dally, M.R., and Borgwardt, R.E., 2001. Artificial selection of rams for sexual performance and its effect on the sexual behaviour and fecundity of male and female progeny. Appl. Anim. Behav. Sci. 72, 41-50.

Bernardini, A., Hozbor, F., Sanchez, E., Fornes, M.W., Alberio, R.H., and Cesari, A., 2011. Conserved ram seminal plasma proteins bind to the sperm membrane and repair cryopreservation damage. Theriogenology. 76(3), 436-447.

Boland, M., Al-Kamali, A., Crosby, T., Haynes, N., Howles, C., Kelleher, D., and Gordon, I., 1985. The influence of breed, season and photoperiod on semen characteristics, testicular size, libido and plasma hormone concentrations in rams. Anim. Reprod. Sci. 9(3), 241-252.

Bousquet, D., Twagiramungu, H., Morin, N., Brisson, C., Carboneau, G., and Durocher, J., 1999. In vitro embryo production in the cow: An effective alternative to the conventional embryo production approach. Theriogenology. 51(1), 59-70.

Bradford, J.M., 1970. Sperm capacitation and fertility in mammals. Biol. Reprod. 2, 128-158.

Brady, D.E., and Gildow, E.M., 1939. Characteristics of ram semen as influenced by the method of collection. J.Anim. Sci. 250-254.

Brito, L.F.C., Silva, A., Rodrigues, L.H., Vieira, F.V., Deragon, L.A.G., and Kastelic, J.P., 2002. Effect of age and genetic group on characteristics of the scrotum, testes and testicular vascular cones, and on sperm production and semen quality in AI bulls in Brazil. Theriogenology. 58(6), 1175-1186.

Buhr, M., Curtis, E., and Kakuda, N.S., 1994. Composition and behavior of head membrane lipids of fresh and cryopreserved boar sperm. Cryobiol. 31(3), 224-238.

Burfening, P.J., Kachman, S.D, Hanford, K.J., and Rossi, D., 1993. Selection for reproductive rate in Rambouillet sheep: Estimated genetic change in reproductive rate. Small Rumin. Res. 10, 317-330.

Buzby, J., Arns, M., and Pool, B.S.K., 1993. Evaluation of different sperm separation techniques for harvesting equine spermatozoa intended for in vitro culture. J. Equine. Vet. Sci. 13(9), 498-501.

Byrne, T.J., Ludemann, C.I., Amer, P.R., and Young, M.J., 2012. Broadening breeding objectives for maternal and terminal sheep. Livest. Sci. 144, 20-36.

Cameron, R.D., and Lauerman, L.H., 1976. Characteristics of semen changes during brucella ovis infection in rams. Vet. Rec. 99(12), 231.

Casey, P.J., Gravance, C.G., Davis, R.O., Chabot, D.D., and Liu, I.K.M., 1997. Morphometric differences in sperm head dimensions of fertile and subfertile stallions. Theriogenology. 47, 575-582.

Chenoweth, P.J., 1981. Libido and mating behaviour in bulls, boars and rams - A review. Theriogenology. 16(2), 155-177.

Ciereszko, A., Ottobre, J.S., and Glogowski, J., 2000. Effects of season and breed on sperm acrosin activity and semen quality of boars. Anim. Reprod. Sci. 64(1-2), 89-96.

Cloete, S., and Durand, A., 1994. Reproduction of merino sheep subjected to divergent selection on maternal values for lambs weaned per ewe joined. S. Afr. J. Anim. Sci. 24(1), 27-29.

Cloete, S., and Scholtz, A., 1998. Lamb survival in relation to lambing and neonatal behaviour in medium wool merino lines divergently selected for multiple rearing ability. Aust. J. Exp. Agric. (38), 801-11.

Cloete, S.W.P., and Olivier, J.J., 2010. South African sheep and wool industries. In: International sheep and wool handbook. Ed.: D.J. Cottle, Nottingham University Press, UK. 95-112.

Cloete, S.W.P., Misztal, I., and Olivier, J.J., 2009. Genetic parameters and trends for lamb survival and birth weight in a Merino flock divergently selected for multiple rearing ability. J. Anim. Sci. 87 (7), 2196-2208.

Cloete, S., Gilmour, A., Olivier, J., and Van Wyk, J., 2004. Genetic and phenotypic trends and parameters in reproduction, greasy fleece weight and liveweight in merino lines divergently selected for multiple rearing ability. Anim. Prod. Sci. 44(8), 745-754.

Coulter, G.H., Cook, R.B., and Kastelic, J.P., 1997. Effects of dietary energy on scrotal surface temperature, seminal quality, and sperm production in young beef bulls. J. Anim. Sci. 75(4), 1048-1052.

DAFF, 2011. A profile of the South African mutton market value chain. Directorate Marketing, Private Bag X15, Arcadia, 0007, South Africa. Email: MorokoloB@daff.gov.za.

De Graaf, S.P., Leahy, T., Marti, J., Evans, G., and Maxwell, W.M.C., 2008. Application of seminal plasma in sex-sorting and sperm cryopreservation. Theriogenology. 70, 1360-1363.

De Kretser, D., Loveland, K., Meinhardt, A., Simorangkir, D., and Wreford, N., 1998. Spermatogenesis. Human Reprod. 13(1), 1-8.

De Lamirande, E., and Gagnon, C., 1984. Origin of a motility inhibitor within the male reproductive tract. J. Androl. 5, 269-276.

De Lamirande, E., Leclerc, P., and Gagnon, C., 1997. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol. Hum. Reprod. 3(3), 175-194.

De Paz, P., Mata-Campuzano, M., Tizado, E.J., Álvarez, M., Álvarez-Rodríguez, M., Herraez, P., and Anel, L., 2011. The relationship between ram sperm head morphometry and fertility depends on the procedures of acquisition and analysis used. Theriogenology. 76, 1313-1325.

Domínguez, M., Falcinelli, A., Hozbor, F., Sanchez, E., Cesari, A., and Alberio, R., 2008. Seasonal variations in the composition of ram seminal plasma and its effect on frozen-thawed ram sperm. Theriogenology. 69(5), 564-573.

Dowsett, K.F., and Knott, L.M., 1996. The influence of age and breed on stallion semen. Theriogenology. 46(3), 397-412.

Doyle, E., Kahn, L., McClure, S., and Lea, J., 2011. Voluntary feed intake and diet selection of merino sheep divergently selected for genetic difference in resistance to *haemonchus contortus*. Vet. Parasitol. 177(3), 316-323.

Drobnis, E.Z., Crowe, L.M., Berger, T., Anchordoguy, T.J., Overstreet, J.W., and Crowe, J.H., 1993. Cold shock damage is due to lipid phase transitions in cell membranes: A demonstration using sperm as a model. J. Exp. Zool. 265(4), 432-437.

Dunnington, E.A., and Siegel, P.B., 1985. Long-term selection for 8-week body weight in chickens – direct and correlated responses. Theor. Appl. Genet. 71, 305-313.

Eady, S., Woolaston, R., and Barger, I., 2003. Comparison of genetic and nongenetic strategies for control of gastrointestinal nematodes of sheep. Livest. Prod. Sci. 81(1), 11-23.

Eddy, E.M., Toshimori, K., and O'Brien, D.A., 2003. Fibrous sheath of mammalian spermatozoa. Microsc. Res. Tech. 61(1), 103-115.

Evans, G., and Maxwell, W.M.C., 1987. Salamons' artificial insemination of sheep and goats. Butterworths, Sydney. pp.194.

Fields, M.J., Burns, W.C., and Warnick, A.C., 1979. Age, season and breed effects on testicular volume and semen traits in young beef bulls. J. Anim. Sci. 48(6), 1299-1304.

Foote, R., 2002. The history of artificial insemination: Selected notes and notables. J. Anim. Sci. 80(2), 1-10.

Frandson, R.D., Wilke, W., and Fails, A.D., 2003. Anatomy and physiology of farm animals. 6th ed. Lippencott Williams & Wilkins, Philadelphia, USA.

Gage, M.J.G., 1998. Mammalian sperm morphometry. Proc. R. Soc. Lond. B. 265, 97-103.

Gallego, V., Peñaranda, D.S., Marco-Jiménez, F., Mazzeo, I., Pérez, L., and Asturiano, J.F., 2012. Comparison of two techniques for the morphometry study on gilthead seabream (*Aparus aurata*) spermatozoa and evaluation of changes induced by cryopreservation. Theriogenology. 77, 1078-1087.

Ganter, M., 2008. Veterinary consultancy and health schemes in sheep: Experiences and reflections from a local German outlook. Small Rumin. Res. 76(1), 55-67.

García-Álvarez, O., Maroto-Morales, A., Martínez-Pastor, F., Garde, J.J., Ramón, M., Fernández-Santos, M.R., Esteso, M.C., Pérez-Guzmán, M.D., and Soler, A.J., 2009. Sperm characteristics and in vitro fertilization ability of thawed spermatozoa from Black Manchega ram: Electroejaculation and post-mortem collection. Theriogenology. 72, 160-168.

García-López, N., Ollero, M., Muino-Blanco, T., and Cebrián-Pérez, J., 1996. A dextran swim-up procedure for separation of highly motile and viable ram spermatozoa from seminal plasma. Theriogenology. 46(1), 141-151.

Garner, D., Thomas, C., Gravance, C., Marshall, C., De Jarnette, J., and Allen, C., 2001. Seminal plasma addition attenuates the dilution effect in bovine sperm. Theriogenology. 56(1), 31-40.

Gatti, J.L., Castella, S., Dacheux, F., Ecroyd, H., Métayer, S., Thimon, V., and Dacheux, J.L., 2004. Post-testicular sperm environment and fertility. Anim. Reprod. Sci. 82-83, 321-339.

Gbore, F.A., and Egbunike, G.N., 2008. Testicular and epididymal sperm reserves and sperm production of pubertal boars fed dietary fumonisin B1. Anim. Reprod. Sci. 105(3-4), 392-397.

Ghaoui, R.E., Gillan, L., Thomson, P.C., Evans, G., and Maxwell, W.M.C., 2007. Effect of seminal plasma fractions from entire and vasectomized rams on the motility characteristics, membrane status, and in vitro fertility of ram spermatozoa. J. Androl. 28(1), 109-122.

Gillan, L., Evans, G., and Maxwell, W., 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology. 63(2), 445-457.

Gordon, I.R., 2004. Reproductive technologies in farm animals. Cabi Pub., Oxfordshire, UK.

Gonyou, H.W., 1984. The role of behaviour in sheep production: a review of research. Appl. Anim. Ethol. 11, 341-358.

Graham, J., 1994. Effect of seminal plasma on the motility of epididymal and ejaculated spermatozoa of the ram and bull during the cryopreservation process*. Theriogenology. 41(5), 1151-1162.

Graham, E.F., Crabo, B.G., and Pace, M.M., 1978. Current status of semen preservation in the ram, boar and stallion. J. Anim. Sci. 47(2), 80-119.

Gravance, C.G., White, C., Robertson, K.R., Champion, Z.J., and Casey, P.J., 1997. The effects of cryopreservation on the morphometric dimensions of caprine sperm heads. Anim. Reprod. Sci. 49, 37-43.

Griswold, M.D., 1998. The central role of Sertoli cells in spermatogenesis. Cell. Dev. Biol. 9, 411-416.

Grubert, M.A., Mundy, C.N., and Ritar, A.J., 2005. The effects of sperm density and gamete contact time on the fertilization success of Blacklip (*Haliotis rubra*; Leach, 1814) and Greenlip (*H. laevigata*; Donovan, 1808) abalone. J. Shellfish Res. 24(2), 407-413.

Gundogan, M., Yeni, D., Avdatek, F., and Fidan, A., 2010. Influence of sperm concentration on the motility, morphology, membrane and DNA integrity along with oxidative stress parameters of ram sperm during liquid storage. Anim. Reprod. Sci. 122(3-4), 200-207.

Hafez, B., and Hafez, E.S.E., 2008. Reproduction in farm animals. 7th ed. Wiley-Blackwell, USA.

Haidl, G., Jung A., and Schill, W.B., 1996. Ageing and sperm function. Human Reprod. 11(3), 558.

Hammerstedt, R.H., Graham, J.K., and Nolan, J.P., 1990. Cryopreservation of mammalian sperm: What we ask them to survive. J. Androl. 11(1), 73-88.

Harrison, R.A., 1996. Capacitation mechanisms and the role of capacitation as seem in eutherian mammals. Reprod. Fertil. Dev. 8, 581-594.

Herman, H.A., Mitchell, J.R., and Doak, G.A., 1994. The artificial insemination and embryo transfer of dairy and beef cattle (including information pertaining to goats, sheep, horses, swine, and other animals). 8th Ed. Interstate Publishers, Inc., USA.

Hewitt, D.A., Leahy, R., Sheldon, I.M., and England, G.C.W., 2001. Cryopreservation of epididymal dog sperm. Anim. Reprod. Sci. 67(1-2), 101-111.

Hidalgo, M., Rodríguez, I., and Dorado, J.M., 2007. The effect of cryopreservation on sperm head morphometry in Florida male goat related to sperm freezability. Anim. Reprod. Sci. 100, 61-72.

Hirai, M., Boersma, A., Hoeflich, A., Wolf, E., Föll, J., Aumüller, R., and Braun, J., 2001. Objectively measured sperm motility and sperm head morphometry in boars (*Sus scrofa*): Relation to fertility and seminal plasma growth factors. J. Androl. 22(1), 104-110.

Ho, H., Granish, K.A., and Suarez, S.S., 2002. Hyperactivated motility of bull sperm is triggered at the axoneme by Ca^{2+} and not cAMP. Dev. Biol. 250(1), 208-217.

Holt, W.V., 1996. Can we predict fertility rates? Making sense of sperm motility. Reprod. Dom. Anim. 31, 17-24.

Holt, W.V, 2000. Basic aspects of frozen storage of semen. Anim. Reprod. Sci. 62(1), 3-22.

http://ahdc.vet.cornell.edu/images/linesmeartechnique.jpg. Last accessed on 7/12/2012

http://nongae.gsnu.ac.kr/~cspark/teaching/images/fig_12_2.gif Last accessed on 2/01/2014

http://www.micropticsl.com. Last accessed on 22/08/2013

Ing, R.M., Li, D.Q., Harding, A.M., and Jones, W.R., 1991. A comparison of swim-down and swim-up methods for the extraction of high motility sperm. Fertil. Steril. 55(4), 817-819.

Jameel, T., 2008. Sperm swim-up: A simple and effective technique of semen processing for intrauterine insemination. J. PMA. 58(2), 71-74.

Janett, F., Burkhardt, C., Burger, D., Imboden, I., Hässig, M., and Thun, R., 2006. Influence of repeated treadmill exercise on quality and freezability of stallion semen. Theriogenology. 65(9), 1737-1749.

Kaabi, M., Paz, P., Alvarez, M., Anel, E., Boixo, J., Rouissi, H., Herraez, P., and Anel, L., 2003. Effect of epididymis handling conditions on the quality of ram spermatozoa recovered post-mortem. Theriogenology. 60(7), 1249-1259.

Kasimanickam, R., Kasimanickam, V., Pelzer, K.D., and Dascanio, J.J., 2007. Effect of breed and sperm concentration on the changes in structural, functional and motility parameters of ram-lamb spermatozoa during storage at 4°C. Anim. Reprod. Sci. 101(1–2), 60-73.

Keck, C., Gerber-Schäfer, C., Clad, A., Wilhelm, C., and Breckwoldt, M., 1998. Seminal tract infections: Impact on male fertility and treatment options. Hum. Reprod. Update. 4(6), 891-903.

Kendall, N.R., McMullen, S., Green, A., and Rodway, R.G., 2000. The effect of a zinc, cobalt and selenium soluble glass bolus on trace element status and semen quality of ram lambs. Anim. Reprod. Sci. 62(4), 277-283.

Keskes-Ammar, L., Feki-Chakroun, N., Rebai, T., Sahnoun, Z., Ghozzi, H., Hammami, S., Zghal, K., Fki, H., Damak, J., and Bahloul, A., 2003. Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. Syst. Biol. Reprod. Med. 49(2), 83-94.

Kidd, S.A., Eskenazi, B., and Wyrobek, A.J., 2001. Effects of male age on semen quality and fertility: A review of the literature. Fertil. Steril. 75(2), 237-248.

Kilgour, R.J., 1985. Mating behaviour of rams in pens. Aust. J. Exp. Agri. 25(2), 298-305.

Kilgour, R.J., and Whale, R.G., 1980. The relation between mating activity of rams in pens and subsequent flock mating performance. Aust. J. Exp. Agric. Husb. 20, 5-8.

Kilgour, R.J., and Wilkins, J.F., 1980. The effect of serving capacity of the ram syndicate on flock fertility. Aust. J. Exp. Agric. Anim. Husb. 20, 662-666.

Kimberling, C.V., 1988. Jenson and Swift's diseases of sheep. 3rd ed. Lea & Febiger, Philadelphia, USA.

Knez, J., 2013. Endocrine-disrupting chemicals and male reproductive health. Reprod. BioMed. 26, 440-448.

Knight, T.W., 1984. Testicular growth and size in rams from flocks of different reproductive potential. NZ J. Agric. Res. 27, 179-186.

Koivisto, M., Costa, M., Perri, S., and Vicente, W., 2009. The effect of season on semen characteristics and freezability in *bos indicus* and *bos taurus* bulls in the southeastern region of Brazil. Reprod. Domest. Anim. 44(4), 587-592.

Krasnow, S.M., and Steiner, R.A., 2006. Physiological mechanisms integrating metabolism and reproduction. In: Knobil and Neill's Physiology of reproduction, 3rd ed. Ed.: J.D. Neill, Elsevier. 2553-2625.

Kuster, C. 2005., Sperm concentration determination between hemacytometric and CASA systems: Why they can be different. Theriogenology. 64(3), 614-617.

Lambrechts, H., 1996. The effect of cryopreservation on the survivability, viability and motility of Merino (*Ovis aries*) and African buffalo (*Syncerus caffer*) spermatozoa. MSc Thesis. University of Stellenbosch, South Africa.

Lambrechts, H., Van Niekerk, F., Cloete, S., Coetzer, W., and Van Der Horst, G., 2000. Sperm viability and morphology of two genetically diverse Merino lines. Reprod. Fertil. Dev. 12(6), 337-344.

Land, R.B., 1974. Physiological studies and genetic selection for sheep fertility. Anim. Breed. Abstr. 42, 155-158.

Langford, G.A., Ainsworth, L., Marcus, G.J., and Shrestha, J. N., 1987. Photoperiod entrainment of testosterone, luteinizing hormone, follicle-stimulating hormone, and prolactin cycles in rams in relation to testis size and semen quality. Biol. Reprod. 37(2), 489-499.

Lavara, R., Vicente, J.S., and Baselga, M., 2013. Genetic variation in head morphometry of rabbit sperm. Theriogenology. 80, 313-318.

Leahy, T., and Gadella, B.M., 2011. Sperm surface changes and physiological consequences induced by sperm handling and storage. Reprod. 142(6), 759-778.

Leahy, T., Evans, G., Maxwell, W., and Marti, J., 2010a. Seminal plasma proteins do not consistently improve fertility after cervical insemination of ewes with non-sorted or sex-sorted frozen–thawed ram spermatozoa. Reprod. Fert. Dev. 22(4), 606-612.

Leahy, T., Marti, J., Mendoza, N., Pérez-Pé, R., Muiño-Blanco, T., Cebrián-Pérez, J., Evans, G., and Maxwell, W., 2010b. High pre-freezing dilution improves post-thaw function of ram spermatozoa. Anim. Reprod. Sci. 119(1-2), 137-146.

Lindsay, D.R., Dunsmore, D.G., Williams, J.D., and Syme, G.J., 1976. Audience effects on the mating behaviour of rams. Anim. Behav. 24, 818-821.

Livestock Development Strategy for South Africa, 2006. Compiled by – Directorate: Animal and Aquaculture Production. Directorate: Agricultural Information Services, Private Bag X144, Pretoria 0001, South Africa.

Lone, F., Islam, R., Khan, M., and Sofi, K., 2011. Effect of transportation temperature on the quality of *cauda* epididymal spermatozoa of ram. Anim. Reprod. Sci. 123(1), 54-59.

Madan, M., 2005. Animal biotechnology: Applications and economic implications in developing countries. Rev. Sci. Tech. Off. Int. Epiz. 24(1), 127-139.

Malo, A.F., Gomendio, M., Garde, J., Lang-Lenton, B., Soler, A.J., and Roldan, E.R.S., 2006. Sperm design and sperm function. Biol. Lett. 2, 246-249.

Mandiki, S., Derycke, G., Bister, J., and Paquay, R., 1998. Influence of season and age on sexual maturation parameters of Texel, Suffolk and Ile-de-france rams: 1. testicular size, semen quality and reproductive capacity. Small Rumin. Res. 28(1), 67-79.

Mapletoft, R., and Hasler, J., 2005. Assisted reproductive technologies in cattle: A review. Rev. Sci. Tech. Off. Int. Epiz. 24(1), 393-403.

Marco-Jiménez, F., Viudes-de-Castro, M.P., Balasch, S., Mocé, E., Silvestre, M.A., Gomez, E.A., and Vicente, J.S., 2006. Morphometric changes in goat sperm heads induced by cryopreservation. Cryobiol. 52, 295-304.

Marco-Jiménez, F., Puchades, S., Gadea, J., Vicente, J., and Viudes-de-Castro, M., 2005. Effect of semen collection method on pre-and post-thaw Guirra ram spermatozoa. Theriogenology. 64(8), 1756-1765.

Maree, L., and van der Horst, G., 2013. Quantification and identification of sperm sub-populations using computer-aided sperm analysis and species-specific cut-off values for swimming speed. Biotech. Histochem. 1-13.

Maree, L., du Plessis, S.S., Menkveld, R., and van der Horst, G., 2010. Morphometric dimensions of the human sperm head depend on the staining method used. Human Reprod. 25(6), 1369-1382.

Maroto-Morales, A., Ramón, M., García-Alvarez, O., Soler, A.J., Fernández-Santos, M.R., Roldan, E.R.S., Gomendio, M., Pérez-Guzmán, M.D., and Garde, J.J., 2012. Morphometrically-distinct sperm sub-populations defined by a multistep statistical procedure in ram ejaculates: Intra- and interindividual variation. Theriogenology. 77, 1529-1539.

Maroto-Morales, A., Ramón, M., García-Alvarez, O., Soler, A.J., Esteso, M.C., Martínez-Pastor, F., Pérez-Guzmán, M.D., and Garde, J.J., 2009. Characterization of ram sperm head morphometry using the Sperm-Class Analyzer. Theriogenology. doi:10.1016/j.theriogenology.2009.10.003.

Martí, J.I., Aparicio, I.M., and García-Herreros, M., 2011a. Sperm morphometric sub-populations are differentially distributed in rams with different maturity age in cryopreserved ejaculates. Theriogenology 76, 97-109.

Martí, J.I., Aparicio, I.M., and García-Herreros, M., 2011b. Head morphometric changes in cryopreserved ram spermatozoa are related to sexual maturity. Theriogenology. 75, 473-481.

Martí, J.I., Aparicio, I.M., Leal, C.L.V., and García-Herreros, M., 2012. Seasonal dynamics of sperm morphometric sub-populations and its association with sperm quality parameters in ram ejaculates. Theriogenology. 78, 528-541.

Martínez-Pastor, F., Anel, L., Guerra, C., Álvarez, M., Soler, A.J., Garde, J.J., Chamorro, C., and de Paz, P., 2006. Seminal plasma improves cryopreservation of iberian red deer epididymal sperm. Theriogenology. 66(8), 1847-1856.

Martins, C.F., Rumpf, R., Pereira, D.C., and Dode, M.N., 2007. Cryopreservation of epididymal bovine spermatozoa from dead animals and its uses *in vitro* embryo production. Anim. Reprod. Sci. 101(3-4), 326-331.

Matás, C., Sansegundo, M., Ruiz, S., García-Vázquez, F.A., Gadea, J., Romar, R., and Coy, P., 2010. Sperm treatment affects capacitation parameters and penetration ability of ejaculated and epididymal boar spermatozoa. Theriogenology. doi:10.1016/j.theriogenology.2010.06.002.

Mathuria, N., and Verma, R.J., 2008. Curcumin ameliorates aflatoxin-induced toxicity in mice spermatozoa. Fertil. Steril. 90(3), 775-780.

Maxwell, W., De Graaf, S., Ghaoui, R.E.H., and Evans, G., 2007. Seminal plasma effects on sperm handling and female fertility. Reprod. Domest. Rumin. 6(1), 13-38.

Maxwell, W., Landers, A., and Evans, G., 1995. Survival and fertility of ram spermatozoa frozen in pellets, straws and minitubes. Theriogenology. 43(7), 1201-1210.

McAlister, D.A., 2010. A comparison of motility and head morphology of sperm using different semen processing methods and three different staining techniques. MSc. Thesis, University of Stellenbosch, South Africa.

Medeiros, C.M.O, Forell, F., Oliveira, A.T.D., and Rodrigues, J.L., 2002. Current status of sperm cryopreservation: Why isn't it better? Theriogenology. 57, 327-344.

Mehmood, A., Anwar, M., and Naqvi, S., 2009. Motility, acrosome integrity, membrane integrity and oocyte cleavage rate of sperm separated by swim-up or percoll gradient method from frozen–thawed buffalo semen. Anim. Reprod. Sci. 111(2), 141-148.

Monteiro, G.A., Papa, F.O., Zahn, F.S., Dellagua, J.A., Melo, C.M., Maziero, R.R.D., Avanzi, B.R., Alvarenga, M.A., and Guasti, P.N., 2011. Cryopreservation and fertility of ejaculated and epididymal stallion sperm. Anim. Reprod. Sci. 127(3), 197-201.

Moore, R.W., 1981. Fertilization rate of ewes mated to high and low prolificacy Romney rams. Proc. N.Z. Soc. Anim. Prod. 41, 224-228.

Moore, R.W., and Whyman, D., 1980. Fertilizing ability of semen from rams of high- and low-prolificacy flocks. J. Reprod. Fert. 59, 224-228.

Muiño-Blanco, T., Pérez-Pé, R., and Cebrián-Pérez, J.A., 2008. Seminal plasma proteins and sperm resistance to stress. Reprod. Dom. Anim. 43, 18-31.

Naqvi, S.M.K., Joshi, A., Das, G.K., and Mittal, J.P., 2001. Development and application of ovine reproductive technologies: an Indian experience. Small. Rumin. Res. 39, 199-208.

Nardone, A., Ronchi, B., Lacetera, N., Ranieri, M.S., and Bernabucci, U., 2010. Effects of climate changes on animal production and sustainability of livestock systems. Livestock Sci. 130(1), 57-69.

Neglia, G., Gasparrini, B., Caracciolo di Brienza, V., Di Palo, R., Campanile, G., Presicce, A.G., and Zicarelli, L., 2003. Bovine and buffalo in vitro embryo production using oocytes derived from abattoir ovaries or collected by transvaginal follicle aspiration. Theriogenology. 59(5), 1123-1130.

Ng, F.L., Liu, D.Y., and Baker, H.G., 1992. Comparison of percoll, mini-percoll and swim-up methods for sperm preparation from abnormal semen samples. Human Reprod. 7(2), 261-266.

Nicholas, F., 1996. Genetic improvement through reproductive technology. Anim. Reprod. Sci. 42(1), 205-214.

Ollero, M., Cebrian-Perez, J.A., and Muino-Blanco, T., 1997. Improvement of cryopreserved ram sperm heterogeneity and viability by addition of seminal plasma. J. Androl. 18(6), 732-739.

O'Meara, C., Donovan, A., Hanrahan, J., Duffy, P., Fair, S., Evans, A., and Lonergan, P., 2007. Resuspending ram spermatozoa in seminal plasma after cryopreservation does not improve pregnancy rate in cervically inseminated ewes. Theriogenology. 67(7), 1262-1268.

Overton, M.W., 2005. Cost comparison of natural service sires and artificial insemination for dairy cattle reproductive management. Theriogenology. 64, 589-602.

Parks, J., and Graham, J., 1992. Effects of cryopreservation procedures on sperm membranes. Theriogenology. 38(2), 209-222.

Parrish, J., Krogenaes, A., and Susko-Parrish, J., 1995. Effect of bovine sperm separation by either swim-up or percoll method on success of in vitro fertilization and early embryonic development. Theriogenology. 44(6), 859-869.

Parrish, J.J., and Foote, R.H., 1987. Quantification of bovine sperm separation by a Swim-up method relationship to sperm motility, integrity of acrosomes, sperm migration in polyacrylamide gel and fertility. J. Androl. 8(4), 259-266.

Pesch, S., and Bergmann, M., 2006. Structure of mammalian spermatozoa in respect to viability, fertility and cryopreservation. Micron. 37(7), 597-612.

PGoh, P., and White, I., 1988. Control of ram sperm adenylate cyclase by divalent cations. Aust. J. Biol. Sci. 41(3), 377-386.

Plas, E., Berger, P., Hermann, M., and Pflüger, H., 2000. Effects of aging on male fertility? Exp. Gerontol. 35(5), 543–551.

Pontbriand, D., Howard, J.G., Schiewe, M.C., Stuart, L.D., and Wildt, D.E., 1989. Effect of cryoprotective diluent and method of freeze-thawing on survival and acrosomal integrity of ram spermatozoa. Cryobiology. 26, 341-354.

Prathalingam, N., Holt, W., Revell, S., Jones, S., and Watson, P., 2006. Dilution of spermatozoa results in improved viability following a 24h storage period but decreased acrosome integrity following cryopreservation. Anim. Reprod. Sci. 91(1), 11-22.

Purdy, P.H., 2006. The post-thaw quality of ram sperm held for 0 to 48h at 5ºC prior to cryopreservation. Anim. Reprod. Sci. 93:114–123

Rege, J.E.O., Toe, F., Mukasa-Megerwa, E., Tembely, S., Anindo, D., Baker, R.L., and Lahlou-Kassi, A., 2000. Reproductive characteristics of Ethiopian highland sheep II. Genetic parameters of semen characteristics and their relationships with testicular measurements in ram lambs. Small Rumin. Res. 37, 173-187.

Rodríguez-Martínez, H., 2006. Can We Increase the Estimative Value of Semen Assessment? Reprod. Dom. Anim. 41(2), 2-10.

Rodríguez-Martínez, H., Saravia, F., Wallgren, M., Roca, J., and Peña, F., 2008. Influence of seminal plasma on the kinematics of boar spermatozoa during freezing. Theriogenology. 70(8), 1242-1250.

Rolf, C., Behre, H.M., and Nieschlag, E., 1996. Reproductive parameters of older compared to younger men of infertile couples. Int. J. Androl. 19(3), 135-142.

Romano, J., and Christians, C., 2009. Sperm loss using different artificial vaginas in rams. Small Rumin. Res. 83(1), 85-87.

Roser, J.F., 2008. Regulation of testicular function in the stallion: An intricate network of endocrine, paracrine and autocrine systems. Anim. Reprod. Sci. 107(3-4), 179-196.

Roser, J.F., 2001. Endocrine and paracrine control of sperm production in stallions. Anim. Reprod. Sci. 68(3-4), 139-151.

Saacke, R., 2008. Sperm morphology: Its relevance to compensable and uncompensable traits in semen. Theriogenology. 70(3), 473-478.

Saez, F., Ouvrier, A., and Drevet, J.R., 2011. Epididymis cholesterol homeostasis and sperm fertilizing ability. Asian J. Androl. 13(1), 11-17.

Salamon, S., 1973. Deep freezing of boar semen III. Effects of centrifugation, diluent and dilution rate, pellet volume, and method of thawing on survival of spermatozoa. Aust. J. Biol. Sci. 26(1), 239-248.

Salamon, S., and Maxwell, W.M.C., 2000. Storage of ram semen. Anim. Reprod. Sci. 62(1), 77-112.

Salisbury, G.W., Van Demark, N.L., and Lodge, J.R., 1978. Physiology of reproduction and artificial insemination of cattle. 2nd ed. San Francisco (& London), W.H. Freeman & Company, USA.

Santiago-Moreno, J., Astorga, R.J., Luque, I., Coloma, M.A., Toledano-Díaz, A., Pulido-Pastor, A., Gómez-Guillamon, F., Salas-Vega, R., and López-Sebastián, A., 2009. Influence of recovery method and microbial contamination on the response to freezing-thawing in ibex (*capra pyrenaica*) epididymal spermatozoa. Cryobiol. 59(3), 357-362.

SAS, 2011. Statistical Analysis Systems user's guide (AS Online Documentation 9.3). SAS Institute Inc., North Carolina, USA.

Schug, T.T., Janesick, A., Blumberg, B., and Heindel, J.J., 2011. Endocrine disrupting chemicals and disease susceptibility. J. Steriod Biochem. Mol. Biol. 127, 204-215.

Shadan, S., James, P.S., Howes, E.A., and Jones, R., 2004. Cholesterol efflux alters lipid raft stability and distribution during capacitation of boar spermatozoa. Biol. Reprod. 71(1), 253-265.

Shamsuddin, M., and Rodriguez-Martinez, H., 1994. A simple, non-traumatic swim-up method for the selection of spermatozoa for in vitro fertilization in the bovine. Anim. Reprod. Sci. 36(1), 61-75.

Simitzis, P.E., Deligeorgis, S.G., and Bizelis, J.A., 2006. Effect of breed and age on sexual behaviour of rams. Theriogenology. 65, 1480-1491.

Simpson, A.M., and White, I., 1986. Effect of cold shock and cooling rate on calcium uptake of ram spermatozoa. Anim. Reprod. Sci. 12(2), 131-143.

Sisson, S., and Grossman, J.D., 1975. Sisson and Grossman's The anatomy of the domestic animals. Saunders Press, Philadelphia, USA.

Smith, B., 2006. The farming handbook. University of KwaZulu-Natal Press, Scottsville, South Africa.

Snook, R.R., 2005. Sperm in competition: not playing by the numbers. Trends. Ecol. Evolut. 20(1), 46-53.

Suarez, S., and Pacey, A., 2006. Sperm transport in the female reproductive tract. Hum. Reprod. Update. 12(1), 23-37.

Suzuki, K., and Nagai, T., 2003. In vitro fertility and motility characteristics of frozen–thawed boar epididymal spermatozoa separated by Percoll. Theriogenology. 8, 1481-1494.

Tamayo-Canul, J., Alvarez, M., López-Urueña, E., Nicolas, M., Martinez-Pastor, F., Anel, E., and de Paz, P., 2011. Undiluted or extended storage of ram epididymal spermatozoa as alternatives to refrigerating the whole epididymis. Anim. Reprod. Sci. 126(1-2), 76-82.

Thurston, L.M., Watson, P.F., and Holt, W.V., 1999. Sources of variation in the morphological characteristics of sperm sub-populations assessed objectively by a novel automated sperm morphology analysis system. J. Reprod. Fertil. 117, 271-280.

Tilbrook, A.J., Cameron, A.W.N., and Lindsay, D.R., 1987. The influence of ram mating preferences and social interaction between rams on the proportion of ewes mated at field joining. Appl. Anim. Behav. Sci. 18, 173-184.

Tohei, A., Tomabechi, T., Mamada, M., Akai, M., Watanabe, G., and Taya, K., 1997. Effects of repeated ether stress on the hypothalamic-pituitary-testes axis in adult rats with special reference to inhibin secretion. J. Vet. Med. Sci. 59(5), 329-334.

Troedsson, M., Desvousges, A., Alghamdi, A., Dahms, B., Dow, C., Hayna, J., Valesco, R., Collahan, P., Macpherson, M., and Pozor, M., 2005. Components in seminal plasma regulating sperm transport and elimination. Anim. Reprod. Sci. 89(1), 171-186.

Tsakmakidis, I.A., Lymberopoulos, A.G., and Khalifa, T.A.A., 2010. Relationship between sperm quality traits and field-fertility of porcine semen. J. Vet. Sci. 11(2), 151-154.

Turner, T.T., and Reich, G.W., 1985. *Cauda* epididymal sperm motility: A comparison among five species. Biol. Reprod. 32, 120-128.

Van der Horst, G., and Maree, L., 2009. SpermBlue®: A new universal stain for human and animal sperm which is also amenable to automated sperm morphology analysis. Biotech. Histochem. 84(6), 299-308

Varisli, O., Uguz, C., Agca, C., and Agca, Y., 2009. Motility and acrosomal integrity comparisons between electro-ejaculated and epididymal ram sperm after exposure to a range of anisosmotic solutions, cryoprotective agents and low temperatures. Anim. Reprod. Sci. 110, 256-268.

Vera-Munoz, O., Amirat-Briand, L., Diaz, T., Vásquez, L., Schmidt, E., Desherces, S., Anton, M., Bencharif, D., and Tainturier, D., 2009. Effect of semen dilution to low-sperm number per dose on motility and functionality of cryopreserved bovine spermatozoa using low-density lipoproteins (LDL) extender: Comparison to Triladyl® and Bioxcell®. Theriogenology. 71(6), 895-900.

Verma, O., Kumar, R., Kumar, A., and Chand, S., 2012. Assisted reproductive techniques in farm animal - from artificial insemination to nanobiotechnology. Vet. World. 5(5), 301-310.

Vicente-Fiel, S., Palacín, I., Santolaria, P., Hidalgo, C.O., Silvestre, M.A., Arrebola, F., and Yániz, J.L., 2013. A comparative study of the sperm nuclear morphometry in cattle, goat, sheep, and pigs using a new computer-assisted method (CASMA-F). Theriogenology. 79, 436-442.

Vishwanath, R., 2003. Artificial insemination: The state of the art. Theriogenology. 59(2), 571-584.

Visser, D., 1974. The effect of freezing method on the survival of ram spermatozoa. S. Afr. J. Anim. Sci. 4, 157-163.

Wani, N., 2009. In vitro embryo production in camel (*camelus dromedarius*) from in vitro matured oocytes fertilized with epididymal spermatozoa stored at 4° C. Anim. Reprod. Sci. 111(1), 69-79.

Watson, P., 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60, 481-492.

Weinbauer, G.F., and Wessels, J., 1999. Paracrine control of spermatogenesis. Andralogia. 31(5), 249-262.

White, I., 1993. Lipids and calcium uptake of sperm in relation to cold shock and preservation: A review. Reprod. Ferilt. Dev. 5(6), 639-658.

Wilkins, J.F., and Kilgour, R.J., 1978. Early reproductive performance in female progeny of rams selected on serving capacity (Abstr). In: Proceedings of the 10th Annual conference on Australian Society of Reproduction Biology. p. 22.

Winfield, C.G., and Cahill, L.P., 1978. Mating competency of rams and flock fertility. Appl. Anim. Ethol. 4, 193-195.

Wulster-Radcliffe, M., Williams, M., Stellflug, J., and Lewis, G., 2001. Technical note: Artificial vagina vs. a vaginal collection vial for collecting semen from rams. J. Anim. Sci. 79(12), 2964-2967.

Yániz, J.L., Vicente-Fiel, S., Capistrós, S., Palacín, I., and Santolaria, P., 2012. Automatic evaluation of ram sperm morphometry. Theriogenology. 77, 1343-1350.

Yeung, C.H., Barfield, J.P., and Cooper, T.G., 2006. Physiological volume regulation by spermatozoa. Mol. Cell. Endocrinol. 250, 98-105.

Appendix A

(Includes information of Chapter 3)

Figure A1 *Photos that show the restrained Merino ewe (left) that was used as a dummy for semen collection with the artificial vagina (AV), and the collection procedure (right) used to obtain the ejaculate*

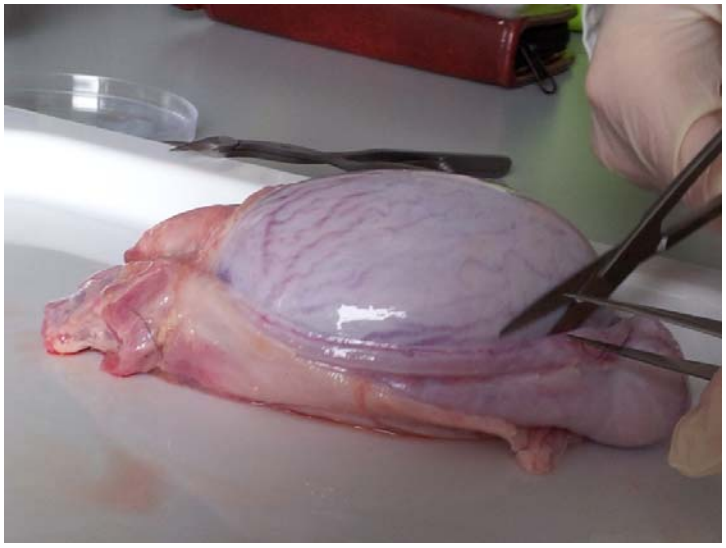

Figure A2 *After the testes were transported to the laboratory, each testis was moistened with Ham's F10 solution. The photo shows the dissection of the cauda epididymis from the entire testis*

A.1 Preparing smears for morphometric analysis

Throughout the current study, the line smear technique was used. Smears were prepared using 10µL of undiluted semen/sperm or when using swim-up sperm, using 10-15µL sperm. The amount of sperm used to make smears depended on the sperm concentration.

Starfrost® slides (Lasec, South Africa) are used, as the slide surface has been treated with a silane-coating. The coating creates an electrostatic attraction, then ensuring a better adhesion of the preferred sample to the slide.

Using a pipette, place a drop of the sperm sample is placed near the frosted end of the slide. The drop can now be spread using another slide, named a spreader slide (need not be a starfrost slide, as it is only used to spread the sperm), by dragging the spreader slide backward into the drop. The spreader slide was then placed at an angle of 45˚ when backed into the drop of sperm (the angle used can be decreased to 20º for samples with low sperm concentrations). As the slide catches the semen drop, it will spread along the edge of the slide by capillary action. The spreader slide is then pushed across the starfrost slide to the far end of the frosted side, thus pulling the sperm sample across to make the smear (see Figure A2). When the smear was made, it was placed horizontally flat in order to air-dry.

A.2 Staining procedure for SpermBlue® stain and fixative kit

SpermBlue® is distributed by Microptic, S.L. (Barcelona, Spain), and was developed by G. van der Horst and L. Maree. This staining procedure was developed to stain all components of sperm (Van der Horst & Maree, 2009). It also stains the different compartments of a sperm cell in different intensities of blue. The procedure for staining slides involves two main steps, namely the fixation of the slide in the one medium, and the staining of the slide in a second medium. Always make sure to wear disposable gloves when using this staining procedure.

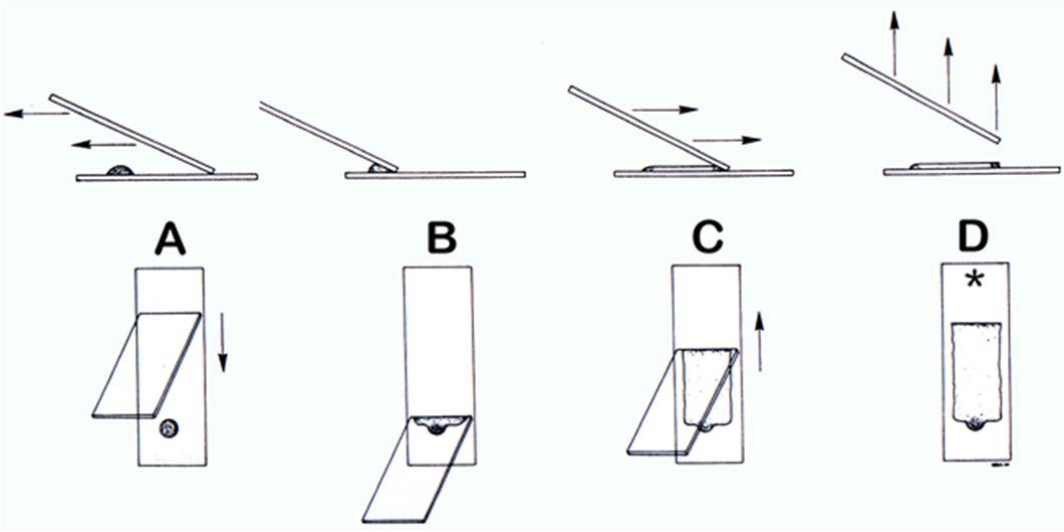

Figure A3 *Representation of spreading a smear on a frosted end microscope slide (http://ahdc.vet.cornell.edu)*

Staining procedure:

1. Make smears of samples (See “Technique for making smears”) and leave to completely air dry.
2. Place dried smears vertically into a Coplin jar containing the SpermBlue® fixative. Take care to immerse slides slowly into the fixative. Leave slides in fixative for 10 minutes at 20-25˚C.
3. Carefully remove the slides from the Coplin jar and place at 60-80˚ angle on tissue paper to dry. No washing needed.
4. After the slide has completely dried, place slides horizontally down onto filter paper after fixation. Using a plastic disposable pipette, put 0.45 - 0.5 mL of stain onto the fixed sperm smear. Make sure that the stain in displaced equally on the whole slide (see Figure A3). Leave stain on slides for 15-18 minutes.
5. After slides are left for time required, the excess stain can be removed by tilting the slide and letting the stain drain off into a separate container. Slides are then dipped into distilled water. It is important to only dip the slide once, and the dip should not exceed 3 seconds. Remove the slides very slowly from the water and leave in an upright position (angle of about 70˚). Let slide totally air dry.
6. When slides are totally dry (preferably left over night), slides can be mounted with DPX or an equivalent synthetic medium used for making permanent slides.
7. If intensity of staining is not clear enough, stain the next slides for another five minutes (or more if required).

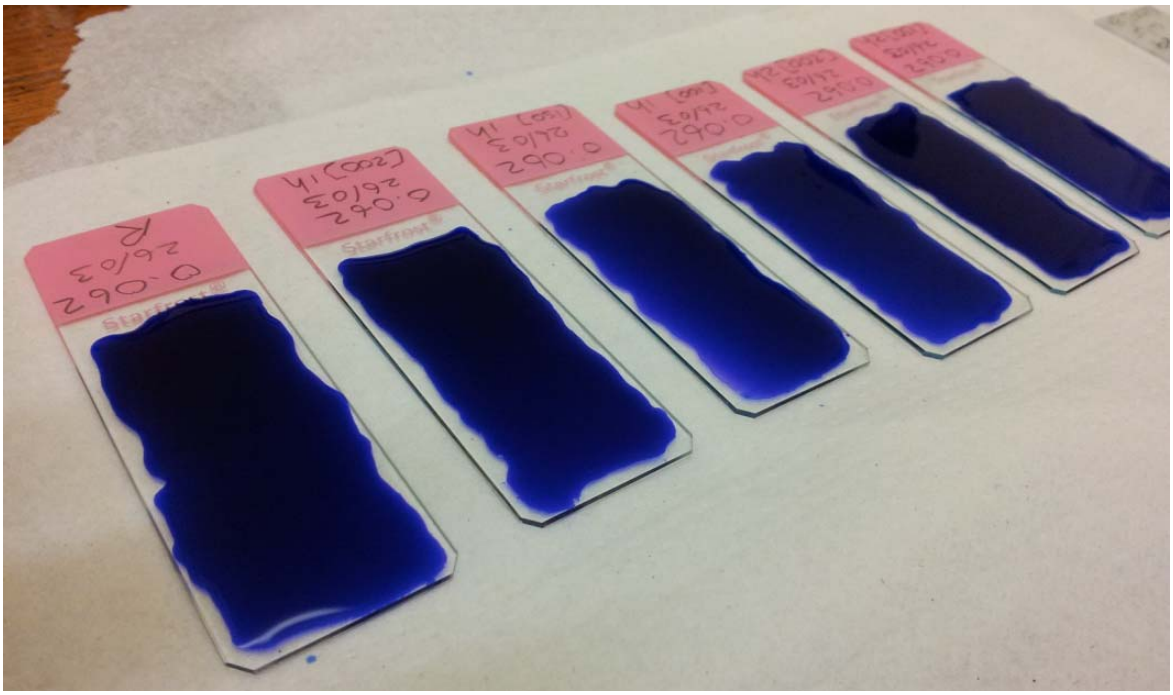

Figure A4 *This photo shows slides that have been placed in the fixative medium, air-dried and are now being stained with SpermBlue®. It also shows the equal spread of stain over the slides*

A.3 Slide mounting procedure:

After slides are stained, they are left for approximately 24 hours before mounting. There are many different mounting mediums that are used in industry, although for the purposes of this study, DPX non aqueous mounting medium for microscopy (Merck, Germany) is used.

1. Slides are placed horizontally on a flat surface covered with tissue paper.
2. A glass rod or plastic pipette can be used to dip into the DPX medium, and then drip two droplets of DPX, approximately one centimetre apart, in the middle of the slide (as shown in Figure B).
3. A long glass coverslip (22 x 50mm) is then placed gently on the two drops, in the centre of the stained slide. Due to capillary action, the DPX will then disperse under the coverslip and cover the intended area for analysis.
4. The slide is then ‘mounted’, and should be left for approximately 24 hours to completely dry. Take care not to tilt slides while DPX is drying.

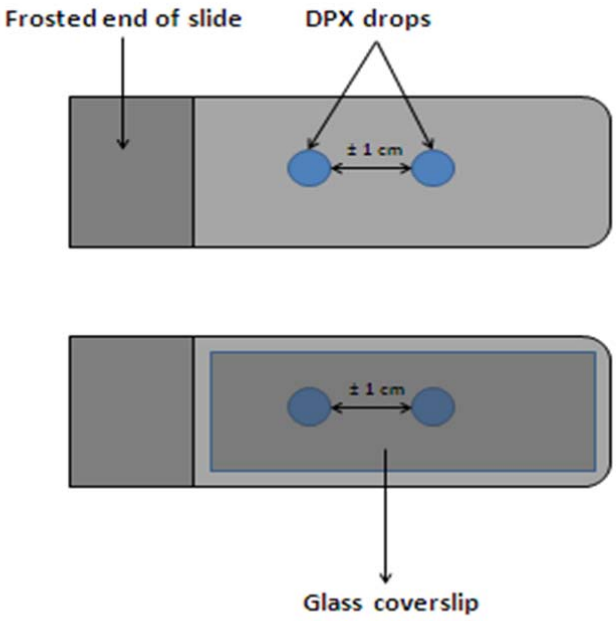

Figure A5 *This figure is a representation of the slide location where the DPX mounting medium is placed on a frosted end slide. It also indicates where the glass coverslip is placed in order to cover the optimum area for analysis with the SCA®*

A.4 Composition of Ramsem® cryopreservation medium

Due to intellectual property rights, for the exact composition of the medium, Ramsem® should be contacted.

Contact information:

RAMSEM
Tel: +27 (0) 82 900 3903/4; 051 412 6327
Fax: +27 (0) 86 554 2612
Wilgelaan 13, Roodewal
Bloemfontein, RSA
PO Box 100600, Renosterspruit,
Bloemfontein, RSA, 9326
http://www.ramsem.com

A.5 Ramsem® cryopreservation medium preparation of the final extender:

The medium is obtained from Ramsem® and is received in aliquots of 42.5 mL each. The medium is then prepared by adding 15% egg yolk, thus an addition of 7.5 mL egg yolk to the 42.5 mL stock.

In order to prepare the ready-to-use extender, egg yolk is to be separated completely from the egg white and extracted with the help of a 10 mL syringe, in order to prevent the egg yolk membrane forming part of the yolk component of the extender. The collected yolk is measured within the syringe, in order to add it to the stock solution. After addition, the solution was divided into three equal parts and stored at -20ºC until needed. Prior to use, the solution was thawed at 4ºC and pre-warmed to 37ºC prior to use as dilution medium for sperm samples.

Appendix B

(Includes information of Chapter 4)

Table B1 *The macroscopic motility scoring of semen samples collected from 15 mature Merino rams of both HL and LL rams for three collected samples via artificial vagina (AV)*

		COLLECTED SAMPLES			
LINE	**RAM ID**	**1**	**2**	**3**	**AVERAGE**
HL	0.019	4	4	4	4.0
	0.032	5	4	4	4.3
	0.232	3	5	4	4.0
	8.064	4	4	3	3.7
	8.065	4	5	5	4.67
	8.121	4	4	4	4.0
	8.166	3	3	4	3.3
	9.029	3	4	5	4.0
LL	0.022	5	4	3	4.0
	0.046	4	4	5	4.3
	0.062	5	4	4	4.3
	7.147	4	4	5	4.3
	8.013	3	3	3	3.0
	8.171	4	3	3	3.3
	8.186	4	4	4	4.0

Table B2 *The sperm concentration of semen samples (x10^6/mL) collected from 15 mature Merino ejaculates of both HL and LL rams for three collected samples each, via artificial vagina (AV)*

LINE	RAM ID	COLLECTED SAMPLES			AVERAGE
		1	2	3	
HL	0.019	4700	4960	3800	4486.7
	0.032	3250	2050	2810	2703.3
	0.232	5340	4020	2810	4056.7
	8.064	4260	5900	2630	4263.3
	8.065	1550	4190	2300	2680.0
	8.121	2000	2570	3550	2706.7
	8.166	3030	2040	2890	2653.3
	9.029	3410	8580	4895	5628.3
LL	0.022	1600	3390	2730	2573.3
	0.046	4030	5410	1990	3810.0
	0.062	3350	6320	2770	4146.7
	7.147	2790	2030	2160	2326.7
	8.013	2260	5900	3840	4000.0
	8.171	4900	2190	2500	3196.7
	8.186	6760	3000	4060	4606.7

Table B3 *The volume of semen samples (mL) collected from 15 mature Merino rams of both HL and LL rams for three collected samples via artificial vagina (AV)*

LINE	RAM ID	COLLECTED SAMPLES			AVERAGE
		1	2	3	
HL	0.019	0.5	0.75	1.1	0.78
	0.032	0.8	1	0.8	0.87
	0.232	1.2	1	0.7	0.97
	8.064	0.6	1.5	1.5	1.20
	8.065	0.75	0.6	1	0.78
	8.121	0.75	1	0.5	0.75
	8.166	0.9	0.5	1	0.80
	9.029	0.5	1	1.2	0.90
LL	0.022	0.75	1	0.8	0.85
	0.046	1.25	1.2	0.8	1.08
	0.062	1.25	1.25	0.75	1.08
	7.147	1.25	1	0.7	0.98
	8.013	0.8	0.75	0.5	0.68
	8.171	0.5	0.5	0.5	0.50
	8.186	0.8	0.75	0.9	0.82

Table B4 *The cumulative lambing rate of ewes served by rams from the HL and the LL for the lambing season of 2012*

DAY LAMBED	HL	LL	DAY LAMBED	HL	LL
1	1	182	**24**	167	182
2	5	182	**25**	170	182
3	8	182	**26**	172	182
4	17	182	**27**	175	182
5	25	182	**28**	175	182
6	35	182	**29**	176	182
7	40	182	**30**	176	182
8	49	182	**31**	176	182
9	55	182	**32**	176	182
10	61	182	**33**	176	182
11	70	182	**34**	176	182
12	84	182	**35**	176	182
13	89	182	**36**	178	182
14	94	182	**37**	180	182
15	98	182	**38**	181	182
16	106	182	**39**	181	182
17	117	182	**40**	181	182
18	135	182	**41**	182	182
19	140	182	**42**	182	182
20	146	182	**43**	182	182
21	151	182	**44**	182	182
22	155	182	**45**	182	182
23	159	182	**46**	182	182

Appendix C

(Includes information of Chapter 5)

Table C1 *The total post-thaw sperm motility (mean ± SEM), including range, observed for epididymal samples when interactions (concentration x equilibration period) were investigated*

Treatment	n	Range (CV)	Mean ± SEM
E1C1	12	0.97 – 3.61 (45.89)	1.88 ± 0.27
E1C2	12	0.43 – 5.78 (84.20)	1.91 ± 0.51
E1C3	12	0.68 – 7.83 (104.57)	2.00 ± 0.63
E2C1	12	0.52 – 3.04 (51.94)	1.55 ± 0.24
E2C2	12	0.43 – 4.59 (64.78)	2.06 ± 0.40
E2C3	12	0.44 – 3.77 (55.16)	1.65 ± 0.26
E3C1	12	0.76 – 3.37 (49.88)	1.96 ± 0.28
E3C2	12	0.74 – 8.72 (79.10)	2.78 ± 0.64
E3C3	12	0.72 – 8.95 (94.90)	2.61 ± 0.74

Table C2 *The influence of sperm concentration and equilibration duration on the range of morphometric measurements observed for fresh epididymal sperm obtained from adult Merino rams*

	C1E1	**C1E2**	**C1E3**	**C2E1**	**C2E2**	**C2E3**	**C3E1**	**C3E2**	**C3E3**
	(n=12)	(n=12)	(n=12)	(n=12)	(n=12)	(n=12)	(n=12)	(n=12)	(n=12)
Length (µm)	7.63 – 8.29	7.60 – 8.49	7.76 – 8.30	7.76 – 8.37	7.76 – 8.37	7.83 – 8.28	7.84 – 8.39	7.76 – 8.24	7.70 – 8.28
Width (µm)	4.30 – 4.64	4.18 – 4.65	4.27 – 4.58	4.31 – 4.62	4.27 – 4.70	4.27 – 4.63	4.32 – 4.62	4.28 – 4.72	4.14 – 4.55
Area (µm^2)	34.66 – 39.86	34.01 – 40.17	35.14 – 39.46	34.69 – 39.62	34.57 – 40.70	35.54 – 39.79	35.93 – 40.64	35.56 – 40.94	34.57 – 39.22
Perimeter (µm)	17.19 – 18.84	16.95 – 19.09	17.41 – 18.68	17.50 – 18.91	17.55 – 18.89	17.26 – 18.79	17.53 – 18.74	17.37 – 19.13	16.97 – 18.55
Ellipticity	1.72 – 1.87	1.76 – 1.86	1.77 – 1.87	1.74 – 1.87	1.75 – 1.88	1.76 – 1.87	1.75 – 1.87	1.73 – 1.87	1.77 – 1.87
Elongation	0.26 – 0.30	0.27 – 0.30	0.28 – 0.30	0.27 – 0.30	0.27 – 0.31	0.27 – 0.30	0.27 – 0.30	0.27 – 0.30	0.28 – 0.30
Roughness	1.35 – 1.48	1.38 – 1.49	1.39 – 1.48	1.36 – 1.51	1.36 – 1.51	1.37 – 1.50	1.37 – 1.52	1.37 – 1.55	1.37 – 1.52
Regularity	0.74 – 0.79	0.74 – 0.80	0.75 – 0.77	0.74 – 0.78	0.73 – 0.78	0.74 – 0.78	0.73 – 0.78	0.72 – 0.78	0.73 – 0.78
Acrosome coverage. (%)	53.46 – 54.85	53.35 – 54.79	53.48 – 54.81	53.89 – 55.11	53.58 – 55.07	53.85 – 54.78	53.96 – 54.76	53.37 – 55.62	53.70 – 54.98

C: Sperm concentration (C1: 100 million sperm/mL, C2: 150 million sperm/mL, and C3: 200 million sperm/mL)

E: Equilibration period (E1: 1 hour, E2: 2 hours, and E3: 3 hours

Table C3 *The influence of sperm concentration and equilibration duration on the morphometric measurements (mean ± SEM) of fresh epididymal sperm obtained from adult Merino rams*

	C1E1	C1E2	C1E3	C2E1	C2E2	C2E3	C3E1	C3E2	C3E3
	(n=12)	(n=12)	(n=12)	(n=12)	(n=12)	(n=12)	(n=12)	(n=12)	(n=12)
Length (µm)	8.10 ± 0.06	8.07 ± 0.08	8.06 ± 0.05	8.01 ± 0.05	8.02 ± 0.07	8.00 ± 0.05	8.08 ± 0.05	8.02 ± 0.05	7.97 ± 0.05
Width (µm)	4.50 ± 0.03	4.45 ± 0.04	4.45 ± 0.03	4.44 ± 0.03	4.43 ± 0.04	4.45 ± 0.03	4.47 ± 0.03	4.45 ± 0.04	4.41 ± 0.03
Area (µm^2)	37.9 ± 0.45	37.30 ± 0.59	37.22 ± 0.40	37.16 ± 0.39	37.06 ± 0.52	37.01 ± 0.35	37.55 ± 0.39	37.28 ± 0.43	36.79 ± 0.44
Perimeter (µm)	18.27 ± 0.14	18.15 ± 0.19	18.12 ± 0.11	18.06 ± 0.12	18.07 ± 0.16	17.97 ± 0.13	18.15 ± 0.12	18.09 ± 0.14	17.93 ± 0.14
Ellipticity	1.80 ± 0.01	1.81 ± 0.01	1.81 ± 0.01	1.81 ± 0.01	1.81 ± 0.01	1.80 ± 0.01	1.81 ± 0.01	1.81 ± 0.01	1.81 ± 0.01
Elongation	0.29 ± 0.00	0.29 ± 0.00	0.29 ± 0.00	0.29 ± 0.00	0.29 ± 0.00	0.29 ± 0.00	0.29 ± 0.00	0.29 ± 0.00	0.29 ± 0.00
Roughness	1.43 ± 0.01	1.43 ± 0.01	1.43 ± 0.01	1.44 ± 0.01	1.43 ± 0.01	1.45 ± 0.01	1.44 ± 0.01	1.44 ± 0.02	1.44 ± 0.01
Regularity	0.76 ± 0.00	0.76 ± 0.00	0.76 ± 0.00	0.76 ± 0.00	0.76 ± 0.00	0.76 ± 0.00	0.76 ± 0.00	0.76 ± 0.01	0.75 ± 0.00
Acrosome coverage (%)	54.29 ± 0.13	54.12 ± 0.13	54.17 ± 0.11	54.50 ± 0.12	54.19 ± 0.18	54.17 ± 0.09	54.48 ± 0.08	54.33 ± 0.17	54.37 ± 0.12

C: Sperm concentration (C1: 100 million sperm/mL, C2: 150 million sperm/mL, and C3: 200 million sperm/mL)

E: Equilibration period (E1: 1 hour, E2: 2 hours, and E3: 3 hours)

Table C4 *The influence of sperm concentration and equilibration duration on the range of morphometric measurements observed for post-thaw epididymal sperm obtained from adult Merino rams*

Morphometric parameter	C1E1	C1E2	C1E3	C2E1	C2E2	C2E3	C3E1	C3E2	C3E3
Length (µm)	7.93 – 8.55	7.81 – 8.55	7.96 – 8.49	7.87 – 8.54	7.88 – 8.24	7.88 – 8.45	7.66 – 8.65	7.86 – 8.55	7.89 – 8.38
Width (µm)	4.48 – 4.87	4.47 – 4.96	4.33 – 4.79	4.41 – 4.79	4.27 – 4.80	4.31 – 4.78	4.25 – 4.86	4.41 – 4.88	4.36 – 4.71
Area (µm^2)	39.01 – 42.47	39.07 – 43.41	38.04 – 42.32	37.90 – 43.22	36.81 – 41.57	36.79 – 41.41	36.19 – 44.10	38.28 – 42.72	37.82 – 40.52
Perimeter (µm)	18.16 – 19.46	17.95 – 19.51	17.73 – 19.30	17.71 – 19.40	17.51 – 18.81	17.69 – 19.15	17.20 – 19.61	17.73 – 19.42	17.88 – 18.96
Ellipticity	1.70 – 1.84	1.67 – 1.83	1.73 – 1.84	1.70 – 1.81	1.70 – 1.85	1.72 – 1.86	1.71 – 1.87	1.69 – 1.82	1.72 – 1.87
Elongation	0.26 – 0.30	0.25 – 0.29	0.27 – 0.30	0.26 – 0.29	0.26 – 0.30	0.26 – 0.30	0.26 – 0.30	0.26 – 0.29	0.26 – 0.30
Roughness	1.41 – 1.52	1.40 – 1.53	1.37 – 1.52	1.42 – 1.52	1.42 – 1.53	1.41 – 1.50	1.41 – 1.54	1.40 – 1.54	1.42 – 1.52
Regularity	0.74 – 0.77	0.73 – 0.77	0.72 – 0.78	0.72 – 0.77	0.72 – 0.76	0.73 – 0.76	0.71 – 0.76	0.72 – 0.77	0.73 – 0.76
Acrosome coverage (%)	53.36 – 54.36	52.92 – 54.38	53.52 – 54.41	52.86 – 54.03	52.63 – 53.89	53.48 – 54.69	53.17 – 54.45	53.24 – 54.58	53.13 – 54.60

C: Sperm concentration (C1: 100 million sperm/mL, C2: 150 million sperm/mL, and C3: 200 million sperm/mL)

E: Equilibration period (E1: 1 hour, E2: 2 hours, and E3: 3 hours)

Table C5 *The influence of sperm concentration and equilibration duration on the morphometric measurements (mean ± SEM) of post-thaw epididymal sperm obtained from adult Merino rams*

Morphometric parameter	C1E1	C1E2	C1E3	C2E1	C2E2	C2E3	C3E1	C3E2	C3E3
Length (µm)	8.20 ± 0.05	8.28 ± 0.07	8.24 ± 0.05	8.19 ± 0.07	8.04 ± 0.04	8.18 ± 0.05	8.14 ± 0.09	8.16 ± 0.06	8.11 ± 0.05
Width (µm)	4.64 ± 0.03	4.70 ± 0.04	4.66 ± 0.04	4.62 ± 0.04	4.54 ± 0.05	4.61 ± 0.03	4.58 ± 0.06	4.64 ± 0.04	4.58 ± 0.03
Area (µm^2)	40.14 ± 0.31	40.82 ± 0.42	40.36 ± 0.41	40.03 ± 0.48	39.03 ± 0.48	39.80 ± 0.39	39.81 ± 0.72	40.10 ± 0.45	39.43 ± 0.26
Perimeter (µm)	18.60 ± 0.12	18.79 ± 0.15	18.66 ± 0.14	18.55 ± 0.16	18.18 ± 0.12	0.00 ± 0.13	18.43 ± 0.23	18.51 ± 0.16	18.38 ± 0.10
Ellipticity	1.77 ± 0.01	1.76 ± 0.02	1.77 ± 0.01	1.77 ± 0.01	1.77 ± 0.02	1.78 ± 0.01	1.78 ± 0.01	1.76 ± 0.01	1.77 ± 0.01
Elongation	0.28 ± 0.00	0.28 ± 0.00	0.28 ± 0.00	0.28 ± 0.00	0.28 ± 0.00	0.28 ± 0.00	0.28 ± 0.00	0.28 ± 0.00	0.28 ± 0.00
Roughness	1.46 ± 0.01	1.46 ± 0.01	1.46 ± 0.01	1.47 ± 0.01	1.49 ± 0.01	1.46 ± 0.01	1.48 ± 0.02	1.48 ± 0.01	1.47 ± 0.01
Regularity	0.75 ± 0.00	0.75 ± 0.00	0.75 ± 0.00	0.75 ± 0.00	0.74 ± 0.00	0.75 ± 0.00	0.74 ± 0.01	0.74 ± 0.00	0.74 ± 0.00
Acrosome Coverage (%)	53.77 ± 0.09	53.69 ± 0.12	53.90 ± 0.07	53.51 ± 0.12	53.48 ± 0.12	53.92 ± 0.09	53.78 ± 0.14	53.83 ± 0.11	53.90 ± 0.14

C: Sperm concentration (C1: 100 million sperm/mL, C2: 150 million sperm/mL, and C3: 200 million sperm/mL)

E: Equilibration period (E1: 1 hour, E2: 2 hours, and E3: 3 hours)

Appendix D

(Includes information of Chapter 7)

Table D1 *All motility parameters of fresh ejaculated sperm obtained from adult Merino rams, evaluated for motility with the SCA®, using either the swim-up technique (SUT) or the flush technique (FT)*

RAM No.	METHOD	NON. PROG. MOT.	PROG. MOT.	TOT. MOT	STAT	RAPID	MEDIUM	SLOW	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
1	FT	42.26	35.47	77.74	22.26	50.94	20.00	6.79	249.59	105.25	140.99	42.17	74.65	56.49	2.77	42.73
	FT	45.42	35.97	81.38	18.62	54.02	20.59	6.77	240.64	101.88	140.11	42.34	72.71	58.22	2.92	39.45
	FT	44.99	48.06	93.05	6.95	62.78	28.02	2.25	238.96	111.92	151.19	46.84	74.03	63.27	2.58	42.04
	SUT	41.11	36.30	77.41	22.59	57.78	14.07	5.56	256.91	107.41	148.04	41.81	72.55	57.62	2.81	45.60
	SUT	60.87	30.69	91.56	8.44	76.09	11.51	3.96	265.60	97.70	149.47	36.79	65.36	56.28	3.28	42.07
	SUT	36.11	37.86	73.96	26.04	40.70	26.26	7.00	229.29	102.36	134.01	44.64	76.38	58.45	2.60	44.37
2	FT	33.13	49.48	82.61	17.39	67.70	8.90	6.00	317.12	139.35	173.97	43.94	80.10	54.86	4.16	53.30
	FT	26.42	58.49	84.91	15.09	56.37	24.76	3.77	250.99	134.70	159.11	53.67	84.66	63.39	2.82	46.60
	FT	44.32	41.35	85.67	14.33	52.39	26.52	6.75	207.69	99.51	131.34	47.91	75.77	63.24	2.55	43.35
	SUT	27.55	51.32	78.87	21.13	70.19	4.15	4.53	313.14	129.59	164.13	41.39	78.96	52.41	4.37	43.49
	SUT	18.86	29.39	48.25	51.75	26.32	14.04	7.89	201.82	106.41	126.41	52.73	84.18	62.64	2.62	49.41
	SUT	43.98	50.21	94.19	5.81	73.03	12.86	8.30	251.51	113.50	148.67	45.13	76.34	59.11	3.18	48.76
3	FT	44.91	51.70	96.61	3.39	70.45	24.20	1.96	232.42	123.79	158.66	53.26	78.02	68.26	2.31	44.52
	FT	26.07	71.78	97.85	2.15	80.37	15.64	1.84	232.65	139.63	165.01	60.02	84.62	70.93	2.32	49.28
	FT	56.58	33.74	90.32	9.68	66.72	17.40	6.20	267.33	103.60	152.53	38.75	67.92	57.06	3.21	41.16
	SUT	42.92	47.28	90.20	9.80	64.65	21.73	3.83	221.33	108.44	144.08	49.00	75.26	65.10	2.64	45.95
	SUT	24.57	55.02	79.58	20.42	65.05	11.76	2.77	250.45	138.44	167.29	55.28	82.75	66.80	2.77	50.87
	SUT	38.60	48.68	87.28	12.72	71.49	11.40	4.39	286.68	127.78	162.06	44.57	78.84	56.53	3.37	43.81
4	FT	45.96	41.15	87.12	12.88	49.81	28.08	9.23	198.47	90.22	121.61	45.46	74.19	61.27	2.44	44.16
	FT	31.91	56.58	88.49	11.51	68.75	13.49	6.25	237.17	117.16	148.10	49.40	79.11	62.45	3.01	48.22

RAM No.	METHOD	NON. PROG. MOT.	PROG. MOT.	TOT. MOT	STAT	RAPID	MEDIUM	SLOW	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
4	FT	65.04	31.59	96.63	3.37	74.93	16.24	5.46	297.79	103.26	160.37	34.68	64.39	53.85	3.79	40.37
	FT	50.08	18.50	68.57	31.43	16.09	35.19	17.29	139.00	46.91	73.71	33.75	63.64	53.03	2.31	27.37
	SUT	26.86	33.47	60.33	39.67	37.19	14.88	8.26	228.78	104.95	134.07	45.88	78.28	58.60	2.99	44.10
	SUT	30.59	51.60	82.19	17.81	69.41	5.94	6.85	292.92	141.16	173.26	48.19	81.47	59.15	3.79	50.11
	SUT	59.79	35.05	94.85	5.15	77.66	13.40	3.78	318.52	122.41	169.14	38.43	72.37	53.10	3.51	45.18
5	FT	33.57	61.37	94.95	5.05	86.46	6.50	1.99	271.56	151.24	187.89	55.69	80.49	69.19	2.66	50.66
	FT	39.96	47.15	87.11	12.89	63.72	17.13	6.26	242.87	118.76	150.82	48.90	78.74	62.10	2.95	46.96
	FT	47.57	43.69	91.26	8.74	73.79	14.56	2.91	284.44	107.29	143.97	37.72	74.52	50.62	3.29	48.22
	SUT	27.27	63.29	90.56	9.44	77.62	8.92	4.02	276.55	145.19	174.98	52.50	82.97	63.27	3.14	50.18
	SUT	34.76	51.87	86.63	13.37	78.07	5.88	2.67	289.67	131.05	169.12	45.24	77.49	58.38	3.15	47.29
	SUT	63.84	27.40	91.24	8.76	68.08	14.12	9.04	293.45	95.58	145.65	32.57	65.63	49.63	3.87	37.71
6	FT	53.96	32.59	86.54	13.46	27.57	50.40	8.58	166.55	73.77	106.09	44.29	69.54	63.70	2.15	37.33
	FT	75.14	18.99	94.13	5.87	69.55	21.04	3.54	263.44	88.22	147.07	33.49	59.99	55.83	3.29	31.96
	SUT	35.08	47.12	82.20	17.80	43.72	27.75	10.73	185.88	93.17	120.24	50.12	77.48	64.69	2.50	42.39
	SUT	22.75	50.26	73.02	26.98	50.26	14.29	8.47	252.95	126.40	148.50	49.97	85.12	58.71	3.37	44.64
	SUT	50.86	38.01	88.87	11.13	55.31	25.51	8.05	231.99	98.40	136.96	42.41	71.85	59.04	2.79	38.96
7	FT	27.98	65.73	93.71	6.29	78.15	13.08	2.48	269.57	149.28	182.27	55.37	81.90	67.61	2.54	52.77
	FT	54.76	37.82	92.58	7.42	67.99	20.02	4.57	235.50	96.49	138.74	40.97	69.55	58.91	3.23	39.87
	FT	23.48	68.41	91.88	8.12	80.58	8.70	2.61	298.27	158.59	188.41	53.17	84.18	63.17	2.89	53.65
	FT	36.32	55.38	91.70	8.30	66.59	22.42	2.69	218.42	118.82	144.59	54.40	82.18	66.20	2.70	45.28
	SUT	11.34	79.83	91.18	8.82	82.35	4.62	4.20	342.65	186.90	205.46	54.55	90.97	59.96	3.57	59.69
	SUT	18.92	67.57	86.49	13.51	70.72	10.81	4.95	284.03	150.18	172.62	52.87	87.00	60.77	3.51	49.85
	SUT	20.08	70.29	90.38	9.62	71.97	13.39	5.02	274.82	151.70	173.16	55.20	87.61	63.01	3.14	54.02
	SUT	19.75	60.91	80.66	19.34	67.90	9.05	3.70	277.96	147.81	172.15	53.18	85.86	61.93	3.42	53.01
8	FT	37.63	12.47	50.10	49.90	7.44	31.59	11.07	123.27	44.00	67.31	35.70	65.37	54.60	2.09	27.15
	FT	35.62	58.58	94.20	5.80	34.04	57.26	2.90	177.39	94.50	117.08	53.27	80.71	66.00	2.17	39.20

RAM No.	METHOD	NON. PROG. MOT.	PROG. MOT.	TOT. MOT	STAT	RAPID	MEDIUM	SLOW	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
8	FT	69.53	13.13	82.66	17.34	51.52	22.90	8.25	224.63	62.25	109.79	27.71	56.70	48.88	2.97	30.20
	SUT	69.91	5.23	75.14	24.86	15.86	49.55	9.73	141.83	46.28	93.15	32.63	49.68	65.68	2.33	18.08
	SUT	35.04	51.09	86.13	13.87	27.37	55.84	2.92	170.55	78.03	102.00	45.75	76.51	59.80	2.26	35.20
	SUT	64.80	8.67	73.47	26.53	32.65	24.49	16.33	191.59	45.48	96.45	23.74	47.15	50.34	2.37	29.67
9	FT	36.49	42.73	79.21	20.79	57.27	14.55	7.39	267.38	123.25	155.20	46.10	79.41	58.05	3.34	51.52
	FT	60.47	35.86	96.33	3.67	74.91	17.99	3.43	306.50	114.90	165.23	37.49	69.54	53.91	4.03	46.92
	FT	44.40	49.50	93.91	6.09	75.12	15.67	3.11	295.86	128.32	170.33	43.37	75.34	57.57	3.64	48.65
	SUT	22.69	73.53	96.22	3.78	80.67	14.29	1.26	243.44	123.01	147.73	50.53	83.27	60.68	3.28	41.35
	SUT	41.91	47.19	89.11	10.89	74.92	9.24	4.95	317.07	128.52	167.43	40.53	76.76	52.81	4.20	47.07
	SUT	29.07	59.91	88.99	11.01	82.38	4.41	2.20	389.61	169.65	205.05	43.54	82.73	52.63	4.78	51.32
10	FT	78.18	14.78	92.96	7.04	45.02	42.44	5.50	189.66	49.49	101.70	26.09	48.66	53.62	2.66	37.05
	FT	47.82	47.82	95.64	4.36	79.80	13.37	2.47	329.41	143.94	191.70	43.70	75.09	58.20	3.63	53.69
	FT	26.05	70.17	96.22	3.78	77.73	18.07	0.42	294.51	148.59	176.75	50.45	84.06	60.02	3.30	50.35
	SUT	26.02	65.04	91.06	8.94	76.42	8.94	5.69	334.99	146.73	178.89	43.80	82.02	53.40	4.52	48.44
	SUT	27.16	54.63	81.79	18.21	63.58	11.82	6.39	309.21	138.59	169.34	44.82	81.84	54.76	3.97	50.67
	SUT	26.98	46.05	73.02	26.98	57.67	9.30	6.05	294.52	132.49	166.49	44.98	79.58	56.53	3.80	47.29
11	FT	36.96	57.12	94.08	5.92	72.96	18.72	2.40	263.40	137.67	173.29	52.26	79.44	65.79	2.68	49.64
	FT	47.70	47.02	94.72	5.28	69.51	21.68	3.52	255.79	115.59	153.96	45.19	75.08	60.19	3.15	47.47
	FT	28.36	63.27	91.64	8.36	61.45	24.36	5.82	254.72	140.09	166.30	55.00	84.24	65.29	2.71	53.20
	SUT	50.42	38.72	89.14	10.86	52.92	27.30	8.91	208.28	90.36	126.38	43.39	71.50	60.68	3.00	40.64
	SUT	34.95	50.97	85.92	14.08	65.53	14.56	5.83	324.07	138.27	174.95	42.67	79.04	53.98	3.62	51.55
	SUT	23.73	61.86	85.59	14.41	69.07	13.98	2.54	283.48	143.84	173.15	50.74	83.07	61.08	3.36	50.89
12	FT	38.06	55.39	93.45	6.55	71.08	18.96	3.41	268.55	137.25	174.36	51.11	78.72	64.93	2.80	50.12
	FT	57.50	31.84	89.35	10.65	66.32	16.49	6.53	268.75	104.66	152.70	38.94	68.54	56.82	3.25	41.19
	FT	21.98	65.68	87.67	12.33	70.24	13.40	4.02	229.92	125.38	147.63	54.53	84.93	64.21	2.96	44.75
	SUT	41.95	40.65	82.60	17.40	58.57	15.45	8.57	246.18	115.62	150.69	46.96	76.72	61.21	3.09	46.41

RAM No.	METHOD	NON. PROG. MOT.	PROG. MOT.	TOT. MOT	STAT	RAPID	MEDIUM	SLOW	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
12	SUT	22.52	57.63	80.15	19.85	56.87	15.65	7.63	252.88	125.55	146.03	49.65	85.97	57.75	3.28	45.60
	SUT	17.12	71.47	88.59	11.41	69.29	16.58	2.72	240.00	128.86	152.76	53.69	84.36	63.65	3.10	44.74
13	FT	41.85	48.40	90.25	9.75	57.53	28.15	4.57	260.17	131.23	166.30	50.44	78.91	63.92	2.76	50.46
	FT	53.47	45.31	98.78	1.22	68.88	27.76	2.14	229.69	113.76	157.38	49.53	72.29	68.52	2.33	41.94
	FT	28.68	61.95	90.63	9.37	75.72	10.52	4.40	301.87	168.14	201.20	55.70	83.57	66.65	2.66	56.98
	SUT	53.35	34.94	88.28	11.72	48.12	29.39	10.77	199.03	86.57	122.94	43.50	70.42	61.77	2.68	42.23
	SUT	16.78	57.34	74.13	25.87	59.44	9.44	5.24	283.60	154.96	175.14	54.64	88.47	61.76	3.06	55.96
	SUT	21.01	69.47	90.48	9.52	76.75	11.20	2.52	318.56	166.61	189.49	52.30	87.92	59.48	3.48	59.23
14	FT	35.01	49.16	84.17	15.83	46.04	34.77	3.36	207.76	103.78	134.21	49.95	77.32	64.60	2.63	40.80
	FT	27.98	65.17	93.15	6.85	63.71	26.18	3.26	241.62	132.82	160.04	54.97	82.99	66.24	2.64	46.47
	FT	61.26	30.31	91.57	8.43	63.27	24.65	3.65	257.94	90.40	144.09	35.05	62.74	55.86	2.92	38.21
	SUT	15.59	42.03	57.63	42.37	33.22	16.27	8.14	187.30	110.52	127.83	59.00	86.45	68.25	2.42	44.14
	SUT	13.39	77.17	90.55	9.45	73.23	15.49	1.84	224.53	132.45	152.15	58.99	87.05	67.76	2.83	45.90
	SUT	75.52	19.86	95.38	4.62	81.06	9.70	4.62	296.66	86.69	159.53	29.22	54.34	53.78	3.15	36.18
15	FT	78.09	9.39	87.48	12.52	53.68	26.92	6.89	224.88	61.13	124.66	27.18	49.04	55.43	3.15	35.44
	FT	50.62	37.39	88.01	11.99	55.91	25.57	6.53	209.23	87.15	127.88	41.65	68.15	61.12	2.70	43.11
	FT	30.70	62.98	93.68	6.32	69.98	16.70	7.00	244.25	131.99	157.88	54.04	83.60	64.64	2.77	52.36
	SUT	81.89	7.82	89.71	10.29	79.84	4.53	5.35	331.06	85.29	170.27	25.76	50.09	51.43	3.45	39.25
	SUT	63.50	33.74	97.24	2.76	74.61	19.71	2.92	252.21	98.97	150.81	39.24	65.63	59.80	3.01	43.43
	SUT	39.22	56.26	95.48	4.52	70.64	21.36	3.49	295.48	133.51	167.07	45.18	79.91	56.54	3.63	49.65