A comparison of Merino and Dormer rams in terms of mating dexterity and sperm subpopulations' characteristics

by

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Summary

Assisted reproductive techniques (ART's) play an increasingly important role in sheep farming systems to ensure the viability and cost-efficiency of production. Sperm quality is a major determinant of the successful application of ART's, and therefore it is important to understand the factors that affect the viability and fertilizing ability of sperm. This study aimed to determine the influence of breed and genetic selection for reproduction potential on mating dexterity, sperm morphometric subpopulation characteristics and fertilizing ability. The technique used to collect semen samples can influence the quality of the sample, with the artificial vagina (AV) method yielding better samples than those collected by means of electro-ejaculation (EE). The use of the AV method requires the prior training of rams, and to date no standard operational procedure (SOP) has been formulated for the training of rams to use the AV. During the training of rams to use the AV, both inexperienced and experienced Dormer rams found mature Dormer ewes more attractive than yearling Dormers ewes, and in the training sessions the Dormer rams did not discriminate between Dormer and Merino ewes (in oestrus), that were used as teaser ewes to stimulate a sexual response in the rams. In contrast, Merino rams in this study were less discriminatory in their choice for either mature or yearling Merino ewes, with experienced Merino rams exhibiting a definite preference for a Merino teaser ewe. There was no conclusive evidence of a breed preference in inexperienced Merino rams. Breed and degree of sexual experience did not influence ease of habituation of a ram to the presence of the semen collector and/or assisting staff. Rams could be habituated within approximately 4 weeks and during a minimum of 8 training sessions when trained by experienced personnel. A higher frequency of training, i.e. 18 training sessions during this 4-week period will result in a more established baseline behaviour that will indicate whether a ram could be successfully trained to use the AV. There was no conclusive evidence that experienced Merino or Dormer rams ejaculated into the AV more readily, when compared to the Dormer and Merino inexperienced rams. It has to be noted that only 50% of the experienced Dormer rams could be successfully trained to use the AV, compared to 90% of the experienced Merino rams. Of the inexperienced rams only 40% of both the Merino and Dormer breeds could be trained to use the AV. Four distinct sperm morphometric subpopulations were identified in semen samples obtained from Dormer and Merino [High reproduction potential line (HL) and Low reproduction potential line (LL)] rams in this study. No significant differences were reported between the breeds in terms of ejaculate sperm subpopulation structure. The sperm subpopulation analysis of the HL and LL ejaculates indicated minor but non-significant differences between certain subpopulations. Breed or genetic selection had no influence on most post-thaw sperm parameters, except for post-thaw sperm viability that differed between HL and LL rams. A significant difference was observed between the sperm binding capacity of Dormer and Merino sperm. Sperm obtained from HL rams tended to have a better binding capacity than sperm

obtained from the LL rams. No conclusive evidence of a correlation between sperm binding capacity and any sperm morphometric subpopulation was obtained. In conclusion, the factors contributing to the difficulty of training experienced Dormer rams, as well as inexperienced Dormer and Merino rams, to use the AV warrants further investigation. Future studies should further investigate the influence of breed and genetic selection on sperm subpopulation traits. Additional research to clarify the relationship between sperm subpopulations traits and the potential role of sperm competition in the determining the fertilizing potential of sperm, is warranted.

Opsomming

Ondersteunende reproduksietegnieke (ORT) speel 'n toenemend belangrike rol in skaapboerdery stelsels om die lewensvatbare en kostedoeltreffende produksie te verseker. Sperm gehalte is 'n belangrike bepalende faktor vir die suksesvolle toepassing van ORT en daarom is dit belangrik om die faktore wat die lewensvatbaarheid en bevrugtingsvermoë van sperme beïnvloed, te verstaan. Die studie was daarop gemik om die invloed van ras en genetiese seleksie vir reproduksiepotensiaal op dekbehendigheid, sperm morfometriese subpopulasie eienskappe en bevrugtingsvermoë te bepaal. Die tegniek wat gebruik word om semen monsters in te samel kan die kwaliteit van die monster beïnvloed, met die kunsvagina (KV) metode wat beter monsters lewer wanneer dit met die elektro-ejakulasie (EE) metode vergelyk word. Die gebruik van die KV metode vereis dat ramme vooraf opgelei moet word en tans is daar geen standaard operasionele prosedure (SOP) geformuleer vir die opleiding van ramme om die KV te gebruik nie. Tydens die opleiding van ramme om die KV te gebruik, is gevind dat beide onervare en ervare Dormer ramme volwasse Dormer ooie meer aantreklik gevind het as jaaroud Dormer ooie. Die Dormer ramme het ook nie tussen Dormer en Merino koggelooie (in estrus) tydens die opleidingsessies gediskrimineer nie. In teenstelling hiermee het die Merino ramme nie tussen óf volwasse of jaaroud en Merino ooie gediskrimineer nie. Ervare Merino ramme het ’n duidelike voorkeur vir Merino koggelooie gehad, in teenstelling met die onervare Merino ramme wat nie ’n voorkeur vir onervare of ervare Merino ooie getoon het nie. Seleksie en mate van seksuele ervaring het geen invloed gehad op die gewoondmaak van die ram aan die teenwoordigheid van die semenkollekteerder en/of ondersteuningpersoneel nie. Ramme kan binne ongeveer 4 weke gewoond gemaak word aan die teenwoordigheid van die semenkollekteerder, wanneer opgelei deur ervare personeel, met 'n minimum van 8 opleidingsessies. 'n Hoër frekwensie van blootstelling, d.i. 18 opleidingsessies gedurende hierdie 4-week periode, sal lei tot 'n meer gevestigde basislyn gedrag wat sal aandui of 'n ram suksesvol opgelei kan word om die KV gebruik. Daar was geen afdoende bewys dat ervare Merino of Dormer ramme meer geredelik in die KV ejakuleer het nie, in vergelyking met die onervare Dormer- en Merino ramme. Dit moet genoem word dat slegs 50% van die ervare Dormer ramme suksesvol opgelei kon word om die KV te gebruik, in vergelyking met 90% van die ervare Merino ramme. Wat die onervare ramme betref, kon slegs 40% van beide die Merino en Dormer rasse opgelei word om die KV te gebruik. Vier afsonderlike sperm subpopulasies is geïdentifiseer in Dormer en Merino semen monsters versamel in hierdie studie. Geen beduidende verskille is aangemeld tussen die rasse in terme van die struktuur van die sperm subpopulasies nie. Die ontleding van die sperm subpopulasies in die semen monsters versamel van die HL en LL Merino ramme het klein maar nie-beduidende verskille tussen sekere subpopulasies uitgewys. Ras of genetiese seleksie het geen invloed op die meeste na-ontdooiing sperm parameters gehad nie. Die uitsondering was na-ontdooiing sperm lewensvatbaarheid, wat betekenisvol tussen HL en LL

ramme verskil het. ‘n Beduidende verskil is waargeneem tussen die bindingsvermoë van Dormer- en Merino sperme, in vergelyking met sperme van die HL ramme wat geneig het om ‘n beter bindingsvermoë as dié van die LL ramme te hê. Geen afdoende bewys van 'n korrelasie tussen die bindingsvermoë en sperm morfometriese subpopulasies is gevind nie. Ten slotte, die faktore wat bydra tot die probleme wat met die opleiding van die Dormer ramme asook onervare Merino ramme om die KV te gebruik, ondervind is, benodig verdere ondersoek. Toekomstige studies behoort verdere ondersoek in te stel na die invloed van ras en genetiese seleksie op sperm subpopulasie eienskappe. Bykomende navorsing om die verhouding tussen die sperm sub-jpopulasie eienskappe en die potensiële rol van sperm kompetisie in die bepaling van die bevrugtingspotensiaal van sperme, word ook benodig.

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Abbreviations

AI	Artificial Insemination
ANOVA	Analysis of Variance
ART's	Assisted Reproductive Techniques
ATP	Adenosine Triphosphate
AV	Artificial Vagina
BCS	Body Condition Score
˚C	Degrees Celsius
cAMP	Cyclic Adenosine Monophosphate
CASA	Computer Assisted Semen Analysis
cm	Centimetre
COC	Cumulus Oocyte Complexes
DAFF	Department of Agriculture, Forestry and Fisheries
DPBS	Dulbecco's Phosphate Buffered Saline
EE	Electro-Ejaculation
FGA	Fluorogestone Acetate
FSH	Follicle Stimulating Hormone
GPS	Global Positioning System
h	Hour
HF	High Frequency
HL	High Line
IVC	*In Vitro* Culture
IVEP	*In Vitro* Embryo Production
IVF	*In Vitro* Fertilization
IVM	*In Vitro* Medium

JIVET	Juvenile *In Vitro* Embryo Transfer
LF	Low Frequency
LH	Luteinising Hormone
LL	Low Line
LOPU	Laparoscopic Ovum Pick-Up
LSM	Least Square Means
m	Meter
min	Minute
MOET	Multiple Ovulation and Embryo Transfer
PC	Principle Component
PCA	Principle Component Analysis
PVC	Polyvinyl Chloride
PVM	Perivitelline Membrane
r	Pearson Correlation Coefficient
SA	South Africa
SE	Standard Error
SOP	Standard Operating Procedure
SP	Subpopulation
UHT	Ultra High Temperature
ZP	*Zona Pellucida*
µL	Microliter
µm	Micrometre

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Chapter 1

General Introduction

Livestock production plays an essential role in the South African agricultural industry (Spies, 2011). In the recent Abstract of Agricultural Statistics (2015) it was reported that livestock production contributed 46% to the gross domestic income of agricultural production. Poultry products contributed the highest percentage (50%) to gross livestock production, followed by beef and veal (24%), fresh milk (13%), sheep and goat meat (6%), pig meat (4%), and small stock fibres (3%). The small stock industry thus contributed 8-10 % of the annual income generated from animal products, where meat (60.6%), wool (31.4%), mohair (7.9%) and karakul pelts (0.2%) all contribute to the income generated (Cloete & Olivier, 2010; Schoeman *et al.*, 2010).

Currently there are 8000 commercial and 5800 communal sheep famers in South Africa, with an estimated sheep population of 21.4 million sheep, and 20 identified breeds. This makes sheep the most common livestock species in numbers, and also an important supplier to global food and fibre industries (Cloete & Olivier, 2010; Amiridis & Cseh, 2012). Overall in South Africa, the highest number of sheep (29%) are found in the Eastern Cape Province, followed by the Northern Cape with 25%, the Free State (20%) and the Western Cape (11%) (Abstract of Agricultural Statistics, 2013; DAFF, 2014).

South Africa has limited agricultural potential, as most of the land is located in arid and semi-arid regions (Cloete & Olivier, 2010). Therefore, more than 80% of agricultural land, covering approximately 71.9 million hectares, is suitable for extensive livestock production only (Livestock Development Strategy for South Africa, 2006). The arid areas are characterized by a low rainfall and poor soil fertility, culminating in a low carrying capacity. Most of the western and central parts of South Africa have a grazing capacity below one livestock unit per 12 hectares. Nevertheless, sheep farming can be sustainable in these arid areas where no other agricultural activities are considered to be viable (Cloete & Olivier, 2010).

It is expected that the global population will grow to an estimated 9.5 billion people by 2050, thus the demand for animal products will increase rapidly in the near future (Thornton, 2010). This increase in the demand for food places sheep farmers under pressure to incorporate management practices and management tools that will allow them to optimise their production practices in a quest to produce mutton more cost-efficiently to meet the increased demand. To assist the small stock industry to increase their livestock production efficiency, sheep farmers can make use of different management tools such as assisted reproductive techniques (ART's) to ensure optimal and cost-efficient production. 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.

The seasonal nature of reproduction in sheep is a major limitations that can affect optimal production, and determine the success of the industry to contribute to food security (Jooste & van Schalkwyk, 2001; Cloete & Olivier, 2010; Nardone *et al.,* 2010). Out-of-season breeding has become a common strategy to increase the supply of product to the marketplace on a year-round basis, and it is assumed that constant production of lamb and mutton will have a positive impact on the viability of sheep production systems (Deveson *et al.,* 1992). The limitation of seasonal breeding can be circumvented by incorporating ART's such as AI and in vitro embryo production (IVEP) into flock management programs.

One of the most important determinants of the success of AI and IVEP is the quality of sperm used in these ART's. It is therefore important to understand factors influencing sperm quality, as well as to how management, the environment the animal is maintained in, and processing can impact on the eventual quality of sperm used for AI or IVEP purposes (Colas, 1983). The method of semen collection can also have an influence on sperm quality. It is commonly known that semen samples collected by means of the artificial vagina method (AV) are superior to samples obtained by means of electro-ejaculation. The former method however, requires the training of rams. Due to the time required for training, this method is not commonly being used in industry when rams are evaluated for breeding soundness (Marco-Jimenez *et al.*, 2005; Palmer, 2005). Recently an increased interest has been demonstrated by sheep producers in the ability of rams to be trained to use the AV, especially where consortiums buy a ram of top genetic merit. There is no formal training protocol or standard operating procedure (SOP) available in South Africa on how to train rams to ejaculate into an AV at present.

The processing of sperm samples for liquid storage or long-term storage can adversely affect the viability and fertilizing competence of ejaculated or epididymal sperm (Salamon & Maxwell, 1995a). Although sperm processing has a damaging effect on sperm viability and fertilizing ability, not much is known on the influence of genetic selection for prolificacy or crossbreeding on sperm quality. In a recent study Boshoff (2014) investigated the impact of selection for prolificacy in two divergently selected lines, which resulted in the establishment of a High line (HL) and a Low line (LL). In the study of Boshoff (2014), it was reported that HL and LL rams differed in their mating dexterity. However and contradictory to what was expected, no difference in the number of offspring sired by the HL and LL rams during the 2012 mating season was observed. Seen against the bigger picture, this potentially implies that there are underlying factors on a physiological level that are affecting the reproductive ability/efficiency of rams. Boshoff (2014) also found differences between the HL and LL rams in terms of sperm head dimensions, which in turn can affect the

fertilizing ability of sperm, as well as their swimming pattern and swimming speed (Malo *et al.*, 2006; Ramón *et al.*, 2013). These morphometric differences are further supported by findings of Sandenbergh (2013), who reported that selection in the HL and LL resulted in different polymorphisms in a SNP marker close to the gene coding for the sperm cytoskeleton, which ultimately can also influence the motility and fertilizing ability of sperm (Beatty & Sharma, 1961; Thurston *et al.*, 1999; Rodríguez-Martínez, 2006). Seen against the abovementioned, the divergently selected Merino resource population was included in the present study as a model to possibly identify what influence divergent selection for reproductive potential could have on sperm traits.

In sheep production systems in South Africa, the Dormer breed is used primarily as a terminal sire (Zishiri *et al.,* 2014). Dormer rams are used commonly on Merino-type ewes in a terminal crossbreeding system (Cloete *et al.,* 2004; 2008). Since a terminal sire breed is expected to mate with ewes of various other breeds and only limited research has been published on Dormer reproduction traits, studies to qualify and quantify reproduction traits in this breed are important.

Compared to the cattle industry, ART's are used to a much lesser extent by commercial farmers in the small stock industry. The optimal application of ART's are influenced by species specific factors, and as most of the protocols used in the small stock industry are based on cattle protocols, successful sperm cryopreservation and post-thaw sperm viability are still considered to be a limiting factor in the optimal application of AI and IVEP (Amiridis & Cseh, 2012). Therefore ovine sperm cryopreservation protocols still require optimization to ameliorate deleterious changes that occur during liquid storage and/or cryopreservation. Cryopreservation inevitably results in ultrastructural, biochemical and functional changes in sperm that reduce the viability and fertilizing competence of sperm (Salamon & Maxwell, 1995b).

When sperm quality is assessed according to standard semen evaluation protocols, three important factors are considered, i.e. acrosome integrity, sperm morphology and sperm morphometry (Martí *et al.*, 2011). Traditionally sperm samples have been considered as a homogenous population of sperm cells, but several studies have reported on the presence of sperm subpopulations within any given sample (Holt & Van Look, 2004). Holt & Palomo (1996) and Druart *et al.* (2009) have postulated that the degree of heterogeneity of sperm subpopulations may be considered as an indicator of ejaculate quality. Each ejaculate consists of a heterogeneous combination of sperm that can be grouped into subpopulations according to different motility and morphometric characteristics (Maree & Van der Horst, 2013). Several authors have also suggested that heterogeneity among sperm subpopulations may have functional relevance, with differences between sperm subpopulations having been linked to fertility (de Paz *et al.*, 2011) and cryotolerance (Thurston *et al.,* 2001; Ortega-Ferrusola *et al.,* 2009). The question now posed is

whether standard semen evaluation protocols provide a reliable enough indication of sperm quality, and whether other sperm traits need to be considered to accurately correlate sperm quality with sperm fertilizing ability and the ultimate conception rate in sheep flocks.

The aims of this study were therefore to conduct a behavioural study to establish a SOP for the training of rams to ejaculate into an artificial vagina. Furthermore, the study also investigated the potential influence of breed and genetic selection for prolificacy on the degree of heterogeneity of ejaculates and to what extent morphometric differences between sperm subpopulations influences the fertilizing competence and cryotolerance of sheep sperm. The findings of the study will contribute to the optimization of current ovine sperm processing protocols, especially for the use in ART's such as AI and IVEP.

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Chapter 2
Literature Review

2.1 Introduction

In South Africa the livestock industry plays an important role in the agriculture sector, contributing 46.3 % to the total agricultural production income in the 2013-2014 period, and of which the small stock industry contributed 8-10 % (Abstract of Agricultural Statistics, 2013). South Africa can be divided into arid and semi-arid regions, with small stock production dominant in these drier areas. Sheep are mainly dependent on natural vegetation as source of grazing (Cloete & Olivier, 2010). In the crop production regions, semi-extensive sheep farming is practiced where sheep utilise crop residues and related by-products (Cloete & Olivier, 2010).

It is expected that the global population will grow to an estimated 9.5 billion people by 2050. With global warming, severe droughts, a fast growing population and the issue of increased production costs, farmers seriously need to consider new or alternative management tools to overcome these challenges to ensure sustainable and cost-efficient production (Jooste & van Schalkwyk, 2001; Smith, 2004). Two such tools that can assist livestock producers to optimise production efficiency include genetic selection and assisted reproductive techniques (ART's). Breeding programs are applied successfully in the livestock industry, promoted by the high accuracy of breeding value estimation, the moderate to high heritability estimates estimated for most production traits and the availability of large reference databases. Breeding programs often focus on selection traits that will contribute to a higher economic production and reproductive efficiency (Rauw *et al.,* 1998). Traits that can be selected for include production traits such as feed conversion and consumption, and reproduction potential, e.g. multiple rearing ability (Cloete *et al.*, 2004; 2009; Doyle *et al.*, 2011). Assisted reproductive techniques can assist livestock producers to increase the intensity and accuracy of selection even more.

Assisted reproductive techniques (ART's) such as artificial insemination (AI), multiple ovulation and embryo transfer (MOET) and *in vitro* embryo production and transfer (IVEP) can be incorporated into management programs to allow sheep farmers to produce lamb and mutton more optimally and cost-efficiently (Nardone *et al.,* 2010).

2.2 Factors affecting cost-efficient sheep production

Currently in South Africa, cost-efficiency of sheep production is of the utmost importance. However, there are various factors in the small stock industry that can affect cost-efficient production. Some of these factors are discussed below.

2.2.1 Mating Systems

When considering a specific sheep mating season there are several factors to consider. Some of these factors include sheep breed, shearing season, ram auctions and performance testing, marketing and management practices. The small stock industry in South Africa traditionally make use of both a spring and an autumn mating season (Van Niekerk & Schoeman, 1993). When comparing the two mating seasons, both of them have advantages and disadvantages. According to Watson (1952), conception rate is much higher during autumn mating, as sexual activity of both the ewe and ram is at a maximum. This results in a higher lambing rate and improved weaning percentages (Van Tonder, 2012). Autumn mating results in spring lambing, when pasture in the winter rainfall regions are fully available, however parasite infestation is severe during these months, with lambs being particularly susceptible to infestation (Van Niekerk & Schoeman 1993). When making use of spring mating, ovulation rates are lower, fewer ewes are in heat, which in turn result in lower lambing percentages. Higher environmental temperatures during spring can also negatively affect a ram's fertility (Ax *et al.*, 2000). In some regions, supplementary feeding may be necessary if pasture availability is limited (Van Tonder, 2012). However, the advantage of spring mating is that lambs will be born during autumn, with lower environmental temperatures resulting in better growth performance of the lambs.

With sexual activity at a maximum during autumn, it would be expected that farmers will normally make use of autumn mating, however this is not always possible. In the Mediterranean part of South Africa, for instance, lambs born in spring will be weaned under unfavourable conditions in the hot, dry summer. From a practical point of view it is not always desirable to only have one mating season per year, as cash flow can become a problem (Midgley, 2009). However, the resources in terms of pasture and feedstuff availability need to be sufficient to sustain two lambing seasons in a calendar year.

2.2.2 Seasonality of reproduction

Sheep are seasonal breeders that depend on a change in daylight length to control reproductive and hormonal rhythms (Dupré & Loudon, 2007; Hashem *et al.*, 2011). In the Southern Hemisphere, the beginning of autumn, characterised by a decrease in daylight length, stimulates the onset of the breeding season (Thiéry *et al.*, 2002). Sheep can thus be regarded as short day breeders as the maximum reproductive activity and exhibition of reproductive behaviour is associated with, or linked to, the shortening daylight hours (De Graaf, 2010).

Seasonal differences in the reproductive activity influence both ewes and rams (Rosa & Bryant, 2003). Seasonality in ewes, stemming from fluctuating circulating hormone levels, can exert an influence on their ovulation patterns (Fogarty *et al.*, 1984; Vázquez *et al.*, 2009). Rosa & Bryant (2003) stated that an ewe's ovulation and oestrus are arrested during certain periods. During

autumn an ewe's ovulation rate is maximal, with the mean number of ova the major determinant of lambing rate and thus production efficiency (De Graaf, 2010). In rams, differences in gonadal activity and sexual behaviour are less noticeable than in ewes, with spermatogenesis and sexual activity being continuous in rams (Rosa & Bryant, 2003; De Graaf, 2010). Seasonality in rams is associated with minor changes in the average scrotal circumference, testosterone levels, sperm concentration and ejaculate volume, with all being higher during the breeding season (Kafi *et al.*, 2004; Sarlós *et al.*, 2013). These changes however, are much more subtle than in ewes.

Seasonal breeding in sheep is a major limitation for the industry, obstructing cost-efficient production. It reduces the effectiveness of accelerated lambing systems, restricts the incorporation of lambing into other farm activities, and limits access to favourable seasonal markets (Notter, 2002).

2.2.3 Disease and parasites

The costs of diseases to the small stock industry are usually underestimated (Besier *et al.*, 2010). Diseases and parasites represent economic and socio-economic threats as it causes losses in production, productivity and profitability. It also causes disruptions to local and the international market, due to the fact that some diseases prevent the export of animal products (FAO, 2009). Control measures are costly and often time-consuming; therefore it is essential to have an effective disease management program in place (Besier *et al.*, 2010).

The occurrence of diseases and parasites varies between regions, and are influenced by factors such as climate, type of pasture and topography (Van Tonder, 2012). In South Africa some of the main diseases that sheep are vaccinated against include bluetongue, pulpy kidney, Rift Valley fever, ovine Johne's disease, pasteurella and brucellosis. Of these diseases, brucellosis and Rift Valley fever affect the reproductive efficiency of sheep. Brucellosis infection results in infertility in rams, and both brucellosis and Rift Valley Fever can cause abortions in sheep (Turton, 2002; Besier *et al.*, 2010).

Sheep can be infested by a number of external and internal parasites, compromising their productive ability, and leading to reduced mutton yields and the downgrading of wool quality (Bates, 2012). The most common parasites in sheep include liver flukes, round worms, tapeworms, blowfly strike, mites, lice and ticks (Van Niekerk & Schoeman 1993). Parasite infestations are largely preventable by a structured annual dosing plan that considers the seasonal risk periods. It is important to provide sheep with optimal nutrition during periods of susceptibility, to reduce the occurrence and effect of diseases and parasites, as well-nourished animals are more disease tolerant (Besier *et al.*, 2010).

2.2.4 Nutrition

The production efficiency of sheep is highly dependent on sufficient nutrition (Johnson, 1984). Nutrition includes the supply of energy, protein, minerals and vitamins. Increasing the amount of feed provided must be associated with the correct balance of nutrients (Speedy, 1980). In South Africa most of the sheep farming practices make use of extensive production systems, where the animals depend on the natural veld and pastures (Ramirez, 1999). The nutritional value and quality of the natural veld varies between seasons (Snyman & Joubert, 2002). During the dry season, sheep grazing on natural veld are more prone to develop serious nutritional deficiencies, and supplementation is crucial during this period (Gertenbach & Dugmore, 2004; Ben Salem & Smith, 2008). Sheep grazing on pastures will in most cases require trace element and vitamin and mineral supplementation (Masters & Thompson, 2016). According to Van Pletzen (2015), sheep in semi-extensive systems grazing on crop residues will require energy, protein, vitamin and mineral supplementation, which can be achieved through the placement of lick blocks, especially when crop residues are of very low quality (Gertenbach & Dugmore, 2004). During severe droughts when drought resistant forages are depleted, supplementation must be increased, as the animals only depend on the additional feed for survival. Therefore, it is important to accumulate feed reserves for these periods, since the buying in of extra feed can be very expensive.

Nutritional deficiencies can have a major influence on the reproductive efficiency of a flock. The nutritional status of the ewe is an important determinant of fertility, fecundity and lamb survival. As example Lupins fed to rams prior to the onset of the mating season resulted in an increase in sperm production caused by an increase in testes weight and seminiferous tubules volume (Jolly & Cottle, 2010).

Effective supplementary feeding practices therefore form an integral part of efficient management and breeding practices and form the basis of profitable sheep farming. The addition of the correct nutrients (protein, energy, minerals and vitamins) in the correct quantity and combination are critical for maximum and cost-efficient production (Coetzee, 2014).

2.3 Methods to overcome limitations

With the ever-increasing demand for animal products and the growing population, farmers face serious challenges to produce as optimally and cost-effectively as possible. To assist the small stock industry to optimise their production practices, sheep farmers can make use of different management tools such as accelerated lambing and ART's to overcome these limitations.

2.3.1 Accelerated lambing

A significant improvement in both productivity and efficiency is possible if reproduction rate can be increased (Fogarty *et al.*, 1984). According to Coetzee (2014), profitability is mainly based on

efficiency and not so much just on product price. Apart from the financial benefit of wool production, a farmer can only generate income from the number of lambs marketed. Therefore the number of lambs marketed per ewe per year is very important to increase the profit margin per hectare. There are several strategies and systems available to increase a sheep flock's lamb production efficiency. Accelerated lambing systems are one of the strategies that may be used to improve a flock's lamb production effectively by increasing the number of lambs marketed per ewe per year (Schoeman & Burger, 1992). Accelerated lambing systems provide the opportunity to produce more lambs throughout the year, as it decrease the lambing interval, creating multiple lambing periods and increasing the annual production per ewe (Beef and Lamb New Zealand, 2007). The two systems that are mostly used are the 8-month system and the Cornel STAR system.

2.3.1.1 8-Month System

This system is the most common system used for accelerating lambing and provides three mating/lambing seasons in two years. The outcome of this system is an 8-month lambing interval and an average of 1.5 births per ewe per year. This system can have two variations, where the whole flock is handled as a single ewe flock or where the flock can be divided into two with either the one or the other lambing every fourth month (Hoque, 1987; Midgley, 2009).

2.3.1.2 Cornel STAR System

This system was developed to maximize the production of market lambs constantly throughout the year. It was designed to have five lambing seasons within each year giving the ewe the opportunity to lamb five times in a three year period (Lewis *et al.*, 1996). A 365-day year is divided into five sections, which represent the points of the star. Ewes can lamb every 73 days in rotation, giving a continuous supply of lamb throughout the year. The star can be rotated to adjust the dates (Figure 2.1).

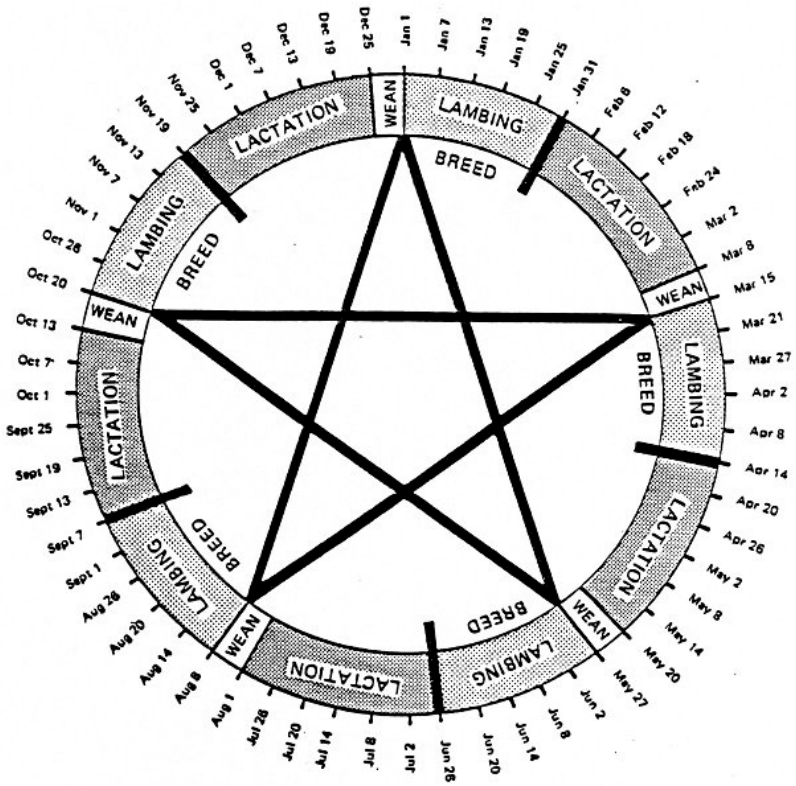

Figure 2.1 The Cornel STAR Accelerated Mating System that allow for five lambing seasons to be accommodated in a 3-year period (Lewis *et al.*, 1996).

These systems will not only increase the reproduction efficiency of a flock, but also supply a more constant supply of lamb during the year and also provide the farmer with a uniform cash flow throughout the year. However, accelerated lambing systems can only be considered if a farmer's management is regulated tightly and if feed is available throughout the year. It is important to consider that these systems cannot compensate for poor management, poor nutrition or low fertility (Coetzee, 2014).

2.3.2 Assisted Reproductive Techniques (ART's)

One of the main limitations of the restricted use of ART's in small ruminants is the seasonal nature of reproduction in sheep (Amiridis & Cseh, 2012). However, with the use of exogenous hormones in combination with certain ART's, farmers can overcome the limitation of seasonal breeding and produce animal products throughout the year (Baldassarre & Karatzas, 2004; Cseh *et al.*, 2012).

The use of ART's allows farmers to produce more optimally, optimise their reproduction efficiency, accelerate genetic progress and also to produce more offspring from animals of a high genetic merit than would have been possible by natural mating (Baldassarre & Karatzas, 2004). Most of the ART's have been developed and applied in large ruminants, but artificial insemination (AI), multiple ovulation and embryo transfer (MOET), *in vitro* embryo production and semen cryopreservation are the main techniques used in the small stock industry (Amiridis & Cseh, 2012).

2.3.2.1 Artificial insemination (AI)

Artificial insemination is the most commonly used ART in livestock production systems, and has made a significant contribution to genetic improvement in the livestock industry (Leboeuf *et al.*, 2000; Baldassarre & Karatzas, 2004). The application of AI, when executed properly, reduces the risk of spreading sexually transmitted diseases and it allows the widespread use of highly genetic superior animals as sires, improving the performance and potential of a flock (Ax *et al.*, 2000). It also increases the selection differential and accelerate genetic progress by shortening the generation interval (Baldassarre & Karatzas, 2004).

Other advantages of AI includes the production of large numbers of offspring and the accurate estimations of breeding values of relatively young animals by progeny testing (Van Arendonk, 2011). Recently, the separation of sperm into different fractions containing X- and Y-chromosomes bearing sperm using flow cytometry opened up new possibilities for livestock production. The sexing of sperm enables farmers to produce more progeny of a specific sex and is useful in production systems, like the dairy industry, where male progeny has little commercial value (Baldassarre & Karatzas, 2004; Alexander *et al.*, 2010).

There are three AI techniques used in sheep, which include vaginal, cervical and laparoscopic insemination as described by Ax *et al.* 2000. Generally, the method of semen preservation determines the method of insemination. The rule of thumb is that the more damaged sperm are, the deeper sperm need to be deposited to achieve good fertilization rate (Baldassarre & Karatzas, 2004). When AI is performed using fresh semen, the vaginal insemination method will be used, whereas chilled or frozen semen are used for intra-uterine or for laparoscopic insemination (Baldassarre & Karatzas, 2004; Cseh *et al.*, 2012).

There are however, disadvantages to consider when deciding to use AI as part of a management program. A trained technician with appropriate knowledge of the technique and experience in heat detection is necessary (Ax *et al.*, 2000). The conception rate with AI is variable and can be influenced by several factors. One of the factors that need to be considered is the method of semen preservation. Chilled semen is generally used for AI, because of the poor fertility results when using frozen-thawed semen. This is as a result of the complex design of the cervix of the ewe, which presents a barrier for the deep deposition of sperm, which thus reduce the efficiency of the technique in sheep. Currently laparoscopic insemination is used as an alternative method when using frozen-thawed semen to achieve higher fertility results. However, laparoscopic AI is a more technical procedure and a veterinarian is required to perform this technique, which contributes to additional costs (Anel *et al.*, 2005). Nevertheless, the correct handling of semen during and after collection, storage and use determine the success of AI (Leboeuf *et al.*, 2000).

2.3.2.2 Multiple ovulation and embryo transfer (MOET)

The genetic gain on the female animal side is limited as a result of a low reproduction rate with only one or two offspring being produced in most ruminant livestock species. Therefore multiple ovulation and embryo transfer has been developed to overcome these limitations and to produce more progeny of genetically superior female animals than would be possible through natural mating (Alexander *et al.*, 2010). The MOET technology is often referred to as the ART that is to the female, what AI is to the male (Baldassarre & Karatzas, 2004).

A standard MOET protocol in small ruminants involves the induction and synchronisation of the oestrus cycle through fluorogestone acetate intra-vaginal sponges and the administration of exogenous gonadotropins to stimulate follicular growth and superovulation (Mayorg *et al.*, 2011). Animals are allowed to mate naturally or inseminated with fresh or frozen-thawed sperm. After fertilization, embryos can be recovered through laparoscopy or embryo flushing, and transferred to oestrus-synchronized recipient ewes (Alexander *et al.*, 2010).

The application of MOET are hampered by results that can be highly variable and unpredictable, due to an inconsistency in the ovarian response of ewes, which can manifest in poor conception rates (Cognié *et al.*, 2003). Therefore this ART is not yet optimally applied in small ruminants, due to the low success rate of obtaining adequate numbers of transferable embryos of a high quality and the associated costs (Menchaca *et al.*, 2009).

2.3.2.3 *In vitro* embryo production (IVEP)

The IVEP technology involves three major stages, i.e. the collection of oocytes and the *in vitro* maturation (IVM) of cumulus-oocyte complexes (COC's), *in vitro* fertilization (IVF) and the *in vitro* culture (IVC) of embryos (Alexander *et al.*, 2010). Prior to IVM, oocytes are generally collected from abattoir derived ovaries, trans-vaginal ultrasound-guided follicular aspiration or laparoscopic ovum pick-up (LOPU), where oocytes are laparoscopically collected from the female animal. However, all the other collection methods are gradually being replaced by LOPU in small ruminants. This procedure is relatively simple, quicker, less costly and can be repeated more times as it is less invasive (Baldassarre & Karatzas, 2004; Holtz, 2005; Tibary *et al.*, 2005). Furthermore, IVEP allows the production of offspring from non-fertile ewes as well as pre-pubertal ewes. The collection of embryos from pre-pubertal ewes is called juvenile *in vitro* embryo transfer (JIVET) and it permits a significant shortened generation interval (Gou *et al.*, 2009).

For IVF, either fresh or frozen-thawed sperm can be used (Amiridis & Cseh, 2012). However, the process of fertilization is complex, requiring pre-fertilization preparation before it can be introduced to the fertilization medium containing the oocytes. For successful fertilization, the most motile and viable sperm are selected by a swim-up technique or discontinuous density gradients (Percoll).

The selected sperm needs to be capacitated (functional maturation of sperm) to achieve successful fertilization (Paramio & Izquierdo, 2016). The process of capacitation allows sperm to undergo the normal acrosome reaction prior to fertilization. For *in vitro* capacitation heparin or caffeine can be used. However, according to Del Olmo *et al.* (2015) the serum of oestrous sheep is the most efficient medium for *in vitro* capacitation of ram sperm.

The use of laparoscopic ovum pick-up and *in vitro* embryo production protocols has great potential to produce more offspring of genetically superior animals more efficiently, but its use is still limited by the requirement for more strict laboratory conditions than is required for MOET (Baldassarre & Karatzas, 2004). A better understanding of oocyte and embryo physiology is needed for this technique to be optimally applied and the production of a large number of good quality embryos is guaranteed (De Souza-Fabjan *et al.*, 2014).

2.3.2.4 Semen collection

A high quality sperm sample must be used in ART's to ensure as high as possible rate of conception. Ram semen is general collected by two methods, either using an artificial vagina (AV) or electro-ejaculation (EE) (Wulster-Radcliffe *et al.*, 2001). Sperm can also be obtained from the epididymis by means of aspiration. The latter method, however, is not commonly used as the sperm needs to be treated differently when used in ART's.

2.3.2.4.1 Artificial Vagina (AV)

The use of the AV for semen collection is the preferred and more humane method, as it mimics natural mating and it is also more hygienic compared to EE (Leboeuf *et al*., 2000; Jiménez-Rabadán *et al.,* 2012; Wulster-Radcliffe *et al.,* 2001). The AV consists of a T-junction polyvinylchloride (PVC) pipe with a latex rubber lining and a glass collection tube at the one end of the AV. The water temperature inside the AV (between the latex lining and PVC pipe) is usually adjusted to 42°C – 45°C, this is critical for successful collection (Ramsem, Bloemfontein, South Africa; Walton, 1945). A restrained ewe is used when collecting semen from a ram with the AV. When the ram mounts the ewe, the penis is gently diverted into the AV to allow for natural ejaculation (Ax *et al.*, 2000).

The quality of sperm collected with the AV are of a much higher quality in terms of a higher sperm concentration, a lower percentage of abnormal sperm and sperm so collected are more resistant to cryodamage (Marco-Jimenez *et al.,* 2005; Batista *et al.,* 2009; Jiménez-Rabadán *et al.,* 2012). However, the AV method requires the training of rams prior to the collection of semen (Bopape *et al.*, 2015). There is variation in how long it can take to train rams to use the AV, and it can take up to three weeks to train rams (Wulster-Radcliffe *et al.*, 2001).

2.3.2.4.2 Electro-ejaculation (EE)

The EE method is a quicker and more convenient method to collect semen in comparison with the AV, as no training of the rams or ewe preparation are necessary (Mattner & Voglmayr 1962; Matthews *et al.*, 2003). The use of EE allows the collection of semen from rams that are not trained or that reject the AV (Marco-Jiménez *et al.*, 2008).

Semen collection is performed by electro-stimulation from an electrode probe connected to a power source (Jiménez-Rabadán *et al.*, 2012). The electrode probe is inserted into the rectum of the ram and the region of the accessory sex glands as well as the sympathetic and parasympathetic nerves associated with ejaculation are stimulated for three to five seconds, followed by rest for three to five seconds and then stimulated again for three to five seconds (Orihuela *et al.*, 2009). However, there are several constraints associated with this method, as it can be stressful to the ram as the ram needs to be constrained and lain down (Ax *et al.*, 2000; Orihuela *et al.*, 2009). The semen quality of a sample collected by EE is often reduced due to urine contamination (Wulster-Radcliffe *et al.*, 2001; Marco-Jimenez *et al.*, 2005). Marco-Jimenez *et al.* (2005) did however find a higher number of stable and functional cells in frozen-thawed sperm samples collected with the EE method. The EE method of semen collection should only be used when collection with an AV is not possible (Bopape *et al.,* 2015).

2.3.2.5 Semen cryopreservation

Assisted reproduction techniques are commonly used in the livestock industry and the application of these techniques mainly depends on the use of frozen semen (Anel *et al.*, 2003). Therefore, techniques have been developed for short term (liquid) and long-term (cryopreserved) semen storage. These techniques are based on the principle of reduced or complete metabolic arrest of sperm, thus extending their lifespan by conserving energy and delaying the processes involved with membrane destabilization (Salamon & Maxwell, 1995, 2000).

The short-term storage of sperm is an alternative method to cryopreserved semen. The semen sample is diluted and stored in a liquid state between 0 – 5˚C in a refrigerator. This technique allows the use of semen for a longer period of time compared to fresh semen (Menchaca *et al.*, 2006). However, with the duration of liquid storage, the quality and viability of the sperm decrease (Salamon & Maxwell, 1995).

The most commonly used technique for semen storage is cryopreservation or long-term storage. This procedure includes the dilution of the sperm sample with a cryodiluent that protects the sperm from cryodamage and the packaging of the cryodiluent and semen in 0.25 or 0.5 mL polyvinyl chloride (PVC) straws sealed with a PVC powder. Finally, the semen straws are stored in liquid nitrogen. This technique is a fast and effective way to store sperm for long periods at -196℃ until

needed (Holt, 2000; Salamon & Maxwell, 2000). The standard protocol for the thawing of ovine semen straws involves the immersion of the straws in a pre-heated water bath at 37˚C for 30 seconds (Ollero *et al.*, 1998).

However, the freezing and thawing processes of sperm, includes ultrastructural, biochemical and functional damage to the sperm, causing a reduction in sperm motility, membrane integrity and ultimately fertilizing ability (Watson, 2000; Purdy, 2006a). This is the result of using cryopreservation protocols mainly based on bovine sperm (Pukazhenthi & Wildt, 2004). However these protocols are not yet optimised for sheep and therefore still need modifications for optimal application (Kouba *et al.*, 2001; Alminana & Cuello, 2015).

2.4 Reproduction in the ram

The male reproductive system consists of a pair of testes that produce sperm and male sex hormones, a duct system for sperm maturation and transport, accessory sex glands that produce the seminal plasma component of semen, and the penis for copulation.

2.4.1 Spermatogenesis

Spermatogenesis is the process through which sperm are produced in the seminiferous tubules of the testes. This is a complex process and includes mitotic cell division, meiosis and the process of spermiogenesis (De Kretser *et al.*, 1998). The process of spermatogenesis is a continuous process and takes approximately up to 6 weeks in a mature ram to be completed (Garner & Hafez, 2000). Spermatogenesis regulation is controlled by the hypothalamo-pituitary-testes axis (HPT), with follicle stimulating hormone (FSH) and luteinizing hormone (LH) the main gonadotropins maintaining and regulating spermatogenesis.

2.4.1.1 Testicular transit

The ram testis predominantly consists of lobules of seminiferous tubules which are surrounded by interstitial tissue. Interspersed between the seminiferous tubules are the Leydig cells that produces testosterone, the steroid hormone that plays an important role in spermatogenesis. Spermatogenesis primarily takes place in the seminiferous tubules that are composed of Sertoli cells and germ cells that are arranged in layers (Kaur *et al.*, 2014). The Sertoli cells play a crucial role in spermatogenesis, with the spermatogonia that are the primordial stage of sperm, originating at the base of the Sertoli cells (Knoblaugh & True, 2012). During spermatogenesis, sperm structure is formed and influenced by genotypic effects (Maroto-Morales *et al.*, 2012). The production of sperm is controlled and regulated by the action of FSH and testosterone on the Sertoli cells (Griswold, 1998). Sertoli cells support the morphological transformation and surface-modifying actions of round germ cells to undergo mitotic cell division, differentiation and meiosis to form haploid, elongated spermatids. These newly formed spermatids are transformed into testicular

sperm and are released into the lumen of the seminiferous tubules (Leahy & Gadella, 2011). Although the sperm released from the testis is morphologically complete, it is functionally incompetent to move and unable to fertilize ova, therefore the sperm are modified further in the epididymis (Cooper 2011; Dacheux & Dacheux 2014).

2.4.1.2 Epididymal transit

The epididymis can be divided into three different regions, the *caput epididymis, corpus epididymis* and the *cauda epididymis.* The major functions of the epididymis are the transport, maturation and storage of sperm prior to ejaculation (Garner & Hafez, 2000). However, although sperm appear normal when arriving from the seminiferous tubules, they are not fully mature and incapable of fertilization (Amann *et al.*, 1993; Leahy & Gadella, 2011). Therefore epididymal transit plays an important role in the maturation of sperm (Williams *et al.*, 1991).

The epididymal maturation process is essential for sperm to undergo some changes to acquire the capability to recognize, bind and penetrate the *zona pellucida* of an ovum during the fertilization process (Axnér, 2006). During epididymal transit, which in the ram is ± 16 days (Garner & Hafez, 2000), sperm undergo major changes that involve the acquirement of their motility and fertilizing ability (Dacheux *et al.*, 2003). Sperm also undergo various biochemical and metabolic changes in the *caput* and *corpus* regions of the epididymis, whereas the *cauda* region is primarily used for sperm storage (Gloria *et al.*, 2011). These biochemical and metabolic changes are influenced and controlled by epididymal secretions (Cooper, 2011). The epididymal transit changes of sperm will be discussed in detail in section 2.5.

2.5 Sperm viability and fertilizing ability

2.5.1 Sperm Morphology

A male animal's reproductive success mainly depends on the ability of its sperm to fertilize an ovum. Mammalian sperm have a highly complex structure that differs in shape and size between and within species (Malo *et al.*, 2006). Mature sperm consists of three highly specialized regions, the head, the midpiece and the tail, with all three components being surrounded by the plasma membrane (Pesch & Bergmann, 2006). The average sperm head dimensions for ram sperm a ram are length = 8.08 µm and width = 4.80 µm (Gravance *et al.*, 1998).

The plasma membrane is a dynamic structure and plays a vital role in sperm-ovum interaction (Flesch & Gadella, 2000). It is characterised by regional specific glycoproteins and lipid components which are important for sperm-ovum binding during fertilization (Pesch & Bergmann, 2006). However, membrane integrity and functional activity is important for sperm metabolism as well as for the fertilization process, therefore the correct changes and modifications of the plasma

membrane are essential for successful sperm-ovum binding (Jeyendran *et al.*, 1984). The sperm head is characterized by the presence of the acrosome and the nucleus, which holds the highly compacted male haploid genome. The acrosome is a large cap-like vesicle covering the anterior part of the sperm head and contains hydrolytic enzymes, i.e. acrosin and hyaluronidase. These hydrolytic enzymes are necessary for the lysis and penetration of the *zona pellucida* during fertilization and are released during the acrosome reaction (Gage, 1998; Flesch & Gadella, 2000; Malo *et al.*, 2006; Pesch & Bergmann, 2006).

The tail of sperm can be divided into three different components: the midpiece and the principal and terminal pieces. The midpiece of the sperm contains many mitochondria that generate energy through oxidative phosphorylation, used for sperm motility (Turner, 2003). The principal and terminal pieces are involved in motility (Flesch & Gadella, 2000). Sperm motility is an important expression of the energy (rate of movement) of sperm and is essential for migration through the female reproductive track to reach the ovum. The tail movements create forces (forward) that assist sperm during the penetration process of the *zona pellucida* (Katz *et al.*, 1989).

Seen against the abovementioned background it is clear that only a functionally intact sperm will successfully fertilize an ovum (Gadella, 2008). The functional viability of sperm depends on progressive sperm motility, membrane and acrosome integrity, an ability to undergo capacitation and the acrosome reaction (Spindler *et al.*, 2004). However, for a sperm to be fully functional and mature it has to be transported through the epididymis, as sperm released from the testis are immotile, the plasma membrane is not yet fully matured and it cannot fertilize a ovum (Flesch & Gadella, 2000; Leahy & Gadella, 2011).

2.5.2 Epididymal maturation

Sperm formed during spermatogenesis and spermiogenesis are fully developed, but are immotile and are thus not yet capable of fertilizing an ovum (Sostaric *et al.*, 2008). Sperm, after leaving the testis, need to undergo a series of physiological changes to reach the ovum and fertilized it (Pérez-Sánchez *et al.*, 1998). Therefore the epididymal sperm maturational processes are essential for sperm development and fertilization competency (França *et al.*, 2005). This maturation process is complex and occurs in a chronological order in the different regions of the epididymis (Figure 2.2) (Bassols *et al.*, 2005).

As mentioned previously, the mammalian epididymis consists out of three regions, the *caput, corpus* and *cauda*. Each of these regions differ in function and environment caused by specific protein secretion and absorption across the epididymal epithelium within each region (Leahy & Gadella, 2011). With the major functions of the *caput* and *corpus* regions being sperm maturation and the *cauda* region primary function sperm storage (Sostaric *et al.*, 2008). The major changes

sperm undergo during epididymal transit are the attainment of sperm motility and the ability to fertilize an ovum (Dacheux & Dacheux, 2014).

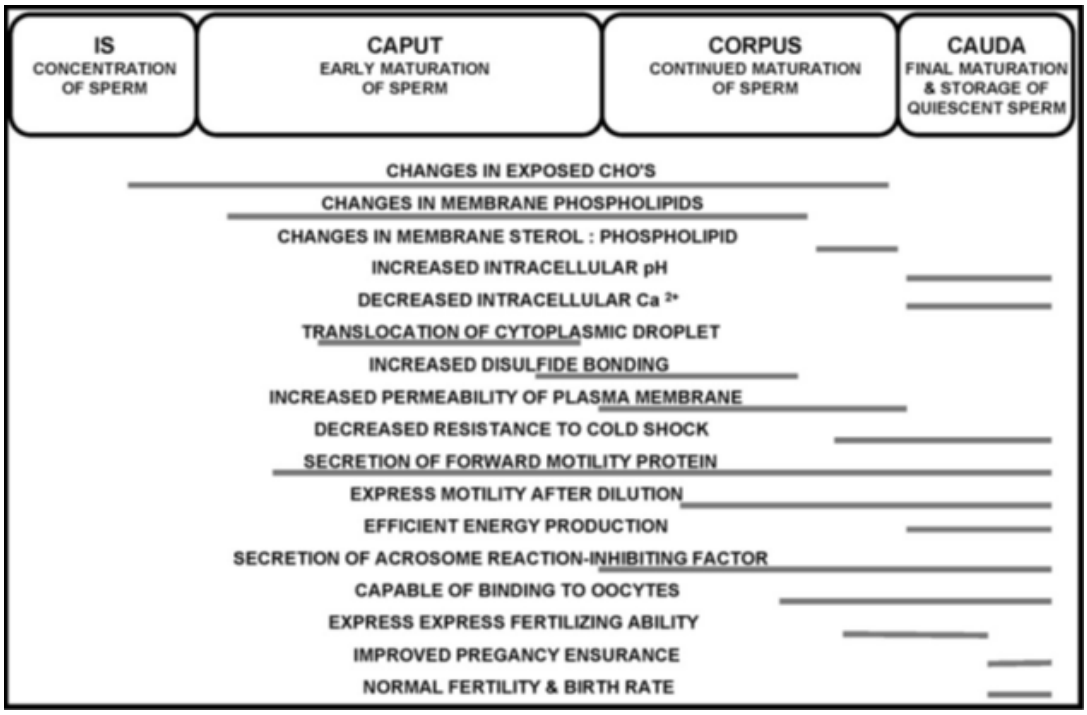

Figure 2.2 A summary of the major maturational changes in the specific regions of the ovine epididymus (Marengo, 2008).

When entering the epididymis the first changes sperm undergo takes place in the initial segment of the epididymis. In this segment luminal fluid reabsorption takes place to make sperm more concentrated which is important for maturation and to make the ejaculate a specific volume (França *et al.*, 2005; Marengo, 2008). During sperm transit in the *caput* and *corpus* regions of the epididymis, all major functional maturation changes take place.

When sperm first enters the epididymis it is either immotile or weakly motile (Sostaric *et al.*, 2008). These immature sperm have a characteristic irregular beating rhythm, resulting in almost immobile heads without any forward motion or with asymmetrical swim-paths. Bassols *et al.* (2005) found that sperm from the *caput* region of the epididymis are mostly immotile or have irregular swim-paths, although the irregular swim-paths develop along the epididymis into more regular and curved paths. Thus it can be said that sperm motility increases progressively from the *corpus* to the *cauda* regions of the epididymis (Dacheux & Dacheux, 2014). It was also found that during epididymal transit, the sperm's intracellular cyclic adenosine monophosphate (cAMP) level increases along with metabolic capacity and adenosine triphosphate (ATP) production from the corpus to the cauda regions of the epididymis. This play an important role in the gradual activation of sperm motility during epididymal transit (Dacheux & Dacheux, 2014). Contri *et al.* (2012) also stated that sperm's propulsive efficiency primarily depends on its mitochondrial function. The

authors also found that sperm from the *cauda* region of the epididymis had much higher mitochondrial activity compared to that of the *caput* region, indicating that mitochondrial activity develops during epididymal transit to provide energy for progressive sperm motility.

Several studies have indicated that modifications occur in the plasma membrane during epididymal transit. Some of the major modifications include changes in the plasma membrane protein, lipid and phospholipid composition as well as the elimination and modification of surface proteins (Dacheux *et al.*, 2003; Caballero *et al.*, 2010). The plasma membrane's sterol and fatty acid composition also undergo significant changes during epididymal transit, which cause major changes in the membrane structure. During epididymal transit the cholesterol content of the sperm plasma membrane decreases and the unsaturated fatty acid ratio increases (Leahy & Gadella, 2011). These lipid changes usually increases membrane fluidity that regulate capacitation (Shadan, 2004). Thus these modifications of the protein and lipid composition of the sperm plasma membrane in the epididymis, support sperm to undergo capacitation, bind to the *zona pellucida* and fuse with the oolema (Leahy & Gadella, 2011). These sperm plasma membrane modifications could be associated with the development of sperm fertilizing ability (Axnér, 2006).

Another important modification occurring during the epididymal maturation process is the development and formation of the acrosome. During spermatogenesis, the acrosome molecules are formed and modified during epididymal transit (Yoshinaga & Toshimori, 2003). Axnér (2006) found that, during epididymal transit, the size of the acrosome decreases from the caput region where the acrosome is still swollen compared to the cauda region where the acrosome becomes more contracted and closer to the sperm head surface.

Sperm cells are constantly being modified on their transit through the epididymis. The most visible morphological change is the migration of cytoplasmic droplets, the remainder of the germ cell cytoplasm (Dacheux & Dacheux, 2014). During sperm maturation sperm head dimensions undergo changes along the *caput* and *corpus* regions of the epididymis. However, no changes in sperm head morphometry takes place in the *cauda* region of the epididymis. Additionally, changes in sperm head morphometry during specific stages of sperm maturation in the different epididymal regions may suggest that the sperm head morphometry of a specific epididymal spermatozoa may be an indication of its relative maturity (Pérez-Sánchez *et al.*, 1998). However, it can be concluded that epididymal transit is essential for the final morphological maturation of sperm.

The majority of the sperm cells are expected to reach their full fertilizing potential when they reach the *cauda* region of the epididymis. Several authors have postulated that sperm from the *cauda* region of the epididymis are mostly mature, having forward motility, are able to undergo capacitation, the acrosome reaction and are fertile when used for IVF or AI (Axnér, 2006). Cooper

(2007) found that sperm from the cauda region had an advantage over sperm from the caput region in binding and penetrating the *zona pellucida*. In the *cauda* region the sperm are stored and accumulated to ensure that a sufficient number of sperm is available at the time of ejaculation (Sostaric *et al.*, 2008). The micro-environment of the cauda region allows the sperm to survive for several weeks and also keeps the sperm in a metabolic inactive state to prevent premature activation (Caballero *et al.*, 2010). To conclude, mature sperm can activate progressive forward motility, be capacitated, bind to the *zona pellucida* and fuse with the oolemma (Dacheux & Dacheux, 2014). The overall sperm motility and fertilizing ability increases substantially from the *caput* to the *cauda* regions of the epididymis, therefore epididymal maturation is essential for sperm maturity (Turner, 1995).

2.5.3 Sperm subpopulations

With variation in sperm morphology originating during spermatogenesis under the possible influence of genotypic effects and with different spermatogenic waves that have matured along the epididymis and stored in the *cauda* region of the epididymis, it is reasonable to assume that a semen sample will be heterogeneous (Thurston *et al.*, 1999; Rodríguez-Martínez, 2006). Traditionally the entire ejaculate has been considered as a homogenous population. However, several studies have reported the existence of sperm subpopulations in the mammalian ejaculate, however the origin and physiological significance of these subpopulations are not yet known (Maroto-Morales *et al.*, 2012). Although the origin of these subpopulations is not yet clear, it has been hypothesised by many researches that their origin is possibly due to differences in the grouping of individual sperm during spermatogenesis as well as to variance in maturational status and age through mixing in the epididymis (Abaigar, 1999).

Maree & Van der Horst (2013) stated that each sperm population consists out of a heterogeneous combination of sperm that can be grouped into subpopulations according to different characteristics. Several studies have described the existence of morphometric as well as kinematic sperm subpopulations within the ram ejaculate (Martí *et al.*, 2011; Maroto-Morales *et al.*, 2012; Garcia-Alvarez *et al.*, 2014). Recent studies have shown that sperm subpopulations may have functional relevance, differences between specific subpopulations being linked to fertility (Maree & Van der Horst, 2013) and that subpopulations may be a good indicator of semen quality (Martí *et al.*, 2011). Yániz *et al.* (2015) found in their study that rams with superior field fertility contained significantly more sperm with rapid and linear movement and sperm with large and elongated heads. The authors conclude that both these sperm subpopulations may possibly be related to more efficient sperm migration and that these specific sperm characteristics may be indicators of fertility. Ramón *et al.* (2013) also postulated that sperm with elongated heads might be faster as they have less resistance and may expend less energy. On the contrary, in a study by Santolaria *et*

*al. (*2015), it was found that a ram's fertility, as defined by the number of ewes lambing after AI, was not related to the distribution of sperm in either morphometric or motility subpopulations.

The existence of morphometric and kinematic subpopulations have also been identified in other livestock species. In a study done by Muiño *et al.* (2008) on Holstein bulls it was found that sperm samples with the highest proportion of subpopulations containing rapid and progressive sperm, were more resistant to cryopreservation damage and also showed the best post-thaw sperm viability. Thurston *et al.* (2001) found distinct sperm morphometric subpopulations within fresh boar ejaculates. Furthermore, the authors observed that the occurrence of these subpopulations is associated with post-thaw semen quality.

Several factors can influence the structure and distribution of sperm subpopulations. Martí *et al.* (2012) found that season has a major influence on sperm morphometric subpopulations. The authors observed significant differences in sperm head dimensions between the breeding and non-breeding seasons, with a reduction in all sperm head morphometric dimensions (area, perimeter, length and width) from the breeding to the non-breeding season. This results in differences in the percentage sperm per subpopulation between seasons. The structure and distribution of subpopulations can also be affected by the maturity and/or age of a ram. In a study by Martí *et al.* (2011), three sperm morphometric subpopulations were identified in yearling rams' ejaculates compared to four in mature rams.

As mentioned previously, the distribution of subpopulations seems to be interrelated with the ejaculate quality, which is a vital criterion of sperm function. Thus, the evaluation of sperm morphometric subpopulation structure together with functional tests may possibly provide valuable information to assess the cryotolerance and potential fertilizing ability of ram sperm (Martí *et al.,* 2011). Furthermore, De Paz *et al.* (2011) stated that the analysis of only the mean values of morphometric parameters of a semen sample is too limited and not sufficient to predict the fertilizing ability of a given sample. Therefore, it is essential to analyse the overall complex structure of a semen sample, by identifying sperm subpopulations by principal component analysis and cluster methods.

The development of computer-assisted sperm analysis (CASA) systems allows the easy characterization of the heterogeneity of a sperm sample (Martinez-Pastor *et al.*, 2011). The presence of sperm subpopulations within a semen sample holds the possibility for new improved semen analysis techniques and is of great potential economic importance as this can lead to the improvement of sperm doses for AI (Martí *et al.*, 2011). Knowledge of different sperm subpopulations within an ejaculate might be of utmost biological importance to improve our knowledge about sperm physiology and cryobiology (Maroto-Morales *et al.*, 2012).

2.6 Factors effecting the reproductive efficiency of rams (sperm production and quality)

The reproductive efficiency of rams is of extreme economic importance and mainly depends on semen quality and quantity (Clément *et al.*, 2012). However, sperm quality depends on a number of biological and environmental factors and therefore a thorough knowledge of these factors affecting sperm production are necessary (Soderquist *et al.*, 1996).

2.6.1 Farming practices/Management of the ram

In South Africa, sheep are mainly farmed under extensive production systems. However, more farmers are making use of semi-extensive production systems these days to increase their profitability (Fourie *et al.*, 2004). The main difference between these systems is the feed resource. In an extensive production system, the main feed resource is natural pastures with minimal supplementation. With the main focus on the ram, the only supplementation in an extensive production system is flush feeding prior to the mating season. In a semi-extensive system sheep can make use of crop residues or pastures. The influence of nutrition on a ram's reproduction efficiency is discussed in detail in section 2.6.5.

However, regardless of the production system, rams represent the largest capital outlay in the flock, therefore ram management is crucial for maximising profitability. It is of utmost importance to examine rams prior to the mating season, to ensure optimally reproductive efficiency. Rams are often neglected apart from the breeding season, but ram nutrition and health management throughout the year are crucial. It is also important that a ram is fit, has an acceptable libido level and is lively. By 60 days prior to the mating season rams should be examined in terms of scrotum circumference, testicular palpation, testicular and penis lesions and sometimes semen evaluation to identify possible problems early (Jooste, 2012).

2.6.2 Behaviour

Sheep are polygamous breeders during the breeding season. The sexual behaviour of a ram establishes the corner stone in the evolutionary development of a cost-effective farming system and its success is mainly affected by the productive potential of the ram (Simitzis *et al.,* 2006). Rams display characteristic mating behaviour signs, by using olfactory signals to identify ewes in oestrus (Roselli *et al.*, 2004). However, the exhibition of sexual behaviour can be influenced by the maturity and age of a ram (Simitzis *et al.,* 2006).

In species like sheep, the choice of the mating partner plays a vital role in sexual selection (Orihuela & Vázquez, 2009). However, it was observed that when an individual ram is exposed to a number of ewes in oestrus, rams will prefer to mate with specific ewes to the exclusion of others.

These mating preferences for certain ewes by a ram indicate that ewes differ in their sexual attractiveness (Tilbrook & Lindsay, 1987). An ewe's sexual attractiveness can be referred to as her stimulus value in eliciting a sexual response from the ram. Tilbrook (1987) stated that the ranking of an ewe's sexual attractiveness is consistent between individual rams. This suggests that mating preferences of a ram is not a component of his own behaviour but rather, his response to specific factors of the ewes surrounding him (Tilbrook *et al.*, 1987). Furthermore it was stated that these factors that contribute to a ewe's sexual attractiveness are stable between oestrus periods (Tilbrook, 1987). It was found that the mating preferences of a ram for a specific ewe is probably the result of differences in the explicit characteristics of ewes in oestrus that attracts rams and causes them to direct their sexual prowess towards this specific ewe (Tilbrook *et al.*, 1987).

A test was developed by Tilbrook & Lindsay (1987) to rank ewes according to their sexual attractiveness, i.e. from the most to the least attractive for each ram. Several studies, based on the Tilbrook & Lindsay (1987) test, confirmed that rams have a specific mating preference; however only some of these factors that contribute to the sexual attractiveness of a ewe have been identified (Tilbrook & Cameron, 1989). Preston *et al.* (2005) concluded that a ram will favour heavier ewes when they were given a choice. It was also observed that a ram will prefer an ewe of his own breed (Owens & Thompson, 1994). In addition, Orihuela & Vázquez (2009) stated that genetic relatedness between a ram and an ewe did not influence the choice of a mate. It was also found that when an ewe was treated with antibiotics, this decreased the normal vaginal flora composition and compromised an ewe's sexual attractiveness. This is caused by the influence the flora has on her pheromone characteristics. Thus rams will prefer ewes that are untreated (Ungerfeld & Silva, 2005). Tilbrook & Cameron (1989) also observed that rams displayed mating preference for ewes with longer wool over shorn ewes under field conditions.

Synnott *et al.* (1981) concluded that sperm will be distributed unequally when ewes are mated under field conditions. Only the more sexual attractive ewes will be mated often, and receive an adequate number of sperm to ensure maximum conception rates. Thus mating preference can have a negative influence on fertility, as some ewes will be mated a large number of times and some infrequently (Tilbrook & Cameron, 1989). Therefore information on mating preferences of rams can be used to derive more appropriate ram: ewe numbers (Abecia *et al.*, 2005).

2.6.3 Seasonal reproduction

It is commonly known that the reproductive activity of rams is influenced by the season, controlled by the changes in the photoperiod, with seasonal fluctuations in hormonal activity, spermatogenesis and also in testicular size. Several studies have observed the seasonal fluctuation in scrotum circumference, with the maximum diameter observed during the breeding season, linked to higher ejaculate volume and sperm concentration (Mandiki *et al.*, 1998).

However, rams are less influenced by the season compared to the ewe as spermatogenesis and sexual activity in the ram are continuous (Rosa & Bryant, 2003; Kafi *et al.*, 2004). However, seasonal variation in sperm quality has been observed to influence specific sperm characteristics, with sperm of superior quality being produced during autumn (the preferred breeding season) (Karagiannidis *et al.*, 2000).

Sperm motility and morphology are both considered as important factors for determining sperm quality and as predictors for potential ram fertility (Martí *et al.*, 2012). However, several studies have shown that these characteristics are influenced by season. In a study done by Kafi *et al.* (2004) on Karakul rams, semen samples had a significantly higher mass motility in autumn compared to the other seasons. Sarlós *et al.* (2013) obtained similar results, with the highest sperm motility found in the late summer and autumn and a lower level of sperm motility observed during winter. The latter authors further observed the highest percentage of morphologically abnormal sperm during winter and the lowest in autumn.

A study on dairy bulls showed an increase in morphologically abnormal sperm numbers during winter, indicating that this might have been affected by the low temperature exposure of the testes (Sekoni & Gustafssont, 1987). Similar results were observed when the scrotum of rams was cooled using ice. An increase in the number of separated sperm heads was observed in ejaculates a couple of days after the cold exposure (Sekoni & Gustafssont, 1987).

According to Bravo *et al.* (2011), sperm morphology may be a good indicator of seasonal effects, as seasonal variation in sperm head shape and size was observed. The authors also found that sperm are longer and more elliptical during autumn and more rounded in spring. Thus, one can assume that the photoperiod influences the sperm head shape and size during the breeding season. The above observations confirm the findings of Martí *et al.* (2012), that sperm morphology are influenced by season resulting in changed sperm morphometric subpopulations. Although many factors can influence sperm quality, it is important to understand how season affects sperm quality, as this knowledge can be used to identify the most suitable season for sperm collection for ART's usage.

2.6.4 Breed

In a country like South Africa, with different climate regions, different breeds of sheep adapted to such regions are used in sheep production systems (Cloete & Olivier, 2010). In addition, some farmers breed with sheep for specific products like meat or wool (Simitzis *et al.,* 2006). It is also known that some breeds are more fertile than other (Kukovics, 1985) and that sperm quality parameters and libido may depend on breed (Boland *et al*., 1985).

In a study by Patel & Dugwekar (1999), significant differences were observed between Patanwadi, Rambouillet x Patanwadi and Merino x Patanwadi rams for microscopic and macroscopic sperm parameters. In another study, Suffolk, Texel and Dorset Horn rams were compared for sperm quality traits. Breed had a significant effect on the mass motility of semen (Boland *et al*., 1985). In contrast, no differences in semen quality traits were observed between Namaqua Afrikaner, Döhne Merino and Dorper rams, when semen was collected by means of electro-ejaculation (Letsoalo *et al.,* 2016).

2.6.5 Selection

Genetic selection for specific traits can increase production levels of livestock markedly. With the increase in global food demands, climate change and high feed costs, farmers are selecting for a population with the highest economic production efficiency (Rauw *et al.,* 1998; Nardone *et al.,* 2010). Lamb production are of utmost importance for production efficiency and economic output (Safari *et al.,* 2005). However reproduction traits have low heritability estimates, are sex-limited, measured late in the life of animals and are composite in nature (Notter, 2012).

Genetic change in the reproduction potential of an animal depends on additive gene action. While the heritability of ovine reproduction traits is generally low, genetic gains may be quite substantial (Cloete *et al*., 2004). These authors emphasised the fact that reproduction traits exhibit higher levels of phenotypic variation compared to other trait complexes, thus facilitating genetic gains. Therefore, the genotype of an animal determines the reproductive physiology and behaviour of that animal within its environment (Krasnow & Steiner, 2006). Selection is often based on an animal's phenotype and specific traits, without considering the potential adverse correlated responses that can have a negative influence on reproduction success. Bench *et al*. (2001) stated that if there is sufficient genetic variation within a sheep population, it is possible to elicit considerable current-flock gains by selecting for ram sexual performance in male offspring within a single generation.

In a study on two genetically diverse Merino lines selected for reproduction potential, Cloete & Scholtz (1998) found that offspring born in the High line (HL) had improved birth weights and a higher overall survival rate, in comparison to the offspring from the Low line (LL). The two genetically diverse lines of Merino sheep were subjected to divergent selection for reproduction since 1986, defined as the ability of ewes to rear multiple offspring. Male and female replacements in the HL were selected from progeny of ewes that reared more lambs than they had joining opportunities. In contrast, LL replacements were descended from ewes than reared fewer lambs than they had joining opportunities (Cloete *et al.*, 2004; 2009).

Genetic selection within a flock, are a slow process in terms of improvement of desired traits. Commercial sheep producers sometimes prefer to mate their ewes to specialist meat sires in a

terminal crossbreeding system where all progeny born are slaughtered (Cloete *et al.,* 2004; 2008). Such crossbreeding systems benefit from the advantages of heterosis and sexual dimorphism. Such systems are competitive at low levels of recording and mostly applies to situations where sheep production complements a more capital and labour intensive cropping enterprise as primary industry. In South Africa (SA), the Dormer breed had been developed in the 1940's to produce slaughter lambs from wool-type ewes (De Villiers & Cloete, 1982). It is still one of the most important terminal sire breeds used in the SA Merino industry (Cloete *et al.,* 2014; Zishiri *et al.,* 2014). According to Olivier (2014), 7.1% of the total weaning weights recorded by the National Small Stock Improvement Scheme consisted out of the Dormer breed.

Due to the above mentioned factors, it was decided to use the Merino in this study as a model to study the impact of divergent selection for reproduction on sperm characteristics. The Dormer was also included, since the breed is used as the primary terminal sire breed in SA. The importance of evaluating the Dormer's sperm characteristics is motivated by this role in the local sheep industry, however research on Dormer reproduction traits.is limited.

2.6.6 Diseases

There are several bacterial and viral infections that may cause male infertility and reduce the reproduction efficiency of the ram. Such infections can cause a decline in spermatogenesis as well as loss of sperm function (Keck *et al.,* 1998). However, one of the major causes of ram infertility is ovine epididymitis caused by the organism *Brucella ovis (B. ovis)* (Burgess, 1982). Ovine epididymitis is usually characterised by an inflamed epididymis that leads to testicular degeneration and infertility of the ram (Swift *et al.,* 1982). *B. ovis* infection leads to poor quality semen with a reduced sperm motility and concentration and a high percentage of morphologically abnormal sperm (CFSPH, 2007).

The mammalian testes functions optimally at a temperature that is lower than core body temperature. The regulation of this specific scrotal temperature is important for sperm production. A fever, generally a symptom of many diseases, may compromise sperm production and integrity (Ax *et al*., 2000). Too high temperatures can adversely affect spermatogenesis, and may also cause the epididymal proteins to undergo degradation, which are important for the processes of sperm maturation, motility and fertilization (Mathuria & Verma, 2008).

2.6.7 Nutrition

It is widely known that the reproduction efficiency of livestock largely depend on their nutritional status and that sufficient nutritional management of utmost importance is for successful mating in sheep (Fernández *et al.*, 2004; Kheradmand *et al.*, 2006). Brown (1994) stated that dietary components such as energy and protein can influence the onset of puberty, expression of libido,

testicular function and endocrinology. The author also mentioned that deficiencies in vitamins and minerals may have a negative impact on normal reproduction function.

In South Africa, sheep are mainly farmed under extensive conditions using natural pastures as the main feed source. These natural pastures quality and quantity varies according to the season and can lead to nutritional deficiencies in sheep (Fourie *et al.*, 2004). Research has shown that an increase in nutritional intake of both energy and protein improved the reproductive performance of rams (Fernández *et al.*, 2004).

Several studies have found that energy and protein deficiencies might adversely affect spermatogenesis and libido in rams (Kheradmand *et al.*, 2006). According to Fernández *et al.* (2004) rams with severe protein deficiencies had a lower sperm quality due to a reduced sperm motility and more sperm with abnormal sperm morphology. In a study done by Braden *et al.* (1974), Merino rams were fed two levels of dietary energy and/or protein and it was found that sperm production was not affected by protein alone, but was higher when the energy level was increased either on its own or in combination with protein. The authors also observed that rams fed a high energy-diet had significantly larger testes and seminal vesicles compared to rams fed a low energy-diet. Therefore adequate levels of both protein and energy are important for maximal sperm production, as it increases testicular size, due to an increase in the volume of seminiferous epithelium and the diameter of the seminiferous tubules (Fernández *et al.*, 2004).

However, rams should not be overfed and allowed to become fat, as overweight rams tend to have a reduced reproductive performance and a lower sperm quality. This could be ascribed to an excess fat in the scrotum neck inhibiting testicular thermoregulation and causing a deterioration in sperm quality (Dance *et al.*, 2016).

Although the intake of energy and protein are of utmost importance, vitamins and minerals also play an important role in the reproductive health of the ram (Zubair *et al.*, 2015). Vitamin A plays an important role in a ram's reproduction efficiency as it increases spermatogenesis, semen quality, libido as well as stimulates testosterone secretion (Coward *et al.*, 1966; Al-Haboby *et al*., 1997 cite Abdulkareem *et al.*, 2005). In a study done by Abdulkareem *et al.* (2005), it was observed that a deficiency of Vitamin A in rams caused the degeneration of the testes and an impaired sperm quality due to an increase in morphologically abnormal sperm. The supplementation of Vitamin A is crucial for rams that had no access to green roughages for an extended period.

Another vitamin that plays an important role in the reproduction efficiency of the ram is Vitamin E. It plays an important role as an antioxidant, preventing cellular damage caused by lipid oxidation. Surai *et al.* (2000) suggested that the dietary supplementation of Vitamin E increases the biological

stability of the sperm plasma membrane. In a study done by Husein *et al.* (2011) on mice, it was found that the supplementation of Vitamin E had a significant impact on sperm quality. The supplementation decreased the percentage of morphologically abnormal sperm when compared to the control group not receiving Vitamin E. Vitamin E and selenium (Se) work together to maintain antioxidant levels in the ram and therefore need to be supplemented together in adequate amounts (Liu *et al.*, 2014).

Minerals also play an important role in sperm production; however, information of the influence of specific mineral deficiencies on the reproductive efficiency of rams is rare. Recently the effect of zinc and selenium supplementation on sperm production was evaluated. Zinc plays an important role in the production of testosterone and gonadotropin releasing hormone (GnRH) and it is also necessary for the attachment of the sperm tail to the head (Kendall *et al.*, 2000). According to Underwood & Somers (1969), zinc supplementation increases daily sperm production and reduces the percentage of morphologically abnormal sperm. As mentioned above, selenium plays an important role in the antioxidant status of rams and a deficiency will influence sperm motility and morphology (Scott *et al.*, 1998). Kendall *et al.* (2000) observed that sperm motility and sperm viability (percentage live sperm) increased with an increase in dietary selenium supplementation. This result suggests that zinc and selenium supplementation may increase sperm quality under marginal and deficient conditions.

2.7 Evaluation of sperm quality

For the successful application of ART's, both female and male components need to be of good quality. Therefore the evaluation of the sperm selected for artificial insemination and *in vitro* embryo production is of utmost importance (Rodríguez-Martínez, 2007). Sperm evaluation is a useful tool to identify infertility or potential sub-fertility. In sheep, ram infertility may have a major impact on the reproduction efficiency of a production system, causing a decreased lambing rate, as a single ram is capable of impregnating multiple ewes by either natural breeding or by artificial insemination (Rodríguez-Martínez, 2007; Tsakmakidis, 2010; Yaniz *et al.*, 2012). Semen evaluation is done together with a physical examination, focussing specifically on the scrotum and testicles, to determine the potential fertility of a ram (Edmondson *et al.*, 2001).

When evaluating semen samples, the sample is subjected to macroscopic and microscopic evaluation. The main aims of semen evaluation is to accurately, objectively, rapidly and where possible, economically predict the fertility of a semen sample, although no single test can accurately predict the fertility of a sample. Evaluating semen samples for specific parameters can only determine greater fertility potential (Ax *et al.*, 2000; Mynhardt, 2011).

The minimum requirements for a possible fertile ram semen sample are more than 80% normal morphology, sperm concentration ranging from 3.5 x 10^9 to 6.0 x 10^9 and more than 50% of progressively forward motile sperm (Ax *et al.*, 2000).

2.7.1 Macroscopic evaluation of semen

The first step in semen evaluation is a simple visual inspection that involves the macroscopic evaluation of a sample and includes the assessment of volume, colour and mass motility.

2.7.1.1 Volume

Semen volume varies between different livestock species and is usually determined by the calibration marks on the collection tube. For example the boar produces a large ejaculate in volume (250-400mL), whereas the ram has a much smaller ejaculate (0.5-2mL) (Ax *et al.*, 2000). A ram's ejaculate volume can be influenced by several factors including breed, age, nutrition, season, collection frequency and method as well as health status (Ghorbankhani *et al.*, 2015; Žaja *et al.*, 2016).

2.7.1.2 Colour

Semen should have a relatively uniform, dense appearance and be free of any contaminants like hair or dirt. The evaluation of colour is not essential for sperm quality, but it can be an indicator of injury or infection (Dhurvey *et al.*, 2012). The colour of ram semen varies from milky-white to a thick creamy colour and is scored on a 0-5 scale (0 watery and 5 thick creamy) (Ax *et al.*, 2000).

2.7.1.3 Mass motility

Mass sperm motility is a rapid and inexpensive test to assess sperm quality (David *et al.*, 2015). The semen sample is assessed immediately after collection for the collective movement of fresh undiluted semen. Ram sperm usually exhibits a wave-like motion when observed on a pre-warmed microscope slide using a light microscope (Ax *et al.*, 2000). Mass motility is subjectively scored according to a scale from 0-5, with 0 indicating no motion and 5 showing many rapid waves (Table 2.1)(David *et al.*, 2015).

Table 2.1 Scoring system used to assess the mass motility of ram sperm (Adapted from David *et al.,* 2015)

Score	Macroscopic appearance
0	No swirl – nil or sporadic oscillation of individual sperm
1	No swirl – generalized oscillation of individual sperm only
2	Very slow distinct swirl
3	Slow distinct swirl
4	Moderately fast distinct swirl
5	Fast distinct swirl

2.7.2 Microscopic evaluation of semen

Microscopic evaluation of sperm is a more accurate way of assessing sperm quality and is used to evaluate sperm concentration, viability, motility, morphology and acrosome integrity.

2.7.2.1 Sperm concentration

Generally, sperm concentration refers to the number of sperm per millilitre of semen. The accurate evaluation of a semen sample is essential to determine how many ewes can be inseminated artificially with an individual semen sample. It is also important to determine to what extent a semen sample needs to be diluted for processing purposes (Graffer *et al.*, 1988; Ax *et al.*, 2000). Several methods are available to assess sperm concentration, some of which include the use of a haemocytometer, spectrophotometer or a Computer-assisted semen analysis (CASA) system.

Sperm concentration can be determined by the haemocytometer method using a Neubauer chamber. The microscope slide is placed under a microscope and the number of sperm per chamber is manually counted. Although this method is very accurate, it is time consuming. A quick method for determining sperm concentration is by means of a spectrophotometer that has been calibrated against a haemocytometer. However, sperm concentration can only be measured within a specific range and the accuracy can be influenced by contaminated semen (Paulenz *et al.*, 1995; Ax *et al.*, 2000).

The problem with the above-mentioned methods is that it is subjective and results may vary. Therefore CASA systems have been developed to allow objective, immediate, consistent, rapid and accurate evaluation of various sperm parameters (Klimowicz *et al.*, 2008). However the use of this method is limited to only specific laboratories due to the high cost of the instruments needed (Verstegen *et al.*, 2002).

2.7.2.2 Sperm viability

Normal intact plasma membranes and intact acrosomes are of utmost importance for sperm capacitation, acrosome reaction and eventually fertilization (Gürler *et al.*, 2016). Therefore, the evaluation of the percentage live sperm in a semen sample is one of the most important sperm parameters to evaluate.

There are several staining procedures available that can be used to determine the percentage live sperm in a semen sample. Most staining procedures used involve the use of fluorescent dyes followed by fluorescent microscope examination. However, the most commonly used staining technique is the combination of eosin-nigrosin. This is a one-step procedure that can be implemented with only ordinary bright-field microscopy and therefore entails the most recommended staining procedure (Björndahl *et al.*, 2003).

2.7.2.3 Sperm motility

Motility assessment is one of the most important semen quality indicators used when evaluating a semen sample for quality and assessing fertilizing capacity. Vigorous and energetic motility is essential for the sperm to rapidly move through the female reproductive tract to reach the ovum for fertilization to take place (Palacin *et al.*, 2013; David *et al.*, 2015). The evaluation of spem motiltity in both raw and extended semen samples is important for the assesment of fertilizing capacity of sperm. The evaluation of raw semen samples is an indication of how well sperm perform in its own accessory gland secretions (Varner, 2008).

Sperm motility has many movement patterns, however many factors can influence sperm motility therefore semen samples should be handled with great care to protect the semen from environmental conditions such as extreme temperatures. Several studies have concluded that ram semen is sensitive to the freeze-thaw proccess which is likely to impair sperm motility (Salamon & Maxwell, 1995). Infertility can also be associated with sperm motility patterns (Ax *et al.*, 2000). Several methods have been developed for the objective evaluation of sperm motility which include time-lapse photomicrography, frame-by-frame playback videomicrography, spectrophotometer or computerized analysis. Computer-assisted semen analysis (CASA) systems based on sperm head movements have been developed to provide more information than subjectively evaluated sperm motility and to increase the accuracy and reliability of sperm motility assessment (Palacin *et al.*, 2013). CASA systems objectively assess specific sperm motion characteristics (Varner, 2008). However, according to Tsakmakidis (2010), the correlation between sperm motion charateristics and *in vivo* and *in vitro* fertility is not yet clear and it is unclear whether the evaluation of specific sperm motiility decriptors are useful for predicting the fertilizing capacity of rams.

2.7.2.4 Morphology

Sperm morphology is one of the most important parameters that can be used to predict the fertilizing ability of semen samples and can be influenced by genetic, environmental and handling factors (Butts *et al.*, 2011). The percentage of morphologically normal sperm within a semen sample are linked to fertility while abnormalities can cause reduced fertility and early embryonic losses (Gravance *et al.,* 1998). Furthermore, the percentage of abnormal sperm in the ram can vary between seasons, with the lowest number of abnormal sperm found during the natural mating season (Ax *et al.*, 2000). However not all morphologic abnormalities have a negative effect on fertility, i.e. bent tails appear to have only a minor impact on fertility (Varner, 2008).

Sperm abnormalities are commonly classified as either primary, secondary or tertiary. During spermatogenesis in the testis, primary abnormalities might arise, while secondary abnormalities can take place during epididymal maturation and tertiary abnormalities during handling and

processing. Sperm head abnormalities are those associated with the head and are classified as primary, with mid piece or sperm tail abnormalities usually classified as secondary (Figure 2.3) (Dhurvey *et al.*, 2012).

There are several staining procedures available to assess sperm morphology of which the eosin-nigrosin stain under a light microscope is commonly used to examine sperm morphology. An eosin-nigrosin stain forms a dark background, highlighting the sperm for easy evaluation (Björndahl *et al.*, 2004).

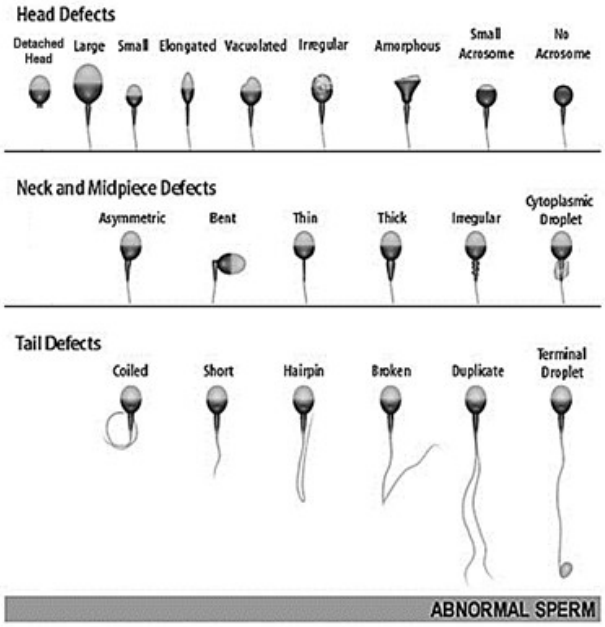

Figure 2.3 Sperm morphology: Abnormal sperm with various head, neck and midpiece and tail defects (Jothipriya *et al.,* 2014).

2.7.2.5 Acrosome integrity

A ram's fertility can also be influenced by the functional competence of the sperm membranes. Intact functional plasma membranes are essential for sperm metabolism, capacitation, acrosome reaction and fertilization. For sperm penetration and fertilization to happen, acrosome integrity is vital. Therefore, the evaluation of sperm acrosome integrity can be valuable when predicting the potential fertilizing ability of a semen sample (Eskandari & Momeni, 2016). There are, however, several factors potentially affecting the acrosome integrity of sperm. During the freeze-thawing process, acrosome integrity is reduced because of cold shock and osmotic stress (Salamon & Maxwell, 2000).

There are several staining techniques that can be used to assess sperm acrosome integrity of which Spermac® and Rapi-Diff® are but a few. These stain all the different components of the sperm in different colours and can be observed under a light microscope.

2.7.2.6 Morphometry

The evaluation of sperm head morphology is essential for predicting the fertilizing capacity of a ram (Öztürkler *et al.*, 2001; Butts *et al.*, 2011). An individual sperm cell is a highly specialized unit that can differ in size and shape and plays an important role in the fertilizing process. Therefore the evaluation of sperm morphometry is an essential part of sperm quality assessment (Yániz *et al.,* 2015). Although standard semen evaluation protocols include the evaluation of sperm morphology, it is subjective and highly variable. Therefore software has been developed to accurately measure sperm head morphometric characteristics (Gravance *et al.*, 1998; Hidalgo *et al.*, 2006).

Computer-assisted sperm morphometry analysis (CASMA) has been developed for accurate and objective evaluation of sperm head morphometry. Sperm head morphometry can be described by different parameters. The four primary parameters that provide information of sperm head dimensions are area, perimeter, length and width (Maroto-Morales *et al.,* 2010; Yániz *et al*., 2015). The most often used technique to assess sperm morphometry is light microscopy together with specific sperm morphometry analysis software (Yániz *et al.*, 2015). CASMA systems like the Sperm Class Analyser (SCA®) have been used to assess ram sperm head morphometry. However, these systems can be expensive. An open source ImageJ software system (https://imagej.nih.gov/ij/docs/guide/user-guide.pdf) that can be used as an alternative to expensive CASMA systems is presently available to automatically measure sperm head morphometry (Butts *et al.*, 2011).

The evaluation of sperm head morphometry can be used for the prediction of the potential fertility of a semen sample. With the existence of different sperm subpopulations within an ejaculate widely accepted, the assessment of sperm head morphometry can be a useful tool to identify possible subpopulations (Yániz *et al.*, 2015). Sperm head morphometric data can be analysed using principal component and multivariate cluster analysis to identify these sperm subpopulations.

2.7.3 *In vitro* fertilization tests

The prediction of an animal fertilizing capacity before being used for breeding is of utmost importance, especially when an animal with a highly genetic merit is used (Larsson & Rodriguez-Martinez, 2000; Losano *et al.*, 2015). Although standard semen evaluation protocols assess sperm quality, information on a more accurate and deeper level of a male animal's fertility is necessary (Larsson & Rodriguez-Martinez, 2000).

Several techniques have thus been developed to predict an animal's fertilizing capacity more accurately. The *in vitro* fertilizing technique involves the *in vitro* maturation, *in vitro* fertilizing of abattoir derived oocytes and the *in vitro* culture of these fertilized oocytes up to the blastocyst

stage. Another technique commonly used is the *zona pellucida* binding assay, based on a spermatozoa's ability to bind to homologous or heterologous *zona pellucida* (ZP), where the total number of sperm bound to the ZP are counted (Larsson & Rodriguez-Martinez, 2000; Waberski *et al.*, 2005). However, these techniques are time-consuming and the availability of abattoir derived oocytes may limit application (Larsson & Rodriguez-Martinez, 2000; De Araujo *et al.*, 2015).

To overcome the abovementioned limitations, researchers have developed an alternative binding assay using the perivitelline membrane of a chicken egg (Barbato *et al.*, 1998; Purdy, 2006b; De Araujo *et al.*, 2015). Due to protein similarities between the chicken egg perivitelline membrane and the mammalian *zona pellucida*, sperm of some species have the ability to bind to this membrane (Losano *et al.*, 2015). For a sperm cell to successfully fertilize an ovum and to bind to the membrane it needs to undergo the processes of capacitation and the acrosome reaction. If unable do to so, membrane binding will not take place (Barbato *et al.*, 1998). According to Purdy (2006b), this is a valuable assay and has been successfully applied in sheep.

2.8 Aims

Against the background that ART's are expected to become more important in the ovine breeding industry as well as the literature surveyed above, the following aims for this study have been identified:

1. To obtain the necessary information to formulate a standard operating procedure (SOP) for training rams to ejaculate into an artificial vagina (AV). It is generally known that sperm used for ART's are mostly collected by means of the AV, due to the better quality of the sperm.
2. To evaluate the influence of ram genotype (Dormer, Merino High line and Merino Low line) on morphometric sperm subpopulation structure.
3. To assess the influence of ram genotype and sperm subpopulation traits on the *in vitro* sperm fertilizing ability of semen.

2.9 References

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Chapter 3
Materials and Methods

Ethical clearance was approved by the Stellenbosch University Animal Ethics Committee (SU-ACUD15-00083). All animal care and procedures used in this study was according to the guidelines provided in the South African National Standards document 10386:2008.

3.1 Experimental location

The study was conducted on the Elsenburg Agricultural Research Farm (GPS: -33.837966; 18.834337) situated near Stellenbosch in the Western Cape Province, South Africa. The farm is the property of the Provincial Department of Agriculture, Western Cape. The average annual rainfall for this region is 486mm, with the average annual temperatures ranging between 6.8 ºC and 29 ºC (CFM, 2016).

3.2 Experimental animals

3.2.1 Rams

The trial animals (n=29) used in the study comprised of 20 Merino rams (10 sexually inexperienced and 10 sexually experienced) and 9 Dormer rams (5 inexperienced and 4 experienced), aged between 1 and 6 years of age. Both breeds form part of the animal genetic resource population maintained on the Elsenburg Research Farm. Rams were considered as sexually experienced when they were used for mating purposes for one of more previous breeding seasons. Rams were considered as sexually inexperienced when they had no previous exposure to ewes, and had no prior history of being used for mating purposes. All rams were managed according to standard industry farming practices, were handled by personnel that had substantial experience in the care and maintenance of sheep, and were subjected to a standard health/disease control programme. All rams were maintained in a single flock and grazed on irrigated kikuyu (*Pennisetum clandestinum*) pastures throughout the study, with water being available *ad libitum*. Animals used for the behavioural study were shorn prior to the onset of the study.

The Merino rams used in this study were selected from the Elsenburg Merino resource flock. The flock originated from a base population established in 1986 on the Tygerhoek Research Farm. The base population was used to produce two genetically diverse lines of Merino sheep, subjected to divergent selection for reproduction, defined as the ability of ewes to rear multiple offspring. Replacements for the High Line (HL) were selected from progeny of ewes that reared more lambs than joining opportunities. In contrast, Low Line (LL) replacements were descended from ewes than reared less lambs than joining opportunities (Cloete *et al*., 2004; 2009). Rams used of both lines were selected in the same way on maternal performance. Selection decisions

were augmented since 2002 by single-trait repeatability model breeding values, resulting in individuals with high breeding values selected within the HL and those with low breeding values within the LL.

3.2.2 Ewes

A total of 24 ewes, consisting of 12 Merino ewes (6 yearling and 6 mature) and 12 Dormer ewes (6 yearling and 6 mature), aged between one and six years, were used for the behavioural study as well as the establishment of a standard operational procedure for the training of rams to use an artificial vagina (AV). Ewes were considered as mature when they have had one or more parities. In contrast, yearling ewes had no prior exposure or contact with rams inside or outside of the breeding season.

For use in the training sessions, the ewes were synchronised using 40mg flugestone acetate (FGA) sponges (Ovakron®, Ramsem, Bloemfontein, South Africa). The intravaginal sponges were inserted using a sponge applicator, and an antibiotic cream (Ramsem, Bloemfontein, South Africa) was applied to each sponge before it was inserted. On day 14 after insertion, the intravaginal sponges were removed, and the ewes came into oestrus 36h later.

The synchronisation protocol of ewes used during the mating preference experiment and the training experiment of the behavioural study will be explained in more detail in section 3.3.1.

3.3 Experimental design

This overall study design incorporated three phases. Phase one consisted of three different experiments that were designed to contribute to the formulation and writing of a standard operational procedure (SOP) for the training of rams to ejaculate in an artificial vagina. Phase two entailed the determination of the influence of breed and genetic selection for reproduction potential on sperm quality parameters and sperm morphometric subpopulation traits. Sperm samples collected during Phase 2 were cryopreserved for the trials in Phase three of the study. The third phase of the study studied the influence of breed and genetic selection on the fertilizing ability of sperm collected from individual Dormer and Merino rams. During the latter phase, the post-thaw sperm quality of the collected samples was also determined. The sections below describe in detail the methodologies used in the respective phases.

3.3.1 Phase 1: Establishment of a standard operational procedure to the training of rams to use an artificial vagina

Phase 1 consisted of three experiments, i.e. a mating preference study, a habituation study, and lastly, the training of rams to ejaculate into an artificial vagina (AV).

3.3.1.1 Mating preference study

In this experiment it was investigated whether a ram of a specific breed and sexual experience category (inexperienced or experienced) has a preference for a mature or yearling ewe of the same breed. The mating preference of 5 Dormer rams and 5 Merino rams of each sexual experience category, i.e. inexperienced or experienced respectively, were determined.

Rams were given an opportunity to demonstrate whether they exhibit a preference for a certain ewe, according to the testing approach described by Tilbrook & Lindsay (1987). During each preference study, a ram was exposed to and given an opportunity to rank six oestrus ewes (i.e. 3 mature and 3 yearling) of the same breed as the ram. For each sexual experience category within a ram breed, a different group of ewes was used. Each group of oestrus ewes within a breed, were ranked either two (Merino) or three (Dormer) times by each ram.

3.3.1.1.1 Exposure protocol

Each ram was individually exposed to the six oestrus ewes in a test pen (6m x 6m) and the time spent by the ram courting and mating each ewe was recorded. Timing started when a ram started showing sexual behavioural signs directed towards a specific ewe. Sexual behavioural signs included sniffing of the perineum, nudging, the Flehmen response, nibbling and/or mounting of the ewe (Banks, 1964). Every five minutes the ewe that the ram has spent the most time with was removed from the pen. Timing stopped if a ram was no longer exhibiting any of the sexual behavioural signs in the presence of a specific ewe. Observations continued until all ewes were ranked. The ewe that was removed first from the pen was ranked as one and was considered as the most “attractive”, whereas the last remaining ewe was ranked as sixth and was considered to be the least “attractive” to the ram.

In cases were after an initial observation period of 5 minutes the observer was unable to discern the most preferred ewe (based on the abovementioned signs of interest), observation continued for another 10 minutes. If after this 15 minutes the observer was still unable to discern the preference priority of the ram, the observations were terminated, and all ewes sharing the attention of the ram receiving the same ranking. In cases where a ram expressed equal interest in two ewes during the same observation interval, thus ignoring the other ewes, both ewes were removed from the pen and received the same ranking.

3.3.1.2 Habituation study

The habituation study investigated the ability of a ram, when exposed to the observer and/or a technical support staff member at a low frequency (LF) or a high frequency (HF), respectively, to become habituated to the presence of the observer and/or support staff member. A LF consisted of the rams being handled on only two days per week (i.e. Tuesday and Thursday),

compared to the HF group that was handled for 5 consecutive days (i.e. Monday to Friday). Inexperienced and experienced rams (n=29) were allocated to either the LF (n=14) and HF (n=15) groups, respectively. Each experience category within each exposure frequency group were further subdivided, with certain rams being exposed to only the observer, and the remainder of the rams exposed to the observer as well as the support staff member.

The habituation procedure used during this study was based on previous work done on the Elsenburg Research Farm (Boshoff, 2014). The habituation procedure was repeated for four consecutive weeks during the morning, and the same observer and support staff member was used throughout the study.

3.3.1.2.1 Handling and exposure protocol

On each handling day the specific sub-group of each exposure frequency group was brought in from the pasture by a support staff member. Each individual ram was placed in a single indoor pen (1m x 2m), and exposed to the student and/or the support staff member. The duration of each handling session throughout the study was five minutes (Waiblinger *et al.,* 2004; Tallet *et al.,* 2005).

During the first week all the rams were introduced to the student and/or the support staff member, by standing in the empty pen next to the holding pen with the ram. No effort was made to enter the holding pen and all handling activities were from outside. In Figure 3.1 the allocation of rams in the holding pens is indicated. This initial exposure was to familiarise the rams with the student and/or the support staff member and to accustom the rams to the presence of humans. The following behaviour patterns were recorded:

- Docility level (score 0 or 1).
    - 0 – the ram was not docile (stormed student and/or the support staff member).
    - 1 – the ram was docile (allowed the student and/or the support staff member to touch him).
- Tolerated the student and/or support staff member to scratch his head or not.

For the remaining weeks, the student and/or the support staff member entered the pen with the ram and the following behaviour patterns were recorded.

- Docility level (score 0 or 1).
- Whether the ram allowed the student and/or support staff member to crouch next to him or not.
- Whether the ram allowed the student and/or support staff member to touch the head, belly and chest area or not.

Each animal within a sub-group (exposure to observer and/or support staff member) was handled individually. After exposure of each sub-group, the group was returned to the pasture and the next sub-group was brought in.

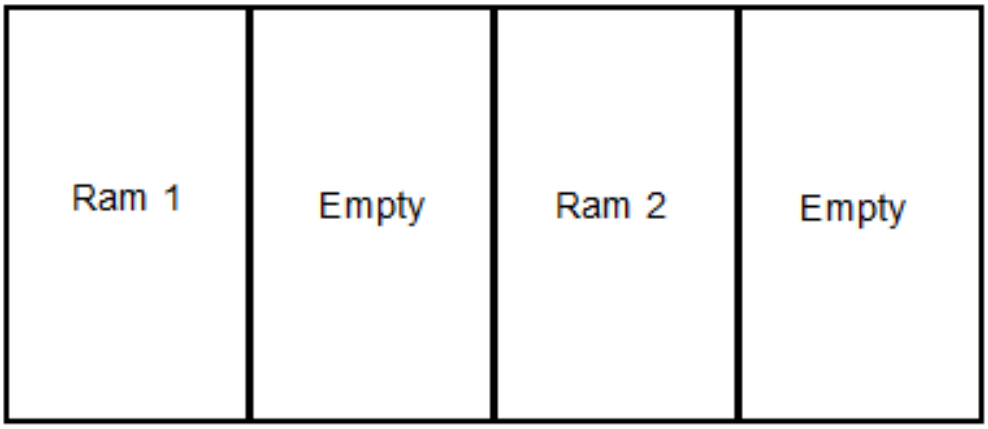

Figure 3.1 Handling pen diagram indicating allocation of rams during a habituation study.

3.3.1.3 Training of rams to use an artificial vagina for semen collection purposes

During this experiment, all of the habituated rams (n=29) were subsequently trained to ejaculate into an artificial vagina (AV) for semen collection purposes. For training purposes, ewes of both breeds (Dormer and Merino) were synchronized and used as dummy ewes to determine whether a ram (inexperienced or experienced) of a particular breed preferred a ewe of his own breed or not.

The rams were trained over a period of two consecutive weeks, with 7 training sessions spread throughout the fortnight. Training of the rams was carried out in the morning, and the training protocol used in this study was according to the method described by Flores *et al.* (2005).

3.3.1.3.1 Training procedure

Throughout the study the AV was prepared prior to each training session as described by Marco-Jimenez *et al.* (2005). Each training session lasted 10 minutes after which the ram was removed and returned to the pasture. On the day of training, all the rams were brought in from the pasture and placed in individual indoor pens until it was their turn to be trained. Each ram was individually exposed to both a restrained Merino and Dormer ewe in oestrus (section 3.2.2) in a large indoor pen (4m x 4m).

With each training session all sexual behavioural signs exhibited by the ram towards each of the ewes were recorded (Banks, 1964). It was recorded whether a ram successfully used the AV, and also if he approached one ewe or both ewes, and which specific ewe he mounted. It was

also recorded when a ram rejected the AV or was frightened by the presence of the support staff member. When a ewe was mounted by a ram, the penis of the ram was gently deflected into the open end of the AV. If a ram ejaculated into the AV, this was considered as the successful usage of the AV.

3.3.2 Phase 2: Semen evaluation and classification of morphometric subpopulations

Phase 2 entailed the collection of semen samples, the evaluation of the samples according to standard semen evaluation protocols, the classification of sperm morphometric subpopulations, and the cryopreservation of the collected samples.

3.3.2.1 Semen collection

Semen was collected for three days per week for three consecutive weeks from 12 Merino rams (6HL; 6LL) and 6 Dormer rams. All rams were aged between 2 to 5 years. The rams were randomly divided into three groups of six rams each and ejaculates were collected once a week from each ram, with a total of three samples per ram for the duration of the study.

Although the rams were trained in Phase 1 to use the AV, all rams except four rejected the AV during semen collection for Phase 2. Due to time constraints, semen samples were collected by means of electro-ejaculation according to a method adapted from Orihuela *et al*. (2009) and Malejane *et al*. (2014). On collection days, rams were brought in from the pasture and placed individually in single indoor pens (2m x 1m) until collection. Semen collection was done in a clean open space, protected from sunlight. Prior to stimulation, each ram was placed in a lateral position on the floor and the penis was gently everted from the sheath and held gently with a piece of sterile gauze. The custom-designed rectal probe that was used in the procedure was 22 cm long, with three longitudinal electrodes along the probe (7cm). Lubricant (liquid paraffin) was applied to the probe and to the anal sphincter before insertion to minimize trauma. The rams were allowed to acclimatise to the sensation, while the operator gently massaged the accessory sex gland area. Electrical stimulation was applied for intervals of three to five seconds and altered with rest periods of three to five seconds. With each stimulation the current was gradually increased, until an ejaculate was obtained. Great care was taken to ensure that the penis was held so that the urethral process was not obstructed or bent. No sedatives or anaesthetics were given during the collection procedure. Any samples contaminated with urine were discarded.

Directly after collection, each collection tube containing the ejaculate was sealed with parafilm (Lasec, South Africa) and transported to the laboratory in a thermal flask (37ºC) within 2 hours after collection for further processing.

3.3.2.2 Evaluation of collected samples

On arrival at the laboratory, the semen samples were transferred to a water bath maintained at 37ºC until further processing.

3.3.2.2.1 Macroscopic evaluation

Macroscopic evaluation of the semen samples included the assessment of volume, colour and mass motility. These parameters of the semen samples were evaluated by the same person throughout the study.

3.3.2.2.1.1 Volume

The semen volume was determined by assessing the volume from the calibrated collection tube. The tube was hold vertically to ensure an accurate determination of semen volume.

3.3.2.2.1.2 Colour

The colour of the semen samples was scored using the grading scale indicated in Table 3.1.

Table 3.1 Grading system used to describe the colour of the semen samples obtained from the rams (adapted from Ax *et al.,* 2000).

Score	Colour
5	Thick creamy
4	Creamy
3	Thin creamy
2	Milky
1	Cloudy
0	Watery

3.3.2.2.1.3 Mass motility

The mass motility of the semen samples (fresh and post-thaw) was determined by placing a 5µL drop of raw semen on a pre-warmed (37ºC) microscope slide and covered with a pre-warmed (37°C) coverslip. Mass motility was scored after viewing the sample's motility under a light microscope (10x objective), according to the scale presented in Table 3.2 (David *et al.,* 2015).

Table 3.2 Grading system to score the degree of mass motility of the semen samples obtained from the rams (adapted from David *et al.*, 2015).

Score	Macroscopic appearance
0	No swirl – nil or sporadic oscillation of individual sperm
1	No swirl – generalized oscillation of individual sperm only
2	Very slow distinct swirl
3	Slow distinct swirl
4	Moderately fast distinct swirl
5	Fast distinct swirl

3.3.2.2.2 Microscopic evaluation

Microscopic sperm traits recorded for the respective samples included sperm concentration, viability, morphology, acrosome integrity and morphometric parameters.

3.3.2.2.2.1 Sperm concentration

The concentration of all semen samples was determined by using the haemocytometer method (Prathalingam, 2006). To determine the concentration, an aliquot (10μL) of each sample was diluted with 990μL distilled water, and mixed gently to ensure that all sperm were immobilised (killed). The Neubauer chamber was then loaded with 10μL of the sperm-water combination, and covered with a coverslip (Lasec, South Africa). Sperm were counted as described by the WHO (2010), and sperm concentration was then calculated by using the following formula:

Number of sperm/mL= N sperm counted x 5 x 10 000 x 100 (dilution factor).

3.3.2.2.2.2 Sperm viability

Sperm viability was determined using the eosin-nigrosin (NE) staining method (Kyron Laboratories, South Africa) (Björndahl *et al.,* 2003). Each NE stain was prepared by mixing a 50μL droplet of sperm with one drop of NE staining material on a pre-warmed (37ºC) microscope slide (Lasec, South Africa) (Ax *et al.,* 2000; Lambrechts *et al*., 2000). Each smear was made as described by Mcalister (2010), and allowed to air-dry. All sperm smears were examined with an Olympus IX70 Inverted Microscope (Wirsam, South Africa) at 40X magnification, and a total of 100 sperm/slide were examined in three fields to determine the percentage of live sperm per sample. The live sperm appeared white/light purple whereas the dead sperm stained dark pink/purple (Figure 3.2).

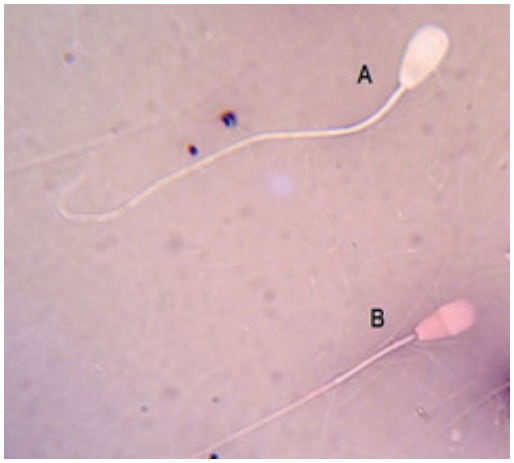

Figure 3.2 Sperm viability: Eosin-nigrosin stain. Live sperm appear white/light purple (A); dead sperm stain dark pink/purple (B) (Moskovtsev & Librach, 2013).

3.3.2.2.2.3 Morphology

For the evaluation of sperm morphology of fresh and post-thaw sperm samples, sperm smears were made and stained with RapiDiff® (Lasec, South Africa). The RapiDiff® staining technique was carried out according to the protocol prescribed by the manufacturer, and allowed to air dry. The air-dried slides were examined with an Olympus IX70 inverted microscope at 40X magnification to determine the percentage abnormal sperm in each sample. A total of 100 sperm were examined in three fields for each slide (Figure 3.3), and expressed as a percentage of the total number of sperm that was counted (Lambrechts *et al.,* 2000).

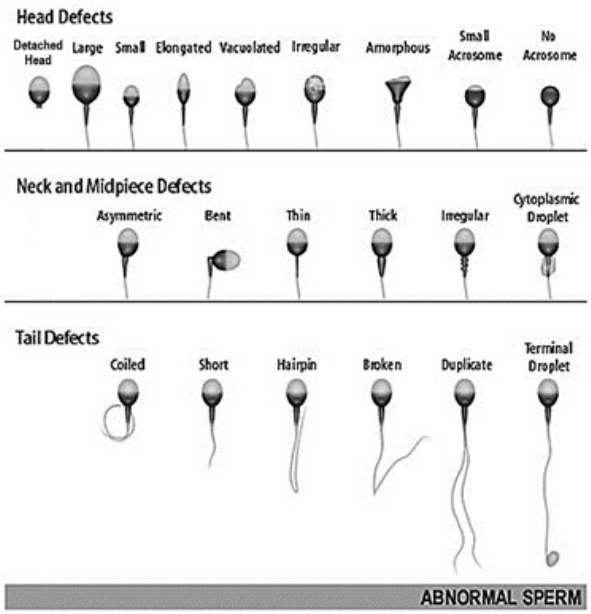

Figure 3.3 Sperm morphology: Abnormal sperm with various head, neck and midpiece and tail defects (Jothipriya *et al.,* 2014).

3.3.2.2.2.4 Acrosome integrity

The same sperm smears that were prepared for the evaluation of sperm morphology (fresh and post-thaw) were also used for the examination of the acrosome integrity of fresh and post-thaw sperm (for more details on sperm smears refer to Appendix A). The sperm smears were examined with an Olympus IX70 inverted microscope at 40X magnification, and 100 sperm in three fields per slide were counted. An acrosome was classified as being intact when no deterioration or detachment (lifting on either side of the sperm head) was observed. Sperm where the acrosome membrane had started to deteriorate of lift halfway or more of the sperm head, were classified as sperm of which the acrosome integrity was compromised. The total number of sperm with intact acrosomes was expressed as a percentage of the total number of sperm counted.

3.3.2.2.2.5 Morphometric parameters

For the identification of sperm morphometric subpopulations, only the fresh sperm samples were evaluated. The smears prepared with the RapiDiff® staining method (see Appendix A), were also used for this purpose.

For the evaluation of the sperm morphometric parameters, micrographs captured with an Olympus IX70 inverted microscope fitted with a Colorview II camera (Wirsam, South Africa), were used. A total of 100 sperm randomly selected in 3 fields were individually measured by using the open-source ImageJ software (https://imagej.nih.gov/ij/docs/guide/user-guide.pdf) (Butts *et al.,* 2011; Ferreira & Rasband, 2012; Yaniz *et al.,* 2012). All digitised images captured were of 3 211 520 pixels and 256 grey levels. The image capturing and morphometric parameter analysis of all the slides was performed by the same person throughout the study.

For each of the 100 sperm the length, width, perimeter and area of the sperm head were measured (Figure 3.4). Three software-calculated indexes, ellipticity, elongation and roundness were included in the sperm head morphometric parameters (Table 3.3). All morphometric measurements of each individual sperm, were saved in a Microsoft Excel file for further analysis of identification of sperm morphometric subpopulations (Maroto-Morales *et al.,* 2012).

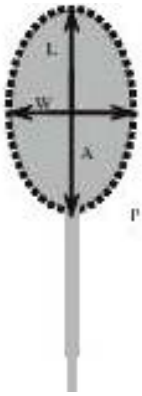

Figure 3.4 Sperm head morphometric parameters measured in this study. The morphometric parameters described for the sperm head are as follows L = Length, W = Width, A = Area, P = Perimeter (Hidalgo *et al.,* 2005; Rubio-Guillen *et al.,* 2007).

Table 3.3 Sperm head morphometric parameters and the formulas used to calculate each parameter (Banaszewska *et al.,* 2015).

Morphometric parameter	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)
Roundness	$P^2/4\pi A$

3.3.2.2.5.1 Sperm morphometric subpopulation identification

All morphometric data, was imported into a single data set (2 969 observations) and analysed by clustering procedures in order to identify sperm morphometric subpopulations. Each spermatozoon was classified according to the individual spermatozoa's morphometric parameters to a specific subpopulation. Each subpopulation was characterised by specific values for each morphometric parameter. For more detail on the clustering procedures used to identify sperm subpopulations, please refer to the relevant section in Chapter 5.

3.3.2.3 Cryopreservation of semen samples

After the macroscopic and microscopic evaluation of the semen samples, samples were processed for cryopreservation.

3.3.2.3.1 Dilution, equilibration and cryopreservation

The cryodiluent used during this study consisted of a cooling and cryopreservation component, and both components were prepared as described by Paulenz *et al.* (2007). For the composition of the respective media, please refer to Appendix A.

Semen samples were diluted with pre-warmed (37 °C) cooling media to obtain a concentration of 600x10^6 sperm/mL, and allowed to equilibrate for 45min at 5 ºC. The equilibrated samples were then diluted after the first equilibration period drop-wise (1:1) with pre-cooled (5 ºC) cryopreservation medium, and allowed to equilibrate for an additional 45min at 5 ºC. After each equilibration interval, the diluted samples were gently mixed to ensure an even distribution of sperm in the sample (Lambrechts *et al.,* 2000). During the cryodilution process, care was taken to minimize temperature fluctuations to minimize the potential of cold shock.

The equilibrated samples, with a final concentration of 300 x 10^6 sperm/mL, were loaded into labelled 0.25cc Cassou straws (IMV®, Taurus Evolution, South Africa) cooled to 5 ºC prior to loading, and sealed with PVC powder. Two straws per ram were cryopreserved for each collection session.

After loading, the semen straws were initially suspended in liquid nitrogen vapour (-80 ºC), 3 cm above the liquid nitrogen level in a Styrofoam cooler box, for 15 min before being plunged directly into the liquid nitrogen (-196 ºC) for 5 min before transferred to a liquid nitrogen tank (Paulenz *et al.,* 2007). 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.

3.3.3 Phase 3: Post-thaw sperm evaluation and sperm binding capacity assessment

Phase 3 entailed the post-thaw quality evaluation of the sperm samples and the use of the cryopreserved sperm from Phase two for the sperm binding assay.

3.3.3.1 Thawing and post-thaw evaluation of samples

The cryopreserved straws were thawed individually in water at 37 ºC for 30 seconds (Paulenz *et al.,* 2007). Each thawed straw was dried thoroughly with a paper towel, cut open at both ends, and the content of each straw emptied into a 2 mL Eppendorf tube (Lasec, South Africa). All thawed samples were evaluated for motility, viability and morphology as previously described in section 3.3.2.2, and the fertilizing ability of sperm was then assessed by a sperm binding assay described below.

3.3.3.2 Sperm binding assay

The binding capacity of cryopreserved ram sperm was determined using a perivitellin membrane binding assay described by Purdy (2006), with minor modifications. The egg yolks of freshly laid, unfertilized chicken eggs were separated from the albumin and the perivitelline membrane (PVM) was punctured to release the egg yolk. Each PVM was then washed delicately with Dulbecco's phosphate buffered saline (DPBS) in a Petri dish (100mm, Lasec,

South Africa) until the membrane became translucent and the DPBS solution was clear of any yolk. The membrane was then gently spread out in the Petri dish and cut into small squares (1cm x 1cm), using a scalpel blade (size no. 11, Lasec, South Africa) and a spectrophotometer cuvette as a guiding tool (Mocé & Graham, 2008). Each PVM section was then transferred to 2mL Eppendorf tubes (Lasec, South Africa) containing 500µL of TALP (for composition, please refer to Appendix A). Fresh perivitelline membranes were prepared for each binding assay.

An aliquot (10µL) of frozen-thawed sperm was added to each Eppendorf tube containing the TALP, and after being gently mixed, each sperm-TALP-PVM combination was incubated at 39 ºC in an HERACell 150i Thermo Fisher CO_2 incubator (SepSci, South Africa), in a controlled atmosphere of 5% of CO2 for 1 h. Each tube was gently shaken every 30min to prevent the membrane folding on itself, thus ensuring that the binding surface was not compromised (Mocé & Graham, 2008). After 1 h of incubation, 5µL of SYBR-14 (prepared according to the LIVE/DEAD® Sperm Viability Kit (L-7011) Protocol provided by the manufacturer, Molecular Probes) was added to each tube to fluoresce the sperm bound to the PVM, and incubated for an additional hour.

After incubation each perivitelline membrane was washed three times in DPBS to remove all unbound sperm. Each PVM square was then gently spread open on a microscope slide to remove any creases, and covered with a coverslip (Mocé & Graham, 2008). The binding ability of the sperm was determined by using an Olympus IX70 inverted fluorescence microscope and at 40X magnification. Sperm bound to each membrane, i.e. sperm that fluoresced, in 10 fields were counted, as described by Purdy (2006) (Figure 3.5). Two replicated analyses per ram per collection day were performed using a separate PVM binding assay for each. The mean sperm bound to each PVM was determined from the two replicated analyses per ram per collection day.

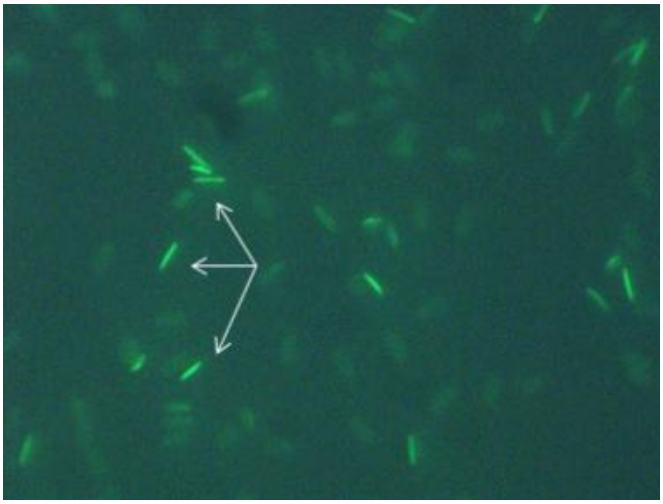

Figure 3.5 Sperm, stained with SYBR-14, bound to the perivitelline membrane (arrows) of a hen's egg observed under an inverted fluorescence microscope (Olympus IX70) at 40 x magnification.

3.4 Statistical analysis

All data were analysed using one of the following statistical programs, ASREML 4, XLSTAT 2016.1 and SAS 9.3. For Phase one the data were analysed using ASREML 4.0. A mixed model factorial ANOVA with day and ewe age (mature or yearling) and ewe as random factors was used to test for significant differences. The results of the mean rankings of the ewes were expressed as mean ± standard error. The same basic methods were used for the habituation and AV training experiments. The fixed effects fitted in the habituation experiment included ram breed, ram experience level, the intensity of exposure as well as the presence of the technical support staff member during exposure sessions. For the AV training experiment, total counts of behaviour attributes during all of the seven training sessions were transformed to square roots after 0.5 was added. The transformed counts were analysed according to a factorial ANOVA, with ewe breed, ram age, exposure frequency, the presence of the assistant during habituation and the ewe breed x ram age interaction included as factors. All findings were considered significant at $p<0.05$.

In Phase two, all the data obtained from the measured sperm parameters were analysed by XLSTAT 2016.1. The least square means (LSM) generated from the Bonferroni post hoc test was used to test for significant differences. For the comparative analyses, the one-way analysis of variance (ANOVA) approach was used. All results were expressed as the mean ± SEM. A 5% level of significance was used.

For the identification of sperm morphometric subpopulations, the morphometric data were analysed by multivariate clustering procedures using XLSTAT 2016.1. The PROC GLM

procedure of SAS 9.3 and ANOVA were used to determine significant differences. The significance level was set at $p < 0.05$.

In Phase three, all data were analysed using XLSTAT 2016.1, to generate least square means (LSM) from the Bonferroni post hoc test. For the comparative analyses, the one-way analysis of variance (ANOVA) approach was used. All results were expressed as the mean ± standard error of the mean. A 5% level of significance was used. Pearson's correlation coefficients were derived with XLSTAT 2016.1. A correlation coefficient value below 0.3 was considered negligible, 0.3 – 0.5 low correlation, 0.5 – 0.7 moderate and > 0.7 was considered as a high correlation (Mukaka, 2012).

3.5 References

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Chapter 4

The influence of ram breed and experience on the ease of training for semen collection purposes

Abstract

The innate behaviour of sheep may influence the ability of rams to be trained for semen collection purposes. Currently there is no formal protocol for the training of rams for semen collection purposes. This study therefore investigated the influence of breed and level of mating experience on the potential of rams to be trained for semen collection purposes. Sexually inexperienced and experienced rams of the Dormer and Merino breeds were used to determine whether a ram of a specific breed and sexual experience level has a preference for a mature or yearling ewe, and also for a ewe of the same breed. In addition the study also investigated the potential of rams to be habituated to a specific or a generic handler, at low and high exposure frequencies over a period of 4 weeks. Rams were trained during 7 sessions to ejaculate into an artificial vagina (AV) by using either Dormer or Merino teaser ewes that were in oestrus. In this study, Dormer rams exhibited a clear preference for mature ewes, with Merino rams being less discriminative in their choice of either yearling or mature ewes. Experienced Merino rams exhibited a definite preference for a teaser ewe of its own breed, with no conclusive evidence of a breed preference in the Dormer rams, irrespective of sexual experience level as well as inexperienced Merino rams. All rams, irrespective of breed or level of sexual experience, were successfully habituated to the handling and training activities within 4 weeks. However, there was no conclusive evidence that experienced Merino or Dormer rams ejaculated into the AV more readily, when compared to the Dormer and Merino inexperienced rams. Only 50% of the experienced Dormer rams could be successfully trained to use the AV, compared to 90% of the experienced Merino rams. Of the inexperienced rams only 40% of both the Merino and Dormer breeds could be trained to use the AV. The study indicated that for the training of rams for semen collections purposes, a mature oestrus ewe of the same breed as the ram should preferably be used. The factors contributing to the difficulty of training experienced Dormer rams, as well as inexperienced Dormer and Merino rams, to use the AV warrants further investigation.

4.1 Introduction

In the livestock industry production and reproduction efficiency are considered as the biggest contributing factors determining the viability of production systems. Optimising reproduction efficiency is considered as a prerequisite for profitable and sustainable animal production (Notter, 2012). The potential application of assisted reproductive techniques (ART's) as part of management programs have received considerable attention in the small stock industry to assist sheep farmers to optimise production and reproduction efficiency of their production systems to meet the increased demand for food (Verma *et al.*, 2012). The use of ART's such as artificial insemination (AI) and multiple ovulation and embryo transfer (MOET) enable farmers to preserve and utilise genetic material of genetically superior animals, allow access to superior genetics that can be propagated nationally and internationally, and can also result in the acceleration of genetic progress in flocks.

Semen samples can be collected by using the artificial vagina (AV) method or by means of electro-ejaculation (EE). In the industry the EE method is mostly used as it is quick and easy, and does not require the prior training of rams. In contrast, the AV method requires prior training of rams to ensure successful collection of semen (Palmer, 2005; Bopape *et al.*, 2015). However, the AV method is preferred as samples collected with an AV are of a much higher quality, which is important when using a semen sample for ART's purposes, as a sample of a high quality is needed (Marco-Jimenez *et al.*, 2005).

It is important to consider the innate behaviour and behavioural requirements of sheep during handling and related management activities. Erhard *et al.* (2006) stated that by habituating livestock to specific handling activities, one can reduce an animal's fear level, and make future handling easier and also safer for both the animal and the handler. According to Grissom & Bhatnagar (2009), the more an animal is handled, the shorter time it will take for the animal to become habituated to handling. In species like sheep, which are polygamous breeders with characteristic mating behaviour signs, the preference for a particular mating partner plays a vital role in the reproductive success of the animal (Orihuela & Vázquez, 2009). It was observed during the exposure of an individual ram to a number of ewes in oestrus, that rams will prefer to mate with specific ewes, resulting in the exclusion of eventual unmated ewes (Tilbrook *et al.*, 1987). This clear mating preference exhibited by rams indicates that ewes differ in their sexual attractiveness to the ram (Tilbrook, 1987a).

An ewe's sexual attractiveness can be referred to as her stimulus value in eliciting a sexual response from a ram. A test was developed by Tilbrook & Lindsay (1987) to rank ewes according to their sexual attractiveness based on the amount of time a ram spent exhibiting certain sexual behavioural signs towards a specific ewe. The authors also find that a ewe's sexual attractiveness was fairly consistent across individual rams. This was further supported by the findings that the factors that contribute to a ewe's sexual attractiveness are stable between oestrus periods (Tilbrook, 1987a). Tilbrook *et al.* (1987) stated that a ram's preference for a particular ewe, is not so much a component of his own behaviour but rather his response to specific characteristics of the ewe. Although it is not yet clear what the specific characteristics are that influence a ram's mating choice, some of these characteristics that contribute to a ewe's sexual attractiveness have been identified. Tilbrook & Cameron (1989) reported that ewes with longer wool are preferred to shorn ewes. It was also observed that when rams were offered a choice they preferred ewes of their own breed and also heavier ewes (Owens & Thompson, 1994; Preston *et al.*, 2005).

In South Africa, there is no formal standard operational procedure for the training of rams to use an AV during semen collection sessions. The formulation of a SOP will assist sheep producers and also students doing research, to collect semen samples of good quality that can be used for ART purposes or for research. The aim of this study was therefore to establish a protocol for the training of rams to ejaculate into an AV. The objectives of this study were to determine the influence of level of reproductive experience and mate choice as well as frequency of exposure to specific farm personnel on the ease of training rams for semen collection by the AV method.

4.2 Materials and Methods

Ethical clearance was provided by the Stellenbosch University Animal Ethics Committee (SU-ACUD15-00083). All animal care and procedures used were consistent with guidelines stipulated in the South African National Standards document 10386:2008.

4.2.1 Experimental location

The experiments were conducted at the Elsenburg Agricultural Research Farm of the Western Cape Department of Agriculture (GPS: -33.837966; 18.834337) located outside Stellenbosch in the Western Cape Province of South Africa.

4.2.2 Experimental animals

A total of 29 rams, consisting of 20 Merino (10 inexperienced and 10 experienced) and 9 Dormer (5 inexperienced and 4 experienced) rams were used in the study. The age of the trial animals ranged from 1 to 6 years of age, and the animals were part of the Elsenburg Dormer and Merino resource flocks.

For the first part of the study, 5 Dormer and 5 Merino rams of each sexual experience category, i.e. inexperienced or experienced, were used to determine if ram breed and sexual experience has an influence on a ram's preference to mate with a specific ewe. All 29 rams were used for the second part (habituation study) and the third part (training of rams to use an AV) of the study. All rams were managed according to normal commercial farming practices at Elsenburg, and were maintained on irrigated kikuyu (*Pennisetum clandestinum*) pastures with water available *ad libitum*.

4.2.3 Behavioural observations

4.2.3.1 Mating preference observations

This part of the study investigated whether a ram of a specific breed and sexual experience category (inexperienced or experienced) has a preference for a mature or yearling ewe of the same breed and whether individual rams ranked six ewes in roughly the same order. The

mating preference of 5 Dormer rams and 5 Merino rams of each sexual experience category, i.e. inexperienced or experienced respectively, were determined.

Rams were given an opportunity to demonstrate whether they exhibit a preference for a certain ewe, according to the testing procedure developed by Tilbrook & Lindsay (1987). During each preference study, a ram was exposed to and given an opportunity to rank six oestrus ewes (i.e. 3 mature and 3 yearling) of the same breed as the ram. For each sexual experience category within a breed, a different group of oestrus ewes was used. Each group of oestrus ewes within a breed, were ranked either two (Merino) or three (Dormer) times by each ram.

Please refer to Chapter 3 for a more detailed description of the mating preference testing procedure used.

4.2.3.2 Habituation observations

This part of the study investigated the ability of a ram, when exposed to the observer and/or a technical support staff member at a low frequency (LF) or a high frequency (HF), respectively, to become habituated to the presence of the observer and/or support staff member. A LF consisted of the rams being handled on only two days per week (i.e. Tuesday and Thursday), compared to the HF group that was handled for five consecutive days (i.e. Monday to Friday). Inexperienced and experienced rams (n=29) were allocated to both the LF (n=14) and HF (n=15) groups, respectively. Each experience category within each exposure frequency group were further subdivided, with certain rams being exposed to only the observer, and the remainder of the rams exposed to the observer and the support staff member. The habituation procedure was repeated for four consecutive weeks during the morning, and the same observer and support staff member were used throughout the study.

Please refer to Chapter 3 for a more detailed description of the habituation procedure used in this experiment.

4.2.3.3 Artificial vagina training observations

During this part of the study, the rams habituated during Part 2 of the study, were subsequently trained to ejaculate into an artificial vagina (AV) for semen collection purposes. For training purposes, ewes of both breeds were synchronized and used as teaser ewes to determine whether a ram (inexperienced or experienced) of a particular breed preferred a ewe of his own breed or not. Training was conducted over a period of two consecutive weeks, with 7 training sessions spread throughout the 2 weeks. All training sessions was carried out in the morning.

With each training session all sexual behavioural signs exhibited by the ram towards each of the ewes were recorded (Banks, 1964). Sexual behavioural signs included sniffing of the perineum, nudging, the Flehmen response, nibbling and/or mounting of the ewe. It was recorded whether a ram successfully used the AV, and also if he approached one ewe or both ewes, and mounted the specific ewe. It was also recorded when a ram rejected the AV or was frightened by the presence of the support staff member. Rams that ejaculated in the AV on four or more of the seven training days were regarded as trained for semen collection. Please refer to Chapter 3 for a more detailed description on the training procedure.

4.2.4 Statistical Analysis

The mating preference data were recorded in four sessions, each for experienced and inexperienced rams of the two breeds. These data were analysed separately, using ASREML software (Gilmour *et al*., 2009). ASREML allow the fitting of various fixed and random effects using traditional mixed model methods. It also allows the prediction of least squares means for desired fixed effects. In analysing the ranks generated by the mating preference tests, a mixed model was fitted, with ewe experience category (yearling or mature) and test days (three for Dormer rams and two for Merinos) fitted as fixed and ewe effects fitted as random. The between ewe variance components yielded by these analyses were used to derive repeatability as described by Turner and Young (1969).

The same basic methods were used for the habituation and training to use the AV studies. Since data recorded on the respective test days were binomial (i.e. 0 or 1) for all traits considered with the exception of number of mounts and number of times semen could be collected in the AV in the latter study, it was decided to total these records to arrive at a count for each ram across the entire evaluation period. Therefore rams that exhibited a specific trait throughout the entire evaluation would have higher counts than those exhibiting the trait sporadically or not at all. The square root of these count data were subjected to least square analyses using ASREML after 0.5 were added to individual data points (Dickson and Sanford, 2005). Fixed effects fitted in the habituation phase included ram breed (Merino or Dormer), ram experience category (experienced vs. inexperienced), the intensity of exposure (high vs. low) as well as the presence of the assistant acting as the semen collector or not (present vs. not present) during exposure sessions. Two-factor interactions were fitted between these main effects, but were not reported as they were not significant ($p>0.05$). The count data for the training sessions for the usage of the AV were analysed by mixed models methods within breeds, fitting ram as a random effect. Fixed effects were ram experience category (experienced vs. inexperienced), the breed of the teaser ewe (Dormer vs. Merino), the intensity of exposure (high vs. low) as well as the presence of the assistant acting as the semen collector or not (present vs. not present) during exposure sessions. The 2-factor interaction between ram

experience category and ewe breed was significant for some traits recorded in Merino rams and was included in the final operational models.

Where appropriate, binomial data were compared between exposure days or the intensity of exposure treatments by using Chi-square procedures (Preacher, 2001). In cases where 2 x 2 contingency tables with frequencies below 5 in more than 1 cell were analysed, Fisher's Exact Test (Preacher & Briggs, 2001) was used instead.

4.3 Results and Discussion

4.3.1 Mating preferences of Dormer and Merino rams

When inexperienced and experienced rams of both breeds were introduced individually to a group of oestrus ewes, each ram immediately started investigating the ewes by sniffing their perineal regions, after which the ram displayed sexual interest in the ewes and exhibited normal sexual behaviour. Signs of sexual behaviour indicating interest in an oestrus ewe included the Flehmen response, nudging, sniffing and mounting. These signs were observed during all of the observation sessions, irrespective of ram experience level and breed. No significant differences were reported between rams in terms of their sexual behaviour repertoires during the study. It was observed that a specific ewe received more attention than her flock mates from a ram. This suggests a greater sexual attractiveness compared to the other ewes.

The comparison of Dormer and Merino rams for their preference for mature or yearling ewes is presented in Table 4.1 and Figure 4.1. When the breeds are compared, it was found that both inexperienced and experienced Dormer rams exhibited a more pronounced preference for mature ewes ($p<0.01$) than for yearling ewes ($p<0.05$), respectively. Merino rams of both the inexperienced and experienced categories did not differ in terms of the sexual interest they displayed in the mature and yearling ewes. The mean ranking position each ewe received were based on when the ewe was removed from the pen during the observations. The ewe that was removed first from the pen was ranked as one and was considered as the most "attractive", whereas the last remaining ewe was ranked as six and was considered to be the least "attractive" to the ram. Thus a lower mean ranking value of a ewe indicated a higher degree of preference by a ram, whereas a higher mean ranking value indicated a low level of interest by a ram.

Table 4.1 The average ranking position (mean ± SE) for mature and yearling ewes, as ranked by experienced and inexperienced Dormer and Merino rams, respectively.

Ram Breed	Ram Experience level	Ewe Age	Mean ± SE
Dormer	**Experienced**	**Mature**	2.28^{a} ± 0.48
		Yearling	4.33^{b} ± 0.48
	Inexperienced	**Mature**	1.40^{c} ± 0.12
		Yearling	2.27^{d} ± 0.12
Merino	**Experienced**	**Mature**	2.27 ± 0.19
		Yearling	2.33 ± 0.19
	Inexperienced	**Mature**	1.93 ± 0.53
		Yearling	2.73 ± 0.53

[a, b] Columns with different superscripts differ significantly ($p<0.05$)
[c, d] Columns with different superscripts differ significantly ($p<0.01$)

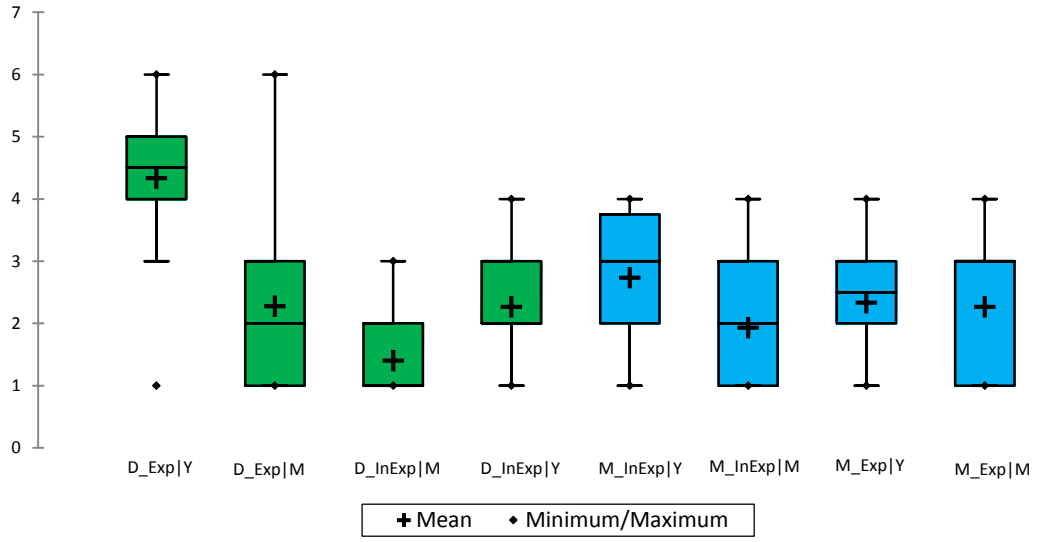

Figure 4.1 Ranking position distribution of experienced and inexperienced Dormer and Merino rams (D: Dormer, M: Merino, Exp: Experienced rams, InExp: Inexperienced rams, Y: Yearling ewes, M: Mature ewes).

Although Merino rams did not exhibit a specific preference for mature ewes, a definite preference for specific ewes, as indicated by the repeatability estimates of ewe rankings presented in Table 4.2, was observed. The derived repeatability estimates for ewe ranking according to experienced (0.70 ± 0.20) and inexperienced (0.68 ± 0.17) Merino and experienced Dormer (0.56 ± 0.17) rams were high. In contrast, the ranking of ewes by inexperienced Dormer rams (0.23 ± 0.14) were moderately repeatable (based on guidelines provided by Turner & Young, 1969). The repeatability estimates was derived from the intra-class correlation of ewe ranks according to the different rams. In the case of Dormer rams, where ewe age affected ram preference, ewe age was excluded from the models used to estimate repeatability.

Table 4.2 Repeatability estimates for the ranking of ewes by inexperienced and experienced rams of the Dormer and Merino breeds.

Breed	Experience category	Repeatability estimate
Dormer	**Experienced**	0.56 ± 0.17
	Inexperienced	0.23 ± 0.14
Merino	**Experienced**	0.70 ± 0.20
	Inexperienced	0.68 ± 0.17

In our study there was a clear indication that ewe age determined their attractiveness to Dormer rams. This was consistent with the findings of Preston *et al.* (2005), who reported that rams will prefer to mate with older ewes that were also heavier than their contemporaries. It was also suggested by Gelez *et al.* (2003) and Abecia *et al.* (2005) that age and a lack of previous sexual experience of ewes influence their ability to attract rams for mating. In the present study, yearling ewes that had no prior exposure to rams, were considered to be less attractive to the Dormer rams, irrespective of the latter's sexual experience level. Previous sexual experience permits maximal endocrine and ovarian responses in ewes (Chanvallon *et al.*, 2010). Therefore, it can be speculated that sexual naivety has a negative effect on a ewe's sexual attractiveness to a Dormer ram.

The finding that inexperienced and experienced Merino rams used in this study did not display a particular preference for yearling or mature oestrus ewes differ from the results reported by Lindsay & Robinson (1961) that Merino rams exhibited more mating and courting behaviour towards yearling ewes compared to mature ewes. To our knowledge, no studies on the influence of ewe age on the mating preference of Merino rams have been done recently. This has however, been investigated in other sheep breeds. Preston *et al.* (2005a) reported that Soay rams prefer to mate with mature ewes. When a breed is genetically selected for a specific criterion, the selection may potentially influence other traits that are not necessarily related to the selection criterion (Gelez *et al.*, 2003). The Merino sheep breed are one of the most widely distributed and extensively genetically selected breeds across the world (Hatcher *et al.*, 2010; Ciani *et al.*, 2015). It may be, however, suggested that this extensive selection over years potentially influenced a ram's ability to distinguish between yearling and mature ewes.

Although Merino rams did not display a specific preference for mature or yearling ewes, the repeatability estimates of the ewe rankings indicated that they indeed exhibited a definite preference for specific ewes. These findings are consistent with those of Tilbrook (1987) and Tilbrook & Lindsay (1987), where it was found that Merino rams will mate to a particular ewe in oestrus to the exclusion of others. In the latter of the two studies, it was found that although Merino ewes differed in terms of their sexual attractiveness to the ram, this feature was not

influenced by the stage or intensity of oestrus. This was contradictory to the findings of Lindsay (1976), who suggested that an ewe's sexual attractiveness to the ram will depend on the stage of oestrus she is in. Thus, although Merino rams did not have a clear preference for either mature or yearling ewes, it is possible that other characteristics of a particular ewe influenced her sexual attractiveness. Unfortunately this study only assessed the influence of ewe age on a ram's mating preference, and so it is impossible to speculate what other specific characteristics may also affect an ewe's sexual attractiveness to rams.

Rams use olfactory stimuli to discriminate between ewes, with the pheromone content of the wool of ewes that can determine attractiveness of ewes to a ram (Tilbrook, 1987b). Pheromones can be defined as semiochemicals used within species for communication and can be classified according to function, i.e. sex or aggregation pheromones (Wyatt, 2003). In a study by Orgeur (1991) it was reported that mature and yearling ewes differed in their sexual odour, as determined from pheromones. According to Ungerfeld *et al.* (2006), an ewe's pheromones may influence a ram's mating preference, which is also supported by the findings of Tilbrook & Cameron (1989) and Vázquez & Orihuela (2001). In the latter two studies it was found that the pheromones secreted through the wool and wax, as well as vaginal secretions, may influence a ewe's sexual attractiveness. It can be speculated that mature ewes have a higher intensity pheromone emission or different pheromone composition which rendered them to be sexually more attractive to rams. A literature survey failed to identify studies that investigated this aspect in sheep. However, it was confirmed in other species like spiders and moths. In a study by Cory & Schneider (2016) on spiders it was observed that young female body shapes and webs attracted hardly any males, whereas mature females attracted more than two males within a given time. The authors also found that mature females had a higher pheromone emission compared to the younger females. These results support the findings of the present study and the hypothesis that mature ewes potentially have a higher intensity pheromone emission, making them more sexually attractive for rams.

Tilbrook & Cameron (1989) also investigated the influence of the wool length on a ewe's degree of attractiveness to rams, and found that rams prefer to mate with ewes with long wool instead of recently shorn ewes. In the present study, all ewes were shorn prior to assessment, and this ensured that wool length could be eliminated as a potential factor influencing ewes' attractiveness to rams. However, Synnott & Fulkerson (1984) suggested that a ewe's sexual attractiveness is not based on her physical appearance. Nonetheless, in the present study we can assume that the preference for mature ewes by Dormer rams was based on their physical appearance as they were physically larger than yearling ewes. This information on mating preferences can be of great value to arrive at optimal ram: ewe numbers (Abecia *et al.*, 2005). Synnott *et al.* (1981) concluded that when ewes are mated under field conditions, spermatozoa

will be distributed unequally, thus only the more sexual attractive ewes will be mated often, and receive an adequate number of spermatozoa to ensure maximum conception rates. Thus mating preference can have a negative influence on fertility, as some ewes will be serviced a large number of times and some quite infrequently (Tilbrook *et al.*, 1987b).

4.3.2 Habituation

No significant interactions were found between breed, animal age, the presence of the technical support staff member or exposure frequency in the habituation study. The traits considered were also independent of ram experience level (inexperienced or experienced) and the presence of the technical support staff member. When the influence of breed was considered, Dormer rams tended to be more likely to allow the student and technical assistant to touch their chest area, when compared to Merino rams (2.56 ± 0.28 vs. 1.19 ± 0.19, p=0.06; Table 4.3). No other obvious breed differences were reported.

Table 4.3 Least squares means (±SE) depicting the influence of ram breed on the traits recorded during the habituation study (geometric means).

Trait	Ram breed	
	Dormer	**Merino**
Docility level	3.34 ± 0.18 (10.64)	3.09 ± 0.12 (9.07)
Scratch of head	3.31 ± 0.19 (10.45)	2.98 ± 0.13 (8.36)
Touch of chest area	2.56 ± 0.28 (6.07)	1.19 ± 0.19 (3.06)
Touch of belly area	2.45 ± 0.27 (5.52)	2.04 ± 0.18 (3.67)
Crouch	2.81 ± 0.11 (7.39)	2.64 ± 0.07 (6.47)

As there were no significant differences for traits measured for breed, age and presence of the technical support staff member, the data obtained for all rams were pooled and analysed to determine the influence of a high and low exposure frequency on the ease of rams to become habituated to the presence of one or more handlers. It was found that exposure frequency had a significant influence on all the traits measured ($p<0.05$; Table 4.4). The HF group was handled 18 times during the 4 weeks of habituation, compared to only 8 handling days for the LF group. This was expected since the longer exposure of rams to the handlers resulted in the rams becoming more used to the presence of the handlers, whereas the LF group's shorter exposure period did not allow complete habituation to the handlers.

Table 4.4 Least squares means (±SE) depicting the influence of exposure frequency on the traits recorded during a habituation study (geometric mean)

Trait	Exposure frequency	
	High	**Low**
Docility level	3.93^{a} ± 0.14 (14.96)	2.42^{b} ± 0.15 (5.35)
Scratch of head	3.78^{a} ± 0.15 (13.78)	2.40^{b} ± 0.16 (5.24)
Touch of chest area	2.76^{a} ± 0.22 (7.13)	1.46^{b} ± 0.23 (1.64)
Touch of belly area	2.73^{a} ± 0.21 (6.94)	1.63^{b} ± 0.22 (2.16)
Crouch	3.25^{a} ± 0.08 (10.06)	2.14^{b} ± 0.09 (4.10)

$^{a, b}$ Different superscripts within rows differ significantly ($p<0.01$)

There was no significant difference between the proportions of rams not docile in either of the HF group or the LF group ($p>0.05$; Table 4.5). In absolute terms, however, the frequencies did increase between intervals, with 100% of the rams in high exposure frequency group calm and docile by the end of the habituation study. For the other traits measured in the high exposure frequency group there were significant differences between Day 1 and 22 for the willingness of rams to allow the operators to touch the head, chest area and belly area ($p<0.05$; Table 4.5). A higher proportion of rams also allowed the operators to crouch next to them by the end of habituation, simulating semen collection in an artificial vagina. No significant differences were obtained between day 15 and 22 of habituation for all traits measured.

No significant differences among exposure events were obtained in the LF group for docility level, as well as for allowing touching of the head and chest area between intervals ($p>0.05$; Table 4.5). However, significant differences were obtained for between exposure events for rams allowing the operators to touch their belly area or crouch next to them between day 1 and 22 ($p<0.05$). As with the high exposure frequency group, no significant differences were obtained between day 15 and 22 for all traits measured.

Table 4.5 The influence of exposure frequencies (HF and LF) on traits measured between weekly calendar days of habituation.

Day	Traits									
	Docility level		Scratch of head		Touch of chest area		Touch of belly area		Crouch	
	HF	LF	HF	LF	HF	LF	HF	LF	HF	LF
1	0.733	0.500	0.200^{a}	0.357	0.000^{a}	0.000	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}
8	0.667	0.643	$0.600^{a,b,c}$	0.643	0.067^{a}	0.071	0.067^{a}	$0.071^{a,b}$	0.067^{a}	0.071^{a}
15	0.933^{1}	0.500^{2}	$0.867^{b,c}$	0.571	$0.467^{a,b}$	0.214	$0.600^{b,1}$	$0.214^{a,b,2}$	$0.867^{b.c}$	0.857^{b}
22	1.000	0.786	0.933^{c}	0.857	$0.800^{b,1}$	0.357^{2}	0.800^{b}	0.571^{b}	1.000^{c}	0.857^{b}

^{a,b,c} Different superscripts within columns differ significantly ($p<0.05$)
^{1,2} Different superscripts within rows differ significantly ($p<0.05$)

The low and high exposure frequency groups were compared in terms of the proportion of rams being docile or allowing touch to a specific area or the operators to crouch next to them (Table 4.5). There were significant differences ($p<0.05$) between the two groups for the proportion of rams being docile on day 15 (H 0.933 vs. L 0.500), for allowing operators to touch the chest area on day 22 (H 0.800 vs. L 0.357) and belly area on day 15 (H 0.600 vs. L 0.214). For all these traits, the HF group had a higher proportion of rams being docile or allowing touch to a specific area compared to the LF group.

No significant differences were obtained between the two groups (HF an LF) at any exposure event for the rest of the traits measured. At the end of the habituation study there were no significant differences for docility level and the ram allowing the operator to crouch next to it between the two groups. Although there were no differences between the two groups for all the traits measured, the results indicated that the frequency of rams expressing desirable behaviour for each trait measured was higher in absolute terms for the HF group (80% or more).

Traits that are considered important during the semen collection procedure include docility level and the ram allowing the operator to crouch next to it, as the ram should be calm during this procedure and also should allow the technical support staff member to crouch next to him to collect the ejaculate, without being startled or frightened. From the results of the present study it was evident that at the end of the habituation study there were no significant differences for docility level and the willingness of rams to allow the operator to crouch next to it between the two groups. These findings are consistent with those reported by Hargreaves & Hutson (1990), who stated that repeated handling can habituate farm animals to specific husbandry procedures or activities.

A literature survey failed to identify similar studies on sheep to support or refute these findings. The results however are in agreement with other studies indicating that repeated handling of

farm animals will reduce fear, increase docility, and eventually result in habituation in response to human presence and handling (Hargreaves & Hutson, 1990; Grandin, 1997; Grissom & Bhatnagar, 2009; Petherick *et al.*, 2009). Boshoff (2014) found that after two weeks of handling, more than 80% of Merino rams were used to the presence of humans prior to training to ejaculate into the artificial vagina. In the present study, most of the rams (80%) were habituated and accustomed to the presence of the handlers within 3 weeks. In a study on wombats (*Lasiorhinus latifrons*), Hogan *et al.* (2011) found that regular handling of this species will increase its level of docility towards the human handler, which support the findings of the present study. The results obtained in the current study, that rams can be habituated to human presence are also supported by findings of Andrade *et al.* (2001) that cattle can be successfully habituated to repeated handling in a squeeze chute after 19 test-days. Boix *et al.* (1988) also found that rats can be easily and effectively habituated by daily handling within 15 days.

In the present study no conclusive differences between the breeds in terms of habituation were observed. This observation is in contrast to results reported by Grandin & Deesing (1998), whom established that breed (genotype) can have an influence on habituation, where some breeds of species habituate more rapidly than others. Despite the fact that the two breeds used in the study are genetically different, it might be suggested that the two breeds are genetically related. Sandenbergh *et al.* (2015) used the OvineSNP50 chip to genotype four South African sheep breeds. Furthermore the authors observed that a principal component analysis indicated that Merino and the SA Mutton Merino tended to cluster together. With these observations in mind and the fact that the Dormer originated from the SA Mutton Merino, it could be assumed that this might explain why no breed differences were found in the current study. A tendency ($p=0.06$) was however found that the Dormer rams would more likely allow the student and technical assistant to touch their chest area, when compared to Merino rams. This was also observed in a recent study by Van Der Merwe (2016) that Dormer rams are more docile and easier to handle.

4.3.3 Breed preference for teaser ewes and ease of AV training

During the training of rams to use the AV, Merino (experienced or inexperienced) and Dormer (experienced or inexperienced) rams were given a choice of either a Merino or a Dormer teaser ewe to mount. All sexual behaviour signs exhibited by a ram as well as if a ram successfully used the artificial vagina (AV) are presented in Table 4.6 and Table 4.7.

Overall, Merino rams showed a definitive preference for a teaser ewe of their own breed by directing significantly more sexual behavioural attention towards the Merino ewe ($p<0.01$; Table 4.6). The only sexual behavioural trait independent from ewe breed was the Flehmen response

(Table 4.6). No conclusive evidence of a ewe breed preference was accordingly observed in Dormer rams, irrespective of sexual experience level (Table 4.6).

Table 4.6 Sexual behaviour signs (LS means ± SE) exhibited by Merino and Dormer rams across experience categories, when exposed to Merino and Dormer ewes across seven training sessions.

Ram breed	Behavioural signs	Ewe Breed	
		Dormer	**Merino**
Merino	Sniff	2.44^{c} ± 0.04 (5.47)	2.63^{d} ± 0.04 (6.44)
	Flehmen response	1.60 ± 0.11 (2.06)	1.70 ± 0.11 (2.40)
	Nudge	1.69^{c} ± 0.10 (2.36)	2.03^{d} ± 0.10 (3.61)
	Mount	1.78^{a} ± 0.11 (2.65)	2.07^{b} ± 0.11 (3.77)
	Number of mounts	3.12^{c} ± 0.32 (9.22)	4.04^{d} ± 0.32 (15.81)
	AV	1.72^{c} ± 0.12 (2.46)	2.03^{d} ± 0.12 (3.64)
Dormer	Sniff	2.62 ± 0.01 (6.34)	2.60 ± 0.10 (6.26)
	Flehmen response	1.69 ± 0.22 (2.36)	1.66 ± 0.22 (2.26)
	Nudge	1.56 ± 0.18 (1.92)	1.60 ± 0.18 (2.06)
	Mount	1.87 ± 0.22 (3.00)	1.73 ± 0.22 (2.49)
	Number of mounts	3.59 ± 0.61 (12.41)	2.88 ± 0.61 (7.81)
	AV	1.70 ± 0.26 (2.38)	1.66 ± 0.26 (2.25)

[a, b] Different superscripts within rows differ significantly ($p<0.05$)
[c,d] Different superscripts within rows differ significantly ($p<0.01$)

It was evident that inexperienced and experienced Merino rams differed in terms of some of the sexual behavioural traits they exhibited. The experienced Merino rams exhibited more nudging behaviour, were more likely to mount a ewe, and also mounted the teaser ewes more frequently during an observation session ($p<0.05$; Table 4.7). In contrast, there were no significant differences in the sexual behavioural traits exhibited by experienced and inexperienced Dormer rams (Table 4.7).

Table 4.7 Sexual behaviour signs (LS means ± SE) exhibited by inexperienced and experienced Merino and Dormer rams, when exposed to Merino and Dormer ewes across seven training sessions.

Ram breed	Behavioural signs	Ram Experience	
		Inexperienced	Experienced
Merino	Sniff	2.50 ± 0.04 (5.77)	2.57 ± 0.04 (6.12)
	Flehmen response	1.49 ± 0.15 (1.73)	1.81 ± 0.15 (2.77)
	Nudge	1.66[a] ± 0.12 (2.24)	2.06[b] ± 0.12 (3.76)
	Mount	1.70[a] ± 0.14 (2.39)	2.14[b] ± 0.14 (4.09)
	Number of mounts	2.96[a] ± 0.40 (8.29)	4.19[b] ± 0.40 (17.07)
	AV	1.69 ± 0.16 (2.36)	2.06 ± 0.16 (3.75)
Dormer	Sniff	2.57 ± 0.13 (6.12)	2.64 ± 0.14 (6.48)
	Flehmen response	1.68 ± 0.26 (2.34)	1.67 ± 0.28 (2.28)
	Nudge	1.50 ± 0.21 (1.75)	1.66 ± 0.23 (2.25)
	Mount	1.79 ± 0.28 (2.72)	1.80 ± 0.30 (2.75)
	Number of mounts	2.90 ± 0.71 (7.90)	3.58 ± 0.78 (12.31)
	AV	1.60 ± 0.34 (2.05)	1.76 ± 0.37 (2.69)

[a, b] Different superscripts within rows differ significantly ($p<0.05$)

Findings regarding the influence of a ram's sexual experience level on the ease of training to use the AV, and subsequent successful use of the AV, are presented in Table 4.7 and Table 4.8. The ease of training the rams to use the AV was independent of previous exposure to the observer and/or a technical support staff member at a low frequency or a high frequency exposure during the habituation phase, therefore only main effects are presented in the tables.

Table 4.8 The percentage of experienced and inexperienced Dormer and Merino rams, successfully trained to use the AV.

Ram breed	Experience Category	Percentage	P-value
Merino	Experienced	90%	0.11
	Inexperienced	40%	
Dormer	Experienced	50%	1.00
	Inexperienced	40%	
Merino vs Dormer	Experienced		0.18
Merino vs Dormer	Inexperienced		1.00

There was no conclusive advantage for experienced Merino and Dormer rams in the successful usage of the AV, compared to inexperienced rams ($p=0.13$ and $p=0.76$ respectively; Table 4.7).

From the results it is evident that there was a considerable absolute difference in the number of rams successfully trained to use the AV, as influence by breed and level of sexual experience. However, none of these differences were found to be significant (Table 4.8). It should be considered that there were only 10 rams in each category for Merinos, with the Dormer rams even lower in numbers. All of the experienced Merino rams (90%) were successfully trained (ejaculated in the AV on 4 or more occasions) during the seven training sessions. In contrast, only 50% or less of the inexperienced rams of both breeds and experienced Dormer rams were successfully trained.

A significant 2-factor interaction was observed between the breed of the teaser ewe and the sexual experience level of the Merino rams for the number of mounts in the given time frame ($p=0.04$) and the number of times the AV was successfully used ($p=0.043$). The total number of mounts and successful usage of the AV were similar for inexperienced rams in response to ewes of both breeds and experienced rams in response to Dormer ewes ($P>0.05$). In contrast, the total number of mounts of experienced rams on Merino ewes exceeded the other groups by a factor of between 2.2 for experienced rams in response to Dormer ewes and 3.2 for inexperienced rams in response to Dormer ewes. The corresponding range for use of the AV was between 1.8 for experienced Merino rams in response to Dormer ewes and 2.2 for inexperienced Merino rams in response to Dormer ewes (Figure 4.2).

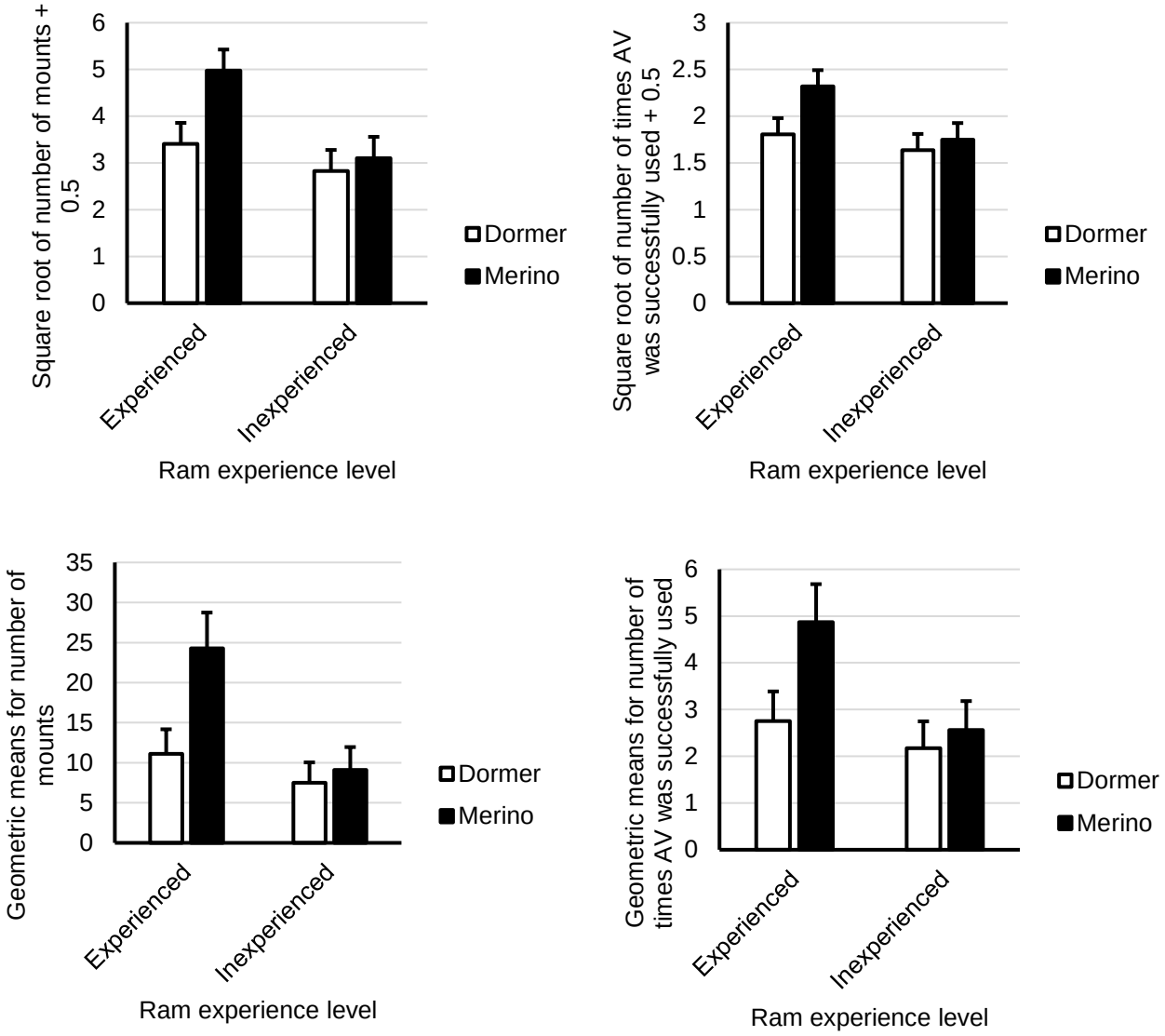

Figure 4.2 Least squares means (±SE) depicting the interaction between ewe breed and ram sexual experience level for number of mounts (left) and the usage of the AV (right). Square root transformed means are given above and geometric means below.

The teaser ewe breed preference of the experienced Merino rams agrees with the findings of Bourke (1967), who reported that Merino rams will preferentially mate with Merino ewes. In a study by Arnold & Dudzinski (1978), it was reported that the genotype of an animal plays an important role in a ram's mating preference, as a ram prefers to mate with a ewe of its own breed. This result lends further support to our findings in the present study. On the contrary, Dormer rams did not exhibit a preference for a ewe of its own breed. It can be speculated that this could be due to the fact that the Dormer and Merino breeds might possibly be genetically related. This preference by a ram for a ewe of its own breed has not been recently reported in the Merino or Dormer breeds but similar studies were done on other sheep breeds. Lees & Weatherhead (1970) found that Clun Forest rams prefer to mate with ewes of their own breed.

Furthermore it was reported by Simitzis *et al.* (2006) that Karagouniki thin-tailed rams exhibited a preference for Karagouniki thin-tailed ewes in comparison to Chios fat-tailed rams, which were willing to mount ewes of both breeds. The results reported for the Dormer rams are supported by the findings of Levine (1978) that Romney rams did not have a preference for a ewe of its own breed, and successfully mated with both Suffolk and Columbia ewes.

The Flehmen response was the only sexual behavioural trait independent of ewe breed. This response facilitates the transfer of pheromones and other odours into the vomero-nasal organ (also known as the organ of Jacobsen) (Keverne, 1999). The results of the present study agree with that found by Simitzis *et al.* (2006), who observed that the breed of a ewe did not influence the exhibition of and the duration of the Flehmen response between Chios and Karagouniki mature rams.

In a study by Borg *et al.* (1992), it was reported that a ram's reproductive behaviour is associated with concentrations of luteinizing hormone (LH), testosterone and prolactin. The authors also found that the overall concentrations of these hormones were much lower in sexually inexperienced rams, and they also exhibited less reproductive behaviour compared to sexually experienced rams. This supports the findings of the present study, where the inexperienced rams did not display as many of the recorded behavioural traits towards the ewes, and also did not display a clear preference for a ewe of its own breed. The fact that the inexperienced rams had never been in contact with oestrus ewes potentially contributed to the observed lack of sexual behaviour indicators displayed during the study (Shackleton, 1991; Simitzis *et al.*, 2006).

Significant differences were observed in the sexual behavioural traits exhibited by experienced and inexperienced Merino rams, however this was not found in the Dormer rams. These findings might be explained by the fact that the Merino is a late maturing breed, whereas the Dormer is an early maturing breed (Snyman, 2014). Therefore, it may be suggested that at the time when the study was conducted, the Dormer rams had already reached the end of the puberty period, and therefore no differences were observed between the experienced and inexperienced Dormer rams in terms of sexual behaviour.

Although the difference in the number of rams trained to use the AV was not significant, there were large absolute differences in terms of the total number of rams trained to use the AV. Almost all experienced Merino rams (90%) were successfully trained, in comparison to less than 50% of the inexperienced Merino and Dormer rams that could be trained. These results are supported by the findings of Katz *et al.* (1988) and Flores *et al.* (2005), where it was found that only 30% of inexperienced rams could be successfully trained to use the AV, compared to 90%

of experienced rams. The current study's results are also consistent with those of Boshoff (2014), who reported that mature Merino rams can be successfully trained to ejaculate into the AV within a period of two weeks. The absolute difference observed between experienced and inexperienced Merino rams can potentially be attributed to sexual inexperience of the young Merinos (Shackleton, 1991; Simitzis *et al.*, 2006).

The observed breed differences suggest that breed may influence libido and thus have an impact on sexual behaviour. This hypothesis is supported by the findings of Boland *et al.* (1985) who reported that Suffolk rams had a higher libido, when compared to Texel and Dorset Horn rams. Letsoalo *et al.* (2016) were unsuccessful to train Namaqua Afrikaner rams to ejaculate into the AV. Furthermore, it should be considered that the Dormer rams were assumingly relatively overweight at the time of the trial, and this could have affected their ability to be trained to use the AV. Both Brown (1994) and Kheradmand *et al.* (2006) suggested that rams that are relatively fat will have a reduced level of sexual activity. This support the hypothesis that the overweightness of the Dormer rams may have had an influence on their libido, potentially also affecting their ability to be trained to use the AV. In a study by Maurya *et al.* (2010), the influence of a ram's body condition score (BCS) on reproductive efficiency in terms of sexual behaviour was assessed. These authors observed that rams with a more moderate BCS (3.0 – 3.5) exhibited more sexual behaviour and were also more likely to mount a ewe.

4.4 Conclusions

This study indicated that Dormer rams had a definite preference for mature ewes, and that these rams also did not discriminate between the two ewe breeds in terms of mating preference. This is in contrast to the Merino (experienced or inexperienced) rams that were less discriminatory in their choice for either mature or yearling ewes, but exhibited a definite preference for a ewe of its own breed during training for AV usage. These results suggest that when using an oestrus teaser ewe for the training of rams to ejaculate into an AV, it is recommended that a mature ewe of the same breed as the ram is used to accommodate the preference of Dormers for experienced ewes as well as Merinos for a ewe of their own breed. Habituation of both Dormer and Merino rams, irrespective of their level of sexual experience, to use an AV can be accomplished within approximately 4 weeks. Training can be done by experienced personnel, and rams can be exposed to handlers for a minimum of eight training sessions within a given period. However, it must be noted that a high frequency of training, i.e. 18 training sessions, will result in more pronounced desirable behaviour repertoires.

Experienced Merino rams can be successfully trained to use the AV within 2 weeks, while extended training periods may be required for experienced Dormer and inexperienced Dormer and Merino rams. Further research on the time to train experienced Dormer rams as well as

inexperienced rams (Dormer and Merino) is required. Further research to establish determinants of a ewe's sexual attractiveness to rams of the same breed as well as other breeds, is also warranted.

4.5 References

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Chapter 5

The influence of breed and genetic selection for prolificacy on sperm subpopulation traits

Abstract

Several studies have indicated the presence of sperm subpopulations in mammalian ejaculates. The quality of sperm is determined by its ability to ultimately fertilize an ovum successfully, and heterogeneous sperm subpopulations are expected to contain sperm that differ in their ability to participate in fertilization. The aim of this study was to characterise the sperm morphometric subpopulation profile of Merino and Dormer rams, and to investigate the influence of selection for prolificacy on the sperm morphometric subpopulation profile. Semen samples were collected by means of electro-ejaculation from 18 rams (6 Dormer, 6 High Line and 6 Low Line). All semen samples were evaluated according to standard macroscopic and microscopic sperm evaluation protocols, and sperm morphometric traits were analysed using ImageJ® open software. Clustering procedures using the sperm morphometric data from both Dormer and Merino rams resulted in the identification of four distinct subpopulations. No significant differences were found between the distributions or classification of subpopulations in either Dormer or Merino rams. The subpopulation analysis of the HL and LL rams indicated minor differences in morphometric parameter values between certain subpopulations. However, no significant differences were found in the distribution of subpopulations between the two genetically diverse lines. Further research is warranted as no noteworthy influences of breed and genetic selection on sperm subpopulation traits were observed. An area of research deserving further study is the possible quantification of the influence of the internal environment of the ewe and the possible contribution of the ewe during the fertilising process.

5.1 Introduction

The use of assisted reproductive techniques (ART's) to assist sheep producers to produce meat and wool as cost-efficiently as possible have received considerably more attention during the last decade. Improved production is enabled by the incorporation of ART's such as artificial insemination (AI) and embryo production into management programs, which in turn hinges on proper assessment of sperm quality. When assessing sperm samples, sperm morphology was identified as one of the most important factors for determining sperm quality and potential fertility (Martí *et al.*, 2012).

Traditionally sperm samples have been considered as homogenous populations of sperm cells, but several studies have reported on the presence of sperm subpopulations within an ejaculate (Holt & Van Look, 2004). The existence of sperm morphometric subpopulations within the mammalian ejaculate is now widely acknowledged. Holt & Palomo (1996) and Druart *et al.,* (2009) postulated that sperm subpopulations may be considered as indicators of the quality of ejaculates, as some subpopulations have been found to withstand cryodamage better. Several authors have also suggested that the degree of heterogeneity of sperm subpopulations may

have functional relevance, with differences between sperm subpopulations linked to fertility (De Paz *et al.*, 2011) and cryotolerance (Thurston *et al.*, 2001; Ortega-Ferrusola *et al.*, 2009). Yániz *et al*. (2015) found in their study on two ram groups differing distinctly in fertility, that the high fertility group had a higher proportion of large and elongated sperm.

Although the physiological significance and origin of subpopulations are not yet properly understood, it has been hypothesised by many researchers that it results from variation in sperm structure formation, caused by genotypic effects during the process of spermatogenesis (Beatty & Sharma, 1961; Thurston *et al.*, 1999; Rodríguez-Martínez, 2006). Another possible interpretation of subpopulations is that it may represent sperm that differ in terms of physiological age or degree of maturation (Abaigar, 1999; Martí *et al.*, 2012). With the knowledge that sperm structure is influenced and controlled by an animal's genotype (Thurston *et al.,* 1999), it may be hypothesised that breed and genetic selection may potentially influence the structure and distribution of subpopulations within a given sample. In a study on the effect of breed on subpopulation structure and distribution, Kondracki *et al.* (2012) found that two pig breeds, Duroc and Pietrain, differed significantly in these traits. This difference can be ascribed to the fact that the two breeds differ genotypically. In a study on two groups of Rasa Aragonesa rams, divergently selected for high and low fertility, significant differences were observed in the distribution and structure of sperm morphometric subpopulations, indicating that genetic selection affects subpopulation structure and distribution (Yániz *et al.*, 2015).

Selection for improved reproduction potential is a dynamic process. In 1986, two divergent Merino lines were established from the same base population. Selection based on number of lambs weaned per ewe mated resulted in the formation of a High line (HL) and a Low line (LL) (Sandenbergh, 2013). Both ewe and ram replacements for the HL were selected from progeny of ewes that reared more lambs than they had lambing opportunities. In contrast, LL replacements were selected from ewes than reared fewer lambs than they had lambing opportunities (Lambrechts *et al.*, 2000; Cloete *et al.*, 2004; 2009).

In a recent study by Boshoff (2014), it was determined that rams from the two lines differ in terms of mating performance, i.e. HL rams exhibited significantly more mating behaviour, as well as mounted ewes more frequently than LL rams. However, when the proportion of ewes that conceived after being mated to rams from the respective lines was considered, no significant differences in number of ewes conceived were observed for the 2012 mating season. Furthermore, when sperm quality of rams from the respective lines were evaluated, microscopic evaluation indicated that most motility parameters did not differ between ejaculated sperm samples collected from HL- and LL rams. However, morphometric analysis indicated that there were differences between the lines in ellipticity and elongation.

Although the presence of sperm morphometric subpopulations within ovine semen samples is widely accepted, a comparison between sheep breeds in terms of the characterisation, structure and distribution of such sperm morphometric subpopulations have never been addressed previously. The aim of this study was therefore to characterise the sperm morphometric subpopulation profiles of Merino and Dormer rams. Furthermore, the study aimed to investigate the influence of genetic selection on the sperm morphometric subpopulation profile, potentially elucidating why the two lines did not differ in terms of the number of offspring sired during the 2012 reproductive season.

5.2 Materials and methods

Ethical clearance was approved by the Stellenbosch University Animal Ethics Committee (SU-ACUD15-00083). All animal care and procedures used in this study was according to the guidelines stipulated in the South African National Standards document 10386:2008.

5.2.1 Experimental location

The experiments were conducted on the Elsenburg Agricultural Research Farm of the Western Cape Department of Agriculture (GPS: -33.837966; 18.834337) situated near Stellenbosch in the Western Cape, South Africa.

5.2.2 Experimental animals

Eighteen mature Dormer and Merino rams aged between 2 and 5 years, were used in this study. Both the Dormer and Merino rams originated from resource populations maintained on the Elsenburg Agricultural Research Farm. The Merino rams were part of a resource population that consists of two genetically diverse lines, divergently selected for multiple rearing ability. For a more in-depth description on the flock history and the selection of the two divergently selected lines, please refer to Chapter 3. For this study, six Merino rams of the HL and LL respectively, and six Dormer rams, were used for semen collection purposes.

All the rams were managed under normal commercial farm practices and maintained on irrigated kikuyu grass (*Pennisetum clandestinum*) pastures with water available *ad libitum*.

5.2.3 Semen collection

Although the rams were trained to ejaculate into an artificial vagina (AV), during the actual collection phase for semen evaluation, almost all rams, with the exception of four rams, rejected the AV. Due to time constraints it was decided to collect semen samples by means of electro-ejaculation (Orihuela *et al.*, 2009; Malejane *et al.*, 2014).

A total of 54 semen samples were collected during three consecutive weeks, with sampling performed on 3 days per week, and a total of six rams was sampled per day. Directly after collection, all semen samples were sealed with parafilm (Lasec, South Africa) to prevent water contamination, and transported to the laboratory in a thermally controlled flask (37ºC), where they were kept in a water bath (37ºC), until macroscopic and microscopic evaluation and processing.

5.2.4 Semen evaluation

5.2.4.1 Macroscopic evaluation

All semen samples were subjected to macroscopic evaluation, which included the parameters volume, colour and mass motility. Please refer to Chapter 3 for a more detailed description of how these parameters were assessed.

5.2.4.2 Microscopic evaluation

After microscopic evaluation, the sperm concentration of each semen sample was determined by using the Neubauer haemocytometer method as described by WHO (2010). For more details on this protocol, please refer to Chapter 3.

After the sperm concentration was determined for each sample, smears were made for each sample to evaluate sperm morphology, acrosome integrity and morphometry. Raw semen was diluted (1:3 in HAM's F10), and a 10µL droplet of the diluted-HAM's F10 sample was spread out evenly over the slide and allowed to air-dry. All smears were stained with RapiDiff® according to the manufacturer's protocol, and examined with an Olympus IX70 inverted microscope (Wirsam Scientific, South Africa) at 40x magnification.

For the evaluation of sperm morphology and acrosome integrity, a minimum of 100 sperm were counted for each smear and expressed as a percentage of the total number of sperm. For the evaluation of the sperm morphometric parameters 100 sperm in 3 fields were captured in photomicrographs by using the analySIS® soft imaging system and an Olympus IX70 inverted microscope fitted with a Colorview II camera (Wirsam Scientific, South Africa). For each semen sample the length, width, perimeter, area, ellipticity and elongation of each of the 100 individual sperm was measured manually by using ImageJ®. The morphometric measurements of each individual sperm were saved in a Microsoft Excel file for further analysis and the identification of sperm morphometric subpopulations. Please refer to Chapter 3 for a more detailed description of the evaluation protocols, parameters and calculations used in this study.

5.2.5 Statistical analysis

All statistical analyses were performed using XLSTAT 2016.1 (Addinsoft, 2016) and SAS 9.3 (SAS, 2006). All data derived from the macroscopic and microscopic sperm evaluation and the sperm morphometric parameters were analysed using XLSTAT. Full models were initially fitted and where the interactions were not significant the analyses were repeated using only the main effects. Due to repeated measures on the same rams being used, the data were initially analysed with ram as a random effect in a mixed model with each of the response variables and breed and lines as the independent variables. This was repeated in a linear model analyses excluding the random effect. The results were unchanged therefore only the ANOVA results are reported. All results were expressed as means ± SEM. The significance level was set at $p<0.05$.

Morphometric data were imported into a single data set (2 969 observations) and analysed by clustering procedures to identify sperm morphometric subpopulations using XLSTAT 2016.1 (Martí *et al.*, 2011, 2012; Yániz *et al.*, 2015). Firstly, a principal component analysis (PCA) was performed, to determine a small number of linear combinations (PC's) that contain as much as possible information of all the initial values. This enabled a summary of a large number of values in a few jointly uncorrelated PC's. The higher the proportion of total variance accounted by a low number of PC's, the better the result. In order to select the number of PC's to be used for further analyses, PC's with an eigenvalue higher than 1 were selected. Secondly, a non-hierarchical analysis using the k-means cluster model was performed to identify the clusters. Finally, multivariate k-means cluster analysis was performed to classify each individual sperm into a reduced number of sperm subpopulations, according to the sperm morphometric parameters.

The PROC GLM procedure of SAS 9.3 and analysis of variance (ANOVA) were used to determine the effect of breed and genetic selection on the structure and distribution of sperm subpopulations and the significant differences in the distribution between breeds and genetically diverse lines. The significance level was set at $p < 0.05$.

5.3 Results and discussion

5.3.1 Macroscopic and microscopic sperm evaluation

The macroscopic and microscopic evaluation results for the semen samples obtained from the Dormer and Merino (HL and LL) rams are presented in Table 5.1.

Table 5.1 Macroscopic and microscopic sperm parameters (mean ± SE) for semen samples obtained from Dormer and Merino (HL and LL) rams.

Sperm parameter	Dormer	Merino	Merino High Line	Merino Low Line
Volume (mL)	1.47 ± 0.09	1.41 ± 0.07	1.32 ± 0.09	1.50 ± 0.08
Concentration (x10^8 sperm/mL)	13.20^a ± 1.12	19.41^b ± 0.80	20.71 ± 1.38	18.12 ± 0.93
Mass motility (1-5 score)	3.67 ± 0.12	3.83 ±0.08	3.94 ± 0.12	3.72 ± 0.12
Abnormalities (%)	12.70 ± 2.22	10.36 ± 1.58	8.30 ± 2.22	12.30 ± 2.22
Acrosome integrity (%)	75.58 ± 1.69	76.66 ± 1.22	78.81 ± 1.69	74.52 ± 1.69

^{a,b} Different superscripts within rows differ significantly ($p<0.05$)

When the two breeds were compared, Merino rams produced samples with a significantly higher sperm concentration compared to Dormers (Table 5.1; $p<0.05$). The average sperm concentration for Merino rams was 19.41 ± 0.80 X 10^8/mL, compared to 13.20 ± 1.12 X 10^8/mL for Dormers. No significant differences were observed between the two breeds for semen volume, mass motility, % abnormalities and % acrosome integrity (Table 5.1). Merino rams however, had in absolute terms, a lower mean percentage of sperm abnormalities (10.36 ± 0.02 vs. 12.70 ± 0.02) and a higher mean percentage of intact acrosomes (76.66 ± 1.22 vs. 75.58 ± 1.69) compared to the Dormer rams. The characterization of Dormer semen samples in terms of sperm abnormalities and acrosome integrity, is to our knowledge, the first reported results for this breed.

Furthermore, no significant line differences were observed for the respective sperm parameters (Table 5.1). However, the HL was consistently favoured in terms of absolute differences for almost all sperm parameters, with the exception of volume.

The findings from this study, with regard to the influence of breed, support the findings of Letsoalo *et al.* (2016), who found no differences in semen quality parameters between Namaqua Afrikaner, Döhne Merino and Dorper rams when semen samples were collected by means of electro-ejaculation. Munyai (2012) also observed no differences in volume, sperm concentration and the percentage sperm abnormalities between Damara, Namaqua Afrikaner, Pedi and Zulu rams. The results of this study are also consistent with those reported by Zargari *et al.* (2016), who found no significant differences between Afshari and Booroola Merino rams for semen volume, mass motility, viability, membrane integrity and sperm morphology.

The findings from this study are however, contradictory to those reported by Patel & Dugwekar (1999), who found significant differences between macroscopic and microscopic sperm

parameters of Patanwadi, Rambouillet x Patanwadi and Merino x Patanwadi rams. The significant difference observed for sperm concentration between Dormer and Merino rams in this study, and with the Dormer rams having a significantly lower sperm concentration, are supported by the findings of Munyai (2012), who reported that sperm concentration is negatively correlated with body weight. In the present study, the Dormer rams appeared relatively overweight, and it is suggested that this potentially affected sperm concentration negatively.

As breed had no conclusive influence on sperm quality parameters, it can be assumed that this may be due to the fact that the Dormer and Merino rams was maintained under the same conditions, thus excluding any environmental factors that might have had an influence on sperm quality. Despite the fact that the two breeds used in the study are genetically different, it might be suggested that the two breeds are genetically distantly related. Sandenbergh *et al.* (2015) used the OvineSNP50 chip to genotype four South African sheep breeds. Furthermore the authors observed that a principal component analysis indicated that Merino and the SA Mutton Merino tended to cluster together. With these observations in mind and the fact that the Dormer is a synthetic breed that originated from the SA Mutton Merino as the female parent, it could be assumed that this might explain why no breed differences were found in the current study.

From the results it were evident that selection had no influence on sperm quality parameters. These results agree with the findings of Boshoff (2014), who reported similar results between fresh ejaculated semen samples obtained from another sample of HL and LL rams originating from the same resource population that was used in this study. Lambrechts *et al.* (2000) also reported similar results with regards to sperm abnormalities, sperm viability and acrosome integrity between HL and LL rams. However, they found significant line differences for some motility parameters, sperm from HL rams exhibiting on average a lower motility compared to LL rams. In a study by Vicente-Fiel *et al.* (2014) on Rasa Aragonesa rams, genetically selected for high and low field fertility, significant differences were observed for semen quality parameters, which are in contrast to the results of the current study.

5.3.2 Morphometric parameters

The results pertaining to the morphometric parameters of fresh sperm samples obtained from Dormer and Merino (HL and LL) rams, are presented in Table 5.2. Sperm head morphometric measurements recorded in this study, are consistent with the ranges of the sperm morphometric parameters reported by Maroto-Morales *et al.* (2010, 2012, 2015).

Table 5.2 Sperm morphometric parameters recorded for fresh samples obtained from Dormer and Merino (HL and LL) rams.

Morphometric parameter	Dormer	Merino	P-value	Merino High Line	Merino Low Line	P-value
Area (µm^2)	43.02 ± 0.64	43.18 ± 0.42	0.68	43.30 ± 0.55	43.07 ± 0.65	0.81
Perimeter (µm)	30.70 ± 0.66	30.71 ± 0.44	0.69	30.97 ± 0.65	30.64 ± 0.61	0.53
Width (µm)	5.92 ± 0.09	5.91 ± 0.06	0.76	5.91 ± 0.08	5.90 ± 0.08	0.87
Length (µm)	9.70 ± 0.10	9.69 ± 0.07	0.89	9.71 ± 0.11	9.68 ± 0.11	0.79
Ellipticity	1.65 ± 0.03	1.65 ± 0.02	0.99	1.65 ± 0.03	1.65 ± 0.02	0.96
Elongation	0.24 ± 0.008	0.24 ± 0.005	0.93	0.24 ± 0.007	0.24 ± 0.007	0.98
Roundness	1.76 ± 0.06	1.77 ± 0.04	0.92	1.78 ± 0.06	1.75 ± 0.05	0.81

No significant differences in sperm morphometric parameters were observed between fresh semen samples collected from Dormer and Merino rams. As mentioned earlier, no research on sperm characteristics of Dormer rams could been found. Furthermore, research comparing sperm morphometric parameters between sheep breeds is also limited. This may suggest that this study is one of the first of its kind, characterising Dormer sperm and also comparing sperm morphometry between sheep breeds. The sample size of Dormers rams was however too small to make generalizations regarding the breed in terms of sperm traits.

When other species are considered, sperm morphometry studies have also been conducted in cattle and pigs. In a study where the sperm morphometry parameters of *Bos taurus* and *Bos indicus* cattle were compared, differences were observed between the two subspecies with regard to sperm morphometry. However, no differences were observed between the cattle breeds within each sub-species (Beletti *et al.*, 2005). In contrast to findings on cattle, Kondracki *et al.* (2012) found significant differences between Duroc and Pietrain pig breeds for sperm morphometry, where the Pietrain boars had significantly smaller sperm head perimeter values and longer sperm head lengths compared to the Duroc boars.

No significant differences with regards to sperm morphometric parameters were observed between HL and LL rams. However, the HL was consistently favoured in terms of absolute differences for sperm morphometric parameters. Boshoff (2014) found similar results, with only significant differences between the HL and LL rams for the ejaculated sperm morphometric parameters, ellipticity and elongation. The HL rams had higher values for ellipticity and elongation, indicating that sperm produced by the HL rams were more elongated and had a less tapered head shape. In the same study, significant differences were found for sperm morphometric parameters of freshly collected HL and LL epididymal sperm for sperm head width, suggesting that the LL rams had broader sperm heads compared to the HL rams. This is in contrast to results obtained in the present study, which suggests that the mean sperm head

width of HL rams and LL rams was similar. Whether this result is of biological significance still needs to be investigated. The current study's results are further supported by the findings of De Paz *et al.* (2011), who found no significant differences between any of the sperm morphometric parameters studied between two Assaf ram fertility groups.

5.3.3 Sperm subpopulations

5.3.3.1 Identification of sperm morphometric subpopulations

All morphometric data of the Dormer and Merino rams were normally distributed by the Kolmogorov-Smirnov normality test. The principal component analysis (PCA) of the morphometric data rendered two principal components (PC's), with eigenvalues above one, representing more than 94% of the cumulative variance (Table 5.3).

Table 5.3 Principal component analysis (PCA) results of the sperm morphometric parameter analysis for ejaculated samples obtained from Dormer and Merino rams.

		Principal components	
		PC1	**PC2**
Initial eigenvalues	**Eigen values**	2.05	1.84
	Individual Variability (%)	49.69	44.52
	Cumulative variance (%)	49.69	94.20
Eigenvectors	**Area (μm^2)**	0.68	-0.27
	Perimeter (μm)	0.86	-0.22
	Length (μm)	0.57	-0.68
	Width (μm)	0.77	0.55
	Ellipticity	-0.27	-0.91
	Elongation	-0.28	-0.93
	Roundness	0.70	-0.14

The two PC's, PC1 and PC2, were used to characterise each individual sperm and also to classify the sperm subpopulations in the cluster analysis. The first PC was positively related to dimension (size) parameters (area, perimeter, length, width and roundness) and negative related to elipticity and elongation (shape parameters). The second PC was positively related to width (dimension parameter) and negatively related to all other dimension and shape parameters (area, perimeter, length, ellipticity, elongation and roundness). The cluster analyses identified four sperm morphometric subpopulations in both Dormer and Merino (HL and LL) rams (Figure 5.1).

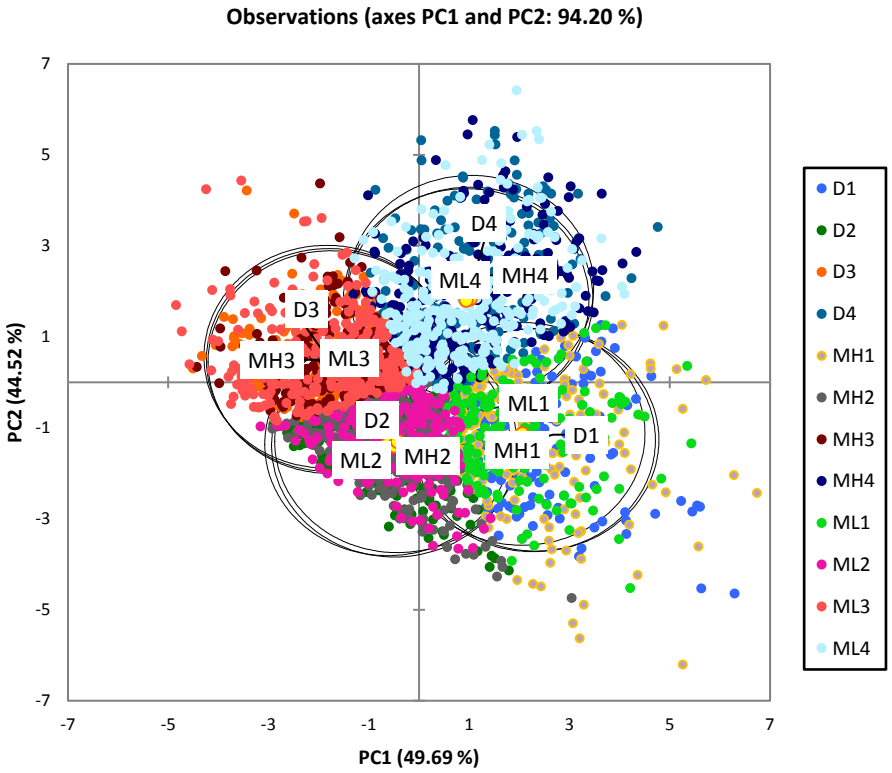

Figure 5.1 A PCA plot indicating the distribution of sperm morphometric subpopulations in ejaculated samples obtained from Dormer and Merino rams (D: Dormer; MH: Merino High line; ML: Merino Low line; 1-4: Subpopulation 1-4).

5.3.3.2 Structure and distribution of sperm morphometric subpopulations

5.3.3.2.1 Influence of breed on structure and distribution of sperm morphometric subpopulations

Four distinct sperm morphometric subpopulations were identified in the semen samples collected from Dormer and Merino rams. Each subpopulation, within a breed, differed significantly in terms of distribution and represented a different sperm head phenotype, characterised by different values for each sperm morphometric parameter ($p<0.05$; Table 5.4). Based on previous studies, the results of the present study was interpreted and four sperm morphometric subpopulations were identified and classified accordingly (Maroto-Morales *et al.*, 2012, 2015; Ramón *et al.*, 2013).

Table 5.4 Sperm morphometric characteristics (mean ± SEM) and distribution of each subpopulation (SP) identified in fresh semen samples collected from Dormer and Merino rams.

		SP1	SP2	SP3	SP4
	Morphometric parameters	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**
Dormer	**Area (μm²)**	$47.57^{a} \pm 0.60$	$42.24^{b} \pm 0.44$	$39.55^{c} \pm 0.34$	$42.72^{d} \pm 0.38$
	Perimeter (μm)	$35.54^{a} \pm 0.56$	$29.23^{b} \pm 0.19$	$27.26^{c} \pm 0.10$	$30.76^{d} \pm 0.33$
	Width (μm)	$6.18^{a} \pm 0.04$	$5.47^{b} \pm 0.05$	$5.56^{c} \pm 0.04$	$6.45^{d} \pm 0.02$
	Length (μm)	$10.38^{a} \pm 0.01$	$9.92^{b} \pm 0.04$	$9.09^{c} \pm 0.03$	$9.41^{d} \pm 0.06$
	Ellipticity	$1.68^{a} \pm 0.01^{a}$	$1.82^{b} \pm 0.01$	$1.64^{c} \pm 0.01$	$1.46^{d} \pm 0.01$
	Elongation	$0.25^{a} \pm 0.003^{a}$	$0.29^{b} \pm 0.003$	$0.24^{c} \pm 0.002$	$0.19^{d} \pm 0.002$
	Roundness	$2.15^{a} \pm 0.09$	$1.62^{b} \pm 0.02$	$1.50^{c} \pm 0.01$	$1.78^{d} \pm 0.03$
	Distribution (%)	**19.01^{a}**	**32.53^{b}**	**$25.85^{a,b}$**	**22.61^{a}**
Merino	**Area (μm²)**	$47.12^{a} \pm 0.32$	$42.91^{b} \pm 0.24$	$39.45^{c} \pm 0.28$	$43.26^{d} \pm 0.36$
	Perimeter (μm)	$35.24^{a} \pm 0.30$	$29.77^{b} \pm 0.16$	$27.11^{c} \pm 0.17$	$31.11^{d} \pm 0.17$
	Width (μm)	$6.18^{a} \pm 0.02$	$5.52^{b} \pm 0.02$	$5.55^{c} \pm 0.02$	$6.38^{d} \pm 0.02$
	Length (μm)	$10.37^{a} \pm 0.05$	$9.92^{b} \pm 0.03$	$9.08^{c} \pm 0.03$	$9.40^{d} \pm 0.04$
	Ellipticity	$1.68^{a} \pm 0.01$	$1.80^{b} \pm 0.01$	$1.64^{c} \pm 0.01$	$1.48^{d} \pm 0.01$
	Elongation	$0.25^{a} \pm 0.002$	$0.29^{b} \pm 0.002$	$0.24^{c} \pm 0.002$	$0.19^{d} \pm 0.002$
	Roundness	$2.12^{a} \pm 0.05$	$1.65^{b} \pm 0.02$	$1.49^{c} \pm 0.02$	$1.80^{d} \pm 0.02$
	Distribution (%)	**16.49^{a}**	**29.69^{b}**	**26.70^{b}**	**27.11^{b}**

^{a,b,c,d} Different superscripts within rows differ significantly ($p < 0.05$)

Subpopulation 1: large and most elongated sperm

This subpopulation was characterised by sperm whose sperm head length and roundness values were the highest (10.38 ± 0.01 and 2.15 ± 0.09, respectively). Therefore, this subpopulation included the longest, largest and the most elongated (i.e. tapered) sperm. This population included 19% of the Dormer and 16% of the Merino total sperm in the data set.

Subpopulation 2: large and round sperm

Subpopulation 2 included sperm with a relatively long head length and relatively low roundness values (9.92 ± 0.04 and 1.62 ± 0.02, respectively). Thus this subpopulation included relatively large and round sperm. Approximately 33% (Dormer) and 30% (Merino) of the total sperm in the data set were assigned to this subpopulation.

Subpopulation 3: short and round sperm

Subpopulation 3 was characterised by sperm with the shortest sperm head length and smallest value for roundness (9.09 ± 0.03 and 1.50 ± 0.01, respectively). Sperm included into this subpopulation, were short and round, therefore small, with a sperm head shape similar in shape to a circle. This population contained approximately 26% of both the Dormer and Merino total

sperm in the data. This population contained 34% of the HL and 26% of the LL total sperm in the data set.

Subpopulation 4: short and elongated sperm

Subpopulation 4 contained shorter sperm with a shorter head length, but with high values of roundness indicating a more tapered shape (9.41 ± 0.06 and 1.78 ± 0.03, respectively). Thus, this subpopulation included short, elongated sperm. This subpopulation represented 23% (Dormer) and 27% (Merino) of the total sperm in the data set.

No significant differences were however found between the two ram breeds with regards to the mean values of each sperm morphometric parameter characterising each subpopulation. This suggest that the classification of sperm subpopulations according to the parameters described above is quite robust and can be used to characterise sperm subpopulations of sheep, regardless of breed.

Significant differences were observed in the percentage of sperm (distribution) included in each sperm subpopulation within each ram breed ($p<0.05$; Table 5.4). In Dormer rams, the contribution of SP2 was significantly greater than those of SP1 and SP4. In contrast, SP2, SP3 and SP4 contributed more than SP1 to the overall number of sperm analysed in Merinos. On the other hand, no significant differences were observed for the distribution of each sperm morphometric subpopulation between the two ram breeds.

The PCA and cluster analysis identified four sperm morphometric subpopulations (large and elongated, large and round, short and round and short and elongated), characterised by different morphometric parameters, within the semen samples collected from Dormer and Merino rams. This is consistent with other studies, which reported the existence of 4 sperm subpopulations within a semen sample collected from different ram breeds (Maroto-Morales *et al.*, 2012, 2015; Martí *et al.*, 2012). However, a few other studies have reported the existence of three or four sperm subpopulations within a ram semen sample (Martí *et al.*, 2011; Yániz *et al.*, 2015). The presence of only 3 subpopulations was observed in yearling rams, while the rams in the current study were all mature rams. Although the biology underlying these sperm subpopulations are not yet clear, it is suggested that each subpopulation represents sperm of different physiological and maturational statuses (Martí *et al.*, 2012).

To our knowledge, this is the first work on the description and characterisation of Dormer semen samples, in terms of sperm morphometric subpopulations. Also, to our knowledge no data exist on the comparison of the structure and distribution of sperm morphometric sup-population between ram breeds. However, the characterisation of each sperm subpopulation agreed with

studies of Maroto-Morales *et al.* (2012, 2015) and Ramón *et al.* (2013), yet no literature could been found to support the finding of no differences between the two breeds.

Considering the distribution and structure that differs between subpopulations, these results are consistent with other findings on ram sperm morphometric subpopulations. However the results obtained in the present study, with regards to the proportion of sperm included into each subpopulation, differed from other studies (De Paz *et al.*, 2011; Martí *et al.*, 2011, 2012; Maroto-Morales *et al.*, 2012, 2015; Ramón *et al.*, 2013; Yániz *et al.*, 2015). A number of factors could have potentially contributed to these differences. Different ram breeds, environmental and climate conditions compared to other studies could have contributed to these differences.

Various studies have reported that sperm quality and fertility might be related to the proportion of sperm allocated to specific subpopulations. In a study by Yániz *et al.* (2015), it was observed that ram fertility may be correlated to the proportion of large and long sperm. Muiño *et al.* (2008) found that the sperm samples with the highest proportion of rapid and progressive sperm in dairy bulls were less damaged by cryopreservation. This may suggest that the subpopulation classification can be used to determine sperm sample quality. Taking all the above-mentioned results into consideration, it can be hypothesised that, when sperm samples of different breeds are analysed for sperm morphometric subpopulations, standard criteria of sperm morphometric subpopulations could be used for semen sample evaluation in both the Merino and Dormer breeds. Additionally, the potential fertility of sperm samples can possibly be predicted (Rodriguez-Martinez, 2003). This holds major possibilities for potential adaptation of semen evaluation protocols for sperm used in assisted reproduction technique

5.3.3.2.2 Influence of selection for prolificacy on structure and distribution of sperm morphometric subpopulations

From the principal component analysis and cluster analysis results, 4 sperm morphometric subpopulations were again identified in the semen samples collected from the HL and LL Merino rams. Each sperm subpopulation, within each genetically diverse line (HL and LL) were structured and characterised by different values for each sperm morphometric parameter, representing different sperm head phenotypes ($p<0.05$; Table 5.5). Based on previous studies, the results of the present study was interpreted and four sperm morphometric subpopulations were identified and classified accordingly for both genetically diverse lines (Maroto-Morales *et al.*, 2012, 2015; Ramón *et al.*, 2013).

Table 5.5 Sperm morphometric characteristics (mean ± SEM) and distribution of each subpopulation identified in fresh semen samples collected from High Line and Low Line Merino rams.

		SP1	SP2	SP3	SP4
	Morphometric parameters	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**
High Line	**Area (μm²)**	46.83^{a} ± 0.47	42.79^{b} ± 0.17	39.90^{c,1} ± 0.25	43.68^{d,1} ± 0.59
	Perimeter (μm)	35.63^{a,1} ± 0.47	29.77^{b} ± 0.24	27.25^{c} ± 0.22	31.25^{d} ± 0.15
	Width (μm)	6.18^{a} ± 0.02	5.50^{b} ± 0.04	5.57^{c} ± 0.02	6.40^{d} ± 0.03
	Length (μm)	10.39^{a} ± 0.08	9.91 ^{b} ± 0.04	9.12^{c} ± 0.05	9.42^{d} ± 0.07
	Ellipticity	1.69^{a} ± 0.01	1.81^{b} ± 0.01	1.64^{c} ± 0.01	1.47^{d} ± 0.01
	Elongation	0.25^{a} ± 0.003	0.29^{b} ± 0.00^{b}	0.24^{c} ± 0.003	0.19^{d} ± 0.003
	Roundness	2.18^{a,1} ± 0.06	1.66^{b} ± 0.02	1.49^{c} ± 0.02	1.79^{d} ± 0.01
	Distribution (%)	**16.67^{a}**	**33.74^{b}**	**24.07^{a,d}**	**25.51^{c,d}**
Low Line	**Area (μm²)**	47.40^{a} ± 0.45	43.04^{b} ± 0.50	38.99^{c,2} ± 0.44	42.84 ^{d,2} ± 0.41
	Perimeter (μm)	34.85^{a,2} ± 0.32	29.779^{b} ± 0.23	26.97^{c} ± 0.25	30.98^{d} ± 0.31
	Width (μm)	6.17^{a} ± 0.04	5.538^{b} ± 0.03	5.54^{c} ± 0.03	6.36^{d} ± 0.02
	Length (μm)	10.35^{a} ± 0.05	9.924^{b} ± 0.05	9.05^{c} ± 0.03	9.39^{d} ± 0.04
	Ellipticity	1.68^{a} ± 0.01	1.80^{b} ± 0.003	1.64^{c} ± 0.01	1.48^{d} ± 0.01
	Elongation	0.25^{a} ± 0.003	0.28^{b} ± 0.001	0.24^{c} ± 0.003	0.19^{d} ± 0.003
	Roundness	2.06 ^{a,2} ± 0.06	1.65^{b} ± 0.04	1.49^{c} ± 0.03	1.80^{d} ± 0.04
	Distribution (%)	**16.30^{a}**	**25.64^{b}**	**29.34^{b}**	**28.72^{b}**

^{a,b,c,d} Different superscripts within rows differ significantly ($p < 0.05$)

^{1,2} Different superscripts within columns differ significantly ($p < 0.05$)

Subpopulation 1: large and most elongated sperm

Subpopulation 1 included approximately 17% of the HL and 16% of the LL total sperm, and were characterised by large and long (i.e. tapered) sperm, as indicated by the highest values for sperm head length and roundness.

Subpopulation 2: large and round sperm

Subpopulation 2 contained relatively long and round sperm, as indicated by the relatively high sperm head length values and relatively low vales of roundness. Therefore this subpopulation included large and round sperm. This population contained 34% of the HL and 26% of the LL total sperm in the data set.

Subpopulation 3: short and round sperm

The sperm in subpopulation 3 were characterised by the shortest sperm head length and the lowest value of roundness. Thus this subpopulation contained the smallest sperm, with a sperm

head shape similar to a circle, indicated by the lowest value of roundness. This population represented 24% of the HL and 29 % of the LL total sperm.

Subpopulation 4: short and elongated sperm

Almost 26% and 29% of the HL and LL, respectively, total sperm were assigned to subpopulation 4. This population contained sperm with relatively low values for sperm head length, but high values of roundness. Therefore this subpopulation included short, elongated sperm.

With regards to the mean values of each sperm morphometric parameter characterising each subpopulation identified in the semen samples obtained from HL and LL rams, significant differences were observed between certain subpopulations in the HL and LL for individual morphometric parameters. The mean values of perimeter and roundness, characterising SP1 differed significantly between HL and LL rams. In SP3 and SP4, the mean value of area, characteristic of these subpopulations, also differed significantly between the HL and LL groups. However, no significant differences were found between the HL and LL groups for any of the other morphometric parameters characterising the subpopulations.

Additionally, significant differences were observed in the percentage of sperm (distribution) included into each sperm subpopulation within each ram breed ($p<0.05$; Table 5.5). With regards to HL rams, there was a significant difference in the total number of sperm included into SP1 and SP2 or SP4, with no significant difference between the percentage of sperm included in SP3 and SP4. For the LL rams, significant differences were observed between SP1 and SP2 or SP3 or SP4. However, no significant differences with regards to the percentage of sperm included into SP2, SP3 and SP4 were observed. Alternatively, no significant differences were observed for the distribution of each sperm morphometric subpopulation between the HL and LL rams. However, the difference in the distribution of subpopulation 2 between the HL and LL rams approached significance ($p=0.051$). Therefore it can be stated that there is a strong tendency for the HL rams to have a higher proportion of sperm included in subpopulation 2, compared to the LL rams. These differences observed in the HL and LL rams, might have an effect on the swimming speed, swimming pattern and fertilizing ability of sperm. Theoretically spoken, the HL rams should actually have a lower proportion of SP2, that is associated with lower fertilizing potential, as the line were genetically selected for reproduction potential (Yániz *et al.*, 2015).

This was the first work done on the classification of HL and LL sperm according to a morphometric subpopulation profile, therefore literature to compare it to could not be found. From the PCA and cluster analysis results it were evident that 4 sperm morphometric

subpopulations, irrespective of genetic line, co-existed in the ejaculates of both the HL and LL rams. These results are consistent with other studies done on high and low fertility groups (De Paz *et al.*, 2011; Ramón *et al.*, 2013; Yániz *et al.*, 2015). Although the origin of the sperm belonging to the respective subpopulations is still unclear, some research seems to indicate that it is possible that the differences in sperm morphometric parameters, are influence by genotypic effects on sperm structure during the process of spermatogenesis (Beatty & Sharma, 1961; Maroto-Morales *et al.*, 2012). These findings might explain the minor differences that were observed between the mean values of individual morphometric parameters (perimeter and area) of certain subpopulations between the two genetic lines. This might also further support the findings of Sandenbergh (2013), whom reported that genetic selection in the HL and LL for reproduction potential resulted in the segregation of a SNP-marker close to a gene coding for the sperm cytoskeleton.

Furthermore, Sailer *et al.* (1996) and Pérez-Sánchez *et al.* (1998) stated that abnormal chromatin structure and condensation, can influence sperm morphometry and fertility. This may be the cause of the differences found between HL and LL groups in the current study in terms of mean sperm head area of subpopulation 3 and 4. This may also potentially elucidate why the HL and LL, did not differ for the number of offspring sired during the 2012 mating season in a previous study (Boshoff, 2014).

In addition, the results of the present study showed a significant difference in the distribution and structure of the subpopulations within each genetic line (HL and LL). These results are consistent with the findings reported by several other authors (De Paz *et al.*, 2011; Martí *et al.*, 2011, 2012; Maroto-Morales *et al.*, 2012, 2015; Ramón *et al.*, 2013; Yániz *et al.*, 2015). According to a few authors, the fertility of a semen sample might be linked to the proportion of sperm within a subpopulation characterised by long and elongated sperm (Yániz *et al.*, 2015). In contrast, Ramón *et al.* (2013) and Maroto-Morales *et al.* (2015) stated that high fertility rates are strongly associated to the proportion of sperm having small and elongated heads. Based on these two theories and the results of the current study, no significant differences were observed in the distribution of subpopulations between HL and LL rams, with specific reference to subpopulation 1 (long, elongated sperm) and 4 (short, elongated sperm). This may suggest why no differences were found in the number of offspring sired in 2012 by HL and LL rams in a previous study (Boshoff, 2014).

On the contrary, the previous statement might be further supported by the tendency of a high proportion of sperm within subpopulation 2 in the HL, characterised by slightly smaller (compared to SP1) and round sperm. It has been reported previously that rams with a high proportion of small and round sperm (represented by subpopulation 3), have sperm sensitive to

cryodamage and poor ejaculates (Esteso *et al.*, 2006; Martí *et al.*, 2011). Despite all the above mentioned, Santolaria *et al.* (2015) reported that fertility is not related to the distribution of sperm in the different subpopulations.

The cumulative evidence reinforces an argument that sperm morphometric subpopulations might clarify why the HL and LL rams did not differ in terms of the number of offspring sired in the 2012 reproductive season. Although the two lines were selected for reproduction potential, it may be hypothesised that the two lines will differ in their subpopulation profile, which were however not the case in the current study. A better understanding of the origin and functional relevance of sperm subpopulations and the complex fertilisation processes are still however needed.

5.4 Conclusion

From this study, it was seen that breed did not influence sperm quality parameters or sperm morphometric subpopulations structure and distribution. However, further research should be conducted with more animals under different environmental conditions as only 6 rams represented each genotype in this study.

With regards to the two divergently selected lines, no significant differences were observed between HL and LL rams in terms of sperm quality or morphometric parameters. The subpopulation analysis showed differences in individual mean values of specific subpopulations between the HL and LL groups. No significant differences were found in the distribution and structure of the morphometric subpopulations. Seen against this background it becomes crucial to understand how selection affected morphometric traits of sperm produced by the HL and LL, which in turn may influence the fertilizing ability of the sperm.

Further research is suggested to determine what the relationship between sperm competition and the reproductive performance of the genetically diverse lines (HL and LL) are. Another possible area of research is the contribution of the ewe during the fertilising process and to the overall reproduction efficiency of a breeding flock.

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Chapter 6

The influence of subpopulation traits on the fertilizing ability of sperm obtained from Dormer and Merino rams, as used in ART protocols

Abstract

The potential fertility of a semen sample can be determined by evaluating the binding capacity of sperm *in vitro*. It has been suggested that *in vitro* and *in vivo* fertility is correlated with the sperm subpopulation structure of an ejaculate. This study investigated whether sperm morphometric subpopulation traits have an influence on the fertilizing ability of sperm obtained from Dormer and Merino rams. Post-thaw semen samples of six Dormer rams and twelve Merino rams were evaluated for post-thaw sperm quality, and subjected to a sperm binding assay to determine the binding ability of the sperm post-thaw. Correlations between the mean number of sperm bound to the perivitelline membrane (PVM) and the proportions of sperm in the different morphometric subpopulations were derived. Post-thaw sperm evaluation indicated no significant differences between Dormer and Merino rams, except for sperm viability between HL and LL rams. A significant difference was found between the Dormer and Merino rams with regards to sperm binding capacity ($p<0.05$). The mean number of sperm bound to the PVM also tended to differ between the HL and LL rams ($p=0.058$). However, no statistically significant correlation between sperm binding capacity and any sperm morphometric subpopulation was observed. These results suggest that no specific morphometric subpopulation or the distribution of sperm in the different subpopulations had an influence on the fertilizing ability of sperm collected from Dormer and Merino rams as determined *in vitro*. Further research is, however, necessary to clarify the findings of the study.

6.1 Introduction

The reproduction success of a breeding flock is determined by the contribution of both the ram and ewe, with male sperm quality being conditional to ensure conception in the ewe. Semen evaluation protocols quantify sperm parameters such as morphology, motility, etc., all of which are considered as important prerequisites to ensure successful fertilization. Although there are several parameters that can be assessed to determine sperm quality, several studies have indicated that all these parameters can vary extensively in predicting the fertilizing ability of sperm (Larsson & Rodriguez-Martinez, 2000; Gadea, 2005).

The fertility of a ram can also be assessed by quantifying the ability of sperm to bind to and penetrate oocytes *in vitro* (Zhang *et al.*, 1998; García-Álvarez *et al.*, 2009; Ferraz *et al.*, 2014). For the fertilization process to be completed, sperm needs to bind and penetrate the *zona pellucida*, making this one of the most important barriers to overcome in order to fertilize an oocyte (Gadea, 2005). In this context, the development of sperm functionality tests have received considerable attention in an attempt to improve the predictive accuracy of the ability of sperm to bind and eventually penetrate the *zona pellucida*. Sperm binding assays have been developed as a tool for identifying the fertility potential of sperm in several species (Purdy, 2006;

De Araujo *et al.*, 2015; Losano *et al.*, 2015).These tests assess among others the potential of sperm to undergo capacitation and the acrosome reaction. If sperm is unable to do undergo capacitation and the acrosome reaction, membrane binding and penetration will not occur (Purdy, 2006).

Correlations between *in vitro* sperm binding capacity and *in vivo* and field fertility have been reported in several species. However, with the knowledge of the mammalian ejaculate consisting of well-defined and distinct subpopulations, a few studies have reported that the sperm subpopulation composition of ejaculates are highly correlated with *in vitro* and field fertility of males (Ramón *et al.*, 2013; Ferraz *et al.*, 2014; Yániz *et al.*, 2015). A study by Yániz *et al.* (2015) reported that the difference in field fertility of two groups of rams selected for prolificacy might be related to differences in their morphometric subpopulation distribution and composition. However, limited information is available on the influence of breed and genotype of rams on sperm subpopulation composition and distribution, fertilizing ability and the correlation of specific subpopulations with fertility.

Since 1986, a resource flock consisting out of two genetically diverse lines of Merino sheep were established from the same base population at the Tygerhoek Research Farm, after selection according to maternal ranking values for the dams of available male and female replacement animals (Cloete *et al.*, 2004; 2009). More details on the selection process for replacements animals can be found in Chapter 3. Studies on this resource population have investigated sperm-related factors that can potentially contribute to the differences in prolificacy between the lines. Lambrechts *et al.* (2000) reported that there were no significant differences in sperm viability and morphology of the two genetically diverse Merino lines. Furthermore, Boshoff (2014) reported minor differences in sperm head morphometry between the two lines. However, the influence of sperm morphometric subpopulations traits on fertilizing ability has not yet been determined.

The aim of this study was therefore to investigate the influence of sperm morphometric subpopulation traits on the fertilizing ability of frozen-thawed sperm obtained from Dormer and Merino rams by means of an *in vitro* sperm binding assay. Furthermore, the study aimed to investigate the influence of genetic selection on the influence of sperm morphometric subpopulation traits on the fertilizing ability of frozen-thawed sperm

6.2 Materials and methods

Ethical clearance for the study was provided by the Stellenbosch University Animal Ethics Committee (SU-ACUD15-00083). All animal care and procedures used in this study was

according to the guidelines stipulated in the South African National Standards document 10386:2008.

6.2.1 Experimental location

The experiments were conducted on the Elsenburg Agricultural Research Farm of the Western Cape Department of Agriculture (GPS coordinates: -33.837966; 18.834337) situated near Stellenbosch in the Western Cape, South Africa.

6.2.2 Experimental animals

Eighteen mature Dormer (n=6) and (n=12) Merino rams, aged between 2 and 5 years, were used in this study. Both the Dormer and Merino rams originated from resource populations maintained on the Elsenburg Agricultural Research Farm. The Merino rams were part of a resource flock, which consists out of two genetically diverse lines, selected for multiple rearing ability. For a more in-depth description on the flock history and the selection of the two divergently selected lines, please refer to Chapter 3. For this study, six rams of the high line (HL), six rams of the low line (LL) and six Dormer rams were used or semen collection purposes.

All the rams were managed under normal commercial farm practices and maintained on irrigated kikuyu grass (*Pennisetum clandestinum*) pastures with water available *ad libitum*.

6.2.3 Collection and processing of semen samples

A total of 54 semen samples were collected by means of electro-ejaculation (Orihuela *et al*., 2009; Malejane *et al*., 2014), during three consecutive weeks, with sampling performed on 3 days per week, and a total of six rams were sampled per day. Directly after collection, all semen samples were sealed with parafilm (Lasec, South Africa) to prevent water contamination, and transported to the laboratory in a thermally controlled flask (37ºC). On arrival at the laboratory, samples were transferred to a water bath (37ºC), and maintained at 37 °C until further processing. All semen samples were subjected to macroscopic evaluation, which include volume, colour and mass motility evaluation as well as microscopic evaluation. Please refer to Chapter 3 for more details on the methods used.

After the microscopic evaluation was completed, all semen samples were diluted to a concentration of $600x10^6$ sperm/mL with cooling media (37 ºC) (Paulenz *et al.,* 2007), and equilibrated for 45min at 5 ºC. The equilibrated samples were then diluted drop-wise (1:1) with 5 ºC freezing media (Paulenz *et al.,* 2007), and again allowed to equilibrate for 45min at 5 ºC. After the second and final equilibration period, two straws were loaded per ram per collection

and cryopreserved for a month according to the technique described in Chapter 3. The end concentration after dilution was 300 x 10^6 sperm/mL.

The cryopreserved straws were thawed at 37 ºC for 30 seconds (Paulenz *et al.*, 2007). The content of each straw was emptied into a 2 mL Eppendorf tube and assessed for post-thaw sperm quality (mass motility, viability and morphology) and sperm binding capacity. For a more detailed description on the sperm evaluation protocols used for the cryopreserved semen samples, please refer to Chapter 3.

6.2.4 Sperm binding assay

The binding capacity of cryopreserved ram sperm was determined using a binding assay described by Purdy (2006), with minor modifications. The egg yolks of freshly laid, unfertilized chicken eggs were separated from the albumin and the perivitelline membrane (PVM) was punctured to release the egg yolk. Each PVM was then washed delicately with Dulbecco's phosphate buffered saline (DPBS) in a Petri dish (100mm, Lasec, South Africa) until the membrane became translucent and the DPBS solution was clear of any yolk. The membrane was then gently spread out in the Petri dish and cut into small squares (1cm x 1cm), using a scalpel blade (size no. 11, Lasec, South Africa) and a spectrophotometer cuvette as a guiding tool (Mocé & Graham, 2008). Each PVM section was then transferred to 2mL Eppendorf tubes (Lasec, South Africa) containing 500µL of TALP (for composition, please refer to Appendix A). Fresh perivitelline membranes were prepared for each binding assay.

An aliquot (10µL) of frozen-thawed sperm was added to each Eppendorf tube containing the TALP, and after being gently mixed, each sperm-TALP-PVM combination was incubated at 39 ºC in an HERACell 150i Thermo Fisher CO_2 incubator (SepSci, South Africa), in a controlled atmosphere of 5% of CO_2 for 1 h. Each tube was gently shaken every 30min to prevent the membrane folding on itself, thus ensuring that the binding surface was not compromised (Mocé & Graham, 2008). After 1 h of incubation, 5µL of SYBR-14 (prepared according to the LIVE/DEAD® Sperm Viability Kit (L-7011) Protocol provided by the manufacturer, Molecular Probes) was added to each tube to fluoresce sperm bound to the PVM, and incubated for an additional hour.

After incubation each perivitelline membrane was washed three times in DPBS to remove all unbound sperm. Each PVM square was then gently spread open on a microscope slide to remove any creases, and covered with a coverslip (Mocé & Graham, 2008). The binding ability of the sperm was determined by using an Olympus IX70 inverted fluorescence microscope at 40X magnification. Sperm bound to each membrane, i.e. sperm that fluoresced, in 10 fields were counted, as described by Purdy (2006). Two replicated analyses per ram per collection

day were performed using a separate PVM binding assay for each. The mean sperm bound to each PVM was determined from the two replicated analyses per ram per collection day.

6.2.5 Statistical analysis

Statistical analysis was performed using XLSTAT 2016.1 (Addinsoft, 2016). All data derived from the macroscopic and microscopic sperm evaluation of fresh and frozen-thawed samples were analysed using XLSTAT. Full models were initially fitted and where the interactions were not significant the analyses were repeated using only the main effects. Due to repeated measure on the same rams being used, the data were initially analysed with ram as a random effect in a mixed model with each of the response variables and breed and lines as the independent variables. This was repeated in a linear model analyses excluding the random effect. The results were unchanged, therefore only the ANOVA results are reported. All results were expressed as mean ± SEM. The significance level was set at $p < 0.05$.

All the morphometric data was imported into a single data set (2969 observations) and analysed by clustering procedures to identify sperm morphometric subpopulations using XLSTAT 2016.1. The PROC GLM procedure of SAS 9.3 and analysis of variance (ANOVA) were used to determine the effect of breed and genetic selection on the structure and distribution of sperm subpopulations and the significant differences in the distribution between breeds and genetically diverse lines. The significance level was set at $p < 0.05$. Please refer to Chapter 5 for more details on the clustering procedures used to identify sperm morphometric subpopulations.

Correlations between sperm binding capacity of the frozen-thawed sperm samples and fresh sperm morphometric subpopulations were analysed using linear regression, to derive Pearson's correlation coefficients. A correlation coefficient value below 0.3 was considered negligible, 0.3 – 0.5 represented a low correlation, 0.5 – 0.7 was moderate, and > 0.7 was considered as a high correlation.

6.3 Results and discussion

6.3.1 Microscopic evaluation

The microscopic sperm parameters recorded for fresh and frozen-thawed sperm samples obtained from Dormer and Merino rams, are presented in Table 6.1. No interactions were observed between breed or line (Dormer vs. Merino HL or LL) and treatment (fresh vs. frozen-thawed) for any of the microscopic sperm parameters, therefore only main effect means are presented.

Table 6.1 The influence of breed and genetic selection and cryopreservation on the microscopic sperm parameters of sperm obtained from Dormer and Merino rams.

Breed	Treatment	Microscopic sperm parameter			
		Mass motility (score)	Abnormalities (%)	Acrosome integrity (%)	Viability (%)
Dormer	**Fresh**	$3.67^{A} \pm 0.12$	$12.70^{A} \pm 2.22$	$75.58^{A} \pm 1.69$	- *
	Frozen-thawed	$2.36^{B} \pm 0.08$	$18.62^{B} \pm 1.99$	$59.90^{B} \pm 1.06$	$24.62^{A} \pm 1.59$
Merino	**Fresh**	$3.83^{A} \pm 0.08$	$10.36^{A} \pm 1.58$	$76.66^{A} \pm 1.22$	-
	Frozen-thawed	$2.56^{B} \pm 0.05$	$15.28^{B} \pm 1.40$	$61.80^{B} \pm 1.01$	$28.07^{A} \pm 1.13$
Merino High Line	**Fresh**	$3.94^{a} \pm 0.12$	$8.30^{a} \pm 2.22$	$78.81^{a} \pm 1.69$	-*
	Frozen-thawed	$1.33^{b} \pm 0.09$	$13.90^{b} \pm 1.99$	$63.51^{b} \pm 1.22$	$31.09^{a} \pm 1.49$
Merino Low Line	**Fresh**	$3.72^{a} \pm 0.12$	$12.30^{a} \pm 2.22$	$74.52^{a} \pm 1.69$	-
	Frozen-thawed	$1.22^{b} \pm 0.09$	$16.70^{b} \pm 1.99$	$60.00^{b} \pm 1.22$	$25.05^{b} \pm 1.49$

^{A,B} Different superscripts within columns differ significantly ($p<0.05$)
^{a,b} Different superscripts within columns differ significantly ($p<0.05$)
*Viability was not assessed in fresh samples

No significant differences were observed between Dormer and Merino rams for microscopic sperm parameters, for either fresh or frozen-thawed samples. The cryopreservation and thawing process markedly impaired ($p<0.05$) mass motility, abnormalities and acrosome integrity in both breeds (Table 6.1).

Furthermore, no significant differences were obtained for fresh ejaculated sperm samples between the two Merino lines for microscopic sperm parameters. However, in the frozen-thawed samples, sperm viability differed between selection lines ($p<0.05$). Sperm produced by HL rams offered more resistance to the detrimental effects of cryopreservation, when compared to sperm produced by LL rams (31.09% vs. 25.05%; $p<0.05$; Table 6.1). No significant differences were found between the two genetically diverse lines in terms of mass motility, acrosome integrity and sperm abnormalities. With regards to cryopreservation treatment, significant differences were observed for all sperm quality parameters between individual rams. All microscopic sperm parameters in the frozen-thawed samples were generally impaired, relative to that of fresh ejaculated samples ($p<0.05$). Overall, absolute differences for post-thaw sperm quality favoured the HL in all instances.

The absence of a breed influence on the microscopic sperm parameters are supported by the findings of Zargari *et al.* (2016), who reported no significant differences in sperm traits of samples collected from Afshari and Booroola Merino rams before and after cryopreservation. These findings are further supported by a study by Cardenas-Gallegos *et al.* (2012) on Dorper and Katahdin rams, where no significant differences in terms of mass motility and acrosome

integrity were reported. In contrast to the above mentioned findings, Kasimanickam *et al.* (2007) observed that Polled Dorset sperm offered more resistance to the damage caused by cryopreservation in a study on Polled Dorset, Suffolk and Katahdin rams.

From the results it were evident that selection had no influence on microscopic sperm parameters, except for viability in frozen-thawed sperm samples. These results are consistent with those reported by Lambrechts *et al.* (2000). On the contrary Lambrechts *et al.* (2000) observed significant differences between the two genetically diverse lines in terms of acrosome integrity of frozen-thawed sperm. Furthermore, no significant difference in the percentage live sperm was observed by the latter authors, as was also found in the present study. The findings of the present study are in agreement with those of Almadaly *et al.* (2016), who reported no significant differences between high and low fertility (determined by using mating records) Barki ram groups, with regards to sperm quality parameters of freshly ejaculated sperm samples. The frozen-thawed results of the current study, agree with the findings of García-Álvarez *et al.* (2009), where no differences were reported for post-thaw sperm parameters of high and low fertility Manchega rams.

In a study on sperm quality parameters of highly and lowly fertile bulls, sperm parameters after thawing were found to be independent of bull fertility (Tanghe *et al.*, 2002). However, the marked impairment of the cryopreservation and thawing process on sperm quality parameters are consistent with that reported by several other studies (Lambrechts *et al.*, 2000; Vicente-Fiel *et al.*, 2014). Although the cryopreservation procedure holds major possibilities for genetic material preservation, dissemination and conservation (Leboeuf *et al.*, 2000), the biggest limitation of the process is that the frozen-thawing process of sperm results in membrane and ultrastructure damage, which in turn impairs motility and viability of sperm (Watson, 2000).

The findings regarding cryodamage in the present study are consistent with various other studies that reported that cryopreservation has detrimental effects on sperm quality, causing changes in sperm structure and function (Ollero *et al.*, 1998; O'Connell *et al.*, 2002; Ahmad *et al.*, 2014).

6.3.2 Sperm binding capacity

The mean number of frozen-thawed sperm, collected from Dormer and Merino (HL and LL) rams, bound to chicken egg perivitelline membrane (PVM) and the correlations between sperm binding capacity and sperm morphometric subpopulations are presented in Table 6.2. Correlations were calculated by using the fresh sperm subpopulation data linked to ultimate sperm binding capacity of the samples. It should be kept in mind that only three values for sperm binding capacity of each ram were considered in the analysis. Although there were

correlations observed in the current study, none were significant due to high levels of variation (Figure 6.1).

Table 6.2 The mean (±SE) number of sperm from Dormer and Merino (HL and LL) frozen-thawed samples bound to a chicken perivitellin membrane and the correlation between sperm binding capacity and sperm morphometric subpopulations.

Breed	Bound sperm		Pearson correlation coefficient			
	Range (CV)	Mean ± SEM	SP1	SP2	SP3	SP4
Dormer	12–33 (26.24)	21.94^{A} ± 1.43	0.08	-0.31	0.11	0.34
Merino	16–43 (23.48)	25.86^{B} ± 1.01	0.28	0.15	-0.15	-0.26
Merino High Line	18.5–43 (23)	27.78 ± 1.40	0.28	-0.01	0.10	-0.24
Merino Low Line	16–33.5 (21.12)	23.94 ± 1.40	0.29	0.26	-0.18	-0.28

^{A,B} Different superscripts within columns differ significantly ($p<0.05$)

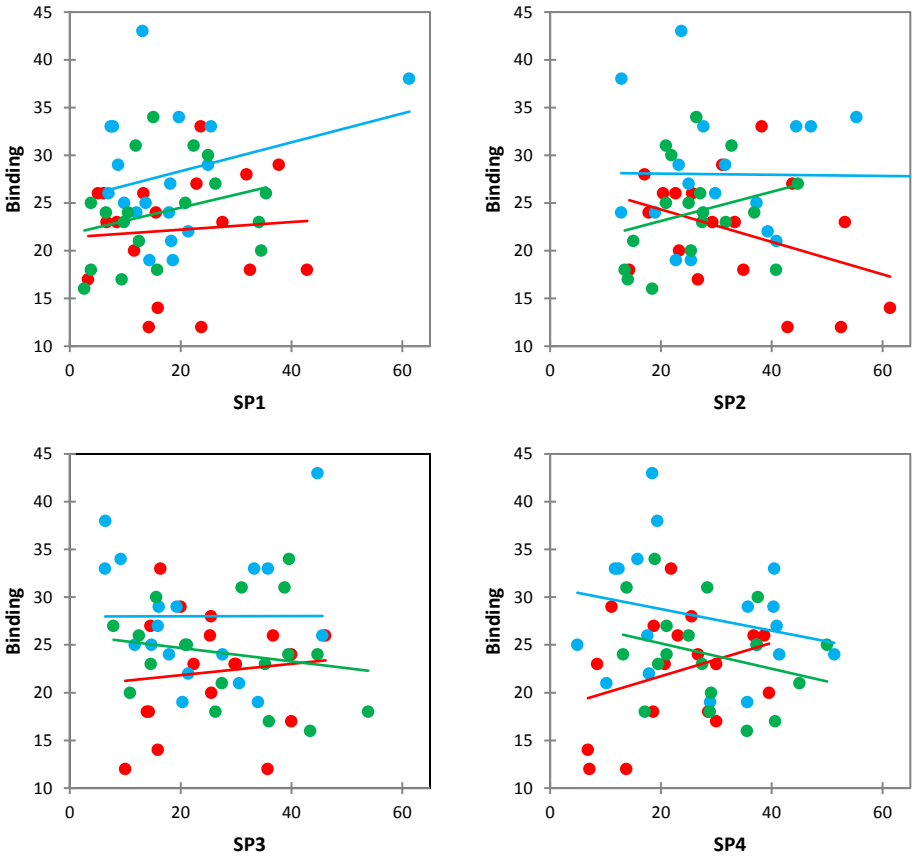

Figure 6.1 Illustrations of breed and line specific regression of sperm binding capacity on the percentage of sperm allocated to four distinct sperm morphometric subpopulations (Red: Dormer; Blue: Merino High Line; Green: Merino Low Line).

When the two breeds were compared in terms of the binding capacity of their frozen-thawed sperm, the Dormer and Merino breeds differed significantly for the mean number of sperm bound to the PVM ($p<0.05$; Table 6.2). The Merino rams had a higher mean value for bound sperm, when compared to Dormer rams (25.86 ± 1.01 vs. 21.94 ± 1.43, respectively). When the two genetically diverse lines were compared in terms of post-thaw sperm binding capacity, the difference in the mean value of sperm bound to the PVM approached significance for frozen-thawed sperm samples obtained from the two lines ($p = 0.058$; Table 6.2). There was thus a strong tendency for frozen-thawed HL sperm to bind more effectively to the PVM than the LL frozen-thawed sperm (27.78 ± 1.40 vs. 23.94 ± 1.40, respectively). In addition, our results indicated that the mean number of sperm bound to the PVM also varied between individual rams within breeds and lines ($p<0.05$; Table 6.3).

Table 6.3 The mean (±SE) number of sperm from Dormer and Merino (HL and LL) frozen-thawed samples bound to a chicken perivitellin membrane and the correlation between sperm binding capacity and sperm morphometric subpopulations.

Breed		Bound sperm	Pearson correlation coefficient			
	Ram ID	Mean ± SEM	SP1	SP2	SP3	SP4
Dormer	1.051	12.67 ± 2.38^{d}	-0.36	0.85	-0.30	-0.54
	3.044	17.33 ± 2.38^{c,d}	0.97	-0.12	-0.98	-0.60
	3.165	24.50 ± 2.38^{a,b,c,d}	0.98	-0.67	-0.58	0.61
	4.055	26.50 ± 2.38^{a,b,c}	0.91	0.74	-0.81	-0.82
	4.061	25.00 ± 2.38^{a,b,c,d}	-0.94	-0.97	0.66	1.00
	4.097	25.67 ± 2.38^{a,b,c,d}	0.84	-0.28	-0.38	-0.99
Merino High Line	3.014	37.50 ± 2.38^{a}	0.09	-0.67	0.29	0.80
	3.018	31.50 ± 2.38^{a,b}	0.44	0.29	0.07	-0.50
	3.145	26.83 ± 2.38^{a,b,c}	0.07	0.94	-0.82	-0.36
	3.208	26.00 ± 2.38^{a,b,c,d}	0.95	-0.26	-0.58	0.19
	4.006	19.67 ± 2.38^{b,c,d}	0.45	0.99	0.28	-0.97
	4.140	25.17 ± 2.38^{a,b,c,d}	-0.17	0.82	0.13	-0.48
Merino Low Line	1.177	25.50 ± 2.38^{a,b,c,d}	-0.48	0.22	0.62	-0.85
	3.092	22.17 ± 2.38^{b,c,d}	0.94	0.81	-0.95	-0.54
	3.150	28.50 ± 2.38^{a,b,c}	0.63	-0.06	-0.06	-0.45
	4.034	21.83 ± 2.38^{b,c,d}	0.54	0.71	-0.71	-0.02
	4.040	19.83 ± 2.38^{b,c,d}	0.89	-0.16	-0.84	0.27
	4.051	25.83 ± 2.38^{a,b,c,d}	0.91	0.40	-0.74	-0.48

[a,b,c,d] Different superscripts within columns differ significantly ($p<0.05$)

The results from the previous section indicated that the Dormer and Merino breeds did not differ in terms of sperm quality parameters for frozen-thawed samples. However, the two breeds differed significantly in terms of sperm binding capacity. It could be speculated that these differences could be related to seminal plasma composition of the two breeds. Muiño-Blanco *et al.* (2008), Leahy & Gadella (2011) and Caballero *et al.* (2012) stated that seminal plasma proteins might be key regulators of sperm functionality in the bull, ram, boar and stallion. In a study by Mendoza *et al.* (2013) it was observed that the presence of certain seminal plasma proteins inhibited capacitation and the acrosome reaction, which resulted in a higher binding capacity of ram sperm. However, seminal plasma composition was not studied in the current study. The differences in the sperm binding capacity of the two breeds may also suggest that sperm of the two breeds respond differently to the TALP medium and Dormers might undergo capacitation differently than Merino sperm (Fraser *et al.*, 2006).

The overall results of the mean number of sperm bound to the PVM are consistent with those found by Mocé & Graham (2008). The binding capacity results in the present study however are lower compared to that observed by Purdy (2006), who reported approximately a 20% higher mean number of sperm bound to the PVM compared to the current study. These differences may be ascribed to that fact that the present study used polyvinyl alcohol (PVA) in the TALP medium instead of bovine serum albumin (BSA) (Biggers *et al.*, 1997; Choi *et al.*, 2003). The low sperm viability of frozen-thawed samples could also potentially have contributed to the lower numbers of sperm bound to the PVM (Luna *et al.*, 2015). Although the Merino showed a higher mean value for binding capacity, it would not be appropriate to state that the semen samples from the one breed have a higher fertilizing ability compared to the other as there are many other factors that can influence the sperm binding capacity of a sperm sample.

With regards to the influence of breed and sperm morphometric subpopulation traits on the fertilizing ability of frozen-thawed sperm samples obtained from Dormer and Merino rams, no significant correlations between the mean number of sperm bound and sperm morphometric subpopulations were observed in any of the breeds ($p>0.05$). However, in the Dormer, sperm binding capacity was positively but lowly correlated to SP4. On the contrary, SP2 also had a low, but negative correlation to sperm binding capacity. Both SP1 and SP3 had a negligible correlation with sperm binding capacity. In the Merino breed, SP1 and SP4 were lowly positively and negatively, respectively, correlated with sperm binding capacity. In contrast, both SP2 and SP3 had negligible correlations with sperm binding capacity (Table 6.2). Additionally, no significant correlation was found for sperm binding capacity and sperm morphometric subpopulations for individual rams (Table 6.3).

It was evident that the mean number of sperm bound from Dormer and Merino rams and sperm morphometric subpopulations were not correlated. This result is consistent with that of Santolaria *et al.* (2015), namely that fertility of a given sample was not related to the distribution of sperm in different subpopulations. In contrast, Ferraz *et al.* (2014) reported significant correlations between sperm motility subpopulations and sperm binding capacity. Several other authors have suggested that the subpopulation containing long, elongated sperm (SP1) is more capable of fertilizing an oocyte (Ramón *et al.*, 2013; Yániz *et al.*, 2015). Based on this theory and the fact that the sperm binding capacity differed significantly between Dormer and Merino sperm samples, it might be speculated that this difference may be due to the fact that Merino's had a low positive correlation of sperm binding capacity with SP1 (long and elongated sperm), whereas for the Dormer rams, this was not observed.

From the sperm binding assay results it was evident that semen samples obtained from HL rams tended ($p=0.06$) to have a higher binding capacity compared to LL rams. These results are consistent with those reported by García-Álvarez *et al.* (2009), who found that the *in vitro* fertility of high reproduction potential rams was significantly higher compared to that of low reproduction potential rams. However, the observed tendency in binding capacity might be explained by the significant difference observed in the post-thaw sperm viability between HL and LL semen samples, LL rams having a significantly lower percentage live sperm compared to HL rams (25% vs 31%, respectively; Table 6.1). Sperm need to be viable to bind to and fertilize an oocyte (Brito *et al.*, 2003; Rodriguez-Martinez, 2003). It may be hypothesised, that as the rams from the two genetically diverse lines were selected for reproduction potential (expressed as ewe multiple rearing ability), a possible difference was expected in terms of in *in vitro* fertility (binding capacity) of semen samples obtained from the two lines (Boshoff, 2014). The tendency observed in the sperm binding capacity of the semen samples obtained from HL and LL rams, might also further support the findings of Boshoff's (2014) study, who found that the area of the sperm covered with the acrosomal cap was significantly larger in HL sperm, when compared to LL sperm. The acrosome contain enzymes, once released during the acrosome reaction, that are essential for the lysis of the zona pellucida and the penetration of the *corona radiata* of the oocyte (Pesch & Bergmann, 2006). These enzymes are responsible for penetrating the *zona pellucida* and thus facilitate fertilization. With the HL sperm having a larger percentage acrosome coverage, it can be postulated that this might be an indicator that semen samples from HL rams might have a higher fertilizing ability compared to the LL rams (Boshoff, 2014). Although this parameter was not determined in the present study, future studies need to investigate this.

The sperm binding capacity of frozen-thawed sperm samples obtained from HL an LL rams was not influenced by sperm morphometric subpopulations traits of the fresh ejaculated sperm samples. No significant correlations were found between the mean number of sperm bound and sperm morphometric subpopulations in either genetic line ($p>0.05$). Although there were no significant correlations between subpopulations and sperm binding capacity, SP1 (positive) and SP4 (negative) were low correlated with sperm binding capacity in HL samples. SP2 and SP3 were negligibly correlated to the mean number of bound sperm. The mean value for sperm binding capacity for LL rams were lowly correlated with SP2 and SP1 (positive) and SP4 (negative), respectively. A negligible correlation of the mean number of bound sperm with SP3 was observed (Table 6.2). In addition, no significant correlation was found for sperm binding capacity and sperm morphometric subpopulations for individual rams (Table 6.3).

When the sperm binding capacity results were compared to the fresh sperm morphometric subpopulations traits, no significant correlations were observed. However, little information is available on studies correlating sperm morphometric subpopulations and fertilizing ability in rams. A few studies have suggested that there might be a positive correlation between fertility (*in vitro* and *in vivo*) and specific sperm subpopulations (Ramón *et al.*, 2013; Ferraz *et al.*, 2014; Yániz *et al.*, 2015). However there are also studies where no clear correlations were found (Quintero-Moreno *et al.*, 2007; Santolaria *et al.*, 2015). In agreement with the results of the current study, Santolaria *et al.* (2015) reported that the distribution of sperm in different morphometric subpopulations was not related to ram fertility. In contrast, in a recent study on bulls, a positive and significant correlation was found between the proportion of sperm in highly motile and progressive sperm subpopulations and the ability of frozen-thawed semen samples to bind *in vitro* to the *zona pellucida* of bovine oocytes (Ferraz *et al.*, 2014). Ramón *et al.* (2013) and Yániz *et al.* (2015) stated that the proportion of sperm with large and elongated heads are the most common indicators of fertility. Based on this theory, it was evident from the results that the binding capacity of both the HL and LL semen samples were lowly correlated ($r=0.3$) to subpopulation 1 (SP1), which were the proportion of sperm with large and long heads, although the correlation was not significant. Although the sperm binding capacity of the HL samples tended to be higher than that of the LL samples, the fact that both lines have a positively low correlation between SP1 and sperm binding capacity, although not significant, support the findings of Santolaria *et al.* (2015), that ferilizing ability of a sample is not only related to a specific morphometric subpopulation.

As mentioned previously, the sperm binding capacity of the frozen-thawed samples was related to the fresh sperm morphometric subpopulations traits. The presence of fat globules of the egg yolk and glycerol used in the cryopreservation medium, influenced the RapiDiff® stain, making the measurement of morphometric parameters of frozen-thawed samples difficult. It is conceded

that the cryopreservation and thawing procedures can modify the structure and distribution of sperm within subpopulations (Muiño *et al.*, 2008). Future studies with an alternative cryodiluent should investigate this.

It is also known that the cryopreservation process has detrimental effects on sperm morphometric parameters (Ollero *et al*., 1998; O'Connell *et al.,* 2002; Ahmad *et al.,* 2014). In contrast, Flores *et al*. (2008) reported that the freezing and thawing process resulted in changes to the subpopulations of boar and donkey semen but it did not affect the general motile-sperm structure present in the ejaculates. Nevertheless, subpopulation traits based on frozen-thawed sperm samples would have been more representative, possibly resulting in more significant correlations.

6.4 Conclusions

This study indicated that cryopreservation and thawing markedly impaired sperm, with no differences reported between the Dormer and Merino breeds for any of the sperm quality parameters. Sperm obtained from Merino rams had a higher binding capacity when compared to sperm obtained from Dormer rams. Sperm from the HL rams also tended to have a higher binding capacity than that of the LL rams. However, no significant correlations were found between any sperm morphometric subpopulation and sperm binding capacity within any of the breeds or genetic lines.

Sperm binding assays might be suitable procedure to assess fertilizing ability of semen samples. However, sperm binding assay results are strictly speaking only applicable to that specific sample analysed (Larsson & Rodriguez-Martinez, 2000). Further research should thus focus on more replicates to predict fertility more accurately. Another field that warrants more attention is the optimization of a cryopreservation protocol for ovine sperm. As mentioned previously, the sperm binding capacity of the semen samples were compared to fresh sperm subpopulations traits, due to the quality of the post-thaw RapiDiff® stains that made it difficult to measure morphometric parameters. Therefore it is recommended that, for future reference, if the RapiDiff® staining technique is used, a different cryopreservation medium to the one used in this study is used.

Further research should focus on sperm morphometric subpopulations of post-thaw sperm samples and their correlation with fertilizing ability of sperm obtained from Dormer and Merino rams. Artificial insemination with sperm obtained from the two genetically diverse lines and comparing the fertility results with sperm subpopulation structure could possibly elucidate the differences observed between the lines. Further studies should also focus on the relationship

between sperm competition and the reproductive performance of the genetically diverse lines (HL and LL).

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Chapter 7

General conclusions and recommendations

With an expected drastic increase in the human population size, the demand for animal products will increase rapidly, placing sheep producers under major pressure to farm more cost-efficiently. The application of assisted reproduction techniques (ART's) such as artificial insemination (AI) and multiple ovulation and embryo transfer (MOET) combined with genetic selection, will potentially enable sheep farmers to increase and optimise their production practice to produce lamb and mutton more cost-efficiently.

One of the most important determinants of the success rate of ART's is the quality of semen samples used. Therefore it is important to understand all aspects concerning the collection of sperm samples, the factors that can influence sperm quality, and to what extent processing, heterogeneity of sperm subpopulations in a given semen sample, and the genotype of the animal can affect sperm quality and eventually fertilizing ability. Against the abovementioned, the influence of breed and genetic selection for prolificacy was studied to determine whether a specific genotype will be more suitable to produce quality semen samples for use in ART's. The overall investigation thus included rams from two Merino lines divergently selected for reproduction, namely a High line (HL) selected in the upwards direction and a Low line (LL) selected against reproduction. The study also included Dormer rams, for the Dormer breed is a preferred choice to be used in commercial terminal crossbreeding systems and limited research has been published on the reproduction traits of this breed.

The overall study investigated important parameters associated with semen collection and evaluation. It is commonly known that semen collected by the artificial vagina method (AV) are of a much higher quality when compared to samples obtained by means of electro-ejaculation (EE). The former method however, requires the training of rams. Currently there is no formal training protocol or standard operating procedure (SOP) available in South Africa on how to train rams to ejaculate into an AV. Therefore a behavioural study was conducted to establish a SOP for the training of rams to ejaculate into an AV. The ability of inexperienced (yearling) and experienced rams (with prior sexual experience) of both breeds to be trained were investigated, and in the training sessions both yearling and mature ewes of both breeds were used. Furthermore, with the presence of sperm subpopulations within a mammalian ejaculate widely accepted, this study also investigated the potential influence of breed and genetic selection for prolificacy on the degree of heterogeneity of ejaculates. Lastly, it was suggested that sperm fertilizing ability are correlated with the structure and distribution of sperm subpopulations within

an ejaculate. It was thus also determined to what extent sperm morphometric subpopulations traits determined the fertilizing competence of sheep sperm.

Phase 1: Behavioural study to determine a ram's ability to be trained to use an artificial vagina

The behavioural component of the overall study investigated whether a ram has a preference for an ewe of its own breed, whether Dormer and Merino rams differed in their ability to be habituated to the presence of the semen collector and/or assisting staff, and whether the ability of rams to be habituated determined the time required to train them to use an AV. Inexperienced and experienced Dormer rams indicated that they have a definite preference for mature Dormer ewes. In the training sessions the Dormer rams did not discriminate between Dormer and Merino ewes used as teasers during semen collection with an AV. On the contrary, in the behavioural study Merino rams were less discriminatory in their choice for either mature or yearling Merino ewes, but exhibited a definite preference for a Merino ewe during training for AV usage.

Furthermore, it was found that breed and degree of sexual experience did not influence ease of habituation of a ram to the presence of the semen collector and/or assisting staff. Rams could be habituated within approximately 4 weeks when trained by experienced personnel, and using a minimum of eight training sessions during this period. However, it must be noted that a high frequency of training, i.e. 18 training sessions during this 4-week period, resulted in more pronounced and desirable behaviour repertoires. In addition, only 50% of the experienced Dormer rams could be successfully trained to use the AV, compared to 90% of the experienced Merino rams. Of the inexperienced rams only 40% of both the Merino and Dormer breeds could be trained to use the AV.

Recommendations: It is recommended that a mature ewe of the same breed as the ram is used when training rams to ejaculate into an AV. When training experienced Merino rams to ejaculate into an AV, this can be done in as short a timeframe as 2 weeks, while extended training periods may be required for experienced Dormer and inexperienced Dormer and Merino rams. Further research on the factors that influence the time required to train experienced Dormer rams as well as inexperienced rams of both breeds, is suggested. Research to qualify and quantify factors that determine and contribute to a ewe's sexual attractiveness to rams of the same breed as well as other breeds, is also warranted to eventually formulate as comprehensive as possible a SOP for the training of rams for semen collection purposes.

Phase 2: Sperm subpopulation characterization of sheep semen samples

The aim of the second part of the overall study was to characterise the subpopulation structure of semen samples collected from Dormer and Merino rams. An additional aspect that was

included was the influence of genetic selection for prolificacy in the Merino breed on sperm subpopulation traits. It is crucial to understand how selection affects morphometric traits of sperm produced by the HL and LL, which in turn may influence the fertilizing ability of the sperm. Neither breed nor genetic line had an influence on sperm quality parameters or sperm morphometric subpopulations structure and distribution in semen samples collected by means of EE from Dormer and Merino (HL and LL) rams.

Recommendations: Further research should be conducted with more animals under different environmental conditions, as only 6 rams represented each genotype in this study. Further research on the possible impact of sperm competition on reproductive performance in the genetically diverse HL and LL rams is also proposed. Another potential area of research is the contribution of the ewe to ensuring fertilisation success and thus to the overall reproduction efficiency of a breeding flock.

Phase 3: Correlation between subpopulation characteristics and sperm binding capacity

The third part of the overall study determined the correlation between sperm subpopulation traits and the ability of sperm to bind successfully, as assessed by means of a perivitelline membrane binding assay. As expected, cryopreservation markedly impaired post-thaw sperm function and binding capacity of sperm to a chicken perivitelline membrane. The ability of sperm to offer resistance to cryodamage was independent of breed and/or genetic line. When the influence of breed and genetic selection on post-thaw sperm parameters was determined, no significant influence of breed or selection was observed for most of the sperm parameters assessed. The exception was post-thaw sperm viability, where it was observed that Merino HL sperm offered more resistance to the damage caused by cryopreservation than Merino LL.

Breed had a significant influence on sperm binding capacity, with Merino sperm exhibiting a higher binding capacity compared to Dormer sperm. Sperm obtained from the HL rams tended to have a higher binding capacity than sperm obtained from the LL rams, with the observed difference approaching conventional significance.

Previous studies have stated that there is a correlation between specific sperm subpopulations and the fertilizing ability of sperm. However, no conclusive correlations were reported between any of the four sperm morphometric subpopulations and the sperm binding capacity as assessed within a breed or between the two genetic lines.

Recommendations: Future research should aim to optimize the cryopreservation protocol for ovine sperm. The sperm binding capacity of the semen samples were compared to the subpopulations traits of the fresh samples only. The fat globules of the egg yolk and the glycerol

component of the cryodiluent complicated the assessment of the post-thaw sperm morphometric traits and thus also the sperm subpopulation traits, and it is therefore recommended that in future studies where the RapiDiff® staining technique is used, a different cryoprotectant is included in the cryodiluent to ensure that it will be possible to assess the morphometric parameters of sheep sperm. In addition, further research should be conducted on the influence of cryopreservation on the potential redistribution of sperm into the respective subpopulations identified in the study, and the correlation of this potential phenomenon on the fertilizing ability of sperm obtained from Dormer and Merino rams. The role of sperm competition in the fertilization success of sperm obtained from HL and LL rams, as determined by the AI of HL and LL ewes and using fresh and post-thaw sperm, also warrants further investigation. When the reproduction performance of the HL and LL rams is considered, with the exception of the 2012 mating season, the two lines differed in terms of number of lambs sired. By elucidating whether sperm compete differently in the environment of the female reproductive tract, given that no differences were reported with standard semen evaluation protocols, may assist in understanding how selection can potentially affected sperm's competitive ability.

To conclude, the abovementioned recommended studies will potentially contribute to better our understanding of the driving forces behind the fertilization success of sperm obtained from a particular ram, and to what extent this can contribute to the improvement and optimization of ovine sperm processing and cryopreservation protocols. A species-specific and optimized sperm protocols for use in ART's will assist sheep farmers to optimise the viability and sustainability of their production systems.

Appendix A

(Includes information of Chapter 3)

A.1 Slide preparation for the analysis of sperm morphology, acrosome integrity and morphometric parameters

Throughout the present study, the RapiDiff® staining technique was used and carried out according to the protocol provided by the manufacturer.

Raw semen samples were diluted (1:3) by mixing 50µL raw sperm and 150µL Ham's F10 solution (37ºC). The smears were prepared by placing 10µL of sperm (diluted or thawed semen samples) on a pre-warmed (37ºC) microscope slide (Lasec, South Africa). The drop of semen was spread out evenly over the slide, by using the edge of another slide to drag the drop of semen along the surface of the slide (Figure A.1) (Mcalister, 2010). The smears were allowed to air dry for at least 3 hours before being stained (Banaszewska *et al.*, 2015).

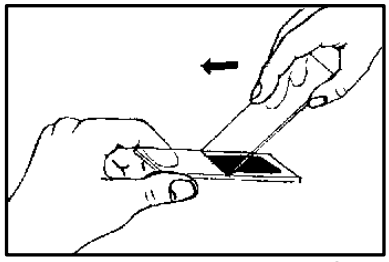

Figure A1 Demonstration of a sperm smear preparation on a microscope slide (FAO, 1994).

A.2 Staining procedure for RapiDiff® stain

The RapiDiff® Differential staining kit is distributed by Lasec, South Africa and consists of three solutions: RapiDiff fix (fixative solution), RapiDiff 1 (stain solution 1) and RapiDiff 2 (stain solution 2). Prior to staining the three solutions in the RapiDiff® staining kit were transferred into three separate Coplin staining jars. Disposable gloves were worn at all times when using the staining solutions. The RapiDiff® staining procedure involved three major steps as described as follows:

1. The dry semen smear was dipped vertically into the fixative for 5 times and allowed to drain the excess and rinsed with distilled water.
2. The procedure was repeated with RapiDiff® 1 and rinsed with distilled water.
3. The procedure was repeated with RapiDiff® 2 and rinsed with distilled water.

All the slides were placed horizontally down onto filter paper (sample side upwards) and allowed to air dry, after the above mentioned steps, and analysed for the different parameters.

A.3 Cryopreservation medium preparation

The semen cryopreservation medium that was used during this study was prepared according to Paulenz *et al.* (2007) and consisted out of two fractions, a cooling medium and a freezing medium. Both of these fractions were prepared prior to the start of the semen collection phase and frozen in aliquots of 2mL in Eppendorf tubes (Lasec, South Africa) until needed.

Prior to the day of use (12 hours) the specific number of tubes of both fractions, necessary for the next day, were removed from the freezer and placed in a refrigerator (5 ºC) to be thawed and used as described in section 3.3.3. The cooling medium was placed in a water bath (37 ºC) prior to use for at least 2 hours.

A.3.1 Cooling media

The cooling medium was prepared by mixing 80 mL of UHT skim milk, at room temperature, with 5 mL egg yolk (freshly laid eggs were used), 1mg/mL penicillin and was filled up to a final volume of 100 mL using distilled water. The solution was gently mixed until homogenous.

A.3.2 Freezing media

The freezing media was created by adding glycerol to an aliquot of the cooling media, so that the final glycerol concentration was 14% by volume. This solution was gently mixed until homogenous.

A.4 TALP composition and preparation (Sperm Binding Assay)

All the chemicals, unless otherwise stated, were purchased from Sigma-Aldrich, South Africa.

The TALP medium was prepared by adding 80 mL of distilled water in a beaker and adding components 1-8 (Table A.1) in order while stirring. The water was filled up to a final volume of 100 mL. This prepared medium was filtered and stored in a refrigerator (5 ºC) for up to two weeks. On the day of use components 9 and 10 were added and the resultant mixture was filtered with 0.2 µm filters (only 5 mL was made on a day to be used that same day).

Component 10 (Na-Pyruvate) was prepared each time on the day of use, by dissolving 22 mg in 10 mL of Saline. After adding component 9 and 10, the pH was measured and adjusted to 7.4 at room temperature, if required

Table A1 Composition and components of TALP (addapted from Sirard & Coenen, 2006).

Component	mM	amount/100 mL
1. $CaCl_2.2H_2O$	2	29.4 mg
2. KCl	3.2	23.9 mg
3. $MgCl_2.6H_2O$	0.5	10 mg
4. $NaH_2PO_4.H_2O$	0.4	5.6 mg
5. Lactic Acid	10	186 µL
6. NaCl	114	666 mg
7. $NaHCO_3$	25	210 mg
8. HEPES	10	238 mg
9. PVA	2	100 mg
10. Na-Pyruvate	0.2	1 mL

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Appendix B

(Includes information of Chapter 5 and 6)

Table B1 The mass motility scoring of fresh and frozen-thawed semen samples collected from Dormer and Merino rams for three collected samples via electro-ejaculation (EE).

		Fresh				Frozen-thawed			
Breed	**Ram ID**	**Collected samples**				**Collected samples**			
		1	**2**	**3**	**Average**	**1**	**2**	**3**	**Average**
Dormer	1.051	3	3	3	**3.0**	1	1	1	**1.0**
	3.044	4	3	4	**3.7**	1	1	1	**1.0**
	3.165	3	4	4	**3.7**	1	1	1	**1.0**
	4.055	4	4	4	**4.0**	1	2	1	**1.3**
	4.061	4	4	4	**4.0**	1	1	1	**1.0**
	4.097	4	3	4	**3.7**	1	1	1	**1.0**
Merino High Line	3.014	5	5	4	**4.7**	2	2	2	**2.0**
	3.018	4	4	4	**4.0**	2	1	2	**1.7**
	3.145	4	4	4	**4.0**	1	1	2	**1.3**
	3.208	4	4	4	**4.0**	1	1	1	**1.0**
	4.006	3	3	3	**3.0**	1	1	1	**1.0**
	4.140	4	4	4	**4.0**	1	1	1	**1.0**
Merino Low Line	1.177	4	4	3	**3.7**	1	2	1	**1.3**
	3.092	4	4	4	**4.0**	1	1	1	**1.0**
	3.150	3	3	4	**3.3**	2	1	2	**1.7**
	4.034	3	4	4	**3.7**	1	1	1	**1.0**
	4.040	4	4	3	**3.7**	1	1	1	**1.0**
	4.051	4	4	4	**4.0**	1	2	1	**1.3**

Table B2 The sperm concentration of semen samples (x10^8/mL) collected from Dormer and Merino rams for three collected samples each, via electro-ejaculation (EE).

Breed	Ram ID	Collected samples			
		1	2	3	Average
Dormer	1.051	18.9	15.90	10.9	**15.23**
	3.044	18.2	12.30	14.7	**15.07**
	3.165	11.9	14.10	11.2	**12.40**
	4.055	5.7	12.50	20.9	**13.03**
	4.061	7.7	12.60	18.1	**12.80**
	4.097	10.9	9.17	11.9	**10.66**
Merino High line	3.014	13.9	18.60	20	**17.50**
	3.018	25.9	25.40	27.3	**26.20**
	3.145	15.1	17.00	20.6	**17.57**
	3.208	20.8	19.30	16.2	**18.77**
	4.006	13.6	19.80	14.4	**15.93**
	4.140	32.4	32.70	19.8	**28.30**
Merino Low line	1.177	15.6	20.10	22.8	**19.50**
	3.092	10.4	13.70	11.6	**11.90**
	3.150	20.1	14.60	19	**17.90**
	4.034	21.7	19.70	15.4	**18.93**
	4.040	23.4	19.50	17	**19.97**
	4.051	23.6	21.10	16.8	**20.50**

Table B3 The volume of semen samples (mL) collected from Dormer and Merino rams for three collected samples each, via electro-ejaculation (EE).

Breed	Ram ID	Collected samples			
		1	2	3	Average
Dormer	1.051	0.5	1.25	1.75	**1.17**
	3.044	1	1.25	1	**1.08**
	3.165	1.5	1.25	1.75	**1.50**
	4.055	2	2	2	**2.00**
	4.061	1	1.25	2	**1.42**
	4.097	1.75	1.5	1.75	**1.67**
Merino High line	3.014	1.25	1.5	1.5	**1.42**
	3.018	1.5	1.5	1.25	**1.42**
	3.145	0.5	1.25	1.5	**1.08**
	3.208	0.5	1.75	1.5	**1.25**
	4.006	1	1.75	1	**1.25**
	4.140	1.75	1.5	1.25	**1.50**
Merino Low line	1.177	1.25	1.5	1.5	**1.42**
	3.092	1	1	1	**1.00**
	3.150	2	1.5	1.25	**1.58**
	4.034	1.5	1.75	2	**1.75**
	4.040	2	1.75	1.5	**1.75**
	4.051	2	1.25	1.25	**1.50**

Table B4 The percentage of abnormal spermatozoa in fresh and frozen-thawed semen samples collected from Dormer and Merino rams for three collected samples via electro-ejaculation (EE).

		Fresh				Freeze-thawed			
Breed	**Ram ID**	**Collected samples**				**Collected samples**			
		1	**2**	**3**	**Average**	**1**	**2**	**3**	**Average**
Dormer	1.051	6%	6%	23%	**11%**	12%	15%	13%	**13%**
	3.044	15%	4%	6%	**8%**	13%	13%	5%	**10%**
	3.165	13%	11%	6%	**10%**	28%	17%	34%	**26%**
	4.055	46%	20%	8%	**25%**	35%	25%	21%	**27%**
	4.061	3%	10%	19%	**11%**	25%	16%	25%	**22%**
	4.097	6%	13%	15%	**11%**	13%	12%	14%	**13%**
Merino High Line	3.014	16%	14%	26%	**19%**	21%	15%	29%	**22%**
	3.018	5%	3%	1%	**3%**	4%	10%	8%	**7%**
	3.145	4%	3%	9%	**5%**	22%	21%	7%	**17%**
	3.208	3%	6%	7%	**5%**	10%	6%	9%	**8%**
	4.006	2%	6%	7%	**5%**	2%	4%	23%	**10%**
	4.140	4%	14%	20%	**13%**	17%	15%	28%	**20%**
Merino Low Line	1.177	7%	31%	36%	**24%**	39%	24%	22%	**28%**
	3.092	4%	5%	20%	**10%**	8%	17%	13%	**12%**
	3.150	6%	3%	5%	**5%**	10%	6%	4%	**7%**
	4.034	9%	8%	15%	**11%**	9%	12%	18%	**13%**
	4.040	6%	13%	4%	**8%**	19%	25%	18%	**21%**
	4.051	7%	7%	35%	**16%**	14%	23%	20%	**19%**

Table B5 The percentage of spermatozoa with intact acrosomes in fresh and frozen-thawed semen samples collected from Dormer and Merino rams for three collected samples via electro-ejaculation (EE).

		Fresh				Freeze-thawed			
Breed	**Ram ID**	**Collected samples**				**Collected samples**			
		1	**2**	**3**	**Average**	**1**	**2**	**3**	**Average**
Dormer	1.051	77%	68%	65%	**70%**	67%	65%	66%	**66%**
	3.044	79%	73%	75%	**76%**	59%	69%	70%	**66%**
	3.165	78%	67%	72%	**72%**	63%	60%	64%	**62%**
	4.055	75%	72%	83%	**77%**	65%	63%	69%	**66%**
	4.061	71%	61%	87%	**73%**	58%	62%	67%	**62%**
	4.097	89%	79%	90%	**86%**	77%	71%	63%	**70%**
Merino High Line	3.014	77%	74%	74%	**75%**	64%	63%	69%	**66%**
	3.018	80%	82%	70%	**77%**	60%	73%	68%	**67%**
	3.145	80%	80%	81%	**80%**	72%	67%	70%	**70%**
	3.208	86%	82%	73%	**80%**	72%	70%	68%	**70%**
	4.006	79%	74%	73%	**75%**	74%	65%	67%	**69%**
	4.140	78%	83%	93%	**85%**	72%	64%	70%	**69%**
Merino Low Line	1.177	81%	63%	65%	**70%**	60%	75%	64%	**66%**
	3.092	87%	70%	70%	**76%**	68%	63%	73%	**68%**
	3.150	80%	62%	67%	**70%**	61%	58%	65%	**61%**
	4.034	85%	79%	75%	**80%**	70%	69%	72%	**70%**
	4.040	71%	75%	76%	**74%**	64%	65%	62%	**64%**
	4.051	86%	71%	78%	**78%**	68%	65%	75%	**69%**

Table B6 The percentage of live spermatozoa in frozen-thawed semen samples collected from Dormer and Merino rams for three samples collected by electro-ejaculation (EE).

Breed	Ram ID	Collected samples			
		1	2	3	Average
Dormer	1.051	24%	22%	18%	**21%**
	3.044	30%	21%	17%	**23%**
	3.165	28%	23%	27%	**26%**
	4.055	43%	36%	28%	**36%**
	4.061	23%	24%	17%	**21%**
	4.097	21%	19%	22%	**21%**
Merino High line	3.014	35%	22%	33%	**30%**
	3.018	40%	31%	22%	**31%**
	3.145	70%	35%	26%	**44%**
	3.208	44%	38%	28%	**37%**
	4.006	31%	32%	24%	**29%**
	4.140	26%	38%	24%	**29%**
Merino Low line	1.177	26%	76%	21%	**41%**
	3.092	30%	26%	25%	**27%**
	3.150	6%	32%	25%	**21%**
	4.034	36%	24%	21%	**27%**
	4.040	31%	19%	13%	**21%**
	4.051	27%	71%	26%	**41%**

Table B7 The average sperm morphometric measurements of fresh sperm samples obtained from Dormer and Merino rams.

Breed	Ram ID	Sperm morphometric parameter						
		Area (μm^2)	Perimeter (μm)	Width (μm)	Length (μm)	Ellipticity	Elongation	Roundness
Dormer	1.051	42.519	30.620	5.659	9.846	1.749	0.270	1.754
	3.044	43.415	31.066	5.940	9.662	1.635	0.239	1.768
	3.165	44.888	29.702	5.915	9.748	1.656	0.245	1.563
	4.055	44.953	30.702	6.018	9.923	1.657	0.245	1.668
	4.061	44.781	29.698	5.962	9.709	1.638	0.239	1.567
	4.097	42.567	29.991	5.899	9.685	1.652	0.243	1.681
Merino High Line	3.014	43.817	31.583	5.876	9.710	1.659	0.246	1.811
	3.018	42.435	29.871	5.800	9.642	1.671	0.249	1.673
	3.145	44.673	31.029	5.882	9.944	1.699	0.256	1.714
	3.208	44.061	31.064	5.776	9.769	1.707	0.257	1.742
	4.006	43.725	30.707	5.914	9.783	1.663	0.246	1.715
	4.140	42.646	30.068	5.902	9.472	1.615	0.233	1.686
Merino Low line	1.177	41.246	30.443	5.747	9.388	1.644	0.241	1.787
	3.092	43.424	31.439	5.928	9.791	1.659	0.246	1.811
	3.150	44.888	29.702	5.915	9.748	1.656	0.245	1.563
	4.034	41.617	29.893	5.912	9.436	1.608	0.230	1.708
	4.040	45.191	30.767	6.003	9.836	1.646	0.242	1.666
	4.051	44.483	31.723	6.053	9.764	1.621	0.235	1.800

Table B8. Frequency of distribution (percentage) of spermatozoa falling in each subpopulation derived from the morphometric analysis within each ram**.**

Breed	Ram ID	Subpopulation 1	Subpopulation 2	Subpopulation 3	Subpopulation 4
Dormer	1.051	19.28	52.41	18.07	10.24
	3.044	25.16	24.53	23.9	26.42
	3.165	18.71	29.5	31.65	20.14
	4.055	19.86	35.62	18.49	26.03
	4.061	15.93	24.18	27.47	32.42
	4.097	15.03	28.76	35.29	20.92
Merino High line	3.014	23.33	30.83	30	15.83
	3.018	12.43	36.22	23.24	28.11
	3.145	18.58	45.36	13.66	22.4
	3.208	15.43	38.29	19.43	26.86
	4.006	16.59	27.8	29.27	26.34
	4.140	13.57	23.57	28.57	34.29
Merino Low line	1.177	17.39	26.63	33.15	22.83
	3.092	23.4	23.4	28.37	24.82
	3.150	14.77	26.7	35.23	23.3
	4.034	4.82	24.7	35.54	34.94
	4.040	15.46	26.09	27.54	30.92
	4.051	21.83	26.06	16.9	35.21

Table B9 The mean number of frozen-thawed sperm obtained from Dormer and Merino rams that bound to a hen's egg perivitelline membrane.

Breed	Ram ID	Collected samples			
		1	2	3	Average
Dormer	1.051	12.00	14.00	12.00	**13**
	3.044	17.50	16.50	18.00	**17**
	3.165	23.50	27.50	22.50	**25**
	4.055	20.00	33.00	26.50	**27**
	4.061	26.00	26.00	23.00	**25**
	4.097	25.50	23.00	28.50	**26**
Merino High line	3.014	37.50	43.00	32.50	**38**
	3.018	32.50	29.00	32.50	**31**
	3.145	21.50	25.00	34.00	**27**
	3.208	23.50	29.00	25.00	**26**
	4.006	19.00	18.50	21.00	**20**
	4.140	23.50	27.00	25.50	**25**
Merino Low line	1.177	19.50	33.50	23.50	**26**
	3.092	18.00	26.00	22.50	**22**
	3.150	30.50	23.00	31.00	**28**
	4.034	16.00	25.00	24.00	**22**
	4.040	16.50	24.50	18.00	**20**
	4.051	21.00	29.50	27.00	**26**