The influence of heat stress on the functionality of ovine spermatozoa

by

Wian Stapelberg

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Supervisor: Dr H. Lambrechts

Co-supervisor: Prof S.W.P. Cloete

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Declaration

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Summary

With the current trend in the global human population growth, the demand for animal production is predicted to increase significantly in the coming years. The increased demand for animal products will coincide with the period in which the world will experience changes in the global climate that will ultimately affect agricultural production. When considering the use of assisted reproductive techniques in the sheep industry, given the expected increase in average global temperatures, it is important to identify rams that produce spermatozoa that are resilient to temperature changes associated with the collection, processing, and preservation. This study aimed to determine the influence of scrotal insulation on sperm sample quality, and the resumption of normal spermatogenic activity in the testes of adult Dohne Merino rams, to potentially classify rams in terms of resilience to heat stress and its related effect on physiological processes such as spermatogenesis. Twelve rams formed part of the scrotal insulation phase, of which 10 rams were subjected to scrotal insulation. Two rams were not subjected to scrotal insulation and represented a Control group. The scrotal insulation devices were fitted after the first semen sampling, and thermographic measurements (Week 0), and remained fitted for 7 consecutive days. Semen samples and thermographic measurements were recorded after removal of the scrotal insulation devices, and thereafter every fortnight at Week 3, 5, 7, 9 and 11. The fitting of scrotal insulation devices in the present study proved to be effective in insulating the testes, as evident in the influence thereof on spermatogenic activity. There were no significant differences between the HR, LR, and Control groups for the percentage of abnormal and live spermatozoa over time during liquid storage (0-48h). There was a difference in the ability of spermatozoa from the rams to offer resilience to heat stress when the entire sampling period of 11 weeks was considered. Sperm morphology took 70 days to achieve pre-insulation values (below 40% for most rams), indicating that early spermatogenesis was most affected by heat stress. Considering the change in ambient temperature at Week 0 and Week 11 of the study as well as the different times of measuring scrotal temperatures during those weeks, scrotal temperatures took approximately 42 days to recover ($18.64°C - 24.84°C$), which coincides with the time it takes for spermatozoa to be produced from spermatogonia. By assessment of the change in iButton and scrotal temperatures, it was found that there were individual differences between rams, which indicates that certain rams are more efficient in thermoregulating testicular temperature than others. The study also aimed to determine whether a sperm hyperactivation functional test is effective in discriminating between Dohne Merino rams in terms of the resilience of their spermatozoa to heat stress. Semen samples were subjected to incubation at $38.5°C$ for 30 minutes after the addition of 10mM procaine hydrochloride. Samples were evaluated at 0min, 15min, and 30min of incubation, and sperm viability, sperm morphology, and sperm acrosome integrity were quantified. There was no significant

difference between the HR and LR groups for percentage live and abnormal acrosomes at all time intervals. There were significant differences between the HR and LR group for percentage abnormal spermatozoa at all time intervals. Significant individual differences were observed between the rams for sperm viability and morphology, but not for acrosome integrity when considering the entire 30 minutes of incubation. There was a faster rate in the reduction of quality for the LR group for abnormal spermatozoa compared to the HR group. The subpopulation structure of ejaculates should be considered when using induced hyperactivation as a sperm functional test, especially when ejaculation frequency is high. Simulations of the *in-vivo* environment of the female reproductive tract in an *in-vitro* environment may assist in more precise determination of fertilizing ability from heat stressed spermatozoa.

Opsomming

Met die huidige tendens in die globale menslike bevolkingsgroei, word voorspel dat die vraag na diereproduksie in die komende jare aansienlik sal toeneem. Die verhoogde vraag na diereprodukte sal ervaar word in die tydperk waarin die wêreld veranderinge in die globale klimaat sal ervaar, wat uiteindelik landbouproduksie sal beïnvloed. Wanneer die gebruik van ondersteunde reproduksietegnieke (ORTe) in die skaapbedryf oorweeg word, gegewe die verwagte toename in gemiddelde globale temperatuur, is dit belangrik om ramme te identifiseer wat sperme produseer wat weerstandbiedend is teen die veranderinge wat met die versameling, verwerking en preservering veroorsaak word. Hierdie studie het ten doel gehad om die invloed van skrotale isolasie op spermmonster kwaliteit, en die hervatting van normale spermatogeniese aktiwiteit in die testes van volwasse Dohne Merino ramme te bepaal, in 'n poging om potensieël ramme te klassifiseer vir veerkragtigheid teen hittestres, en die verwante effek daarvan op fisiologiese prosesse soos spermatogenese. Twaalf ramme het deel gevorm van die skrotale-isolasie-fase, waarvan 10 ramme aan skrotale-isolasie onderworpe was. Twee ramme was nie aan skrotale-isolasie onderworpe nie en het 'n Kontrole groep verteenwoordig. Die skrotale isolasie sakke was op die skrotums geplaas na die eerste semenmonster en termografiese metings (Week 0) geneem was. Die -sakke het in posisie gebly vir 7 opeenvolgende dae. Semenmonsters is versamel en termografiese metings is geneem na verwydering van die skrotale isolasie sakke, en was daarna elke twee weke geneem op Week 3, 5, 7, 9 en 11. Die passing van skrotale isolasie sakke in die huidige studie was doeltreffend om die testes te isoleer, soos blyk uit die invloed daarvan op spermatogeniese aktiwiteit. Daar was geen beduidende verskille tussen die HR-, LR- en Kontrolegroepe vir die persentasie abnormale en lewende sperme oor tyd, vir monsters gestoor by 4°C (0-48h) nie. Daar was 'n verskil in die vermoë van sperme van die ramme om weerstand teen hittestres te bied, wanneer die hele monsternemingsperiode van 11 weke in ag geneem was. Sperm morfologie het 70 dae geneem om waardes soortgelyk aan waaardes voor skrotale isolasie te bereik (onder 40% vir die meeste ramme), wat aandui dat vroeë spermatogenese die meeste deur hittestres beïnvloed was. Met inagneming van die verandering in omgewingstemperatuur by Week 0 en Week 11 van die studie, asook die verskillende tye van meting van skrotumtemperature gedurende daardie weke, het skrotumtemperature ongeveer 42 dae geneem om te herstel (18.64˚C – 24.84˚C), wat ooreenstem met die tyd wat dit neem vir sperme om geproduseer te word vanaf spermatogonia. Die veranderinge aangeteken in iButton en skrotale temperature tydens die isolasietydperk, het aangetoon dat daar individuele verskille tussen ramme was, wat daarop dui dat sekere ramme meer doeltreffend is in termoregulering van testikulêre temperatuur as ander. Die studie het ook ten doel gehad om vas te stel of 'n sperm hiperaktivering funksionele toets effektief was om tussen Dohne Merino ramme te onderskei in die veerkragtigheid

van hul sperme teen hittestres. Semenmonsters was onderworpe aan inkubasie by 38.5˚C vir 30 minute na die byvoeging van 10mM prokeinhidrochloried. Monsters is geëvalueer op 0min, 15min en 30min van inkubasie, en lewensvatbaarheid, morfologie en akrosoomintegriteit van sperme was gekwantifiseer. Daar was geen betekenisvolle verskil tussen die HR- en LR-groepe vir onderskeidelik persentasie lewende en abnormale akrosome op alle tydsintervalle nie. Daar was beduidende verskille tussen die HR- en LR-groep vir persentasie abnormale sperme op alle tydsintervalle. Beduidende individuele verskille was waargeneem tussen die ramme vir lewensvatbaarheid en morfologie, maar nie vir akrosoom integriteit van sperme wanneer die hele 30 minute van inkubasie in ag geneem word nie. Daar was 'n vinniger tempo in die vermindering van kwaliteit vir die LR-groep vir abnormale sperme in vergelyking met die HR-groep. Die subpopulasiestruktuur van ejakulate moet in ag geneem word wanneer geïnduseerde hiperaktivering as 'n sperm funksionele toets gebruik word, veral wanneer ejakulasiefrekwensie hoog is. Simulasies van die *in vivo* omgewing van die vroulike voortplantingskanaal *in vitro* kan help met meer presiese bepaling van bevrugtingsvermoë van sperme wat hittestres ervaar.

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Chapter 1

General Introduction

Global warming is responsible for increasing the global average temperature by approximately 0.8°C over the past century (Hansen *et al.*, 2006). This increase in temperature has mainly occurred in two phases, with the first being from 1910 – 1945, and the second from 1976 and onwards (Walther *et al.*, 2002). Up to 1975, global warming occurred at a slow rate, but then started to increase rapidly, resulting in a 0.6°C increase in the next three decades (Hansen *et al.*, 2006). According to the meta-analyses study of Root *et al.* (2003), long-term, large-scale alterations in animal and plant populations are observable as a result of recent increases in climatic temperatures. These alterations could be in the form of; density changes in species at specific locations, shifts in phenology, changes in morphology, and shifts in genetic frequencies (Root *et al.*, 2003). It is thus of major concern that such considerable changes are observed with only 0.6°C global warming when the Intergovernmental Panel on Climate Change (IPCC) predicts a total global temperature increase of 6°C, by 2100 (IPCC, 2001).

South Africa's entire Western and North-Western regions fall in the arid zone, with the South-Western, Southern, Central and Northern regions falling in the semi-arid zone, therefore limiting the country's potential for agriculture (Cloete and Olivier, 2010). Thus, only 20% of the agricultural land available in South Africa is suitable for non-extensive livestock farming (DAFF, 2020). The arid pastoral areas are however suitable for sustainable, extensive sheep production (Cloete and Olivier, 2010).

Sheep farming is practised throughout South Africa, with farms mainly concentrated in the arid regions of the country, with the Eastern Cape province having the largest number of sheep (30%) from the estimated 22.06 million, followed by the Northern Cape (24%), the Free State (20%) and the Western Cape (12%), respectively (DAFF, 2020). Sheep used in commercial farming practices are however most abundant in the Northern Cape (31%), followed by the Free State (23.2%), the Eastern Cape (22.4%) and then the Western Cape (11.4%) (Cloete and Olivier, 2010). Regardless of the naturally dry climate in most parts of South Africa, the drought in recent years has affected livestock farming significantly, with the 53% of agricultural land utilised by cattle, sheep and goat farmers being reduced even further, negatively affecting grazing capacity and forming pockets of livestock mortality (DAFF, 2018). Sheep farming combines well with crop farming, as drought and disease often lead to crop failure, and with widely varying grain prices, sheep farming remains the only source of income to these farmers (Cloete and Olivier, 2010).

Even though the gross production of mutton (average over the last 10 years) only contributes 3.76% to the total gross value generated from animal products (R141 321.54 million) in South Africa, sheep still

remain as the most abundant livestock species (besides poultry), regardless of the severe decrease from 30 million sheep in the 1980's, to the estimated 21.605 million sheep in 2020 (Cloete and Olivier, 2010; DAFF, 2021b). Semi-extensive meat (60.6%) and wool (31.4%) production are the two main contributors to this income, valued at R72.38/kg for 6 145 000 animals (sheep, lambs and goats) slaughtered and R4.8 billion for 48 200t wool produced, from July 2017 to June 2018 (Cloete and Olivier, 2010; DAFF, 2020). The main South African sheep breeds that contributed to the National Small Stock Improvement Scheme from 2005 – 2008 include; Merino (31.4%), Dorper (24.2%), Dohne Merino (23.9%) and SA Mutton Merino (6.1%) (Cloete and Olivier, 2010).

While sheep are the most abundant livestock species in South Africa (besides poultry), mutton imports have exceeded exports tremendously between 2010 and 2019, indicating that South Africa is a net importer of mutton (Cloete and Olivier, 2010; DAFF, 2021a). The decline in sheep numbers in the country over the last few decades together with a rapid human population growth, exceeding 100% since 1970 to 2011 (21.794 million to 51.770 million), have resulted in a severe shortage of mutton and lamb, and thus a high increase in demand (DAFF, 2020). This has caused the price of mutton and lamb to increase (also as a result of inflation) from R14.62/kg in 2001 to R72.38/kg in 2018, averaging at an increase of R4.32/kg per annum for the past 10 years (DAFF, 2020; DAFF, 2021a). Over the 10 years from 2010 to 2019, South Africa has thus imported an average of 11 000 tons of mutton, valued at R352 million (DAFF, 2021a). The decline in sheep numbers is mainly attributed to stock theft as well as losses due to predators (DAFF, 2021a). Global challenges such as increasing international competition, and consumer organisations that are continuously hammering on animal welfare issues, also negatively affect the South African small stock industry (Cloete and Olivier, 2010).

When farmers select livestock for farming in a specific environment, the effect of global warming, thus increased ambient temperatures, needs to be taken into consideration as it affects the physiological processes associated with animal production and reproduction. Heat stress disrupts the physiology and reproductive performance of animals, and is a major factor that limits the success of animal production in arid and semi-arid regions, as found in most of South Africa (Hansen, 2004). Elevated temperatures adversely affect reproduction and thus production in sheep, as it causes a decrease in body weight, growth rate, average daily gain (ADG), and total body solids (Marai *et al.*, 2007). This reduction in reproduction happens as a result of the biological functions of sheep that are negatively affected due to heat stress (Marai *et al.*, 2007). These functions include; decreased feed intake and utilization, disturbance of the metabolism of protein, energy, water and mineral balances, hormonal secretions, enzymatic reactions and blood metabolites (Marai *et al.*, 2007).

By understanding the effects of heat stress associated with animal production and reproduction, we would be able to refine current ovine-specific protocols for assisted reproductive techniques (ART's) and increase the development of such protocols. Many existing techniques have been adapted from the respective ART's developed for the cattle industry, which is a major limiting factor for sheep ART's (Boshoff, 2014). Assisted reproductive technologies in sheep also have a relatively short history of application, when compared to the cattle industry (Boshoff, 2014).

Artificial insemination (AI), and multiple ovulation and embryo transfer (MOET) are two ART's that allow small stock producers to produce higher numbers of livestock, and to do so more cost-efficiently. In sheep, AI is however still limited due to the convoluted anatomy of the ovine cervix that prevents the passage of an insemination catheter, and due to fertility being low after cervical insemination with ram spermatozoa stored in the liquid state, as well as in the frozen state (Martí *et al.*, 2003; Yániz *et al.*, 2005; Falchi *et al.*, 2018). To preserve spermatozoa, their metabolism must be reduced or arrested to ensure a prolonged fertile life (Maxwell and Salamon, 1993). For successful fertilization, several factors play a role in the fertilizing ability of spermatozoa which include motility, viability, and the ability to undergo capacitation and the acrosome reaction in the female reproductive tract (Martí *et al.*, 2003). It is thus of utmost importance that a diluent needs to maintain these factors while being cooled to sub-normal temperatures. It is evident that the cryopreservation and thawing of spermatozoa cause deleterious damage to spermatozoa, as evident in a compromise in motility, viability, and fertilizing capacity, when compared to spermatozoa in the liquid state. Storing spermatozoa in a cryopreserved state allows for a longer storage period, compared to spermatozoa in the liquid state (Coyan *et al.*, 2010).

The quality of spermatozoa is not only compromised when spermatozoa are cooled to sub-normal temperatures, but also when spermatozoa in the testes are exposed to temperatures above the normal temperature range at which spermatogenesis occurs optimally (Mieusset *et al.*, 2004). For normal testis function to be maintained in the animal, the testicular temperature needs to be kept at 2 to 6˚C lower than the body temperature (Fleming *et al.*, 2004; Rocha *et al.*, 2015; Alves *et al.*, 2016; Kastelic *et al.*, 2017). Testicular thermoregulation is made possible by the actions of the cremaster muscle, pampiniform venous plexus, tunica dartos, scrotal sweat glands and scrotal superficies (Alves *et al.*, 2016). These actions can be disrupted by high environmental temperature as well as inflammation, fever and cryptorchidism, resulting in testicular degeneration (Setchell, 1998). Heat stress in any of these forms will thus have a negative impact on sperm motility, concentration, viability, morphology and acrosome integrity (Naqvi *et al.*, 2012). This results in poorer fertility in females due to either, a failed fertilization, a normal fertilization, but increased embryonic deaths, or failed fertilization or an increase in embryonic death (Mieusset *et al.*, 2004). It has also been found that

spermatozoa from rams with an insulated scrotum not only suffer with regards to quality characteristics whilst being exposed to heat stress while they are within the testis, but spermatozoa are also less able to withstand further stress when exposed to freezing and thawing or when stored in the liquid state (Mieusset *et al.*, 2004; Arman *et al.*, 2006).

An effective way of studying the effects of increased testicular temperature on the production of spermatozoa and semen quality is by scrotal insulation. This is done by attaching a scrotal insulation device around the scrotum, which minimises the thermoregulatory capacity of the testes (Cruz Júnior *et al.*, 2015). Rocha *et al.* (2015) observed a significant reduction in the percentage of motile spermatozoa as early as 4 days after the beginning of scrotal insulation, with almost no motile spermatozoa seen on day 8. A significant reduction in morphologically normal spermatozoa was also found on day 4. Animals do however respond differently to heat stress, which can be attributed to the time of scrotal insulation, the environmental temperature, and individual characteristics (Alves *et al.*, 2016).

Even though testicular degeneration occurs during scrotal insulation, the testis has the ability to recover and efficiently re-establish the mechanisms associated with thermoregulation, once the thermal insult is over (Alves *et al.*, 2016). According to Rocha *et al.* (2015), it takes 106 days for ejaculates to contain the same amount of morphologically normal spermatozoa as observed prior to scrotal insulation. The concentration of spermatozoa reaches normal levels on day 92, after rams became azoospermic from day 29 until day 71 (Rocha *et al.*, 2015). Spermatogenesis and epididymal transit takes approximately 42 to 53 days and 13 to 15 days respectively, thus coinciding with the time it takes for the scrotal circumference (71 days) to recover to normal (Cruz Júnior *et al.*, 2015; Rocha *et al.*, 2015). Recovery of efficient testes temperature in rams takes at least a day since the period of heat stress has ended.

The first aim of this study was therefore to classify rams into two groups of high or low resilience based on the ability of their spermatozoa to handle the stress associated with sample collection, processing, cryopreservation, and thawing. The aim was then to determine the resilience of the ram spermatozoa to heat stress, induced by scrotal insulation. The final aim was to assess the influence of heat stress caused by scrotal insulation on the hyperactivation potential of ram spermatozoa. The findings from this study will contribute to having a refined protocol for the processing of spermatozoa obtained from sheep from arid areas as well as having an improved understanding of how heat stress affects spermatogenesis and ultimately, the fertilizing potential of ram spermatozoa.

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Chapter 2

Literature Review

2.1 Introduction

It is well established that the potential for agriculture in South Africa is limited. Most of the country is characterized by arid and semi-arid zones, with the grazing capacity being as low as one livestock unit per 12 ha in the entire Central and Western parts of the country (Cloete and Olivier, 2010). For the 2018/2019 production year, agricultural production had a total gross value of R319 732 million, which was 11.9% more than the previous production year of R285 604 million (DAFF, 2021b). Animal products contributed the most to the total gross value of agricultural production with 44.2%, followed by horticultural products and field crops with 31.5% and 24.3% respectively (DAFF, 2021b).

In 2020 there was a total of 21.6 million sheep in South Africa, which are mainly kept for the production of mutton and wool, contributing 60.6% and 31.4% to the industry, respectively (Cloete and Olivier, 2010; DAFF, 2021b). The 21.6 million sheep is constituted by approximately 8000 commercial sheep farms as well as 5800 communal sheep farmers (DAFF, 2021a). Income generated from the production of mutton however, only contributed 3.76% to the total income generated from animal products in South Africa (R141 321.54 million) (DAFF, 2021b). Sheep farms are found all over South Africa, but they are concentrated in the more arid regions of the country, with the Eastern Cape (30%), Northern Cape (24%) and the Free State (20%) having the largest numbers of sheep (DAFF, 2020). South Africa is a net importer of sheep meat and most of the mutton and lamb produced in the country is therefore consumed locally, whereas most of the wool produced in South Africa is exported (Cloete and Olivier, 2010). In 2017/18 a total of 6.145 million sheep, lambs and goats were slaughtered, which was 7.94% lower than in 2016/17, while around 4000 tons of mutton was imported in 2019 (DAFF, 2021a). South Africa produced 48 300 tons of wool during 2019/20, consisting mostly of a Merino clip (62.66%) that is used as for apparel production (DAFF, 2020; DAFF, 2021b). South Africa exports most of the wool produced (94%) in the greasy or semi-processed form and mainly exported to China, the Czech Republic and Bulgaria during 2019/20 (DAFF, 2021b).

Besides sheep having a relatively small contribution to the gross production of animal products in South Africa, sheep farming allows for sustainable production in the extensive pastoral areas where it is difficult to successfully produce crops or other animal products (Cloete and Olivier, 2010). These extensive pastoral areas account for 80% of the agricultural land that is available in South Africa (DAFF,

2018; Cloete and Olivier, 2010). Besides these low carrying capacities, drought in recent years has caused severe problems for cattle, sheep and goat farmers in these already dry areas as grazing areas have been negatively affected (DAFF, 2020). Drought remains one of the major limiting factors for agriculture in South Africa, together with diseases and unstable grain prices, causing farmers to rely on sheep production as an alternative or added source of income to make a living (Cloete and Olivier, 2010). Sheep farming is therefore seen as one the best farming options and in some cases the only way of generating an income in these dry areas where no alternative farming ventures are possible (Cloete and Olivier, 2010).

Breeds in South Africa are registered by the South African Studbook Association and it ranks breeds according to the number of weaning weights recorded by the National Small Stock Improvement Scheme (Cloete and Olivier, 2010). According to these records the most important breeds are Merino and Dohne Merino (55%) which are the major wool breeds. The Dorper (24%), which is a meat breed was next, where the Black-headed Dorpers accounted for 20.3%, and the White Dorper for the other 3.9%. The white-woolled South African Mutton Merino and white-woolled Afrino (both dual-purpose breed) contributed 6.1% and 1.6% respectively. The Dormer, Ile de France, and Merino Landsheep (all terminal sire breeds) contributed 6%, 2.9%, 1.6%, respectively, with Meatmaster, Damara and Suffolk accounting for the rest.

With global average temperatures predicted to increase to a total of 6°C by 2100, it is important to ensure that animals farmed in specific environments would be capable of withstanding and adjusting to increased ambient temperatures, while still producing optimally (Root *et al.*, 2003). Studies suggest that small ruminants show higher resilience towards increased temperatures, as well as having a higher adaptability to wide ranging climatic conditions with regards to rainfall (Cloete *et al.*, 2014). The number of sheep and goats may possibly increase as climate change continue to show rising temperate levels (Cloete *et al.*, 2014).

Sheep numbers have however declined by approximately 8.5 million since the 1980's, even though the South African human population has increased significantly from approximately 22 – 52 million people over the 40 years from 1970 (Cloete and Olivier, 2010; DAFF, 2020; DAFF, 2021b). This resulted in a severe shortage in mutton supply, thus increasing the demand, and ultimately causing the price of mutton to increase as well (DAFF, 2021a). The increase in the demand for mutton, together with the poor agricultural potential in South Africa and changing climatic conditions favouring sheep production, demands for improved assisted reproductive techniques in sheep production. A key focus

point is the effect of heat stress and how it affects the animal's physiological processes associated with reproduction.

Assisted reproductive techniques to implement oestrus out of season, thereby enhancing reproductive performance and genetically improving sheep populations, which will ultimately optimize production efficiency and thus profitability, is therefore gaining popularity amongst sheep farmers (Amiridis and Cseh, 2012). The most popular assisted reproductive techniques include artificial insemination (AI), multiple ovulation and embryo transfer (MOET), and *in vitro* embryo production and transfer (IVEP) (Amiridis and Cseh, 2012).

2.2 Global warming and its impact on food security

When referring to global warming, the term 'climate change' is often used synonymously, even though the two terms have different meanings. Global warming specifically refers to increasing global temperatures, thus being directional to warmer temperatures. According to Seijan *et al.* (2015), global warming is an enhanced greenhouse effect caused by increased atmospheric concentrations of greenhouse gasses that mainly originate from anthropogenic activity. Climate change is non-directional, and includes unusual weather of any kind, thus leaning towards the extremes of both cold and warm temperatures, and does not focus on temperature alone (Sejian *et al.*, 2015). Earth is subjected to a natural greenhouse effect that allows the average surface temperature to be 15˚C instead of -19˚C. The latter temperature would be the surface temperature in the absence of a greenhouse effect. This latter scenario would make life on earth impossible as everything would be frozen (Sejian *et al.*, 2015).

Carbon dioxide, methane and nitrous oxide are the three main and most long-lived greenhouse gasses in the atmosphere and are all partly due to the livestock production sector that contributes 18% of all global greenhouse gas emissions (Sejian *et al.*, 2015). Carbon dioxide has the most significant contribution to greenhouse gas emissions due to the great volumes it is produced in (Koneswaran and Nierenberg, 2008). Despite the large volume of carbon dioxide that is produced, methane and nitrous oxide have much greater global warming potentials (GWP's) (Koneswaran and Nierenberg, 2008). By assigning a value of 1 GWP to carbon dioxide, methane and nitrous oxide have GWP's of 23 and 296, respectively (Steinfeld *et al.* 2006).

Agriculture and food systems are expected to be put under severe pressure in the future due to an increased demand for food as a result of the expected increase in the global population (Thornton *et*

al., 2011). This will consequently result in a reduction of the land available for agricultural activities (Abbasi and Abbasi, 2016). Contributing to this pressure is that the world will experience a changing global climate at approximately the same time, which will change local climates of various regions and affect local and global agriculture, and the associated food systems (Nardone *et al.*, 2010). It is predicted that the impact of climate change will be much more severe in the warmer regions of the world relative to other regions (De *et al.*, 2017). Food security, according to the United Nations, is when all people always have safe and nutritious food available, meeting their dietary requirements and food preferences, so that a healthy and active lifestyle can be lived (Smith and Gregory, 2013). With food security being one of the most important factors for a healthy population, the environmental impact of food production should be reduced and minimised, especially with the increase in demand for food (Smith and Gregory, 2013).

With industrialized livestock systems, the indirect effects of global warming such as soil infertility, water scarcity, grain yield, and quality and diffusion of pathogens may have a far greater effect on production than the direct effects such as heat stress (Nardone *et al.*, 2010). These systems are able to control the direct effects to a certain extent, for example adjusting the diet or lowering the temperature within the facility as well as following other specific management strategies (Nardone *et al.*, 2010). In contrast, such cooling techniques actually contribute to global warming as they cause high energy consumption and also increase production costs (Nardone *et al.*, 2010). Extensive or semi-intensive farming systems will be much more affected by global warming than the intensive farming systems. This is due to the direct effects such a high temperature and solar radiation on the animals, as well as climate change causing lower rainfall and droughts, which will result in poorer pasture growth (Nardone *et al.*, 2010).

It is important to note that livestock animals are selected for improved production that increases their metabolic rate, which in return diminishes their ability to regulate body temperature during heat stress (Hansen, 2009). Therefore, the direct impact of global warming would be much more severe on domestic animals than it would be on wild mammalian species (Hansen, 2009).

2.3 Assisted reproductive techniques (ARTs)

Through the use of assisted reproductive techniques, farmers can now enable genetically superior animals to produce more offspring than would normally be possible with natural mating (Baldassarre and Karatzas, 2004; Andrabi and Maxwell, 2007; Rahman *et al*., 2008). In sheep farming, genetic improvement is however limited due to sheep being seasonal breeders. It can however be accelerated

by using assisted reproductive techniques that allow farmers to initiate oestrus out of season, as well as to enhance reproductive performance and genetic improvement of their sheep populations (Amiridis and Cseh, 2012).

Techniques such as artificial insemination (AI) and embryo transfer (ET) are used to increase the selection differential while other techniques such as juvenile *in vitro* embryo technology (JIVET) increase the rate of progress by shortening the generation interval (Baldassarre and Karatzas, 2004). Artificial insemination and heat synchronization work very well together as mating and parturition can take place over a more concentrated time-period, but also have animals ready for the meat supply chain in specific times of the year (Baldassarre and Karatzas, 2004). These benefits are therefore of great importance to the management aspect of production systems. Despite assisted reproductive techniques having been developed and applied in the global sheep industry, the commercial application of these techniques are still far less successful in the ovine industry, when compared to the successes achieved in the cattle industry (Amiridis and Cseh, 2012).

2.3.1 Artificial insemination

Artificial insemination can be regarded as a first generation ART, and is the most commonly used ART (Baldassarre and Karatzas, 2004; Rahman *et al.*, 2008). Artificial insemination is not a single specific assisted reproductive technique, but rather involves various techniques with the same aim, which is to artificially place semen in the female reproductive tract (Ogbuewu *et al*., 2010). The technique is particularly popular as it is quite simple to apply, and inexpensive, resulting in the dissemination of superior genes (Baldassarre and Karatzas, 2004; Rahman *et al.*, 2008).

The first person to investigate this technique was Ivanov (1907, 1912), but it was only after the First World War that intensive studies in this field continued, this time being led by Milovanov in the former Soviet Union, with large scale sheep breeding programmes being active by the early 1930's, using fresh and diluted semen (Salamon and Maxwell, 2000).

Hafez and Hafez (2008) and Edmondson *et al.* (2012) identify several advantages of AI which include amongst others, firstly from a management and financial perspective, that it eliminates the need for rams on the farm, thus reducing costs as well as minimising the risk of spreading sexually transmitted diseases. Artificial insemination makes it possible to use males with incapacitated or oligospermic semen. Artificial insemination is a necessity for large groups of ewes after oestrus synchronization and is a valuable research tool for investigating various aspects of male and female reproductive

physiology. From a genetic point of view, AI also maximises the use of outstanding sires. This also means that frozen semen can be used for AI from donors that have died, thus preserving specific lines. Artificial insemination allows for crossbreeding to improve production traits, and it improves the potential and performance of the national herd as well as permitting accelerated introduction of new genetics. Males with desirable genetic markers can be used in specific genetic pairings. Progeny testing can also be done under various environmental and managerial conditions which thus improves selection.

There are however several disadvantages to AI (Edmondson *et al.*, 2012). The equipment and liquid nitrogen that is needed is expensive. With AI, there is an increased demand in labour for oestrus detection and insemination. Artificial insemination also lacks in standardization of procedures needed for packing and quality control of semen. There is often a lack of suitable sire proofs for production traits, and less desirable traits could spread quickly due to the ease at which AI is applied on large numbers of ewes. The equipment used for AI should be clean and straws must be bought from registered centres to prevent the spread of diseases.

One of the first challenges associated with AI is that semen harvested from superior rams must be used to inseminate large numbers of ewes which are often long distances away from the point of semen collection, meaning that semen transport is required (Salamon and Maxwell, 2000). There is also the desire to use rams over extended periods as well as during different times of the year when rams would not produce spermatozoa optimally (Salamon and Maxwell, 2000). These challenges have thus increased the need for research on the storage of spermatozoa under artificial conditions (Salamon and Maxwell, 2000).

There are different methods of preserving semen for AI and include, fresh, refrigerated and frozen, which is generally the determinant for the AI method that will be used, with a general rule of thumb being that more damaged spermatozoa will require a method that allows for semen to be deposited deeper within the female reproductive tract to achieve good fertilization rates (Baldassarre and Karatzas, 2004). The different techniques used for artificial insemination in animals include vaginal, cervical, transcervical, laparoscopic and intrauterine insemination (Hafez and Hafez, 2008).

Sperm samples collected for AI can be stored for use in the long- or short-term. Long-term storage of spermatozoa refers to the cryopreservation of spermatozoa, whereas short-term storage refers to liquid storage.

The cryopreservation of spermatozoa makes it is possible to extend the spread of genetic material internationally, and it also allows for artificial insemination during both the reproductive season as well as the non-reproductive season, and therefore makes it possible for genetically superior males to have an extended reproductive life even after the male's death (Baldassarre and Karatzas, 2004; Rahman *et al.*, 2008; Ogbuewu *et al.*, 2010).

The poor fertility being achieved with frozen semen limits the use of AI in sheep (Yániz *et al.*, 2005). The anatomical characteristics of the ewe's cervix does not allow for the use of an insemination catheter, and lower fertilization rates with frozen-thawed semen therefore limits cervical insemination to fresh semen, and frozen-thawed semen is thus only used for intrauterine insemination (Domínguez *et al.*, 2008; Falchi *et al.*, 2018). Even though laparoscopic intrauterine insemination results in good fertilization rates with frozen-thawed semen, it is an expensive procedure and is impractical to perform on a routine basis as it would result in welfare issues (Lloyd *et al.*, 2012; Ntemka *et al.*, 2018). It is said that when fresh semen is used for AI, the fertilization rate is equal to that of natural mating, being higher than 80%, but with frozen semen it is lower, being above 60% (Rodriguez-Martinez, 2012).

Artificial insemination in a normal farming environment is generally performed within a short period since the semen samples have been collected (Kasimanickam *et al.*, 2007; Falchi *et al.*, 2018). The liquid storage of spermatozoa is therefore a good alternative for frozen storage. Liquid storage is more practical and less expensive than cryopreservation, it allows for cervical insemination, and is a popular storage method when farmers within the same area have an agreement to use the same ram for breeding (Baldassarre and Karatzas, 2004; Mara *et al.*, 2005; Kasimanickam *et al.*, 2007; Falchi *et al.*, 2018).

One limitation of liquid storage for AI, specifically for cervical insemination, is that fertility is reduced when the spermatozoa are stored for longer than 24 hours (Maxwell and Watson, 1996). The same as for frozen storage, liquid storage accelerates the maturation of sperm membranes, therefore increasing the number of capacitated and acrosome reacted cells (Salamon and Maxwell, 2000b). The usage of liquid extenders causes cell sedimentation to occur, whereas cells are more homogeneously spread in extenders in a more solid form (Gheller *et al.*, 2018). This is important as it allows the contents in the extender to optimally play their role as energy source, buffer, or protecting agent (Gheller *et al.*, 2018).

It is commonly known that the quality of spermatozoa decreases as the storage time increases and happens irrespectively of the type of diluent, dilution rate, conditions or temperature of storage (Salamon and Maxwell, 2000; Falchi *et al.*, 2018). While spermatozoa are being stored, motility and morphological integrity becomes reduced, with a subsequent decrease in survival within the female reproductive tract, reduced fertility and increased embryonic loss (Paulenz *et al.*, 2002). This can largely attributed to the fact that the spermatozoa accumulate toxic products, known as reactive oxygen species (ROS), when the sperm membranes undergo lipid peroxidation (Salamon and Maxwell, 2000).

The sex-sorting of spermatozoa for AI is also receiving considerable attention as it is another method of increasing reproductive efficiency in animals, especially with species or breeds where males have lower commercial value (Baldassarre and Karatzas, 2004; Rahman *et al.*, 2008).

2.3.2. Multiple ovulation and embryo transfer (MOET)

Multiple ovulation, also known as superovulation, is where females are treated with specific hormones that increases the number of ova that is released by the ovary (Rahman *et al.*, 2008). Embryo transfer (ET) is when fertilized eggs are collected from genetically superior females, that have either undergone normal ovulation, or mostly from females that have been induced to superovulate (Sejian *et al*., 2010). Embryo transfer makes it possible for embryos to be transferred from females with superior genetics, to females who are genetically, less desirable (Sejian *et al*., 2010). These two methods are generally used together. Multiple ovulation and embryo transfer thus make it possible to accelerate the rate of genetic improvement of domestic animals as well as preserving the genetic resources of endangered wild animals (Mayorg *et al.*, 2011). In small ruminants there are three methods used for embryo transfer and include laparoscopic ET, surgical ET, and transcervical ET, with laparoscopic ET achieving the most successful pregnancies (Rahman *et al.*, 2008).

Despite MOET being comparative with AI for males, this technique is often frustrating as it is sometimes extremely successful, while at other times it completely fails without any changes in the operating procedures, making it unpredictable (Baldassarre and Karatzas, 2004). The three main factors affecting the success of this technique include the high variability of the super-ovulatory response, poor fertilization achieved with high ovulatory responses, and the corpora lutea undergoing early regression (Baldassarre and Karatzas, 2004). This unpredictability together with high cost and the collection and transfer of embryos requiring surgical procedures, prevents the large-scale application of this method (Baldassarre and Karatzas, 2004). The high variability can be attributed to

several endogenous factors. These include, genetics, nutritional status, follicular status and season of the year, as well as exogenous factors such as the type of superovulatory treatment, and the nature and possible 'contamination' of the gonadotropin administered (Amiridis and Cseh, 2012).

Embryos that are morphologically of the highest quality, originate from donors whose functionality of the genital tract is at an optimum (Stroud and Hasler, 2006). This entails that the processes of folliculogenesis, oocyte maturation, multiple ovulation, sperm transport, fertilization, and subsequent early embryonic development, operate smoothly (Stroud and Hasler, 2006). The quality of the spermatozoa used to produce embryos through MOET is thus of critical importance, and the starting point to ensuring that a high quality and quantity of embryos are produced with this ART.

2.4 Reproduction in sheep rams (*Ovis aries*)

2.4.1 Anatomy of the male reproductive system

2.4.1.1 Scrotum

The scrotum forms from the skin and fascia of the abdominal wall, and is suspended away from the body as in Figure 2.1 (Hafez and Hafez, 2008; Edmondson *et al.*, 2012). The spermatic cord is positioned in the vaginal process and encapsulates blood vessels, nerves, and the *ductus deferens* as they lead into the scrotum (Hafez and Hafez, 2008). The *ductus deferens* however parts away from the vessels at the orifice of the vaginal process to join with the urethra (Hafez and Hafez, 2008).
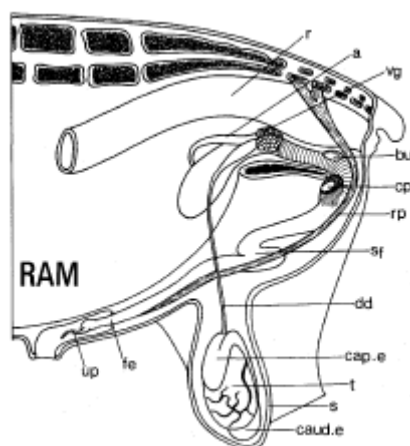
Figure 2.1 Reproductive tract of the ram. *up,* urethral process; *fe,* free part of the penis; *caud. e*, cauda epididymis; *s,* scrotum; *t*, testis; *cap. e*, caput epididymis; *dd*, ductus deferens; *sf,* sigmoid flexure; *rp,* retractor penis muscle; *cp,* left crus of penis; *bu*, bulbourethral gland; *vg,* vesicular gland; *a*, ampulla; *r*, rectum (Hafez and Hafez, 2008).

The scrotum of most terrestrial mammals is a pouch of perineal skin that is situated on the outside of the body and is lined with the peritoneum, which is in direct contact with the peritoneal cavity,

allowing for continuous communication between the two (Foster, 2016). The scrotum is often covered by hair or wool, but this depends on the breed and husbandry practices (Edmondson *et al.*, 2012). The scrotum is able to contract itself owing to the fibroelastic tissue and smooth muscle that constitute the dartos tunic and is divided into two halves by the scrotal septum (Edmondson *et al.*, 2012; Foster, 2016). The scrotum, together with two other thermoregulators from the reproductive system, the cremaster muscle and the pampiniform venous plexus, are responsible for maintaining the testes and epididymis at 2 – 8˚C below core body temperature (Fleming *et al.*, 2004; Foster, 2016).

Scrotal circumference (SC) is a measure of testicular size and is seen as a superior index for sperm production in rams as it is a highly heritable trait (Chellaet *et al.*, 2017). According to the study of Chella *et al.* (2017), SC and body weight are highly and positively correlated. They added that the quantity of spermatogenic material within the testes is a direct reflection of SC, which can be used to indicate the animal's capacity to produce spermatozoa.

2.4.1.2 Testes

In mammals, the testes are located inside the scrotum (Cruz Júnior *et al.*, 2015). Each testis consists of tubular and extra-tubular compartments as shown in Figure 2.2, with the tubules containing Sertoli cells and germinal cells, called spermatogonia, spermatocytes, spermatids and spermatozoa (Edmondson *et al.*, 2012; Foster, 2016). Between the seminiferous tubules there are interstitial cells, known as Leydig cells, that are responsible for the secretion of testosterone into the testicular veins and lymphatic vessels (Hafez and Hafez, 2008). From the seminiferous tubules, spermatozoa move through the straight tubules and into the intratesticular rete, from where they leave the testis through the rete testis to enter the efferent ductuli that form into the epididymis (Foster, 2016). The testes normally have a pale pink or whitish appearance (Foster, 2016). The testes are housed in the scrotum within the vaginal process, which is a separate extension from the peritoneum that pass through the abdominal wall at the inguinal canal (Hafez and Hafez, 2008). The testis and epididymis both receive blood via the testicular artery, which is accompanied by afferent and efferent nerves (Hafez and Hafez, 2008).
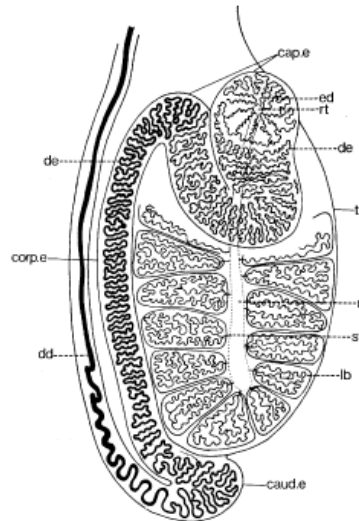
Figure 2.2 The tubular system of the testis and epididymis of a bull (the duct system of the rete testis is not shown for clarity purposes). *t*, testis; *lb*, lobule with seminiferous tubules; *st*, straight tubule; *rt*, rete testis; *cap. e*, caput epididymidis; *ed.*, efferent ductule; *de*,duct of the epididymis; *corp. e*, corpus epididymidis; *caud. e*, cauda epididymis; *dd*, ductus deferens (Hafez and Hafez, 2008).

2.4.1.3 Epididymis

The epididymis is a long and tortuous part of the male reproductive system and can be divided into three main areas. These include the *caput epididymis* (head) that forms a flattened structure at the dorsal pole of the testis, the *corpus epididymis* (body) and the *cauda epididymis* (tail), that is situated at the opposite pole of the testis, as can be seen in Figure 2.3 (Hafez and Hafez, 2008). The cauda epididymis terminates in the *vas deferens* (ductus deferens) (Foster, 2016). The spermatic cord encapsulates and joins several blood vessels and nerves to the testis and lies within the vaginal process, with the ductus deferens also forming part of these vessels, but it goes in its own direction at the orifice of the vaginal process to join the urethra (Hafez and Hafez, 2008). The spermatozoa will eventually be ejaculated from the urethra (Hafez and Hafez, 2008).

The testis and the caput epididymis are the two areas where immotile and immature spermatozoa are housed, with spermatozoa that reach the cauda epididymis being motile and mature, thus being capable of fertilization (Lone *et al.*, 2011). Spermatozoa are stored in the cauda epididymis until ejaculation occurs, and around 75% of epididymal spermatozoa are stored here (Hafez and Hafez, 2008). Depending on species, spermatozoa take 9-13 days to be transported through the epididymis (Hafez and Hafez, 2008). During the movement of spermatozoa through the epididymis, their plasma membrane experiences the modification or addition of surface glycoproteins due to secretions from the epididymis and luminal cells (Parkinson, 2018). These changes aid in the stabilisation of the acrosome and prevents immunogenicity against the spermatozoa when the spermatozoa are in the

female reproductive tract, and enhances the sperm membranes to bind to the zona pellucida (Parkinson, 2018).
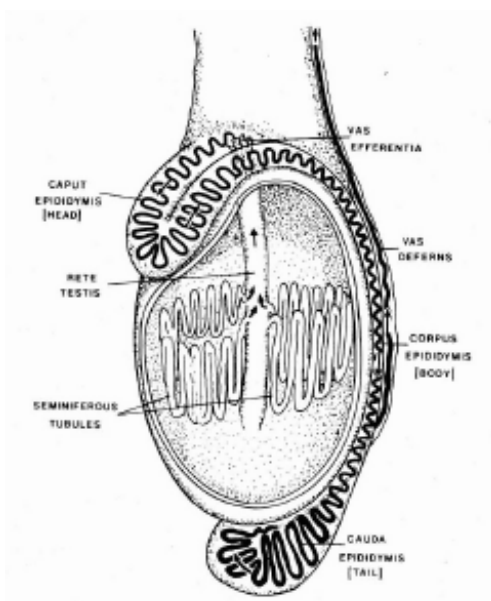
Figure 2.3 The pathway that spermatozoa follow to be released from the penis, starting at the seminiferous tubules, to the rete testis and then the excurrent duct system of the ram (Hafez and Hafez, 2008).

2.4.1.4 Penis

The penis consists of two major structures, with the first being the corpus spongiosum penis that surrounds the penile urethra in its entire length, and also the corpus cavernosum penis, the most prominent structure of the penis (Edmondson *et al.*, 2012). A pair of crura from the ischial arch forms the corpus cavernosum penis and is enclosed by ischiocavernosus muscle (Hafez and Hafez, 2008). The *tunica albuginea* surrounds the entire penis (Edmondson *et al.*, 2012). The retractor penis muscles are responsible for keeping the penis in an S-shaped bend, called the sigmoid flexure, except during erection and ejaculation (Edmondson *et al.*, 2012). There are two retractor penis muscles and they emerge from the coccygeal vertebrae from where they pass around the anus so that they become two distinct muscles that attach to the ventrolateral surface of the penis at the distal bend of the sigmoid flexure (Edmondson *et al.*, 2012).

The ischiocavernosus muscle pumps blood from the cavernous spaces of the crura into the rest of the corpus cavernosum penis which allows it to stiffen and straighten during erection (Hafez and Hafez, 2008). The bulbospongiosus muscle compresses the penile bulb during ejaculation, resulting in the flow of blood into the remainder of the corpus spongiosum penis (Hafez and Hafez, 2008).

2.4.1.5 Accessory sex glands

When the ram ejaculates, spermatozoa moving from the tail of the epididymis and protein-rich plasma from the accessory sex glands mix as it is being discharged into the ductus deferens and into the pelvic portion of the urethra (Hafez and Hafez, 2008; Leahy and de Graaf, 2012). Seminal plasma is thus a combination of the secretions from the testes, epididymis, and accessory sex glands. The seminal plasma acts as transport and as a buffer for the spermatozoa when deposited within the female reproductive tract (Leahy and de Graaf, 2012).

The accessory sex glands consist of the ampulla, the vesicular, prostate, and bulbo-urethral glands, that all require androgens to be functional (Parkinson, 2018). Figure 2.4 indicates the order from the testes to the urethra in which the accessory sex glands contribute to the composition seminal plasma. The vesicular gland is large in the ram and bull, with the ampulla, prostate and bulbourethral glands being small and disseminated (Leahy and de Graaf, 2012). Eutherian species show major differences in their seminal plasma, both physically and biochemically, as a result of the variation in the amount and in the relative contribution of the accessory sex glands (Leahy *et al.*, 2019).

The main function of the ampullae is to act as reservoirs for spermatozoa before the spermatozoa enter the pelvic urethra (Parkinson, 2018). The vesicular glands are positioned distally from the ductus deferens and open into the urethra, supplying secretions containing large quantities of citrate, as well as fructose, contributing significantly to the volume of the semen (Parkinson, 2018). The secretions from the vesicular glands assist in semen coagulation, sperm motility and capacitation, and supresses the immune response in the female reproductive tract (Parkinson, 2018). The prostate surrounds the urethra, and its secretions are watery and include large amounts of chloride ions (Parkinson, 2018). These secretions are highly alkaline and protect spermatozoa from the cervical mucus and vaginal secretions that are acidic, and help coagulate semen in the reproductive tract of the female directly after ejaculation (Flint *et al.*, 2015). The bulbourethral glands are positioned between the anus and urethra and release a watery secretion, known as the pre-ejaculate, that clean the urethra of urine prior to coitus, and also neutralizes the acidity of the urine inside the urethra and vagina (Flint *et al.*, 2015; Parkinson, 2018).
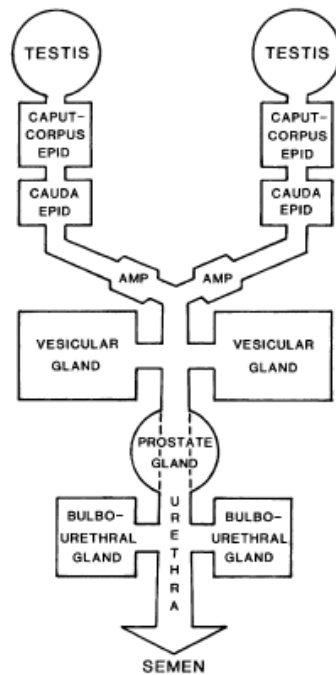
Figure 2.4 The testis, epididymis, and accessory sex glands and the order in which they contribute to the seminal plasma composition (Hafez and Hafez, 2008).

2.4.2 Spermatogenesis

A synchronized and regulated process, known as spermatogenesis, is responsible for the production of mature spermatozoa, as illustrated in Figure 2.5 (Tapia and Peña, 2009). Sperm production occurs in the testis after which the spermatozoa are transported through the caput and corpus epididymis (Mortimer, 1997). The spermatozoa is then stored in the proximal cauda epididymis, before being released from the body by means of ejaculation (Mortimer, 1997). The process of spermatogenesis begins when stem cells in the testes proliferate by means of mitosis and is then followed by meiotic phase so that they can differentiate (Foster, 2016). Each stage respectively consists of spermatogonia, spermatocytes and spermatids (Foster, 2016). The production of spermatozoa from spermatogonia takes between 42 and 53 days, with epididymal transit taking a further 13 to 15 days (Cruz Júnior *et al*., 2015).

The development of spermatozoa happens in the seminiferous tubules of the testes, after which they pass through the straight tubules into intratesticular rete that is in the mediastinum testis, with Figure 2.6 showing an enlarged view of these structures (Foster, 2016). The seminiferous epithelium lines the inside of the seminiferous tubules and consists of the Sertoli cells and the developing germ cells (Hafez and Hafez, 2008). The seminiferous tubules consist of a complex series of developing germ cells

that continuously divide and undergo developmental changes, starting at the periphery and then moving towards the lumen of the tubule to ultimately form the male gametes, spermatozoa (Hafez and Hafez, 2008). The stem cells are known as spermatogonia and divide several times to form spermatocytes that undergo meiosis, thus reducing the DNA content to half of that of somatic cells, making them haploid cells, called spermatids (Hafez and Hafez, 2008). Spermatids then undergo further structural and developmental changes to form spermatozoa (Hafez and Hafez, 2008). Some of the spermatogonia do however renew themselves to become spermatogonial stem cells (Tapia and Peña, 2009).

From here the spermatozoa leave the testis via the rete testis, then entering the efferent ductules (usually between 13 and 20, depending on species) and eventually join the single duct of the epididymis, with some however ending blindly (Foster, 2016). During sperm transport and storage in the epididymis, the spermatozoa become mature and functional due to a series of morphological, biochemical and physiological changes (Mortimer, 1997). These changes give them the potential to fertilize the ova (Hafez and Hafez, 2008). Roughly 70% of the spermatozoa from the excurrent ducts are stored in the cauda epididymis, with the vas deferens containing about 2% (Hafez and Hafez, 2008). The liquid cellular suspension containing the spermatozoa and secretions from the accessory sex glands is called semen, with the complete fluid portion that is formed at ejaculation being called seminal plasma (Hafez and Hafez, 2008).

To be able to sustain spermatogenesis at a physiological level, there should be a dynamic balance between the regeneration and elimination of germ cells, which is controlled by the Sertoli cells as they nurture a specific number of germ cells (Tapia and Peña, 2009). During the different phases of development, some germ cells undergo apoptosis as part of a natural phenomenon, while the Sertoli cells are responsible for the uptake of these degenerative and dead cells (Foster, 2016). Apoptosis, in contrast to necrosis, is an important outcome as it does not normally result in an inflammatory response, which cause damage to the testicular function (de Kretser *et al.*, 2015). These apoptotic events are thus crucial for successful spermatogenesis. Apoptosis mainly targets spermatogonia and spermatocytes, both under physiological and pathological circumstances, but it does occur in all stages of spermatogenesis (Tapia and Peña, 2009). Even though spermatozoa are stored in an environment favourable to their survival, they cannot survive forever (Hafez and Hafez, 2008). Unejaculated spermatozoa are usually gradually excreted via urination, or deteriorate, starting with a loss in fertility, followed by motility and eventually disintegrate (Hafez and Hafez, 2008).

Apoptosis can be triggered by two major pathways and include a pathway involving mitochondria, or one involving death receptors, with the former being intrinsic, and the latter being extrinsic (Tapia and Peña, 2009). Cell surface receptors mediate the death receptor pathway through the activation by soluble ligands that lead to intracellular effects through adaptor proteins, whereas, intrinsic mechanisms, not involving cell death receptors activate the mitochondrial pathway (Tapia and Peña, 2009). These two pathways can work together, as the death receptor pathway can use the mitochondrial pathway to amplify apoptotic signals when the receptors are activated (Tapia and Peña, 2009). Germ cells undergo cell death through various pathways. These include pathways mediated by the Bcl-2 family proteins such as Fas ligand, tumour necrosis factor (TNF), as well as tumour necrosis factor alpha-related apoptosis-inducing ligand (TRAIL) that activates the receptors of its counterpart that belong to the tumour necrosis factor receptor superfamily (TNF-R); and through tumour suppressors like p53.

Even though apoptosis occurs naturally, it can also be induced or enhanced by factors affecting the spermatozoa *in-vivo* (Foster, 2016). These factors include, amongst others, exposure to heat, radiation, or toxic compounds, depletion of growth factors, changes in hormonal support, transient ischemia, free radical status, or drug treatments (Foster, 2016). Various stimuli and stressors have been found to induce apoptotic-like events in ejaculated spermatozoa (Tapia and Peña, 2009). These include, amongst others, heat stress, cryopreservation, progesterone, androgen receptor activation, oxidative stress, staurosporine, betulinic acid, active lipids, and tumour necrosis factor-alpha (TNFα). Caspase activation, loss of MMP, PSE, and DNA fragmentation have also caused ejaculated spermatozoa to show signs of apoptosis, and factors modulating both the intrinsic and extrinsic pathways have appeared in these spermatozoa (Tapia and Peña, 2009). It therefore indicates that these pathways can be activated independently in ejaculated spermatozoa (Tapia and Peña, 2009). It is however still unclear whether the causes of apoptosis are purely a detrimental action, or if they have a physiological significance (Tapia and Peña, 2009).

The neuroendocrine system is responsible for physiologically controlling spermatogenesis, which is directly influenced by the thermoregulation of the testis and scrotum (Cruz Júnior *et al.*, 2015). Spermatogenesis in mammals is a continuous process since puberty has been reached, until the time of death (Foster, 2016).
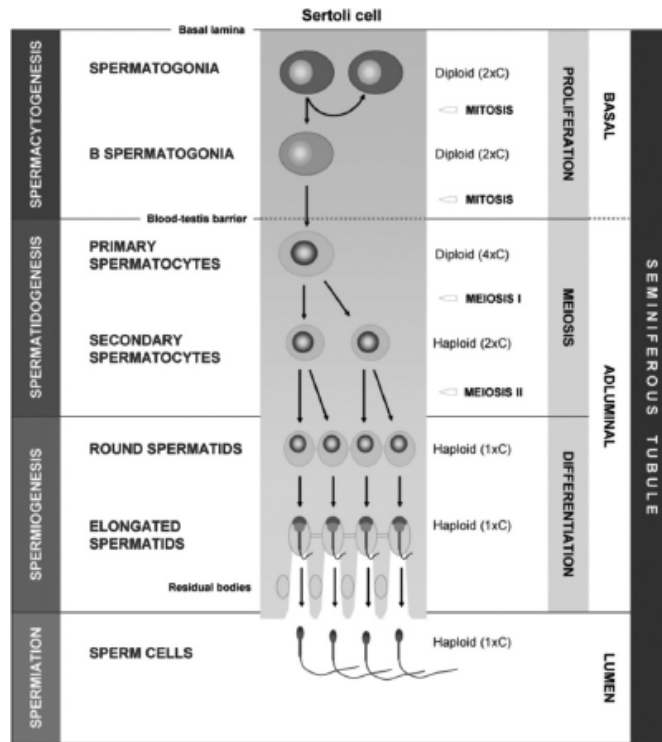
Figure 2.5 A summary of the different phases of spermatogenesis, the germ cell characteristics in each stage, and the different compartments of the seminiferous tubules (Tapia and Peña, 2009).
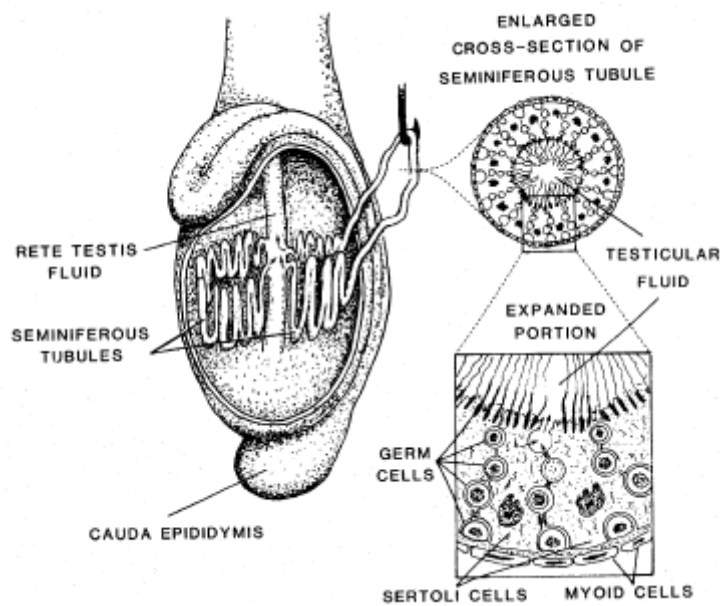
Figure 2.6 Ram testis with an enlarged cross-section of a seminiferous tubule to show the microanatomy thereof (Hafez and Hafez, 2008).

2.4.3 Hormonal control of spermatogenesis

Puberty in males is reached when the animal becomes capable of producing gametes and exhibit sexual behaviour (Hafez and Hafez, 2008). The onset of puberty is determined by the degree of synchronized communication between the different components of the hypothalamic-pituitary-gonadal axis (Hafez and Hafez, 2008). The endocrine system and central nervous system (CNS) collectively play an important role in the neuroendocrine control of spermatogenesis (Hafez and Hafez, 2008). The nervous system makes use of electric nerve impulses whereas the endocrine system relies on chemical messengers or hormones to regulate slow body processes (Hafez and Hafez, 2008). The respective hormones involved in reproduction in the ram are produced in the hypothalamus, the anterior pituitary, and the testes, respectively (Hafez and Hafez, 2008). These hormones include, gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone, inhibin, activin, and oxytocin.

Reproductive activity starts with the release of gonadotropin-releasing hormone (GnRH). Gonadotropin-releasing hormone is responsible for the humoral link between the neural and endocrine system and releases pulses of GnRH into the hypophyseal portal system, as a response to neural signals for the synthesis LH and FSH, the pituitary gonadotropins, to be released from the anterior pituitary (Hafez and Hafez, 2008; Sejian *et al*. 2010; Parkinson, 2018).

Luteinizing hormone is secreted in pulses as the pituitary is episodically stimulated by GnRH, and its main role is to control testosterone synthesis by acting upon the Leydig cells (Parkinson, 2018). The secretion of LH is controlled by means of a negative-feedback system through the effect that testosterone has on the hypothalamic GnRH, and by the synthesis and secretion from the pituitary (O'Donnell *et al.*, 2006). The Sertoli cells also produce activin that stimulates the production of FSH, but it is considered to mostly function as a paracrine agent in the testis (de Kretser *et al.*, 2004). Follicle stimulating hormone mainly targets the Sertoli cells (Parkinson, 2018). Follicle stimulating hormones stimulate the Sertoli cells to secrete androgen-binding protein (ABP) and is responsible for aromatising testosterone into oestrogens, as well as for activating several genes that regulate or assist spermatogenesis (Parkinson, 2018). The release of FSH is controlled by a negative feedback mechanism that acts on gonadal steroids and inhibin (Parkinson, 2018).

The enzyme, 5α-reductase, converts testosterone into 5α-dihydrotestosterone (DHT) in the Sertoli cells and accessory sex glands (Parkinson, 2018). The Sertoli cells secrete ABP that binds the testosterone and DHT in the tubule lumen, and thus maintains high concentrations of androgen in the

lumina of seminiferous tubules as well as in the epididymis (Parkinson, 2018). Testosterone is required for sperm production and the subsequently for the maturation of the spermatozoa in the epididymis (Parkinson, 2018). Testosterone also plays an important role in; the function of the accessory sex glands, the development of the secondary sexual characteristics in males, sexual behaviour, late stages of spermatogenesis, prolongs the life of epididymal spermatozoa, and has anabolic effects (Hafez and Hafez, 2008; Parkinson, 2018).

Inhibin is produced by the Sertoli cells and inhibits the release of FSH, but it does not alter the release of LH (Hafez and Hafez, 2008; Parkinson, 2018). Growth factors are chemical messengers that play a role in the control of reproduction, but do not form part of the endocrine or central nervous system (Hafez and Hafez, 2008).

Oxytocin plays a greater role in the female than in the male. In the male, oxytocin is responsible for peristaltic contractions and relaxations of the peritubular tissue of the seminiferous tubules to move spermatozoa towards the rete testis (de Kretser *et al.*, 2015). In the female, in terms of spermatozoa, oxytocin stimulates the uterus to contract, therefore facilitating sperm transport to the oviducts during oestrus (Hafez and Hafez, 2008). Figure 2.7 provides an overview of these hormones and the negative feedback mechanisms that regulate hormone concentration.
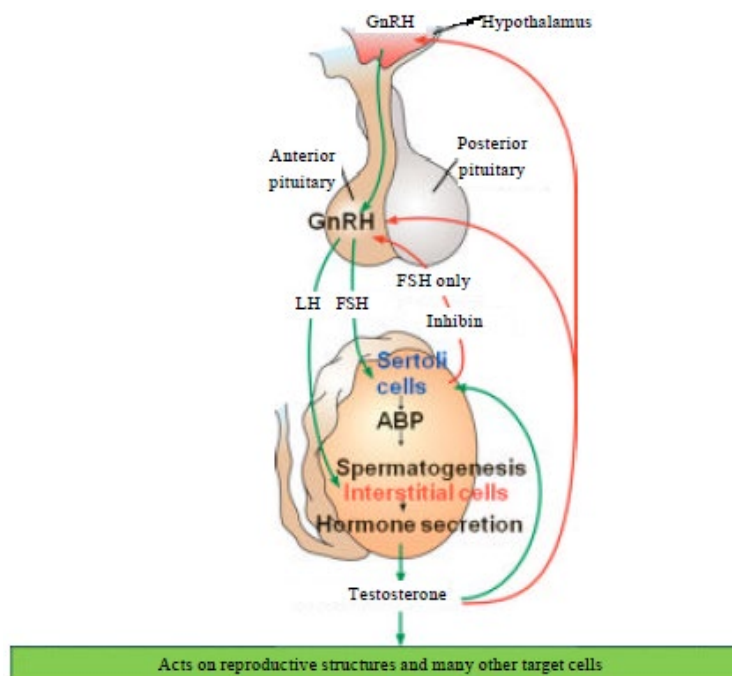
Figure 2.7 Overview of the reproductive hormones and the negative feedback mechanisms regulating hormone concentration (Sejian *et al.*, 2010).

2.4.4 Sperm morphology

Spermatozoa are morphologically, the most diverse cell type in the animal kingdom (Vicente-Fiel *et al.*, 2013). Spermatozoa that are completely formed are elongated cells with flat heads that contain the nucleus and have a tail that makes the cells motile, as can be observed in Figure 2.8 (Hafez and Hafez, 2008). Figure 2.9 shows the differences in shape and size of spermatozoa from farm animals and other vertebrates.
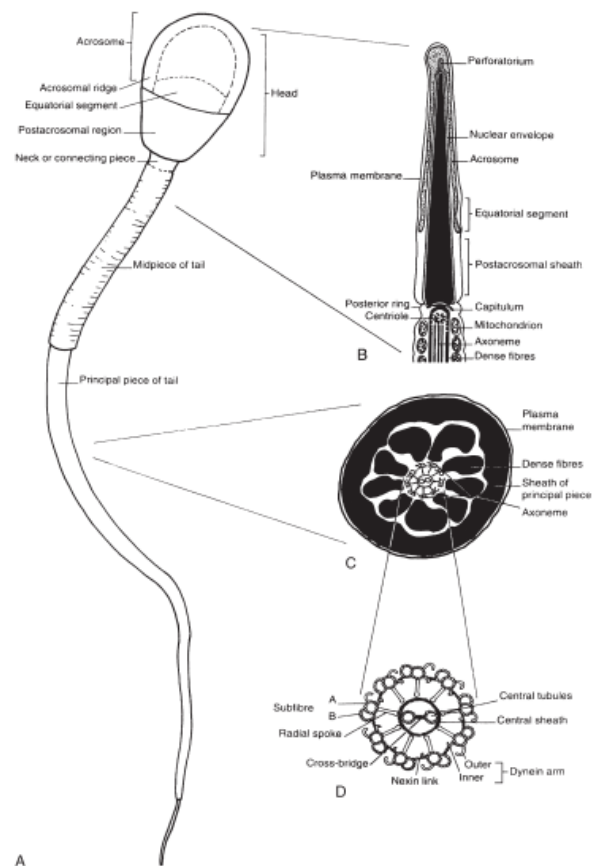
Figure 2.8 Diagram of the ultrastructure of a spermatozoon. (A) The main structural components as viewed with alight microscope. (B) A generalised illustration of the ultrastructural components of the head and connecting piece. (C) Ultrastructure of the proximal principal piece of the tail. (D) Generalised enlargement of the ultrastructure of the exoneme of the tail (Parkinson, 2018).

2.4.4.1 Head

The spermatozoon head mainly consists of the nucleus that is filled with highly compact chromatin, but also contains the acrosome, with the entire spermatozoon being surrounded by the plasma membrane (Hafez and Hafez, 2008; Yániz *et al.*, 2012). The condensed chromatin is made up of deoxyribonucleic acid (DNA) that is complexed to basic proteins called protamines (Hafez and Hafez, 2008). Spermatozoa are haploid, meaning that they contain half of the DNA content/chromosomes that somatic cells of the same species contain (Hafez and Hafez, 2008). Male mammals are

heterogametic, meaning that one half of the spermatozoa carry the X chromosome and the other half the Y chromosome (Hafez and Hafez, 2008). This results in equal chance of male or female offspring, as spermatozoa carrying the X-chromosome produce female embryos and spermatozoa carrying the Y-chromosome producing male embryos (Hafez and Hafez, 2008). In other words, the somatic cells of the female carries a pair of X chromosomes and the somatic cells of males carry XY chromosomes (Hafez and Hafez, 2008). There is a double walled structure between the plasma membrane and the anterior region of the spermatozoon head called the acrosome or acrosomal cap (Hafez and Hafez, 2008). The spermatozoon head can be divided into two domains, being the acrosomal and post-acrosomal domains, of which the acrosomal domain is separated into two segments, the anterior and equatorial segment (López Armengol *et al.*, 2012). The anterior segment is more susceptible to chemical and physical effects as well as ionic changes, when compared with the equatorial segment, as this part of the membrane is more delicate and fragile (López Armengol *et al.*, 2012).

The plasma membrane of spermatozoa, also known as the plasmalemma, is a delicate and unstable layer that physically protects the cell, selectively controls the passage of substances and interacts with other cells, like oocytes and epithelial cells within the female reproductive tract (Yániz *et al.*, 2008). Due to the plasma membrane of spermatozoa being rich in polyunsaturated fatty acids, spermatozoa are susceptible to peroxidative damage (Coyan *et al.*, 2010). This is due to the presence of reactive oxygen species during aerobic storage that leads to poorer membrane integrity, reduced sperm motility and ultimately a loss in fertility (Coyan *et al.*, 2010).

For the spermatozoa to survive within the female reproductive tract, the plasma membrane must be intact and active so that spermatozoa can bind to the zona pellucida and the vitelline membrane, as well as undergo the process of capacitation and the acrosome reaction (Martí *et al.*, 2003). The plasma membrane remains stable, until capacitation starts, by means of seminal plasma proteins that are adsorbed onto the ejaculated spermatozoon surface, with their removal being a prerequisite for fertilization (Domínguez *et al.*, 2008). Capacitation is the process where acrosomal changes occur that will allow the spermatozoa to attach to and penetrate the ovum (Hafez and Hafez, 2008). This process requires that spermatozoa are present in the female reproductive tract for some time, usually the isthmic region of the oviduct, before being able to do so (Hafez and Hafez, 2008). Capacitation takes place so that the acrosome reaction is not prematurely activated and only starts once the spermatozoa reach the site of fertilization where it is in contact with the ovum (Hafez and Hafez, 2008). The acrosome reaction is when the plasma membrane of a spermatozoon fuses with the outer acrosomal membrane (Hafez and Hafez, 2008). Once the spermatozoon has undergone the acrosome reaction,

the equatorial segment plays a pivotal role in the initiation of the spermatozoon fusing with the plasma membrane of the oocyte during fertilisation (López Armengol *et al.*, 2012).

The effect of heat stress on mammalian germ cells have several consequences and include, amongst others, the miss-folding of proteins, damaged DNA, the inability of DNA repair systems to function properly, inhibition of various processes associated with DNA replication and chromatin maturation (Salces-Ortiz *et al.*, 2015). The chromatin of spermatozoa has been found to be impaired in men (Ahmad *et al.*, 2012; Rao *et al.*, 2016), mice (Paul *et al.*, 2008; Pérez-Crespo *et al.*, 2008), bulls (Karabinus *et al.*, 1997), rams (Hamilton *et al.*, 2018) and stallions (Love and Kenney, 1999) exposed to testicular and epididymal heat stress. Rao *et al.* (2016) also observed higher percentages of spermatozoa with impaired mitochondrial membrane potentials, which is an early event of apoptosis and is due to damaged caused by reactive oxygen species. Houston *et al.* (2018) further reported that even mild heat stress results in increased oxidative DNA damage of germ cells in mice.

2.4.4.2 Middle piece and tail

The middle piece and tail of spermatozoa is often described together as it can be considered as a single functional structure (Parkinson, 2018). A neck connects the spermatozoon head to the tail (flagellum) that can be divided into three regions, the middle, principle, and end piece. These regions can be seen in Figure 2.9 where the morphometry can be compared between various livestock species, humans, and rats (Hafez and Hafez, 2008).

The core of the middle piece and tail of spermatozoa is known as the axoneme (Hafez and Hafez, 2008). The axoneme is constructed from nine pairs of doublet microtubules that surround two central filaments in a radial manner (Hafez and Hafez, 2008; de Kretser *et al.*, 2015). For the middle piece, the 9+2 formation is also surrounded by 9 outer dense fibers that are associated with the 9 doublets of the axoneme (Hafez and Hafez, 2008; de Kretser *et al.*, 2015). The midpiece is enclosed in a helix of mitochondria (Parkinson, 2018). The flagellar movement of the tail and thus the bending of the tail is made possible by the forces created from adjacent peripheral doublets of the axoneme (Parkinson, 2018). An unbinding process, using adenosine triphosphate (ATP), is repeated to result in the progressive bending of the flagella (Parkinson, 2018). This happens when the dynein arms of the doublet, that are normally bound to the adjacent doublet in their resting form, unbind and elongate to bind to a different site further down the filament (Parkinson, 2018). An important aspect of this mechanism is that the doublets on opposing sides of the axoneme work in opposition to each other,

creating the beat of the tail in alternate directions, thus allowing forward movement (Parkinson, 2018).

Heat stress inflicts various forms of damage on the head of spermatozoa, mostly associated with DNA damage, as the head contains the nucleus that holds the chromatin (Salces-Ortiz *et al.*, 2015). The effect of heat stress on changes in the structural composition of the middle piece and tail, and thus, the sensitivity of these components to heat stress, is currently unknown.

Against this background, it is important to understand the resilience of spermatozoa (i.e., the ability of spermatozoa to withstand changes induced by processing) and how it influences the use of spermatozoa in ART's.
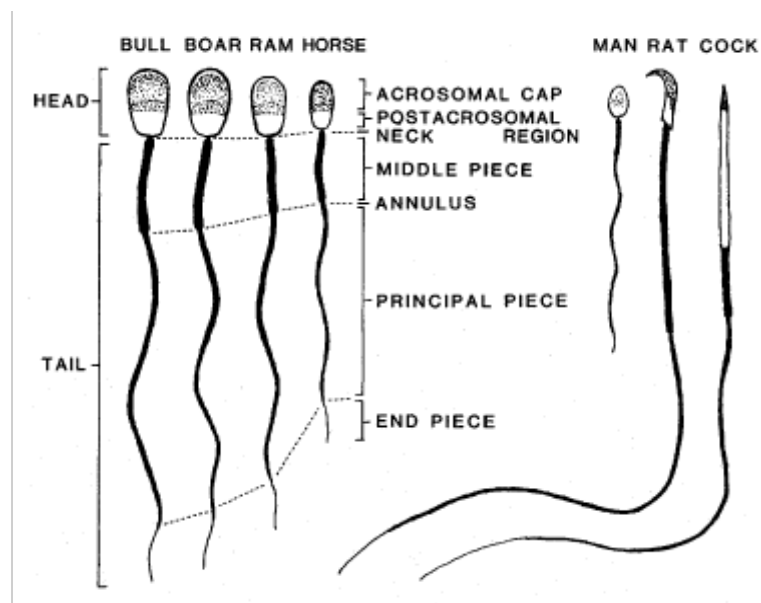
Figure 2.9 Comparison of spermatozoa from farm animals and other vertebrates (Hafez and Hafez, 2008).

2.5 Collection of semen samples

The first important step in cryopreservation of semen is the method of semen collection, and is known to affect post-thaw quality of spermatozoa (Jiménez-Rabadán *et al.*, 2016). In small ruminants, the use of an artificial vagina (AV) or electro-ejaculation (EE) are the two most common methods of semen collection (Hafez and Hafez, 2008; Jiménez-Rabadán *et al.*, 2016). With good management and feeding practices, semen can be collected from bulls at 12 months of age, rams, goats and boars at 12 months and stallions at 24 months (Hafez and Hafez, 2008).

2.5.1 Ejaculate collection

The use of an AV is most often the preferred technique for semen collection, but requires that males undergo training to be able to ejaculate in the AV (Hafez and Hafez, 2008; Jiménez-Rabadán *et al.*, 2016) Thus, when males were not trained to ejaculate in an AV, electro-ejaculation is a safe and repeatable alternative method for semen collection (Jiménez-Rabadán *et al.*, 2016).

Electro-ejaculation is done by placing a lubricated electrical bipolar probe, connected to a power source, in the rectum of the male, with the electrodes facing the ventral region of the animal (Marco-Jiménez *et al.*, 2005; Hafez and Hafez, 2008). Low voltage electric stimuli are then applied for a specific time interval (2-4s), with a certain rest period in between stimuli (10-20s), and with the voltage generally being increased by 1 volt per stimulus until the male ejaculates (Marco-Jiménez *et al.*, 2005; Hafez and Hafez, 2008). The increasing voltage should however be limited. A graduated collection vial is used for the collection of semen (Marco-Jiménez *et al.*, 2005).

When using an AV for semen collection, rams are penned with oestrual ewes, with an experienced handler ready for collection with an AV (Marco-Jiménez *et al.*, 2005). Artificial vaginas are made of a 20-25cm hose with a 5-7cm diameter and is lined with rubber (Hafez and Hafez, 2008). It is important to lubricate this lining during semen collection (Hafez and Hafez, 2008). The AV has water in its lining and should have a temperature between 40 to 44˚C at the time of semen collection (Marco-Jiménez *et al.*, 2005). The collection vial should be heated to 37˚C to avoid cold shock and after ejaculation the vial is placed in a waterbath at 30˚C (Hafez and Hafez, 2008).

With electro-ejaculation, Marco-Jiménez *et al.* (2005) reported a lower recovery efficiency compared to an AV, as the samples are often contiminated with urine or rams fail to respond to the electrical stimulus. The collection methods (AV vs. EE) did not show significant differences for fresh semen quality. There was however a significant difference between the two methods for sperm concentration, but the amount of spermatozoa present in the samples did not differ between the methods and thus, possible seminal doses for AI were similar (Marco-Jiménez *et al.*, 2005). Electro-ejaculation also resulted in more stable and functional spermatozoa for frozen-thawed cryopreservation than AV did (Marco-Jiménez *et al.*, 2005).

Ejaculates have higher volumes and lower concentration when collected by EE compared with an AV, which could be attributed to the electrical stimulis causing excessive secretion by the accessory sex glands (Mattner and Voglmayr, 1962; Jiménez-Rabadán *et al.*, 2012; Jiménez-Rabadán *et al.*, 2016). According to the study of Jiménez-Rabadán *et al.* (2012), the spermatozoa that were collected with an

AV had a higher resistance to cold shock compared to those collected by EE, whereas the study of Marco-Jiménez *et al.* (2005) shows that spermatozoa obtained by EE had higher resistance to cold shock than those collected with an AV. Electro-ejaculation could thus change the secretory function of the accessory sex glands which would result in a change in the total volume and chemical composition of the seminal plasma (Jiménez-Rabadán *et al.*, 2012). Various factors are able to affect the stimulation and secretion of the accessory sex glands and include age, season, previous management of rams and the specific EE protocol that is followed (Marco-Jiménez *et al*., 2008). The ejaculate volume will also decline if collection of semen takes place more than three times per day for several days in a row (Hafez and Hafez, 2008).

2.5.2 Epididymal collection

The post-mortem collection of spermatozoa from genetically superior animals that have suddenly died is a valuable technique for the preservation of viable spermatozoa from the epididymis as well as for the genetic contribution of these animals after their death, that would otherwise have been lost (Kaabi *et al.*, 2003; García-Álvarez *et al.*, 2009). Besides animals dying unexpectedly, a rapid and cheap alternative way of preserving valuable genetic stock and maintaining genetic variability in livestock is thus to collect epididymal spermatozoa from slaughtered animals (Ehling *et al.*, 2006).

Epididymal sperm collection is performed by removing the testes from the scrotal sac, followed by the removal of the parietal tunic, and then making several incisions on the cauda epididymis with a surgical blade (Blash *et al.*, 2000; García-Álvarez *et al.*, 2009). Slight pressure is then applied to the cauda epididymis to let the spermatozoa form droplets, which are then collected and pipetted into an extender (Blash *et al.*, 2000; García-Álvarez *et al.*, 2009).

Epididymal spermatozoa are fully capable of fertilizing ova and allow for normal embryo and fetal development (Ehling *et al.*, 2006). The study of García-Álvarez *et al.* (2009) showed that with fresh sperm samples, electroejaculated and epididymal spermatozoa were of similar quality, with viability however, being higher for epididymal spermatozoa. The study of Kaabi *et al.* (2003) found that there was a significant decrease in the quality parameters, total motility, progressive motility and normal spermatozoa, when the epididymis was stored at 5˚C for 48h in comparison with 24h, with these parameters not showing significant variations during the first 24h. Varisli *et al.* (2009) compared epididymal spermatozoa and electro-ejaculated spermatozoa with regards to their sensitivity towards cryobiologically relevant stressors such as chilling, osmotic stress and CPA (cryoprotective agents) addition and removal, and found that epididymal spermatozoa are more resistant. Lone *et al.* (2011) found that the epididymis can be transported at both refrigeration temperature and at ambient

temperature for collection and preservation. The sperm quality, motility and liveability did not difffer significantly between the two methods as the results from both were higher than the minimum acceptable limit. A drastic deterioration in quality was however observed as time progressed for preservation at ambient temperature in comaprison with refrigeration temperature.

2.6 Processing of semen samples

The evaluation of semen is a useful tool that allows for the selection of males and ejaculates that can be used for assisted reproduction (Yániz *et al.*, 2012). The fertility of a sperm sample can however not yet be accurately determined by a single test and various physical characteristics should be assessed to achieve the most precise fertility potential (Hafez and Hafez, 2008).

2.6.1 Concentration

The concentration of spermatozoa in a sample can be measured with a haemocytometer, spectrophotometer (Memon *et al*., 2006; Hafez and Hafez, 2008), calorimeter (Hafez and Hafez, 2008) and a commercial Unopette (Memon *et al*., 2006; Edmondson *et al.*, 2012). A haemocytometer is a microscope slide that has precisely scored chambers that are used to count the number of spermatozoa within each chamber, and therefore takes a lot of time (Hafez and Hafez, 2008). This method is however very accurate in determining sperm concentration (Hafez and Hafez, 2008). The spectrophotometer and calorimeter allow for determining sperm concentration fast while still being precise, but they must be calibrated with a haemocytometer (Hafez and Hafez, 2008). Sperm concentration is given as the number of spermatozoa per milliliter (mL) and total number of spermatozoa per ejaculate and generally range from $3.5 \times 10^9 - 6.0 \times 10^9$ spermatozoa/mL for rams (Memon *et al*., 2006; Hafez and Hafez, 2008).

2.6.2 Macroscopic evaluation of semen

When semen is collected from rams the semen samples are immediately placed in a warm-water bath with a temperature of 37˚C, after which it is evaluated (Edmondson *et al.*, 2012). The main aspects that are macroscopically evaluated include the following: volume, consistency, colour, mass motility, and foreign material present in the ejaculate (Edmondson *et al.*, 2012). The samples are directly evaluated with the naked eye with only mass motility requiring the use of a microscope.

2.6.2.1 Volume

Graduated collection vials are used to directly measure the volume of semen produced during an ejaculation (Memon *et al.*, 2006; Edmondson *et al.*, 2012). It is however said that the evaluation of the volume of semen produced is of more value for semen collected via an AV than from electro-ejaculation (Edmondson *et al.*, 2012). The volume of the ejaculate is affected by a few factors, namely: ram age, ram condition, season, skill of collector and collection frequency (Hafez and Hafez, 2008). Mature rams have ejaculate volumes of 0.5 – 2.0mL and young rams 0.5 – 0.7mL (Hafez and Hafez, 2008).

2.6.2.2 Colour

Semen usually has a whey-like to milky-white to creamy colour and depends on the concentration of spermatozoa (number of spermatozoa per mL) (Edmondson *et al.*, 2012). When sperm samples exhibit any other colour than this, it indicates that the sample is inferior and that there are potential problems associated with the reproductive system of the ram, for instance; when the semen is the normal colour but with tinges of pink (blood contamination, possibly due to injury of the penis during collection), brown or grey (reproductive tract infection/contamination) and, yellow and dilute with a strong odour (urine contamination, which often happens when an electro-ejaculator is used) (Hafez and Hafez, 2008; Edmondson *et al.*, 2012). Samples that are contaminated must be discarded (Hafez and Hafez, 2008).

2.6.2.3 Mass motility

Mass motility, also known as gross motility, is evaluated by taking a small droplet of fresh, undiluted semen and placing it on a glass slide (without a coverslip) that was pre-warmed to 37˚C, and is then observed through a 10x phase contrast objective with magnification of x100 (Memon *et al*., 2006). The vigour of the wave is estimated and graded accordingly as: very good (++++, vigorous swirls), good (+++, slow swirls), fair (++, no swirls, but generalized oscillation), or poor (+, sporadic swirls) (Memon *et al.*, 2006).

2.6.3. Microscopic evaluation of semen

Light microscopy is not a popular method for the analyses of sperm morphology due to its limited magnification, whereas scanning and/or electron microscopy (SEM/TEM) can detect subtle abnormalities in spermatozoa (Hafez and Hafez, 2008). These methods are expensive, but SEM allows

for viewing of the spermatozoa in its three-dimensional form, while TEM allows for cross sectional viewing that reveals ultrastructural detail (Hafez and Hafez, 2008).

2.6.3.1 Sperm viability

A valuable measure of sperm quality is to assess the plasma membrane integrity (viability) of sperm (Yániz *et al.*, 2008). It is of vital importance that sperm have an intact and functional plasmalemma as sperm cannot survive in the female reproductive tract, bind to the zona pellucida and vitelline membrane if the sperm plasmalemma is damaged (Martí *et al.*, 2003). Plasma membrane integrity can be measured by use of several different methods. These methods include, measuring how permeable the membrane is to water in a hypo osmotic solution (hypo-osmotic swelling test, HOST) (Revell and Mrode, 1994), by stains (eosin-nigrosin method), or by fluorescent probes (Hoescht, or propidium-iodide (PI), alone or combined with the permeable fluorochromes CFDA or SYBR) (Garner *et al.*, 1986; Harrison and Vickers, 1990; de Leeuw *et al*., 1991; Garner and Johnson, 1995; Pintado *et al*., 2000).

2.6.3.2 Sperm motility

The evaluation of sperm motility is done before cryopreservation and after thawing (Hafez and Hafez, 2008). Mammalian spermatozoa generally showcase two different types of physiological motility. The first is motility found in fresh ejaculated semen samples, and second, the motility we observe in spermatozoa recovered from the site of fertilisation, respectively known as activated motility and hyperactivated motility (Katz and Yanagimachi, 1980; Suárez and Osman, 1987). With activated spermatozoa the flagellum allows movement of the spermatozoa in a relatively straight line by generating a symmetrical lower-amplitude waveform, given that the extender is not too viscous (Turner, 2006). Once spermatozoa reach a certain point in the oviduct, they become hyperactivated, which is when the flagellar beat changes to one that is asymmetric and of higher amplitude (Turner, 2006). This results in the spermatozoa to move in a figure-eight-like pattern in the extender (Turner, 2006).

A microscope with a built-in stage warmer and phase-contrast optics, generally with 200X or 400X magnification, is used to observe a microscope slide with a drop of extended semen smeared with another slide for motility (Hafez and Hafez, 2008). Parameters of motility include the percentage of motile spermatozoa (normal is 70 – 90% motile), percentage of progressively motile spermatozoa, sperm velocity (0, stationary to 4, fast), and longevity of sperm motility in raw semen at room temperature (20-25˚C), or at refrigerated temperature (4-6 ˚C) (Hafez and Hafez, 2008).

2.6.3.3 Sperm morphology

Spermatozoa are morphologically the most diverse cell type in the animal kingdom (Vicente-Fiel *et al.*, 2013). One of the most meaningful measures of determining the quality of spermatozoa is through the assessment of sperm morphology, which allows for a good prediction of the male's fertility potential (Martí *et al.*, 2012). All semen samples have some abnormal spermatozoa, and it is generally accepted that samples with abnormalities higher than 15% should not be used for AI (Hafez and Hafez, 2008).

The spermatozoon head is the provider of either an X or a Y chromosome and will determine the genetic sex of the offspring at fertilization in the oviduct (Hafez and Hafez, 2008). The neck is roughly 1µm in length and its anterior region attaches to the basal plate and the posterior region to the outer dense fibres of the flagellum, thus linking the spermatozoon head and flagellum (Pesch and Bergmann, 2006). The tail makes up the longest part of the spermatozoon and consists of a midpiece, principal piece and end piece (Pesch and Bergmann, 2006).

Visual observations have led to subjective assessment of sperm morphology. This causes results to vary substantially intra- and inter-laboratories and for technicians, thus making accurate data interpretation difficult and emphasising the need for objective, precise and repeatable techniques (Hidalgo *et al.*, 2006). The perfect method to analyse the morphometry of sperm heads is one that is automatic, consistent, accurate, usable in different sperm presentations, such as wet mounts and smears, and that is inexpensive (Yániz *et al.*, 2012).

To limit the subjectivity associated with the assessment of sperm morphology, computer-assisted morphometry analysis (CASMA) systems were developed (Yániz *et al.*, 2013). Standardization of morphometric evaluation when using CASMA is however only possible to a certain extent as various factors could affect the morphometric traits of the spermatozoa being analysed. These factors include, sample preparation, fixation, staining of spermatozoa, and the settings of the analyser, to list a few (Vicente-Fiel *et al.*, 2013). This still makes direct comparison of results from studies between different laboratories difficult (Vicente-Fiel *et al.*, 2013). Besides the many different and new methods used for sperm analyses, semen smears remain as a popular and routinely used method for morphological evaluation by light microscopy (Pesch and Bergmann, 2006).

Eosin-nigrosin is a stain that is used to assess sperm morphology, usually with a high magnification (400x) where 150 spermatozoa are examined and then classified into various categories (Hafez and

Hafez, 2008). These categories include several abnormalities associated with the sperm head, midpiece, and tail, respectively.

2.6.3.4 Sperm morphometry

Computer-Assisted Morphometry Analysis Fluorescence was recently developed and is an automated system with open-access software that evaluates sperm nuclear morphometry and combines fluorescence microscopy and image analysis that reduces factors that can potentially affect morphometric results (Yániz *et al.*, 2012). In the study of Yániz *et al.*, (2014), CASMA-F was used to quantify the different morphometric dimensions of the spermatozoon head, nucleus and acrosome in rams. This study was the first to separately analyse the morphometry of the different components of the spermatozoon head, the acrosome and nucleus.

2.6.3.5 Acrosome integrity

The acrosome is essential for spermatozoon function. Therefore, it is important that the acrosome integrity is evaluated, as successful fertilization relies on spermatozoa that are motile, capacitated, and that develop the acrosome reaction for the penetration of the zona pellucida of the oocyte (Yániz *et al.*, 2013). Spermatozoa with intact acrosomes are classified as those where the acrosomal membrane is completely smooth, perfect in shape, with no uneven dark spots or light patches along the edges, and no edges lifting where the acrosome reaches the post-acrosomal region.

2.7 Semen storage

The goal of semen preservation is to achieve pregnancies after AI as close to those obtained through natural mating (Hafez and Hafez, 2008). Extensive studies on semen storage started as the location of semen collection and AI are often large distances from one another. This problem occurs as semen is collected from rams on one farm and must then be transported to distant farms where the ewes are kept (Salamon and Maxwell, 2000). Contributing factors are the desire to use rams over extended periods of time and at different times of the year (Salamon and Maxwell, 2000). Methods that reduce or arrest the metabolism of spermatozoa and thus ultimately prolonging the fertile life of the spermatozoa would make these desires possible (Salamon and Maxwell, 2000; Hollinshead *et al.*, 2004).

The two most studied semen storage methods are liquid and frozen storage. A third method has also been receiving more attention in recent years and is the method of semen storage in a solid state. It

is important that *in vitro* handling aims at mimicking the signals and protective aspects as close as possible to those of the *in vivo* environment (Leahy *et al.*, 2019). This allows the spermatozoon to maintain form and function, as semen processing can cause fluctuations of temperature, pressure, osmolarity and pH, that could be harmful to the sperm plasma membrane, therefore limiting the fertilising lifespan of the processed spermatozoa (Leahy *et al.*, 2019).

2.7.1 Short-term storage

With short-term storage, semen is stored in the liquid state, and two main methods are generally being used and include storage at reduced (0 – 5˚C or 10 – 15˚C), and at ambient temperatures by reversible inactivation of spermatozoa (Salamon and Maxwell, 2000). Paulenz *et al.* (2002) found that the viability parameters studied for semen stored at 5˚C were less influenced than those of semen stored at 20˚C. Given that AI takes place under farming conditions within a short time since semen collection, liquid storage is a good alternative for frozen storage as it is practical, inexpensive, and can be used for cervical insemination (Mara *et al.*, 2005; Kasimanickam *et al.*, 2007; Falchi *et al.*, 2018). This method of semen storage is therefore often used when several farmers in a community make use of the same ram for breeding (Baldassarre and Karatzas, 2004).

With cervical insemination, liquid storage has the limitation that fertility is reduced when the semen is stored for periods longer than 24 hours (Maxwell and Watson, 1996). It is commonly known that the quality of spermatozoa decreases as the storage time increases, and happens irrespectively of the type of diluent, dilution rate, conditions or temperature of storage (Salamon and Maxwell, 2000; Falchi *et al.*, 2018). While spermatozoa are being stored, motility and morphological integrity becomes reduced, with a subsequent decrease in survival within the female reproductive tract, reduced fertility and increased embryonic loss (Paulenz *et al.*, 2002). This reduction in sperm quality and functionality is largely attributed to the fact that the spermatozoa accumulate toxic products, called reactive oxygen species (ROS), when the sperm membranes undergo lipid peroxidation (Salamon and Maxwell, 2000). Liquid extenders are also known to cause cell sedimentation, whereas spermatozoa extended in a more solid form are more homogeneously spread, therefore allowing the contents in the extender to fulfil their role as energy source, buffer, or protecting agent (Gheller *et al.*, 2018).

The processes of capacitation, hyperactivation, and the acrosome reaction do however require ROS in physiological amounts (Falchi *et al.*, 2018). When there is an imbalance in the production of ROS and the intrinsic scavenger methods that counteract them, movement and survival in the female reproductive tract becomes difficult and led to reduced fertilizing ability and increased embryonic loss

(Salamon and Maxwell, 2000; Paulenz *et al.*, 2002; Falchi *et al.*, 2018). The spermatozoa of mammals contain high amounts of polyunsaturated fatty acids (Hashem and Eslami, 2018). The sperm membranes from small ruminants have even higher ratios of unsaturated to saturated fatty acids than other species (Hashem and Eslami, 2018). These higher ratios make their susceptibility to peroxidative damage higher, and consequently cause a reduction in membrane integrity, impaired cell function, and reduced motility and fertilising ability (Alvarez and Storey, 1995).

2.7.2 Long-term storage

With long-term storage, semen is stored in a frozen state, thus being preserved at sub-zero temperatures (Salamon and Maxwell, 2000). According to records, Bernstein and Petropavlovsky (1937) were the first people to successfully store mammalian (stallion, bull, boar, ram, rabbit and guinea pig) and avian (fowl and duck) spermatozoa and have done so using a glycerol solution (1 M, 9.2%) stored at -21˚C (Salamon and Maxwell, 2000). At first, the same method and diluent for freezing of bull semen was used for freezing ram semen, but fertility results were extremely poor and therefore, a more ram-specific method for freezing semen was needed (Salamon and Maxwell, 2000). By using frozen semen in AI programs, genetic material can be spread internationally, semen can be used in both the reproductive and non-reproductive seasons, and genetically superior males are able to remain productive even after their death (Baldassarre and Karatzas, 2004; Rahman *et al*., 2008; Ogbuewu *et al*., 2010).

When semen is frozen and thawed, the spermatozoa become injured in these processes and there is thus a reduction in fertility when compared to refrigerated storage (Kasimanickam *et al.*, 2007). Ice forms inside the cells when dilute aqueous solutions are frozen and the ice consists purely out of crystalline water as the ice has a very poor ability to dissolve solutes (Pegg, 2002). Extracellular ice crystals are formed when the cell suspension is slowly cooled to below 0˚C which causes the solutes to be highly concentrated in the much smaller volume of liquid water that remains (Hafez and Hafez, 2008). A very important aspect of cooling semen for preservation is the rate at which semen is cooled through the critical temperature range (around -5 ˚C to -50˚C), as this can be defined as the range when ice crystal formation and consequent cell dehydration occurs (Kumar *et al.*, 2003). The rate of cooling these spermatozoa will determine whether they remain in equilibrium with their extracellular environment or if they will be progressively supercooled, which increases the potential of intracellular ice formation (Kumar *et al.*, 2003). A protocol that is often recommended for freezing semen, is where minimal concentrations of glycerol is used that still allows for preservation of fertilising ability, with a cooling rate of -30˚C/min from -5 ˚C to -50˚C (Kumar *et al.*, 2003).

The two-factor hypothesis of Mazur *et al.* (1972) says that when cells are cooled rapidly, intracellular ice is formed which is then followed by recrystallization when they are warmed up again, causing cell death to occur (usually with slow warming). Factor two is that cells that are cooled slowly, die because they are exposed to major alterations produced in extra- and intracellular solutions when water is transformed to ice over longer periods of time. These alterations or solution effects include a changing pH, dehydration, the concentration of solutes and precipitation thereof (Mazur *et al.*, 1972). According to Fiser *et al*. (1986), the rate at which the semen samples are frozen at must be slow enough to allow water to leave the cells by osmosis, but fast enough so that extracellular water freeze, as the formation of intracellular ice causes irreversible damage to the spermatozoa.

2.7.3 Solid storage

Solid storage of semen was first practiced in the first half of the 20th century by workers from the former Soviet Union by the supplementation of gelatine to the semen extender so that the viscosity would be increased and sperm motility would be reduced (Yániz *et al.*, 2005). The study of Yániz *et al.* (2005), found that storage of ram semen in the solid state at 15˚C with the supplementation of gelatin to the extender improves motility, viability (membrane integrity) and the capicity to penetrate lamb oocytes that were matured *in vitro*. Motility and acrosome integrity was also improved in the study of Gheller *et al.* (2018) when semen was stored at 5˚C for 72h and thus allows for storage over longer intervals, as semen is generally not stored for longer than 48h.

For both, short- and long-term storage of spermatozoa, semen collection, transport, processing, storage, and the preparation of semen for use, from refrigerated, or frozen conditions, all make semen samples susceptible to deviations from the temperatures required for the maintenance of high quality. The level of deviation in the required temperatures is however not the only determining factor that negatively affects sperm quality during these porcesses, but so does the time that the spermatozoa experience the deviations.

2.8 Functional tests

An effective way of studying how resilient spermatozoa is to the deviations in the required temperatures of the various aspects of processing, is to make use of functional tests. Functional tests are used to determine whether spermatozoa possess the biological capability to perform the needed steps to reach and fertilize the ova, and to ultimately result in the birth of offspring (Vasan, 2011).

2.8.1 Computer Assisted Sperm Analysis

Computer Assisted Sperm Morphometry Analysis (CASMA) systems provide a series of objective parameters that facilitate the standardization of morphological semen evaluation, thus also reducing the subjectivity thereof (Yániz *et al.*, 2012; Vicente-Fiel *et al.*, 2013; Yániz *et al.*, 2013; Yániz *et al.*, 2014). These systems originally focussed on human semen but were then adapted for use on animal species too (Yániz *et al.*, 2013). Various factors such as sample preparation, fixation, staining of spermatozoa, and settings of the sperm morphometry analyser, could however affect the morphometric traits of the spermatozoa while using CASMA, consequently making comparison between laboratories and in different studies with the same species difficult (Vicente-Fiel *et al.*, 2013). Computer-Assisted Sperm Analysis systems also allow for population studies of the sperm kinematics as the movement characteristics of several hundreds of spermatozoa could automatically be determined within minutes (Mortimer, 1997).

2.8.2 Flow cytometry

A flow cytometer is an instrument that can measure the physical and multicolour fluorescence properties of particles of cells that flow in a flowing stream (Hossain *et al.*, 2011). Flow cytometers consist of four main systems. The first three include fluidics, optics, and software handling. Lastly, the cell suspension flows through a tubular system while being exposed to laser illumination on specific areas on the cells and then records these emissions from the cells that are illuminated, and digitally stores and processes it on a computer to provide understandable results (Hossain *et al.*, 2011).

The two main types of flow cytometers are analytical or sorter. Analytical flow cytometers are exclusively used to analyse cells or other particles, whereas sorter flow cytometers have the same function, but can also physically isolate the cells or particles of interest based on the results obtained from the analysis (Hossain *et al.*, 2011).

2.8.3 Hypo-Osmotic Swelling Test (HOST)

The hypo-osmotic swelling test is a test that assesses the functional integrity of the spermatozoon membrane and the test score is the proportion of spermatozoa that have functionally intact membranes (Buckett *et al.*, 1997). When the spermatozoa are incubated in a hypo-osmotic solution, spermatozoa with intact membranes experience an influx of the hypo osmotic solution, causing the cytoplasmic space to swell, which results in the spermatozoon tail curling (Casper *et al.*, 1996; Buckett *et al.*, 1997). It is easy to identify these changes under a light microscope and the spermatozoa that have damaged or chemically inactive plasma membranes do not show any swelling or curled tails

(Casper *et al.*, 1996). This test is simple, repeatable, cost effective, and non-invasive (Hossain *et al.*, 1998; Tartagni *et al.*, 2002; Stanger *et al.*, 2010).

2.8.4 Perivitelline membrane binding assay

Another way of predicting the fertilising capacity of a semen sample is by doing a perivitelline membrane binding assay. Bird eggs have a vitelline envelope that consists of two layers that differ with regards to structure and composition (Monné *et al.*, 2006). These layers are separated by a thin and continuous membrane (1- 3.5μm thick) called the inner or perivitelline membrane that consists of a network of fibers (Monné *et al.*, 2006). The glycoproteins of the chicken egg perivitelline membrane has been found to be homologous to the zona pellucida glycoprotein 3 (ZP3) of mammals, known as chicken ZPC (chZPC) in hens, that allows spermatozoa from several mammalian species to bind to it (Waclawek *et al.*, 1998; Bausek *et al*. 2000). Zona pellucida glycoprotein 3 is the glycoprotein that is responsible for inducing the acrosome reaction which is when the spermatozoon is able to penetrate the zona pellucida and bind to the secondary receptor, ZP2 (Monné *et al.*, 2006).

2.8.5 Hyperactivation

For successful fertilization to take place in the female reproductive tract, mammalian spermatozoa must undergo the changes associated with capacitation and subsequent hyperactivation (Arai *et al.*, 2019). Hyperactivation specifically occurs within the oviduct and is characterised by its vigorous flagellar beating (Chang and Suarez, 2011). Hyperactivation is needed for spermatozoa to detach from the epithelium of the oviduct so that they can escape from oviductal storage reservoir (Chang and Suarez, 2011). Spermatozoa generate a much more powerful thrusting force that allows them to swim through the mucus in the oviducts and to penetrate the viscoelastic cumulus matrix that surrounds the oocyte (Chang and Suarez, 2011). Spermatozoa must also be hyperactivated for penetration of the zona pellucida of the oocyte, and then to reach and ultimately fuse with the plasma membrane of the oocyte (Chang and Suarez, 2011).

For spermatozoa to fertilize an oocyte, they must undergo several functional changes, and therefore, it is required that sperm functional tests are performed to accurately determine abnormalities in the fertilizing ability of the spermatozoa (Mortimer and Maxwell, 1999). Hyperactivation can be applied as a functional test in mammalian spermatozoa by hyperactivating spermatozoa in *in vitro* experiments by simple incubation in capacitation mediums, and then determining the proportion of spermatozoa that showcase hyperactivated motility (Mortimer and Maxwell, 1999; Arai *et al.*, 2019).

2.9 Standard cryopreservation protocols

2.9.1 Dilution

To be able to store semen at reduced temperatures it must be extended in special diluents (Salamon and Maxwell, 1995a). Diluents, also known as extenders, are generally empirically formulated and allows the semen to be protected and maintained during the processing and storage thereof (Paulenz *et al.*, 2002). Diluents used to preserve semen from any species should have a suitable osmolarity, pH, buffering capacity, and must have the ability to protect spermatozoa from cryogenic injury (Salamon and Maxwell, 1995a; Salamon and Maxwell, 2000). It is very important that any new methods of semen processing are tested prior to being applied in practice (Paulenz *et al.*, 2002).

One of the first diluents to be used for ram semen was a citrate buffer combined with sugar as it proved successful for freezing bull semen (Salamon and Maxwell, 1995a). This adaptation however does not work well for ram semen and had to be adjusted to be more suitable for freezing ram semen (Salamon and Maxwell, 1995a). Investigators originally had contrasting opinions on the best suited sugar for the citrate medium, and as glycerol causes a reduction in the osmotic pressure in the extender, hypertonic citrate-glucose-yolk or citrate-fructose-yolk diluents with an osmotic pressure of 8 – 12 atm were generally used (Salamon and Maxwell, 2000).

Milk extenders, with skim, whole, or reconstituted milk, have been used for ram semen for many years and is an isotonic medium that contains several components that are favourable to maintain sperm viability (Salamon and Maxwell, 1995a; Salamon and Maxwell, 2000). Milk as a diluent is successful due to the protein fraction thereof that acts as both a buffer against pH changes, and as a chelating agent against heavy metals that are present (Salamon and Maxwell, 2000a). It may also have protecting abilities at reduced storage temperatures (Salamon and Maxwell, 2000). It is important that the whole, skim, or reconstituted milk is heated to 92 – 95˚C for 8 – 10 min so that the lactenin in the protein fraction can be inactivated as it is toxic to spermatozoa (Salamon and Maxwell, 2000).

Saccharose-, lactose-, raffinose- and tris-based diluents have all been studied comprehensively and act as good diluents (Salamon and Maxwell, 1995a; Salamon and Maxwell, 2000). One of the most used and highly recommended diluents is the tris-based diluent, known as tris-glucose (Salamon and Maxwell, 1995a). It is commonly known that ram spermatozoa have a reduced viability following cryopreservation, which can largely be attributed to its chilling sensitivity and therefore demands the need for protective compounds to be added to extenders (Falchi *et al.*, 2018).

The two most commonly used protective substances for freezing ram spermatozoa are egg yolk and glycerol, with glycerol being able to penetrate cells and has been the most favoured protective substance of the two (Salamon and Maxwell, 1995a). Glycerol protects spermatozoa during freezing as it has colligative or water-binding properties. Glycerol also acts as a diluent and can reduce the osmotic pressure of the freezing medium by lowering the ability of the salts to dissociate (Salamon and Maxwell, 1995a). The addition of glycerol may however play its part in the damage caused by freezing and thawing (Fahy, 1986). Addition of glycerol to the semen can be done in two ways, one being in a separate diluent fraction where glycerol is added at $2 - 5°C$, known as two-step dilution, or by adding the diluent containing glycerol at once at $30°C$, known as the one-step method (Salamon and Maxwell, 1995a; Salamon and Maxwell, 2000). The latter is the most used method for dilution of ram semen for frozen storage, with both methods requiring a cooling period to $2-5°C$ varying between $1 – 3$ hours (Salamon and Maxwell, 1995a; Salamon and Maxwell, 2000).

Egg yolk preserves the motility and integrity of acrosomal and mitochondrial membranes of spermatozoa, thus protecting the cells against cold shock and damage caused by freezing and thawing (Salamon and Maxwell, 1995a; Salamon and Maxwell, 2000). According to Salamon and Lightfoot (1969), a 15% egg yolk concentration is optimal for pellet freezing semen, but it depends on the diluent composition, and for freezing semen in ampoules a $3 – 6\%$ concentration seems sufficient (Salamon and Maxwell, 2000).

Fructose is the only simple carbohydrate present in ram semen, but when the sugars glucose and mannose are added to extenders, spermatozoa are also able to metabolise them (Salamon and Maxwell, 2000). These are the only sugars that act as energy sources during sperm storage (Salamon and Maxwell, 2000).

2.9.2 Equilibration

Once the semen has been diluted it is cooled to a temperature close to $0°C$ and allows the spermatozoa to adapt to a slower metabolism before it is frozen (Salamon and Maxwell, 2000a). Equilibration refers to the total time that the spermatozoa are in contact with the cryodiluent before it freezes and is the period in which the cryoprotective agent enters the spermatozoon so that the intracellular and extracellular environment can be balanced (Salamon and Maxwell, 1995a; Salamon and Maxwell, 2000). It is however not only the intracellular and extracellular concentration of the cryoprotective agent that should be balanced, but also that of other osmotically active components in the diluent (Salamon and Maxwell, 1995a; Salamon and Maxwell, 2000).

2.9.3 Cryopreservation protocols

When spermatozoa are frozen and later thawed for use, the decrease and increase in temperature in both processes respectively, causes spermatozoa to become less motile, and ultrastructural, biochemical and functional damage is observed (Salamon and Maxwell, 1995b; Salamon and Maxwell, 2000). Ultrastructural damage refers to the damage that is caused to the plasma and acrosomal membranes, the acrosome, mitochondrial sheath and axoneme (Salamon and Maxwell, 2000). Freezing ram semen in ampoules is seldomly used today and freezing has shifted to PVC straws and minitubes, or to the pellet form (Salamon and Maxwell, 2000).

When semen is frozen in straws, the straws are suspended in the vapour from liquid nitrogen in a liquid nitrogen tank and the cooling velocity can be regulated by keeping the straws varying distances from the liquid nitrogen in the tank (Salamon and Maxwell, 1995a; Salamon and Maxwell, 2000). The best distance for cooling is at 4 – 6cm above the liquid nitrogen which allows the straws to cool down in a parabole shaped curve instead of linearly (Salamon and Maxwell, 2000). With pellet-freezing the droplets of semen are generally placed into depressions on dry ice or on a polymer plate that is cooled to between -80 ˚C and -95˚C (Salamon and Maxwell, 2000). For pellet freezing the cooling rate is determined by the volume of the semen as well as the temperature of the freezing agent (Salamon and Maxwell, 2000).

2.9.4 Thawing protocols

Spermatozoa that are to be used after frozen storage has to go through the critical temperature zone ($-15˚C$ to $-60˚C$) twice, as it must firstly survive cooling to $-196˚C$ and then be warmed up and thawed for use (Salamon and Maxwell, 1995a). The cooling rate through the critical temperature range is of utmost importance as this is the period when ice crystals are formed, and cell dehydration occurs (Kumar *et al.*, 2003). This rate determines whether the spermatozoa remain in equilibrium with their extracellular environment or become progressively supercooled, increasing the possibility that intracellular ice crystals will be formed (Kumar *et al.*, 2003). After spermatozoa have been frozen and thawed, about 40-60% of spermatozoa maintain their motility, but only 20-30% are undamaged, meaning that the spermatozoa may be motile, but the level of damage to the spermatozoa may prevent successful fertilization (Salamon and Maxwell, 1995b; Salamon and Maxwell, 2000). It has been found that ram spermatozoa are more susceptible to damage during freezing and thawing than spermatozoa from bulls (Salamon and Maxwell, 1995b). When semen straws are thawed, a temperature between 38˚C and 42˚C is generally used (Salamon and Maxwell, 1995a; Salamon and Maxwell, 2000).

2.10 Factors affecting sperm quality in rams

Various factors can exert an influence on the quality of spermatozoa of rams, and include amongst others, age, season, temperature, breed, as well as individual differences between animals of the same breed (Memon *et al*., 2006). There are various potential causes of testicular degeneration in rams and include factors such as advancing age, nutritional disorders, heat, stress/corticosteroid therapy, trauma, radiation, ultrasound, epididymitis, viral infection, neoplasia, and certain chemicals, plants and hormones (Foster, 2016) .

2.10.1 Hormones

As sheep are seasonal breeders, the photoperiod is important in controlling their seasonal reproductive pattern. The influence of season on sexual activity in rams can be observed through changes in the quality of semen, testicular diameter, as well as the level of hormone secretion (Aller *et al.*, 2012). Seasonal changes cause varying levels of testosterone secretion that regulate spermatogenesis, epididymal function, and the composition of the seminal plasma (Flores-Gil *et al.*, 2020). The change in testicular size is related to the change in seasons, which is influenced by the concentration of luteinizing hormone (LH), which is determined by the frequency of LH pulses (Aller *et al.*, 2012). According to D'Alessandro and Martemucci (2003), the seasonal variation observed in semen cryo-resistance might be due to high testosterone concentrations that modify the functional and/or morphological properties of spermatozoa, and therefore impacting their level of sensitivity to freezing and thawing.

Any condition that would negatively influence the release of gonadotropin-releasing hormone (GnRH), would have a negative effect on the quality of spermatozoa produced. This is because GnRH stimulates the secretion of luteinizing hormone (LH) and follicle stimulating (FSH) from the pituitary, that respectively, play major roles in the function of the Leydig cells and spermatogenesis (de Kretser and Fracp, 1979). Other hormones that can cause testicular degeneration include dexamethasone, estrogen, and zearalenone (Foster, 2016).

2.10.2 Genetics

In the study of Vicente-Fiel *et al.* (2014), rams previously classified as high or low fertility were used to see whether their fertility status was reflected in differences for nuclear sperm morphometry, the basal rates of sperm DNA fragmentation, spermatozoa viability and motility. An increase in sperm viability, motility, sperm nuclear size and a reduction in DNA fragmentation at an incubation temperature of 37°C was observed for the high fertility group of rams. This makes it possible for

genetically superior rams to be selected for AI based on the assessment of the quality of their spermatozoa.

2.10.3 Breed

Cruz Júnior *et al.* (2015) studied the effects of testicular insulation (heat stress) on two local Brazilian sheep breeds and four exotic sheep breeds in a tropical environment. During the pre-insulation phase, no differences were found for sperm concentration and motility between the breeds. A decrease in sperm concentration and motility was observed in all breeds within the first week post-insulation. Azoospermia was seen in Hampshire Down, Dorper, and Texel breeds from the first week post-insulation, with Santa Inês, Bergamasca and Ile de France showing azoospermia from the fourth week post-insulation, with all six breeds maintaining azoospermia until week 8 post-insulation. Sperm motility recovered at week 10 post-insulation for all breeds except the Dorper, with recovery of motility values at week 11 post-insulation.

The spermatozoa with normal morphology were significantly less for the Santa Inês, Bergamasca and Texel in the first week post-insulation in comparison with the values of pre-insulation. The values of normal spermatozoa for Ile de France and Texel recovered at week 10 post-insulation, while the other four breeds recovered at week 11. The recovery of plasma membrane integrity was worse for the Dorper and Hampshire Down breeds. Similar values were observed to those from pre-insulation, from week 11 post-insulation, with the other breeds having the plasma membrane integrity recovering from week 10 post-insulation. Recovery of acrosome integrity was slowest for the Dorper and Ile de France.

The study of Aller *et al.* (2012) showed that there were breed differences between Pampinta and Corriedale rams. Sperm concentration and total output of spermatozoa was higher in Pampinta rams, but percentage of abnormal spermatozoa was lower in Corriedale rams.

The study of Kasimanickam *et al.* (2007) also compared breeds with regards to the quality of their spermatozoa, but during storage for 8 days at 4˚C. It was found that polled Dorset had better quality spermatozoa under these conditions in comparison with Suffolk and Katahdin.

2.10.4 Age

Ram age plays an important role in their reproductive abilities as it influences how many ewes a ram can service as well as the quality of semen that is produced (Chella *et al.*, 2017). In the study of Chella *et al.* (2017) on Zulu rams, a higher quality of spermiogramic and seminal parameters (semen volume,

sperm concentration, sperm motility, and percentage live spermatozoa) were observed for the 2-3 year old rams in comparison with the 1-2 year old rams. From the results it was evident that optimal reproductive potential was reached at 3 years of age, with a decline in the quality of these parameters being observed from 4 years of age.

The study of Mandiki *et al.* (2002) showed similar results as the volume and sperm concentration for the first ejaculate evaluated did not differ for rams of 2 and 3 years of age. The 3-year old rams did however show higher sperm production than the 2-year old rams when evaluated with the exhaustion test. The percentage of live and morphologically normal spermatozoa were also better for the 3-year old rams than for the 2-year old rams.

Yankasa rams in the study of Osinowo *et al*. (1988) also showed increased sperm concentration and sperm output per ejaculate from 1 to 3 years of age, with ejaculate volume increasing from 1- to 2-year old rams and decreasing slightly from the 2- to the 3-year old rams. The 3-year old rams had significantly lower sperm motility compared to the younger rams. All three age groups showed similar percentages for morphologically normal spermatozoa.

Based on the results from these three studies, adult rams thus have a higher spermatogenesis efficiency than pubertal rams (Mandiki *et al.*, 2002). There is however a decline in certain parameters as rams get older than 3 years.

2.10.5 Seminal plasma composition

Seminal plasma is formed from various secretions from the different reproductive glands that mix with the epididymal spermatozoa during ejaculation (Bergeron *et al.*, 2004; Domínguez *et al.*, 2008). It therefore contains various substances that protect spermatozoa from aging prematurely during storage, thus creating the optimal storage environment (Kasimanickam *et al.*, 2007a; Zamiri *et al.*, 2010). The seminal plasma acts as a buffer and fluid vehicle to the spermatozoa which allows them to swim freely and interact with the fluid and cells of the female reproductive tract (Leahy and de Graaf, 2012).

Bergeron *et al.* (2005) found that the major protein in ram seminal plasma accounts for 45% of all the seminal plasma proteins and is a sperm adhesin of 15.5 kDa. The other major ram seminal plasma (RSP) proteins account for 20% of the total seminal plasma proteins, and form part of the BSP protein family. The RSP proteins are known as RSP-15 kDa, RSP-16 kDa, RSP-22 kDa and RSP-24 kDa. These

ram seminal plasma proteins are capable of binding to gelatin, meaning that they have type II domains just as BSP proteins do and can also bind to the low-density lipoprotein fraction (LDF) isolated from hen's egg yolk, as well as heparin, but the 22 and 24 kDa RSP proteins have higher affinity for heparin than the 15 and 16 kDa RSP proteins. Seminal plasma containing BSP proteins are detrimental to bull spermatozoa during sperm processing (Bergeron *et al.*, 2004). Ram seminal plasma proteins however have beneficial effects during sperm processing (Leahy and de Graaf, 2012). This detrimental effect in bulls is due to the BSP proteins that bind to the bovine spermatozoa at ejaculation, stimulating cholesterol and choline phospholipid efflux from the spermatozoon membrane, with the lipid efflux being time and concentration dependant (Bergeron *et al.*, 2004). It was however found by Pini *et al.* (2018) that BSP's have beneficial effects on frozen-thawed ram spermatozoa when they are added prior to freezing.

Besides acting as a transport mechanism for spermatozoa, seminal plasma plays another crucial role in the female reproductive tract as illustrated in Figure 2.10. Seminal plasma has an effect on the immune response so that the female immune system can 'tolerate' the embryo/foetus for a successful pregnancy, as it is genetically foreign (Schjenken and Robertson, 2014). The seminal plasma causes biological signalling of cytokines and chemokines that initiates the immune tolerance (Schjenken and Robertson, 2014). At the time of insemination in mammals there is a rapid and dramatic influx of inflammatory cells into the site of semen deposition (Robertson, 2005). Neutrophils are the first to be released and is then followed by other leucocytes such as macrophages, dendritic cells, and T cells (Schjenken and Robertson, 2014). All of these move into the reproductive tissues and partly respond directly to chemotactic agents in the seminal plasma, but showcase an amplified response due to cytokines and chemokines being produced by the epithelial cells lining the reproductive tract because of the presence of seminal plasma (Schjenken and Robertson, 2014). The cytokines and chemokines force the leucocytes out of the blood stream and into the reproductive tissues (Schjenken and Robertson, 2014). This inflammatory response to seminal plasma ensures that excess spermatozoa and seminal debris are cleared from the female reproductive tract and that the tract can become a sterile environment again after the micro-organisms have entered during mating (Schjenken and Robertson, 2014).
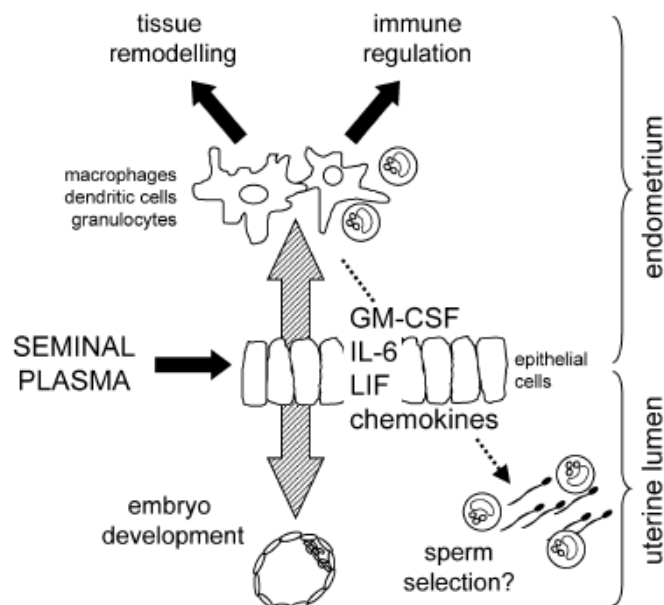
Figure 2.10 Seminal plasma signals in the female reproductive tract to facilitate implantation of the embryo (Robertson, 2005)**.**

2.10.6 Other physiologically related factors

2.10.6.1 Cryptorchidism

Normal descent of the testes is when the testes migrate from the abdominal region to the internal inguinal ring, then passing through the inguinal canal, and lastly into the scrotum (Hafez and Hafez, 2008). One of the most common disorders of sexual development observed in males is the incomplete descent of the testes and associated structures and is known as cryptorchidism (Foster, 2016). It could be a case where one or both testes fail to descend from the abdominal cavity into the scrotum and could be retained at any point along the normal path of descent (Hafez and Hafez, 2008; Pugh and Baird, 2012).

Animals that have bilateral cryptorchidism are sterile due to spermatogenesis being thermally supressed, with unilateral cryptorchid animals generally having normal spermatogenesis, thus being fertile, but having reduced sperm concentrations (Hafez and Hafez, 2008). These unilateral cryptorchid animals display normal secondary sex characteristics as their testes still produce testosterone at close to normal levels due to the elevated levels of LH (Hafez and Hafez, 2008). Even though these unilateral cryptorchid animals can breed successfully, it is not advised to use them for breeding as most cases of cryptorchidism is presumed to be heritable (Foster, 2016). With individual cases of cryptorchidism the

cause may be chromosomal, hormonal, structural, or environmental, whereas with outbreaks of cryptorchidism the cause is expected to be due to hormonal or environmental factors (Foster, 2016). Cryptorchidism most often has an autosomal recessive mode of inheritance in rams, but can also occur due to a dominant gene with incomplete penetrance (Hafez and Hafez, 2008; Foster, 2016). In rams the right testis is more often the one that is retained and unilateral cryptorchidism is more common than bilateral cryptorchidism (Foster, 2016).

2.10.6.2 Testicular hypoplasia

One of the most common disorders of sexual development of the ram reproductive tract is testicular hypoplasia, which is a sporadic disease where the testes do not grow to the normal size and can be unilateral or bilateral, resembling Klinefelter syndrome and an XXY genotype (Foster, 2016). This disorder is identified when an immature testis does not grow to the expected normal size (Foster, 2016). This results in a reduced amount of seminiferous epithelium, meaning there are less Sertoli cells, spermatogonia, spermatocytes, elongated spermatids, and spermatozoa, thus causing failure of spermatogenesis (Hafez and Hafez, 2008; Foster, 2016). The reduced amount of seminiferous epithelium is however due to a reduced number of tubules, or due to the tubules being very short in length or small in diameter (Foster, 2016).

This disorder can be caused by abnormal sex chromosomes, a gonadotropin deficiency or an unsuitable environment during the development of the testes (Foster, 2016). The diagnosis is usually only made once the animal has reached puberty when it is found that the animal is less fertile than it should be, or even sterile (Hafez and Hafez, 2008). Semen, libido, and mating ability is not affected in the less severe forms of testicular hypoplasia, but due to the reduced amount of seminiferous epithelium, sperm concentration may be low (Hafez and Hafez, 2008). Due to the lack of, or reduced amount of seminiferous epithelium of the affected testis, the testis is much smaller in size than it would normally be (Hafez and Hafez, 2008).

2.10.6.3 Segmental aplasia of the Wolffian ducts

Segmental aplasia of the Wolffian ducts is characterised by small or large segments of one, or both wolffian ducts, consisting of the epididymis, vas deferens or ampulla, that are absent (Hafez and Hafez, 2008). Unilateral tubal deficiencies or occlusions allow males to still be fertile, but bilaterally affected males are sterile (Hafez and Hafez, 2008). When the epididymis shows segmental aplasia, spermatozoa accumulate at the occluded point in the epididymis and is then known as a spermatocele (Hafez and Hafez, 2008).

2.10.7 Disease

When the testes, epididymis and seminal vesicles function under pathologic conditions, fertilization may be negatively affected due to spermatogenesis or sperm maturation being hindered, resulting in abnormal sperm characteristics, or blocking spermatozoa from moving from the testes to the urethra (Hafez and Hafez, 2008).

2.10.7.1 Epididymitis

Epididymitis in older males is caused by the organism *Brucella Ovis* and is a clinically important disease in rams (Pugh and Baird, 2012). The organism spreads by means of contact with the mucous membranes, resulting in bacteraemia and localisation of the epididymis and the accessory sex glands (Pugh and Baird, 2012). The epididymis swells and upon gross examination, localized inflammation, hyperplasia, and obstruction of the epididymal ducts can be found (Pugh and Baird, 2012). This causes the passage of spermatozoa to be blocked, as well as the development of sperm granulomas and eventually necrosis due to a pressure build-up (Pugh and Baird, 2012).

In young rams, epididymitis is caused by a few organisms, but generally not by *B. ovis* as in older males and is diagnosed by palpating the enlarged epididymis (Pugh and Baird, 2012). Epididymitis in young rams is spread between each other by the nasal or oral route (Pugh and Baird, 2012).

2.10.7.2 Orchitis

Orchitis is a rare disease found in domesticated animals and is most often found in bulls from regions endemic for *Brucella abortus* or tuberculosis, and possibly canine epididymitis too (Foster, 2016). Orchitis is however often diagnosed incorrectly as it mistaken for epididymitis (Foster, 2016). There are three types of orchitis; interstitial, intratubular or granulomatous and necrotizing orchitis (Foster, 2016).

Interstitial orchitis is difficult to identify macroscopically, but with histological examination, lymphocytes can be found in the interstitial stroma, followed by, or coinciding with fibrosis. With intratubular orchitis, the seminiferous tubules are inflamed and is often due to an ascending infection, but it does occur without the presence of epididymitis. Necrotic orchitis can result from conditions causing severe trauma or ischemia of the testes, as well as various infections, but *brucellosis* is one of the main characteristics of necrotic orchitis.

In rams infected with the ovine-lentivirus maedi-visna virus, chronic interstitial orchitis is often present at the same time (Foster, 2016). Orchitis in rams is generally not found in the absence of epididymitis and is caused by the same bacteria (Foster, 2016).

2.10.8 Nutrition

Domestic ruminants grazed on pasture and bred by means of natural mating systems are highly dependent on the quality and quantity of feed available, especially in the period prior to mating which has the greatest influence on successful mating and fertilization (Brown, 1994). However, grazing livestock experience large fluctuations in the quality and amount of feed and water available throughout the year in the hot semi-arid zones and need to walk vast distances in search of water and food. It is therefore not only one stressor affecting the animal in these extensive farming systems used for sheep, but multiple, simultaneous stressors (Kumar *et al.*, 2017). Kumar *et al.* (2017) thus evaluated the effects of multiple stressors (thermal, nutritional, and walking stress) on several biological functions of Malpura rams, simultaneously. Undernutrition is primarily an energy deficiency that is associated with a loss of body weight, which is enhanced by other stress factors and not just nutritional limitations, causing more energy usage and placing the animals at risk (Brown, 1994).

The rams exposed to multiple stressors showed significant reductions in their body weight and body condition scores, which can most likely be attributed to the nutritional restriction and the stress of walking vast distances as it has been said that heat stress on its own does not influence bodyweight when the animals are fed *ad libitum*. These three stressors did not affect the production of semen, but it did however affect the semen quality. It is important that immature rams receive adequate nutrition as growth rate, rather than age, will determine the rate of sexual development, as malnutrition before puberty retards sexual development, the onset of puberty, the appearance of external genitalia, and supresses spermatogenesis (Brown, 1994). Dietary changes affect immature animals more than mature animals as their reproductive organs are more sensitive towards such change (Brown, 1994).

2.10.9 Season

Various factors play a role in the quality characteristics of spermatozoa of which environment (ambient temperature, humidity and photoperiod) changes in conjunction with the season (Al-Anazi *et al.*, 2017). Sheep have a distinct breeding and nonbreeding season, and are referred to as seasonal breeders, where photoperiod is the main factor controlling the seasonal reproductive pattern (Aller *et al.*, 2012). The effect of photoperiod can be attributed to complex hormonal interactions that differ

between the breeding and nonbreeding seasons as the day length changes (Martí *et al.*, 2012). The season of birth or the season in which the animal finds itself can effect growth and reproductive development of rams as these environmental factors differ according to the season and also result in variation in feed availability and quality (Brown, 1994). Season therefore also influences nutritional plane.

In the study of Martí *et al.* (2012), it was confirmed that during the breeding and nonbreeding season, morphometric subpopulations of spermatozoa exhibited a seasonal effect, which was also related to sperm quality. These authors also reported a significant increase in sperm concentration from the breeding season to the late nonbreeding season, possibly due to a decrease in sexual activity after the breeding season. Spermatozoa obtained a more elliptic shape during the nonbreeding season due to over-maturation, as spermatozoa remain in the epididymis for longer due to a lower mating frequency in comparison with the frequent depletion and restoration of spermatozoa during the breeding season.

Chella *et al.* (2017) conducted a study on Zulu rams in South Africa, with results for semen volume and concentration being similar in autumn, winter, and spring, significantly differing for semen volume, but not for sperm concentration, with summer exhibiting the lowest values for both parameters. However, the different seasons, did not have a significant effect on the mass and progressive motility, percentage of spermatozoa with abnormal morphology and pH of ram semen. Overall, rams showed better performance during autumn and winter, which makes sense, because if ewes conceived during winter with a gestation period of 4-5 months, lambing would take place in early spring or summer, which provides the most favourable climate for the progeny the grow up in. Scrotal circumference was also lowest for summer in comparison with the other months. Seasonal differences in this specific study were however not large enough that it would compromise year-round breeding, which is also the case in the study of Aller *et al.* (2012), the study of Kafi *et al*. (2004) and the study of Karagiannidis *et al.* (2000).

In the study of Aller *et al.* (2012) on Pampinta and Corriedale rams in Argentina, ejaculate volume, sperm progressive motility, sperm output and percentage of abnormal spermatozoa were all significantly different according to the season of semen collection. Season had a significant effect on scrotal circumference of both breeds and was greatest during summer and autumn, being positively correlated with bodyweight. Summer and autumn showed the highest ejaculate volume (Aller *et al.*, 2012). Sperm concentration did not differ between the seasons, which means that there were

differences in the secretion and release of seminal plasma, possibly due to changing testosterone concentrations in the plasma. Significantly higher values were found in winter and spring for the percentage of abnormal spermatozoa than in summer and autumn. Summer and autumn (breeding season) proved to be the two seasons that provide the best quality semen in this region for these breeds.

Similar to Aller *et al.* (2012), the study of Karagiannidis *et al.* (2000) on Chios and Friesian rams in Greece, the study of Kafi *et al.*, (2004) on Karakul sheep in Iran, and the study of Zamiri *et al.* (2010) on Moghani rams in Iran, resulted in semen characteristics generally being better in summer and autumn than during winter and spring, with optimal results found during autumn.

Semen volume and sperm motility were both significantly lower in summer than in the other seasons, with sperm concentration being significantly lower in both winter and summer in the study of Al-Anazi *et al.* (2017) with Naimi and Najdi rams in Saudi Arabia. Spring and autumn resulted in the highest quality semen, but summer still showed significantly high values for semen pH, volume, concentration, and total spermatozoa per ejaculate. Scrotal circumference was also reported to be significantly influenced by season, with summer showing the highest mean value and autumn the lowest. When sperm motility is low in summer, heat stress due to high temperatures is the main causing factor (Al-Anazi *et al.*, 2017).

In the study of Domínguez *et al.* (2008) it has been established that seminal plasma collected during autumn or winter results in a significant improvement in the progressive and total motility of frozen-thawed spermatozoa. The composition and concentration of seminal plasma proteins were much higher in autumn (31 mg/mL) in comparison with winter, spring, and summer (21, 10.5 and 21.2 mg/mL respectively) and thus, as expected, proteins that bound to the surface of spermatozoa during autumn differed from those that bound during winter, spring, and summer, which had similar proteins bound to the surface of these spermatozoa. Seminal plasma from autumn is therefore either beneficial because of the higher seminal plasma protein concentration, or due to the specific proteins that bind to the surface of the spermatozoa.

Seminal characteristics, testicular circumference and plasma testosterone concentrations was significantly affected by season in Moghani rams found in Iran (Zamiri *et al.*, 2010).

Different breeds and different latitudes at which the breeds are kept, are said to be the cause for variation in quantitative and qualitative sperm production and also varying times of the year at which the unfavourable effect of photoperiodism occurs (Karagiannidis *et al.*, 2000).

2.10.10 Heat stress

One of the current greatest threats to sustainable livestock production in many parts of the world is climate change, where exposure to high ambient temperature results in heat stress that limits animal productivity. Heat stress results in a disruption of the physiological and reproductive performance of animals, especially in arid and semi-arid regions, as more energy needs to be used for the dissipation of body heat, which results in an increased respiration rate and body temperature (Marai *et al.*, 2007; De *et al.*, 2017). This then results in a reduction in profitability (Kumar *et al.*, 2017).

In sheep, respiratory evaporation is one of the main methods of heat dissipation as the wool coats of sheep make it difficult to dissipate heat through sweating (Marai *et al.*, 2007). In mammals that have scrotal testes, spermatogenesis is often negatively affected when the testicular temperature and whole-body temperature increases to levels higher than normal (Mieusset *et al.*, 1992). Such severe increases result in a decreased sperm concentration, motility and percentage of morphologically normal spermatozoa, which leads to a reduction in fertilizing capacity (Arman *et al.*, 2006, Cruz Júnior *et al.*, 2015). The amount of time that the animal is exposed to heat stress determines how severe the impact is on semen quality and seminiferous tubule cells, and directly relates to sperm recovery and the reestablishment of spermatogenesis (Alves *et al.*, 2016).

Even though heat stress has major effects on the quality parameters of spermatozoa, sperm production recovers to normal levels between 30 and 42 days after exposure, with testes temperature recovering to normal as soon as 24 hours after the heat stress has ended (Moule and Waites, 1963; Alves *et al.*, 2016). Besides the direct effects of increased ambient temperature on spermatogenesis, other biological functions are also negatively affected. These ultimately affect reproductive efficiency and include, a decreased efficiency of feed intake and utilisation, and disturbed protein, energy and water metabolism, mineral imbalances, hormonal secretions, enzymatic reactions and blood metabolites (Marai *et al.*, 2007). While the quality parameters of spermatozoa are negatively affected due to heat stress, these spermatozoa are also less able to withstand additional stress associated with freezing/thawing and storage at refrigerator temperature after being harvested (Arman *et al.*, 2006). Mieusset *et al.* (1992) conducted a study where rams were exposed to moderate, but sustained increases in scrotal temperature, followed by insemination of normal female sheep with semen from

this period of increased scrotal temperature to assess the fertilization rate and embryonic mortality. By increasing the subcutaneous scrotal temperature by 2˚C for 16h/day, embryonic loss increased as soon as day 4 of treatment, but the fertilization rate was not significantly affected until day 21, whereafter the quality and the quantity of spermatozoa were impaired (Mieusset *et al.*, 1992) .

Rocha *et al.* (2015) also evaluated the effects of increased scrotal temperature by means of scrotal insulation on testis morphology, semen parameters, and the seminal plasma proteome of sheep rams. The testis size and sperm parameters were affected after 8 days, rectal temperature decreased after 4 days, and the seminal plasma proteome changed significantly. All the above have however returned to normal levels within a few days after the thermal insult.

2.11 Testicular thermoregulation

Various mechanisms play a key role in testicular thermoregulation, such as the pampiniform plexus, tunica dartos, cremaster muscle, scrotal superficies and scrotal sweat glands (Alves *et al.*, 2016). For the mammalian testes to function effectively, it must be maintained at $2 - 8$˚C lower than normal body temperature (Fleming *et al.*, 2004; Hafez and Hafez, 2008).

According to Kastelic *et al.*, (1997), the scrotum has a positive temperature gradient (warmer at the top than at the bottom), whereas the testis has a negative temperature gradient (warmer at the bottom than at the top), when the testis was removed from the scrotum. The exposed testis also had considerably cooler intratesticular temperatures at every measured location when compared to the testes covered by the scrotum for both bulls and rams. Therefore, the scrotum has a significant effect on intratesticular temperature.

The scrotal skin contains many large adrenergic sweat glands, and together with the tunica dartos and the cremaster muscle, the surface area and the thickness of the scrotum can be changed to have the testes at the appropriate distance from the body wall for optimal thermoregulation (Hafez and Hafez, 2008; Ahmad Para *et al.*, 2018). The testicular artery is highly convoluted and in the shape of a cone, with the base resting on the dorsal pole of the testis (Hafez and Hafez, 2008). The network of veins from the pampiniform plexus is enmeshed with the coils from the testicular artery and forms a counter-current heat exchange mechanism that cools the arterial blood entering the testis by means of the cooler venous blood leaving the testes (Hafez and Hafez, 2008; Para *et al.*, 2018). This allows the testes temperature to be kept lower than the body temperature.

2.11.1 Response of spermatozoa to heat stress

The cells in the testis that are most easily affected by heat are the pachytene spermatocytes and spermatids, as germ cells have high mitotic activity which makes them more susceptible to heat stress (Setchell, 1998; Lue *et al.*, 1999; Shiraishi *et al.*, 2012). Other cell types in the testis can also be affected depending on the severity of the heat exposure, such as an increase in temperature or in the duration thereof (Setchell, 1998; Alves *et al.*, 2016). The Sertoli cells are suspected to be the region where the action of heat is most drastic as they are situated in the seminiferous epithelium where they can have an effect on all germ cells as they pass through the blood-testis barrier, thus reducing their capability of supplying these germ cells with nutrients and restricting proper development (Setchell, 1998).

Germ cells can be damaged in various ways by heat stress and include germ cell apoptosis, autophagy, damaged DNA due to synapsis being altered and strand breaks, and by the generation of reactive oxygen species (Durairajanayagam *et al.*, 2015). The physiological status in cells and tissues of an animal can be used to indicate the oxidative stress that the animal is experiencing (Takahashi, 2012). When testicular temperature increases, the metabolism also increases as a result and this is followed by an increase in the demand for oxygen (Kastelic *et al.*, 2017).

After a period of heat stress the testicular weight decreases, which can be attributed to the loss of germ cells by means of apoptosis (Durairajanayagam *et al.*, 2015). Apoptosis is process of physiological cell death through which unwanted cells are removed or eliminated (Vaux and Korsmeyer, 1999). Paul *et al.* (2008) found that even though the testes is eliminating germ cells with abnormalities by means of apoptosis, it is not entirely efficient by doing so, causing DNA damaged germ cells to develop into mature spermatozoa, which would ultimately be able to fertilize egg cells. Autophagy is another mechanism of cell death, but the specific pathway, either autophagy or apoptosis, that is chosen by the cells, depend on the type of stimulus and circumstances of the cell environment (Eisenberg-Lerner *et al.*, 2009). Autophagy and apoptosis are not mutually exclusive processes and can work in synergy as well as counter each other, and also share several of the same molecular regulators (Eisenberg-Lerner *et al.*, 2009).

In mice, the extent to which the testicular histology was disrupted is dependent on ambient temperature and that the higher the temperature the more severe the effects (Paul *et al.*, 2008). Testicular heat stress at 42˚C had several consequences including the formation of giant degenerating germ cells, multinucleated giant cells, with germ cell loss and gaps forming within the seminiferous epithelium. Spermatocytes also showed an increased number of DNA strand breaks due to the heat

stress, especially in the pachytene spermatocytes. In men, chromatin damage is observed with testicular and epididymal heat stress and occurs even before the sperm counts drop to levels lower than that required for natural conception (Ahmad *et al.*, 2012). The study of Hamilton *et al.* (2018) also showed that in rams, heat stress causes increased DNA fragmentation and chromatin alterations in spermatozoa, but does not change the protamination process or cell types undergoing apoptosis significantly. In Holstein bulls, testicular spermatozoa showed reduced chromatin stability after scrotal insulation (Karabinus *et al.*, 1997). Spermatocytes exposed to heat stress in mice caused subfertility, whereas spermatids that experienced heat stress showed DNA fragmentation, and mature spermatozoa experiencing heat stress while in the epididymis, had an altered sex ratio (Perez-Crespo *et al.*, 2008).

Heat shock proteins (HSP's) can be found in the cytosol, mitochondria, endoplasmic reticulum, and nucleus, and is specifically present in cells when the cells experience sublethal heat stress (Kiang and Tsokos, 1998). These proteins can protect cells, tissues, organs, and animals from a subsequent and normally, a lethal period of heat stress, as well as from other harmful conditions such as hypoxia and ischemia or reperfusion. Again, the degree at which these HSP's are induced is based on the duration and level of exposure to heat stress. Heat shock proteins can be classified into two main categories, being, constitutive or inducive (Durairajanayagam *et al.*, 2015). Constitutive HSP's are normally molecular chaperones that ensure the correct transportation and assembly of polypeptides and play a role in other cellular processes, such as the stabilization of proteins in their inactive forms and inhibiting degeneration. Cells pause normal protein synthesis when experiencing heat stress and divert the available resources to produce inducible HSP's that protect the cells by binding to the proteins, preventing them from denaturing and incorrect folding.

2.12 Scrotal insulation

Three different methods exist for local heating of the testes and include: induced cryptorchidism, short-term heating by immersion in a water bath, and scrotal insulation (Setchell, 1998). The experimental method that is most commonly used to identify the effects of increased testicular temperature on sperm production and quality is scrotal insulation, as it induces testicular degeneration (Alves *et al.*, 2016; Kastelic *et al.*, 2017) . Very few studies have investigated the effect of scrotal insulation on the quality and functionality of spermatozoa in rams (Mieusset *et al.*, 1992; Arman *et al.*, 2006; Cruz Júnior *et al.*, 2015; Rocha *et al.*, 2015; Alves *et al.*, 2016; Hamilton *et al.*, 2016; Kastelic *et al.*, 2017).

Heating the testes can have direct effects on specific cell types within the testes, as well as have indirect effects which occur as a result of cellular changes due to the heat experienced (Setchell, 1998). These direct effects can occur on five different levels and include: effects on the testes itself, effect on the number, motility and structure of spermatozoa that exit the testes in rete testis fluid or in semen, the fertilizing ability of spermatozoa that seem to be normal with regards to appearance and motility, and the *in vivo* or *in vitro* fertilization and subsequent development of embryos in females fertilized by spermatozoa originating from heat treated males (Setchell, 1998).

Various scrotal insulation methods have been tested in the past. One of the more popular methods used is where the insulating bag is made of one layer of aluminium foil that is placed between two layers of cotton cloth, with another layer of waterproof cotton cloth on the outside, and four tapes running over the back of the ram to support the bag in its position (Mieusset *et al.*, 1992; Arman *et al.*, 2006). Rocha *et al.* (2015) however, used two layers of plastic that surrounded an inner layer of cotton, with cords at the bag opening to keep the bag in position. Cruz Júnior *et al.* (2015) used two overlapping disposable diapers that also consist of two layers of plastic and an inner layer of cotton, but these devices were fixed with masking tape. In the study of Kastelic *et al.* (2017), the layer in direct contact with the skin was Insul-Bright and was secured to the scrotum with tag cement, then covered by a disposable diaper and lastly a tubular medical bandage to hold everything in place in conjunction with adhesive tape at the scrotal neck.

The environmental temperature and the length of heat exposure are the two main dependent factors on the effect that scrotal insulation has on spermatogenesis (Alves *et al.*, 2016). Scrotal insulation does have deleterious effects on spermatogenesis and the maturation of spermatozoa in the epididymis, but these are however reversible, given that scrotal insulation is no longer applied (Martí *et al.*, 2003). When the testes is exposed to either acute or chronic heat, one of the most obvious consequences is that the weight of the testes decreases (Setchell, 1998). The recovery time for the production of spermatozoa, following a thermal insult, coincides with chronology of spermatogenesis (Martí *et al.*, 2003).

With whole-body heating, the effects of high testicular temperature on spermatogenesis makes it difficult to distinguish from other physiological reactions that the ram could have in response to the heat stress that may affect testicular function indirectly (Moule and Waites, 1963). Scrotal insulation is however a more focussed thermal insult that specifically heats the testis, but it does not mean that

physiological processes within the animal do not work together with the testis to minimise the effect of damage on spermatogenesis through thermoregulation.

It is of interest to discover whether spermatozoa sampled from specific rams from the same breed are capable of better withstanding the stress associated with sample collection, processing, cryopreservation, and subsequent thawing, compared to other individuals. Following these findings, it is important to observe if these higher resilient rams with regards to how their spermatozoa handled processing associated with cryopreservation, are also more resilient in withstanding the effect of heat stress on spermatogenesis, and subsequent storage of heat stressed spermatozoa, better than the lower resilient rams.

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Chapter 3

Materials and Methods

Ethical clearance (#ACU-2019-9835) for this study was obtained from the Stellenbosch University Research Ethics Committee (Animal Care and Use). The animal care and procedures used in this study were consistent with the guidelines stipulated in the South African National Standards document 10386:2008.

3.1 Experimental location

The study was conducted on the Welgevallen Experimental farm (33°56'33"S, 18°51'56"E) that is situated in Stellenbosch in the Western Cape, South Africa. Stellenbosch has warm/hot and dry summers, with cold and rainy winters, thus having a Mediterranean climate.

3.2 Experimental animals and husbandry

A total of 13 Dohne Merino (*Ovies aries*) rams, aged between two and three years, were used in the study. The rams were allowed to graze in paddocks during the day and were brought inside and kept in pens during the night. The paddocks consisted of irrigated kikuyu pasture for grazing, while water was available *ad libitum* for the entire duration of the study. All animals were kept under similar conditions. All 13 rams were used for Phase 1 of the study, and 12 rams were used during Phase 2 and Phase 3, respectively. Prior to Phase 2, the group was reduced to 12 rams to have two groups consisting of 6 rams each.

The rams were carefully monitored during each sampling session for any health- or injury related problems. The rams were also monitored before and after sampling, and in the paddocks for any deviations from normal behaviour. If rams showed any severe problems that could not be dealt with by the farm management, a veterinarian was contacted to assist. Specific attention was paid in Phase 2 where scrotal insulation devices were fitted on the rams. The rams were carefully monitored during scrotal insulation to ensure that the effects of heat on the testes did not affect normal locomotion or behaviour.

As part of standard husbandry practices, the hooves of the rams were examined regularly, and trimmed when required. Rams were sheared every six months, at the end of January and the beginning of August. Rams were however not sheared during the duration of the study.

3.3 Experimental Layout

The overall study was structured into three distinct phases to evaluate the resilience of ovine spermatozoa to tolerate heat stress. Table 3.1 presents the experimental layout followed for Phase 1, 2, and 3, and is followed with notes on the respective phases.

Table 3.1 Experimental layout of Phase 1, 2, and 3, of a study on the effect of heat stress on ovine sperm functionality.

Phase	Date	Number of animals	Description of trial activities	Repetitions
ONE	11 November 2020 – 9 December 2020	13	• Classification of rams into high-resilience (HR) and low-resilience (LR) groups, based on resilience of spermatozoa to tolerate processing and cryopreservation.	3
			• Semen samples collected using electro-ejaculation.	
			- Sperm viability, morphology, and acrosome integrity recorded for fresh, equilibrated, and cryopreserved samples.	
TWO	24 March 2021 – 14 June 2021	12 (HR=6; LR=6)	• Five rams of each group fitted with scrotal insulation devices. Remainder ram of each group retained as Control ram	7
			• Semen samples collected using electro-ejaculation.	
			- Before scrotal insulation devices were fitted.	
			- After 7 days of scrotal insulation.	
			- Every fortnight – up to week 11.	
			• Semen samples subjected to liquid storage at 5°C, for up to 48h.	
			- Sperm viability, morphology, and acrosome integrity recorded at 0h, 12h, 24h, 36h, 48h.	
			• Recording of weather data, infrared thermographic measurements, and iButton data, and testicular measurements.	
THREE	5 August 2021 – 19 August 2021	12 (HR=6; LR=6)	• Use of functional test to assess influence of scrotal insulation on sperm fertilizing ability.	5
			• Hyperactivated semen samples subjected to incubation at 38.5°C, and 5% CO_2 for 30min.	
			- Sperm viability, morphology, and acrosome integrity recorded at 0min, 15min, 30min.	

Notes:

Phase 1: The allocation of rams to the HR and LR groups was based on the percentages of abnormal spermatozoa recorded for fresh, equilibrated, and post-thaw samples, respectively. Based on the evaluation of the fresh, equilibrated, and post-thaw samples, a value of 10.85 ± 2.41% was used as cut-off point to allocate rams to the respective treatment groups. Rams that produced semen samples with an average percentage of abnormal spermatozoa of more than 10.85 ± 2.41% post-thaw, was allocated to the high-resilience (HR) group, whereas rams that produced semen samples with an average percentage of abnormal spermatozoa lower than 10.85 ± 2.41%, were allocated to the low-resilience (LR) group.

Phase 2: Care was taken to ensure that the harness fitted snugly to prevent heat loss through radiation. The scrotal insulation bag was kept in position during normal activity by stabilising using a custom-designed harness (refer to Appendix 1 for design details).

3.4 Methodologies used in Phases 1, 2 and 3

3.4.1 Design and fitting of the scrotal insulation bag

3.4.1.1 Design of the bag

Scrotal insulation devices were designed to insulate the scrotums of the rams in the best possible way to limit heat loss through convection or radiation (Figure 3.1). The design was based on a combination of designs originating from studies that involved scrotal insulation of rams to assess the effect of heat stress on quality and functionality of spermatozoa (Mieusset *et al.*, 1992; Arman *et al.*, 2006; Cruz Júnior *et al.*, 2015; Rocha *et al.*, 2015; Alves *et al.*, 2016; Hamilton *et al.*, 2016; Kastelic *et al.*, 2017). Devices were made by using three different insulating fabrics. The inside layer consisted of a block-out fabric, generally used on the back side of curtains to block UV rays. The middle layer consisted of batting ($310g/m^2$) with a high density and thus insulative property. The outside layer consisted of Oxford Nylon, which is a waterproof fabric that is the same type of fabric used for the outer layer of rain jackets. The devices consisted of seven individual panels stitched together. A non-elastic draw string was included in the design to ensure that the bag could be closed sufficiently to prevent heat loss, but not to cause any discomfort of chaffing during the insulation phase. The final design had the shape of a rugby ball, thus fitting very well and closely around the scrotum. The bags also had a length that limited the natural response during period of elevated temperatures, i.e., descend of the testes, and thus ensured true scrotal insulation as best possible.

3.4.1.2 Design of the harness

Four nylon straps were attached to each bag. These straps were positioned two-two on either side at the top of the bag, leading into a 45˚, upward direction. These straps were clipped into a rectangle that was stitched from nylon strapping by means of release-buckles and was positioned on the front part of the sacrum to ensure that it does not slide off the back. A single strap went from the rectangle, also attached with a release-buckle, to the region where the thoracic vertebrae meet the cervical vertebrae. Two straps were stitched onto the end of this strap at a 45˚ angle and crossed over the front legs, around the chest area, and back up from under the legs. These straps then attached 15cm behind the starting point of the two straps by means of a release-buckle on each side positioned at 45˚. All straps where a release-buckle was involved, had a slider buckle behind it which ensured that the straps were maintained in the positioned that they were fixed in. For positioning of harness straps, see Figure 3.1.

Figure 3.1 Rear and top view of a ram fitted with a scrotal insulation bag, indicating the position of the harness bands to ensure effective insulation.

3.4.1.3 Fitting of the scrotal insulation devices and placement of iButtons

A total of 10 scrotal insulation devices were fitted to 10 rams (HR=5; LR=5) that were randomly allocated to two groups.

The devices were fitted on a Wednesday and Thursday, to ensure the timeous collection and processing of the samples. Due to a limited number of active IButtons (Hygrochron DS1923-#F5) available (n=5), the iButtons were placed inside the scrotal insulation devices fitted to the rams allocated to the second group. The iButtons were calibrated to log temperature (˚C) and relative humidity (%) in 5-minute intervals, for the duration of scrotal insulation period.

3.4.2 Testicular measurements

Testicular measurements (i.e., length and circumference) and thermographic measurements were recorded during each sample collection session of Phase 2. The length and circumference of the testes was recorded using a measuring tape, with the length of the scrotum measured from the base of the spermatic cord to the most distal point of the scrotum. Circumference was measured around the widest part of the scrotum, and care was taken to not place a thumb on the neck of the scrotum (which may result in separation of the testes and thus inaccurate measurement).

3.4.3 Thermographic measurements

Rectal temperature was taken with a Kruuse Vet Digital Thermometer (Kyron Laboratories, South Africa). Rectal temperatures and thermographic images were taken in the mornings (between 7am and 8am) before the ram was subjected to semen sampling. The rectal thermometer was placed in the rectum until the device made a sound around 5 – 10s later that indicated that the temperature has been established and could be read.

Infrared thermal images were captured using a FLIR E75 Advanced Thermal Imaging camera (Teledyne FLIR, Wilsonville, Oregon, USA). Figure 3.2 indicated the locations on the body where thermal images were recorded. Each thermal image was recorded 2m away from a ram, and by the same operator throughout the study. Temperatures were taken on three different locations, i.e., just above the hind and front legs respectively, and in the middle of these two locations (Figure 3.2). Care was taken to position the camera at the same level of the ram to ensure as accurate as possible recording of body surface temperature.
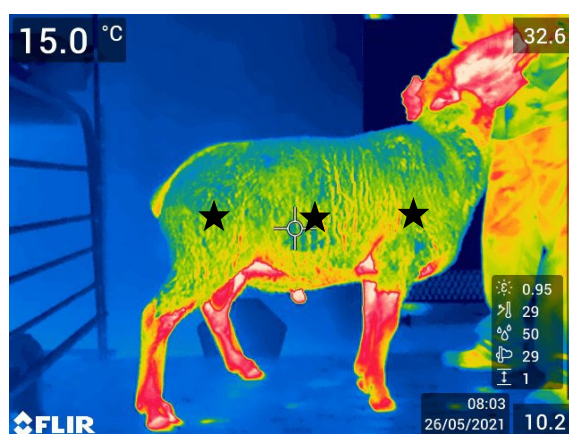
Figure 3.2 A thermographic photo taken of the side profile of a Dohne Merino ram. The stars present the three locations where the temperatures were taken.

Figure 3.3 indicated the different locations where scrotal temperatures were recorded. The locations were selected based on the study of Cruz Júnior *et al.* (2015), and included the top, bottom, left, right, and centre of the posterior region of the scrotum.

The scrotal thermal measurements were recorded 1m away from a ram, and by the same operator throughout the study. Care was taken to position the camera at the same level of the ram to ensure as accurate as possible recording of respective scrotal temperatures.
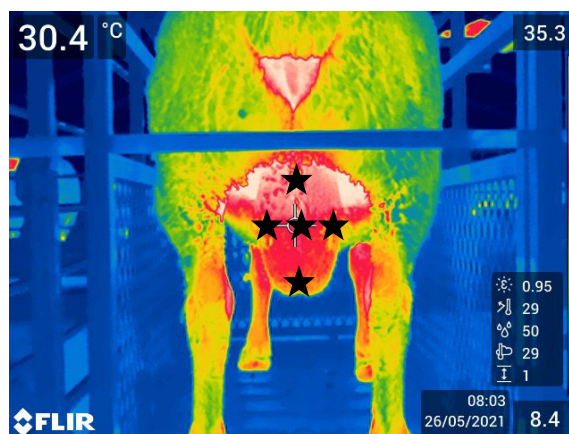
Figure 3.3 A thermographic photo taken of the posterior scrotal view of a Dohne Merino ram. The stars present the five locations where the temperatures were taken.

3.4.4 iButton data

The iButtons were calibrated to log temperature (˚C) and relative humidity (%) in 5-minute intervals. After removal of the scrotal insulation devices, the iButtons were recovered, and the data were downloaded using the associated software programme, ColdChain ThermoDynamics, and exported to Excel.

3.4.5 Weather data

Weather data was recorded by a CR800 weather station, which is located on the Welgevallen Experimental Farm of Stellenbosch University. The weather data was recorded at hourly intervals and included wind speed (including maximum wind speed) (m/s), wind direction (degrees), average air temperature (˚C), relative humidity (%), and rain fall (mm). During Phase 2, the minimum temperature recorded was 6.59˚C, and the maximum temperature recorded was 36.34˚C.

3.4.6 Semen collection

Semen samples were collected by means of electro-ejaculation with an EL Toro 3 Ejaculator (Electronic Research Group, South Africa). The stimulation process followed was similar to those used in the studies of Jiménez-Rabadán *et al.* (2012), Jiménez-rabadán *et al.* (2013), Jiménez-rabadán *et al.* (2015), Jiménez-Rabadán *et al.* (2016). The probe of the ejaculator was lubricated with liquid paraffin and placed inside the rectum of the ram, with the electrodes facing the ventral region of the ram. The prostate was then stimulated with the front part of the probe once the stimulation process has begun. The power was set to level between one third and a half to start off with, which was adjusted according to the response of the ram as the sampling process continued. The device is programmed with two modes. The sampling was started on Mode 1 and is the preferred starting mode, as it has a gentle effect in comparison with Mode 2. The handle of the probe has a switch which allowed for pulses to be applied in a rhythmic pattern. Electrical pulses were applied for 2-4s at a time, with rest periods of 10-20s. Ejaculation normally occurred within 4 to 6 pulse cycles.

A minimum of four people were required for safe handling during sample collection. Two people were responsible for getting the ram out of the pen, handling it, and picking it up onto a table where the sampling would be performed. One person held the ram firmly by the front region of the body and legs, from behind the head area, and the other held the back legs, facing the scrotal region of the ram. Prior to stimulation, the ram was held up-right so that it sat on its bottom region, which allows the penis to be extruded from the prepuce by the person responsible for stimulation. The person responsible for sample collection then held the penis behind the glans penis with a paper towel folded to a width of 2cm, after which the ram could be positioned on its side again. The glans penis was then held inside a 50ml test tube into which the ram ejaculates with successful stimulation.

The 50mL test tube as well as a 10mL test tube was kept inside the pocket of the person collecting the sample to keep it warm. Once the sample was collected, it was transferred to the 10mL test tube, which was calibrated for the determination of ejaculate volume. The 10mL tubes were sealed with a strip of parafilm before placement in a thermos flask (Stanley thermos, Stanley, USA), containing water at 37°C.

3.4.7 Sample processing and evaluation

Upon arrival at the laboratory from the farm, the temperature of the water inside the flask was measured again. Samples were then removed from the flask and placed in a Memmert water bath

(Lasec, South Africa), with water temperature being maintained at 37˚C. All semen samples were subjected to macroscopic and microscopic evaluation, respectively.

3.4.7.1 Macroscopic evaluation

The samples were evaluated macroscopically immediately upon arrival at the laboratory, firstly by measuring the volume of the sample from the graduated test tube and then evaluating the colour/consistency. Mass motility was observed by placing 10µL of the sample on a pre-heated microscope slide (Lasec, South Africa) and covered with a cover slip (22mm X 22mm; Lasec, South Africa), and then left for two minutes on a warming plate (set at 35 °C) before being viewed with an Olympus light microscope (Olympus, Japan) under a 10x magnification. The same person was responsible for evaluating the colour and mass motility of the samples after every collection during the study so that the evaluation was carried out consistently.

3.4.7.1.1 Volume

Graduated 50mL test tubes (Lasec, South Africa) were used to collect the sperm samples during electro-ejaculation, after which the samples were immediately transferred to graduated, 10mL collection vials (Lasec, South Africa) to directly measure the volume of semen produced from an ejaculation (Memon *et al*., 2006; Edmondson *et al.*, 2012).

3.4.7.1.2 Colour

A scale ranging from 0 to 5 was used to grade the colour/consistency of the sample, with 0 being clear/watery and 5 being thick and creamy as can be seen in Table 3.2 (Hafez and Hafez, 2008). This grading was done while the semen was still inside the 10mL collection vial.

Table 3.2 Scoring system used for semen colour/consistency of semen samples for all phases of the study (Hafez and Hafez, 2008).

Score	Consistency
5	Thick creamy
4	Creamy
3	Thin creamy
2	Milky
1	Cloudy
0	Clear (watery)

3.4.7.1.3 Mass motility

The mass/gross motility was evaluated by taking a 10µL droplet of fresh, undiluted semen and placing it on a microscope slide that was pre-warmed on an electrothermal slide drying bench (Lasec, South Africa) set at 35˚C. A square cover slip with a width of 22mm was then placed on top of the droplet from an angle so that the droplet spread evenly. The microscope slide was observed through an Olympus light microscope with 10x objective. The mass motility was then scored according to a scoring system with values of 0 to 5, with 0 indicating total immobility and 5 indicating rapid wave motion with eddies present as seen in Table 3.3 (Hafez and Hafez, 2008).

Table 3.3 Scoring system used for grading the mass/gross motility by evaluation of the wave motion of semen samples for all phases of the study (Hafez and Hafez, 2008).

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

3.4.7.1.4 Sperm concentration

Sperm concentration was determined using a Neubauer haemocytometer (Lasec, South Africa) (Prathalingam, 2006). A 1.5mL Eppendorf (Lasec, South Africa) tube was used to gently dilute 10µL of the sperm sample with 990µL of distilled water. Besides diluting the spermatozoa with water, the mixing also ensures that all spermatozoa are immobilised and killed. A total of 10µL was loaded into the Neubauer haemocytometer and was covered with a square cover slip with a width of 22m. Spermatozoa were counted in 5 of the 25 large squares, positioned diagonally from the upper left to bottom right, with each larger square containing 16 smaller squares. The total number of spermatozoa counted was then used in the following equation to calculate the sperm concentration/mL:

Number of spermatozoa counted x 5 x 100 (dilution factor) x 10 000 = number of spermatozoa/mL of sample.

3.4.8 Sample dilution

For Phase 1, the desired sperm concentration was 300 x 10^6 spermatozoa/mL. The sperm samples were diluted with Fraction A (Minitube, Germany, Ref. 13500/0004) and Fraction B (Minitube, Germany, Ref. 13500/0006) of a glycerol-based diluent, Biladyl. This was done in two steps, as per instructions of the manufacturer. A total of five 0.25cc Cassou straws (IMV®, Taurus Evolution, South Africa) were desired for each sample. Therefore, the total volume of sample needed was 1.25mL as each straw is 0.25mL. The amount of semen sample that was needed according to the desired sperm concentration was multiplied by 1.25, and this answer was subtracted from 1250µL to produce the volume needed from Fraction A of the diluent. Fraction A was added very slowly to the sperm sample and microscope slides (labelled 'Fresh') were prepared for each diluted Fraction A sample. The diluted samples were then placed in the refrigerator for 2 hours at 4-5˚C. After the 2 hours, the diluted Fraction A samples were removed from the refrigerator and slides were prepared again (labelled 'Eq'). For further dilution with Fraction B, 1250µL was added very slowly to the diluted Fraction A samples and placed in the refrigerator for a further 2 hours. After the 2 hours, microscope slides were prepared again (labelled 'EqAB').

In Phase 2 the desired sperm concentration was kept as 300 x 10^6 spermatozoa/mL, but in Phase 3 it was changed to 600 x 10^6 spermatozoa/mL. In both these phases, the samples were diluted with a milk-yolk extender (MYE). The MYE was prepared by adding 1.25mL egg-yolk to 48.75mL of skimmed milk (Parmalat, South Africa) into a 50mL test tube and gently agitating it until a homogenous mixture was achieved. This extender was prepared before sampling in the mornings and was placed in a Memmert water bath maintained at 37˚C. The individual sperm concentrations from each sample, given the desired sperm concentrations, determined how much of a sample was added to the MYE so that a desired 1 mL diluted sample was achieved in Phase 2. The same calculation methodology was followed for Phase 3, except that the amount of sample determined according to the above-mentioned method was divided by two so that the sample and MYE added up to a desired 500µL. In Phase 3, Procaine hydrochloride (Sigma-Aldrich, United States of America, PCode. 102370538) (500µL) was then added to the diluted sample to achieve a ratio of 1:1.

3.4.9 Equilibration time

In Phase 1, the semen samples were diluted in two steps with a glycerol diluent (Biladyl) to achieve a final concentration of 300 x 10^6 spermatozoa/mL. Fraction A was slowly added to the samples as the first step of the dilution. Once the samples were diluted with Fraction A, the samples were placed in the refrigerator, set to 4-5˚C, to equilibrate for 2 hours. When the first equilibration was done, the

samples were removed from the refrigerator and Fraction B of the Biladyl diluent was added to the samples. Once the samples were diluted with Fraction B, the samples were placed in the refrigerator for a final 2-hour equilibration. After the final 2-hour equilibration, the samples were ready for cryopreservation.

3.4.10 Loading, cryopreservation, and thawing of straws

After the diluted samples were in the refrigerator for the final 2 hours, the samples were loaded into 0.25cc Cassou straws and sealed with a PVC sealing powder (Poudre de Bouchage Violette; IMV Technologies, France, Ref. 018819).

The sealed straws were placed 3cm above the level of the liquid nitrogen in a freezer box, for a period of 5 minutes, whereafter it was plunged into the liquid nitrogen for a further 2 minutes. Thereafter, the straws were transferred directly to a canister within the liquid nitrogen tank, and canister number was recorded. Straws were stored inside the liquid nitrogen tank at -196˚C until it was needed for analysis. The level of the liquid nitrogen inside the tank was carefully monitored weekly and, when required, topped up to the required level.

For thawing of the straws for evaluation during Phase 1, a small polystyrene box was filled halfway with liquid nitrogen. This box was used to place the canisters with the straws in immediately after they have been removed from the liquid nitrogen tank. The straws were then sorted inside the polystyrene box, under the liquid nitrogen, so that the straws from each ram, for each semen collection date, were grouped together. Once the group was complete, the straws were thawed at 37˚C for 30 seconds to thaw (Paulenz *et al.,* 2007). The straws were then removed and gently dried with a paper towel, and the contents of the straws were then emptied into an Eppendorf tube.

3.4.11 Microscopic evaluation

For microscopic evaluation, the microscope slides that were prepared during sample processing were placed under an Olympus IX70 inverted microscope (Olympus, Japan) fitted with an Olympus SC50 camera (Olympus, Japan). This camera was connected to a laptop which was used to run an imaging software program, cellSens, that enabled photos being taken of the microscope slides. The photos of the slides were then saved onto an external hard drive and assessed at a later stage.

Photos were taken at a 20x and 40x magnification. For the 20x magnification a maximum of 10 photos were used, which in most cases, allowed for 100 spermatozoa to be counted. This magnification was

used for the assessment of sperm abnormalities and sperm viability. For the 40x magnification, a maximum of 15 photos were used, which in most cases, allowed for 100 spermatozoa to be counted. This magnification was specifically used to assess the acrosome integrity of the spermatozoa. If a sperm-count of 100 was achieved before the 10 and 15 photos were taken for the 20x and 40x magnifications, respectively, then that number of photos where the 100th spermatozoon was counted, was used. Photos were labelled with specific codes, starting with the ram number, followed by the monster type/time, the magnification, the sample date, and lastly the photo number, for example, R1-0h-20X-26-5-2021(1).

3.4.11.1 Sperm viability

After the semen samples were diluted with the storage medium to have a standardised sperm concentration, microscope slides were prepared by placing 50µL of the diluted sperm sample (mixed with procaine in Phase 3) on the microscope slide, and then adding two drops of an Eosin-Nigrosin stain (Kyron Laboratories, South Africa) (Björndahl *et al.,* 2003). The sperm-stain mixture was agitated gently and left for 30 seconds to react with the spermatozoa. After the 30 seconds, a smear was made for evaluation. Spermatozoa that were alive at the time and not having any damage to their acrosome or post-acrosomal region remained completely light in colour (Figure 3.4). Spermatozoa that were dead and had damaged acrosomes or/and post acrosomal regions, allowed for the purple/pink Eosin-Nigrosin stain to enter the sperm head, thus giving them a purple/pink appearance (Figure 3.4).
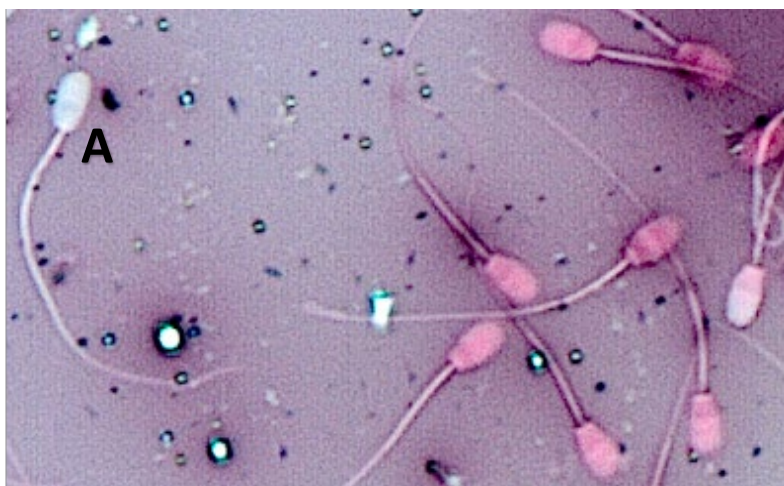
Figure 3.4 Spermatozoa from this study stained with Eosin-Nigrosin. Live spermatozoa are completely white in colour (A); Dead spermatozoa are stained pink/purple (all the rest).

The microscope slides were viewed with an Olympus IX70 inverted microscope connected to an Olympus SC50 camera that was operated through a laptop. The necessary number of photos were taken with cellSens imaging software on the laptop at 20X magnification as explained in Chapter two.

A minimum of 100 spermatozoa were counted per sample, and the percentage live expressed as a percentage, using the following equation:

Number of live spermatozoa/total number of spermatozoa counted x 100 = % live spermatozoa

3.4.11.2 Sperm morphology

Various morphological abnormalities were analysed for each of the different sperm components (head, midpiece and tail). These morphological abnormalities are presented in Table 3.4.

Table 3.4 The morphological abnormalities analysed in this study for the head, midpiece, and tail of the sampled spermatozoa.

Head	Midpiece	Tail
Microcephalic	Elongated	Coiled
Macrocephalic	Abaxial	Short
Round	Thickened	Double
Pyriform	Double	Protoplasmic droplet
Elongated	Protoplasmic droplet	Bent
Double	Bent	Other
Detached	Other	

The viability smears were also used for the assessment of morphology. A minimum of 100 spermatozoa were counted per sample, and the percentage abnormal spermatozoa expressed as a percentage, using the following equation:

Number of abnormal spermatozoa/total number of spermatozoa counted x 100 = % abnormal spermatozoa

3.4.11.3 Acrosome integrity

The same microscope slides that were prepared for sperm viability and sperm morphology were used for acrosome integrity. An Olympus IX70 inverted microscope connected to an Olympus SC50 camera, and a laptop was used to take photos with cellSens imaging software, at a magnification was adjusted to 40x. Spermatozoa with intact acrosomes were classified as those where the acrosomal membrane was completely smooth, perfect in shape, with no uneven dark spots or light patches along the edges, and no edges lifting where the acrosome reaches the post-acrosomal region. If the acrosome showed

any of these defects, then the acrosome integrity was compromised. A minimum of 100 spermatozoa were counted per sample, and the number of abnormal acrosomes expressed as a percentage, using the following equation:

Number of spermatozoa with abnormal acrosomes/total number of spermatozoa counted x 100 = % spermatozoa with abnormal acrosomes

3.4.12 Hyperactivation functional test

In Phase 3, Procaine hydrochloride was used to hyperactivate the spermatozoa (Van der Horst *et al.*, 2020). The following formula was used to calculate the amount of procaine hydrochloride needed to make 50mL of a 10mM stock solution:

m = c x V x M
c = 10mM, V = 50mL, and the molecular weight(M) of Procaine hydrochloride is 272.77g/mol.

Therefore, m = 0.01 x 0.05 x 272.77 = 0.136g procaine hydrochloride was required for preparation of the stock solution.

The procaine hydrochloride was added to the diluted sample in a ratio of 1:1 into a 2mL Eppendorf tube (Lasec, South Africa). The Eppendorf tubes were then incubated at 5% CO_2 for 15 minutes in a HERACell 150i Thermo Fisher CO_2 incubator (SepSci, South Africa) set at 38.5˚C. Microscope slides were prepared at intervals, 0 min, 15 min, and 30 min, since the addition of procaine hydrochloride. The Eppendorf tubes were thus placed in the incubator for a further 15 minutes after the preparation of the microscope slides at the 15 min-interval. The microscopic parameters were assessed as mentioned above.

3.4.13 Statistical analysis

The data in this study were analysed using Microsoft XLStat Annual Version 2021.4.1 and SAS Enterprise Guide 5.1 software, Version 9.3 for Windows. The HR, LR, and Control groups were compared in terms of the respective sperm parameters (percentage abnormal spermatozoa, percentage abnormal acrosomes, and percentage live spermatozoa) with general linear models, using PROC GLM of SAS Enterprise Guide 5.1 software, Version 9.3. The intercept of the regression equations was used as a covariate to adjust for differences in the starting values. The resulting

regression coefficients were then used in an analysis of covariance (ANCOVA) in XLStat. Significant differences were noted at a significance level of $p \leq 0.05$ (95%).

A principal component analysis (PCA) was performed using XLStat to determine the relationship between thermographic data and rectal temperature recordings for the sampling weeks during Phase 2.

The data in Phase 3 of the study were analysed using Microsoft XLStat Annual Version 2021.4.1. A one-way analysis of variance (ANOVA) was used for the percentage of abnormal spermatozoa, percentage of abnormal acrosome, and percentage live spermatozoa for the resilience groups over the 30 minutes of incubation. The one-way analysis of variance (ANOVA) approach was used for the percentage of abnormal spermatozoa, percentage of abnormal acrosome, and percentage live spermatozoa for the resilience groups at every time interval, 0 min, 15 min, and 30 min, respectively.

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Chapter 4

Resilience of ram spermatozoa to heat stress, induced using scrotal insulation

Abstract

The study investigated the influence of scrotal insulation on the resilience of ram spermatozoa to heat stress. Twelve adult Dohne Merino rams were classified according to fresh and post-thaw sperm morphology as high resilience (HR), or low resilience (LR), and were subsequently subjected to scrotal insulation for a period of seven consecutive days. Semen samples were collected using electro-ejaculation prior to the fitting of the scrotal insulation devices (Week 0), on the last day of scrotal insulation (Week 1), and then every fortnight for a further 10 weeks. Temperature loggers (iButtons) that logged humidity as well, were placed in the scrotal insulation devices to monitor the immediate scrotal environment during insulation. Infrared thermography was used to measure body temperature and scrotal temperatures of all rams during each sampling session. Rectal and ambient temperatures were also collected on all sampling days. Semen samples were stored at 4-5°C for 48h, and sperm viability, sperm morphology, and acrosome integrity were assessed at 0h, 12h, 24h, 36h, and 48h of liquid storage. The scrotal insulation approach in the present study proved to be effective in insulating the testes, as evident in the influence thereof on spermatogenic activity. No significant differences were observed between the HR, LR, and Control groups for the percentage of abnormal and live spermatozoa over time during liquid storage (0-48h). Rams differed in the ability of their spermatozoa to offer resilience to heat stress when the entire sampling period of 11 weeks was considered. The morphology of spermatozoa took 70 days to achieve pre-insulation values (below 40% for most rams), which indicates that early spermatogenesis was significantly affected by the heat stress. Considering the change in ambient temperature at Week 0 and Week 11 of the study as well as the different times of measuring scrotal temperatures at these weeks, scrotal temperatures took approximately 42 days to recover (18.64°C – 24.84°C), which coincides with the time it takes for spermatozoa to be produced from spermatogonia. By assessing the change in iButton and scrotal temperatures, individual differences were also found between rams, indicating that certain rams are more efficient in thermoregulating testicular temperature than others.

4.1 Introduction

The global human population faces one of its greatest challenges in the history of humankind, known as climate change, which involves increasing ambient temperatures, rising sea levels due to melting icecaps and glaciers, as well as major shifts in weather patterns and ocean currents (Steinfeld, 2006). Global warming can be defined as the increase in the average temperature of Earth, as a result of the accumulation of greenhouse gasses in the atmosphere (Para *et al.*, 2020).

It is well documented that a large increase in the demand for animal production in the following decades is predicted given the trend in the global human population growth and this also coincides with the period in which the world will experience drastic changes in the global climate that will ultimately effect agriculture on a local and global scale (Nardone *et al.*, 2010; Sejian *et al.*, 2015). Global warming will have a far more drastic effect on livestock systems following a grazing and mixed farming approach compared to industrialised systems. This will be mainly due to predicted lower rainfall, increased incidence and severity of droughts, and the resultant effect on crop and pasture production, as well as an increased solar radiation phenomenon (Nardone *et al.*, 2010).

Animal production has shown significant growth and improvement in recent years as the use of ARTs have received much greater interest from farmers (Kordan *et al.*, 2013). Economies in developing countries, specifically those exposed to tough climatic conditions and sub-fertile lands, rely largely on the contribution from small ruminants (Amiridis and Cseh, 2012). Due to sheep being seasonal breeders, farmers had to start implementing ARTs, as it permits animals with high genetic value to produce more offspring than would be possible through a natural mating approach (Rahman *et al.*, 2008; Amiridis and Cseh, 2012). Assisted reproductive technologies offer solutions to producers to mitigate seasonal breeding effects of small stock. Artificial insemination (AI) is the most common and most used ART in the world and entails the artificial placement of spermatozoa in the female reproductive tract (Ogbuewu *et al.*, 2010).

Semen samples must however be preserved under artificial conditions after collection, either in a liquid state (unfrozen) at temperature lower than body temperature, or in a frozen state at sub-zero temperatures for subsequent use for AI (Salamon and Maxwell, 1995a). Spermatozoa are therefore exposed to changes in temperatures during the process of sample collection and transport to the site of processing. When semen is frozen and thawed, it also experiences a further reduction in temperature, followed by an increase in temperature, resulting in a reduced number of motile sperm

due to ultrastructural, biochemical, and functional changes to the spermatozoa (Salamon and Maxwell, 1995b).

Semen sample quality is not only negatively affected by low temperatures associated with processing, but also through exposure to temperatures higher than normal *in vivo*, as has been observed by the use of scrotal insulation, where spermatogenesis is significantly affected by both ambient temperature and the length of heat exposure (Alves *et al.*, 2016). In mammals, the testes should be maintained at $2 - 8°C$ lower than normal body temperature (Fleming *et al.*, 2004). When rams are exposed to high ambient temperatures, sperm sample quality degeneration occurs as a direct effect, as the rams lose the ability to thermoregulate locally, resulting in increased testicular temperatures (Moule and Waites, 1963).

The testes can be locally subjected to above-normal temperature, i.e. by induced cryptorchidism, immersion in a water bath (short-term), or by scrotal insulation (Setchell, 1998). When studying the ability of animals to manage physiological processes such as gametogenesis, scrotal insulation is a valuable method of determining whether specific male animals are genetically better adapted to offer resilience and thus be better capable of alleviating the effects of heat stress on spermatogenesis (Setchell, 1998). A well-designed scrotal insulation bag does not only increase testicular temperature by means of insulation, but also impacts the two muscles responsible for thermoregulation of the testes, thus minimising the actions of cremaster muscle that determines the distance of the scrotum relative to the body, as well as the tunica dartos that regulates the scrotal surface area (Para *et al.*, 2020).

When considering the use of ARTs in the sheep industry, it is important to identify rams that produce spermatozoa that are resilient to temperature changes associated with the collection, processing, freezing, and thawing of spermatozoa during ARTs. To enable livestock producers to select rams that are resilient to heat stress and its effect on spermatogenesis, it is important to understand how spermatogenesis is ultimately affected. It is also important to determine if rams differ in their ability to resume normal spermatogenic activity in the testes. The aim of the study was therefore to determine the influence of scrotal insulation on sperm sample quality, and the resumption of normal spermatogenic activity in the testes of adult Dohne Merino rams, to potentially classify rams towards resilience to heat stress and its related effect on physiological processes such as spermatogenesis.

4.2 Materials and methods

Ethical approval for this study was obtained from the Animal Care and Use Ethics Committee of the University of Stellenbosch (#ACU-2019-9835). The animal care techniques and procedures in this study followed the guidelines stipulated in the South African National Standards document 10386:2008.

4.2.1 Experimental location

The Welgevallen Experimental farm (33°56'33"S, 18°51'56"E) served as the location for the sheep in this study, and is situated in Stellenbosch, South Africa. Stellenbosch has an average temperature of 16.2˚C, and an average annual rainfall of 787 mm.

4.2.2 Experimental animals and husbandry

A total of 13 Dohne Merino rams (*Ovies aries*), ranging between two and three years of age, were used in the study. A total of 13 rams were used in Phase 1, and 12 rams were used in Phase 2, respectively. Rams grazed on kikuyu grass paddocks during the day and were kept inside a shed during the evenings.

The rams were carefully monitored at each sampling session for any health- or injury related problems. The rams were also monitored prior to and after sampling, and in the paddocks for any abnormal behaviour. If a ram showed signs of any severe problems, a qualified veterinarian was contacted for assistance. Specific attention was paid to the behaviour and comfort levels of rams during the period of scrotal insulation in Phase 2, ensuring that the heat stress on the testes did not cause abnormal behaviour of the rams. It was ensured that the scrotal insulation devices were not too tightly fitted around the scrotums, and that the straps were tight enough without causing any rash where it went around the legs. Rams were sheared at the end of January and at the beginning of August in 6-month cycles. Rams were therefore sheared approximately one month prior to scrotal insulation. The scrotums of the rams were sheared before scrotal insulation to ensure that all scrotal insulation devices fitted snugly around the scrotum. The hooves of the rams were examined regularly and trimmed when required.

4.2.3 Experimental design

4.2.3.1 Phase 1 – Classification of rams in terms of high or low resilience based on the ability of their spermatozoa to handle stress caused by sample processing, cryopreservation, and thawing

Phase 1 involved the collection of semen samples from 13 Dohne Merino rams, aged between one and two years. The rams were subsequently allocated into high resilience (HR) and low resilience (LR) groups based on the percentages of abnormal spermatozoa in fresh, equilibrated, and post-thaw samples. In this study, resilience refers to the identification of rams according to the ability of their spermatozoa to offer resistance or to tolerate changes induced by processing, cryopreservation, and thawing. The collection of semen samples in Phase 1 started on 11 November 2020 and finished on 9 December 2020. The cryopreserved samples were thawed and processed in January 2021.

The rams were thus only placed in the two resilience groups once the assessment of percentages of abnormal spermatozoa recorded for fresh, equilibrated, and post-thaw samples, has been done, respectively. The HR group consisted of rams with the lowest percentage of abnormal spermatozoa, whereas the LR group consisted of rams with the highest percentages of abnormal spermatozoa. Rams allocated to the HR group (n=6) produced samples with LS Means values lower than 10.85 ± 2.41%, whereas the LR group (n=6) produced samples with LS Means values higher than 10.85 ± 2.41%. The LS Means value of 10.85 is the value precisely between the LS Means value of the LR ram with the highest average percentage abnormal spermatozoa (10.785 ± 2.41%) and the LS Means value of the HR ram with the lowest average percentage abnormal spermatozoa (10.922 ± 2.41%). The group of 12 rams could then be divided into two groups of 6 rams each.

4.2.3.2 Phase 2 – Effect of scrotal insulation on the viability, morphology, and acrosome integrity of ram spermatozoa

Twelve Dohne Merino rams, aged between one and two years, were involved in Phase 2 of the study. Prior to Phase 2, the group size was reduced to have two groups consisting of 6 rams each. These 12 rams were randomly divided into two groups of 6 rams each, and not according to the HR or LR groups. Ten of the 12 rams were subjected to scrotal insulation, with the other two rams not subjected to scrotal insulation, being named the Control group. One ram from the HR group and one ram from the LR group formed the Control group.

A semen sample was collected from each ram on the morning before the scrotal insulation devices were fitted on the rams (week 0). The scrotal insulation devices were fitted after collection of the first

samples, and it was ensured that the devices fitted snugly around the scrotums to prevent heat loss through radiation. The rams were carefully monitored during normal activity to ensure that the scrotal insulation bag was kept in position by stabilising the custom-designed harnesses (refer to Appendix 1 for design options and details).

Scrotal insulation devices remained fitted for seven consecutive days, after which the devices were removed. A semen sample was collected from each ram on the last day of scrotal insulation, with this sampling interval being considered as Week 1. Semen samples were collected on Wednesday and Thursday mornings for the two groups, respectively. Semen samples were then collected every fortnight up to 10 weeks (Week 11) since the removal of the scrotal insulation devices. The samples were collected in the same order throughout the sampling period, and each ram provided seven semen samples during this phase.

Testicular measurements (length and circumference) and thermographic measurements were taken on the mornings of semen collection. Week 1 was the only exception, where the measurements were taken in the afternoon after the scrotal insulation devices were removed.

In Phase 2, the collection of semen samples and thermographic measurements started on 24 March 2021 and finished on 14 June 2021. The scrotal insulation devices were fitted on the 14th and 15th of June 2021, respectively for the two groups, and were removed 7 days later, on the 31st of June 2021 and the 1st of July 2021, respectively.

Semen samples were subjected to the same processing methods, which included macroscopic and microscopic evaluation. Macroscopic evaluation consisted of the recording of sample volume, sample colour, sperm mass motility, and sample viscosity (Hafez and Hafez, 2008). The sperm concentration of each sample was also determined. Following concentration determination, semen samples were diluted with a milk-yolk extender (MYE) (Paulenz *et al.*, 2002). Microscopic evaluation involved the assessment of the parameters, sperm viability (% live), sperm morphology (% abnormality), and acrosome integrity (% intact acrosomes). Diluted samples were then subjected to liquid storage at 4-5 °C for 48h. Sperm viability, morphology, and acrosome integrity was determined at 0h, 12h, 24h, 36h, and 48h after dilution.

4.2.4 Experimental procedures

4.2.4.1 Scrotal insulation

In Phase 2 of the study, scrotal insulation devices were designed similar to the study of Cruz-Júnior *et al.* (2015) and fitted on 10 of the 12 rams (for more details on the design of the scrotal insulation devices, refer to Chapter 3, Sections 3.4.1.1 and 3.4.1.2). The scrotal insulation devices were fitted on the rams on the first day of sampling, which was then considered as Week 0 of Phase 2. The scrotal insulation devices were kept on the rams for 7 consecutive days.

4.2.4.2 Testicular and thermographic measurements

Testicular measurements (length and circumference) and thermographic measurements were taken in the morning before every sampling session, except Week 1, where measurements were taken in the afternoon after the devices were removed from the scrotums (for more details on the thermographic measurements, please refer to Chapter 3, Section 3.4.3). The rectal temperature of each ram was also determined before every sample collection.

4.2.4.3 iButton data

Five iButton (DS1923#F5 Hygrochron) temperature and humidity loggers were placed inside the scrotal insulation bag of rams allocated to the respective treatment group. The iButtons were pre-set to log the temperature and humidity at 5-minute intervals for the 7 days of scrotal insulation. The data was logged and captured in Microsoft Excel, and summarized with the software programme, ColdChain ThermoDynamics.

4.2.4.4 Weather data

During Phase 2, weather data was gathered from the weather station (CR800 Series) located on the Welgevallen experimental farm. The weather data was recorded at hourly intervals and included wind speed (including maximum wind speed) (m/s), wind direction (degrees), average air temperature (˚C), relative humidity (%), and rain fall (mm). The minimum temperature recorded was 6.59˚C, and the maximum temperature recorded was 36.34˚C.

4.2.4.5 Semen collection

Semen samples were collected by means of electro-ejaculation in Phase 1 and Phase 2 of the study (for a detailed explanation on the specific procedure followed, please refer to Chapter 3).

Semen samples were collected from 13 rams during Phase 1 of the study. The rams were randomly allocated into three groups of 3, 5, and 5 rams per group. Rams were subjected to one semen collection per week, for three consecutive weeks. Each ram provided a total of 3 semen samples for this phase (n=39).

Semen samples were collected from 12 rams during Phase 2 of the study. The rams were randomly allocated into two groups of 6 rams each. One semen sample was collected per ram at respectively week 0, 1, 3, 5, 7, 9, and 11 of the scrotal insulation study (n=84).

4.2.4.6 Semen evaluation and processing

Once the samples arrived at the laboratory, samples were first subjected to macroscopic evaluation that involved the assessment of volume, colour, and mass motility. The concentration of the samples was then determined.

Phase 1

The samples in Phase 1 were diluted with Fraction A (Minitube, Germany, Ref. 13500/0004) and Fraction B (Minitube, Germany, Ref. 13500/0006) of a Biladyl diluent. The diluted samples were subjected to cryopreservation. Microscope slides were prepared for fresh, equilibrated, and thawed, diluted samples. Sperm viability, morphology, and acrosome integrity were determined with microscopic evaluation of the microscope slides. Once the samples were diluted with Biladyl in Phase 1, the diluted samples were subjected to cryopreservation in liquid nitrogen and were stored until it was needed to be thawed for further assessment (please refer to Chapter 3 for more details on the cryopreservation protocol followed).

Phase 2

The samples in Phase 2 were diluted with a milk-yolk extender (MYE) (see Chapter 3 for protocols on the respective dilution methods). Diluted samples were subjected to liquid storage at 4-5 °C for 48h. Sperm viability, morphology, and acrosome integrity were determined at 0h, 12h, 24h, 36h, and 48h after dilution (please refer to Chapter 3 for details about the microscopic evaluation).

4.2.5 Statistical analysis

The data in this study were analysed using Microsoft XLStat Annual Version 2021.4.1 and SAS Enterprise Guide 5.1 software, Version 9.3 for Windows. The HR, LR, and Control groups were compared in terms of the respective sperm parameters (percentage abnormal spermatozoa,

percentage abnormal acrosomes, and percentage live spermatozoa) with general linear models, using PROC GLM of SAS Enterprise Guide 5.1 software, Version 9.3. The intercept of the regression equations was used as a covariate to adjust for differences in the starting values. The resulting regression coefficients were then used in an analysis of covariance (ANCOVA) in XLStat. Significant differences were noted at a significance level of $p \leq 0.05$ (95%).

A principal component analysis (PCA) was performed using XLStat to determine the relationship between thermographic data and rectal temperatures recording for the sampling weeks during Phase 2.

Scatter plots were created in XLStat for the relationship between iButton data and ambient temperatures. Scatter plots were also created for % abnormal spermatozoa, % live spermatozoa, rectal temperature, and scrotal temperature, for the sampling weeks.

4.3 Results

4.3.1 Descriptive statistics

Table 4.1 presents the descriptive statistics for the sperm viability, sperm morphology, and sperm acrosome integrity measured for the resilience groups (HR and LR), and control group, of Dohne Merino rams over a period of 11 weeks.

Table 4.1 Descriptive statistics for sperm viability, sperm morphology, and sperm acrosome integrity measured for resilience groups, and control group, of Dohne Merino rams over a period of 11 weeks.

Parameter	Mean ± SE	Range	Coefficient of variation
Control group (n=2)			
Sperm viability (% live)	1.846 ± 0.489	0.000 – 23.358	2.119
Sperm morphology (% abnormal)	20.819 ± 2.435	1.980 – 79.762	0.936
Acrosome integrity (% abnormal)	99.985 ± 0.015	99.029 – 100.000	0.001
High-resilience group (n=5)			
Sperm viability (% live)	2.586 ± 0.381	0.000 – 38.095	1.885
Sperm morphology (% abnormal)	19.124 ± 1.355	0.730 – 64.602	0.907
Acrosome integrity (% abnormal)	99.912 ± 0.026	98.058 – 100.000	0.003
Low-resilience group (n=5)			
Sperm viability (% live)	2.336 ± 0.287	0.000 – 26.549	1.597
Sperm morphology (% abnormal)	31.172 ± 1.864	1.563 – 92.000	0.777
Acrosome integrity (% abnormal)	99.921 ± 0.025	97.143 – 100.000	0.003

4.3.2 Sperm viability, sperm morphology, and acrosome integrity during liquid storage

A linear regression over time (0-48h) was fitted for each treatment group for each variable, percentage abnormal, percentage live, and percentage abnormal acrosomes. The percentage abnormal acrosomes displayed no change over time due to values being close to 100 for all samples. The resulting coefficients for percentage live and percentage abnormal were used in an ANCOVA to determine the gradients for percentage live and percentage abnormal spermatozoa, and where the coefficient for the gradient of the first sampling was included as a covariate, are presented in Table 4.2.

Table 4.2 The ANCOVA-calculated gradients for percentage live and percentage abnormal spermatozoa during a scrotal insulation study using adult Dohne Merino rams.

Treatment group	Gradient % Live	Gradient % Abnormal
Control (n=2)	-0.013 ± 0.037	0.084 ± 0.047
HR (n=5)	-0.043 ± 0.023	0.111 ± 0.036
LR (n=5)	-0.047 ± 0.23	0.033 ± 0.034

4.3.3 Correlation between thermographic, ambient temperature, and iButton measurements

A Principal Component Analysis (PCA) was carried out to determine the correlation between the thermographic parameters and rectal temperature. A PCA analysis was performed for each sampling week, i.e., week 0 to week 11. The data recorded for the Control group (that contained only two rams), were not included in the PCA analyses. The results presented below are only based on the data recorded per week. The PCA analysis for the groups and each sampling week did not display a significant difference between the HR and LR group and is therefore not presented.

Table 4.3 presents the correlations calculated for the respective thermographic parameters and rectal temperature measurements on the day before rams were fitted with scrotal insulation devices in the scrotal insulation study.

High correlations were reported for the respective body measurements and the scrotal thermographic parameters during Week 0. The highest correlation for the side measurements was between the front and the back (0.929, Table 4.3). The highest correlation for the scrotal measurements was between the top and middle of the scrotum (0.887, Table 4.3). The highest correlation between the side and

scrotal measurements was between the front of the ram and the bottom of the scrotum (0.873, Table 4.3) Rectal temperature for most parameters, was positively and relatively low correlated with the respective body and scrotal measurements (Table 4.3). The only exception was the correlation between rectal temperature and thermographic measurement on the posterior end of the flank of rams, which was negative and low (-0.016, Table 4.3).

Table 4.3 The correlation matrix calculated for the thermographic parameters and rectal temperature measurements at Week 0 of a scrotal insulation study using adult Dohne Merino rams.

Parameter	Side Middle (˚C)	Side Back (˚C)	Side Front (˚C)	Scrot Middle (˚C)	Scrot Left (˚C)	Scrot Right (˚C)	Scrot Top (˚C)	Scrot Bottom (˚C)	Rectal temperature (˚C)
Body									
Side Middle (˚C)	**1**	**0.803**	**0.922**	**0.675**	**0.623**	**0.625**	**0.710**	**0.803**	0.394
Side Back (˚C)	**0.803**	**1**	**0.929**	0.486	0.422	**0.670**	0.542	**0.854**	-0.016
Side Front (˚C)	**0.922**	**0.929**	**1**	**0.619**	0.499	**0.731**	**0.669**	**0.873**	0.154
Scrotum									
Middle (˚C)	**0.675**	0.486	**0.619**	**1**	0.521	**0.821**	**0.887**	**0.730**	0.204
Left (˚C)	**0.623**	0.422	0.499	0.521	**1**	0.519	0.237	0.453	0.338
Right (˚C)	**0.625**	**0.670**	**0.731**	**0.821**	0.519	**1**	**0.632**	**0.793**	0.077
Top (˚C)	**0.710**	0.542	**0.669**	**0.887**	0.237	**0.632**	**1**	**0.699**	0.136
Bottom (˚C)	**0.803**	**0.854**	**0.873**	**0.730**	0.453	**0.793**	**0.699**	**1**	0.253
Rectal temperature (˚C)	0.394	-0.016	0.154	0.204	0.338	0.077	0.136	0.253	**1**

Values in bold are different from 0 with a significance level alpha=0.05

Table 4.4 presents the correlations calculated for the respective thermographic parameters and rectal temperature measurements on the last day of scrotal insulation in a scrotal insulation study.

A drastic change from a high to a low correlation is observed between the temperatures of the side of the ram and temperatures of the scrotum of the ram from Week 1 (Table 4.4 and Figure 4.1). The measurements taken for Week 1 were taken 7 days since the measurements of Week 0, and was thus taken on the day that the scrotal insulation devices were removed from the scrotums of rams. The correlations between the temperatures of the side of the ram and temperatures of the scrotum of the ram only started to return to initial correlation from Week 7, thus being positively correlated, which was not seen for all these correlations between Week 1 and Week 5.

Table 4.4 The correlation matrix calculated for the thermographic parameters and rectal temperature measurements at Week 1 of a scrotal insulation study using adult Dohne Merino rams.

Parameter	Side Middle (˚C)	Side Back (˚C)	Side Front (˚C)	Scrot Middle (˚C)	Scrot Left (˚C)	Scrot Right (˚C)	Scrot Top (˚C)	Scrot Bottom (˚C)	Rectal temperature (˚C)
<u>Body</u>									
Side Middle (˚C)	**1**	**0.979**	**0.987**	0.236	0.120	0.282	-0.150	0.496	0.398
Side Back (˚C)	**0.979**	**1**	**0.954**	0.280	0.080	0.308	-0.059	0.525	0.432
Side Front (˚C)	**0.987**	**0.954**	**1**	0.237	0.102	0.240	-0.232	0.489	0.355
<u>Scrotum</u>									
Middle (˚C)	0.236	0.280	0.237	**1**	0.554	**0.743**	**0.584**	**0.693**	0.491
Left (˚C)	0.120	0.080	0.102	0.554	**1**	**0.813**	0.518	**0.576**	0.354
Right (˚C)	0.282	0.308	0.240	**0.743**	**0.813**	**1**	**0.704**	**0.750**	0.475
Top (˚C)	-0.150	-0.059	-0.232	**0.584**	0.518	**0.704**	**1**	0.415	0.438
Bottom (˚C)	0.496	0.525	0.489	**0.693**	**0.576**	**0.750**	0.415	**1**	0.548
<u>Rectal temperature</u> (˚C)	0.398	0.432	0.355	0.491	0.354	0.475	0.438	0.548	**1**

Values in bold are different from 0 with a significance level alpha=0.05

For Week 1, the correlations of rectal temperature were more correlated with the temperatures of the side of the rams and temperatures of the scrotum of the rams, and all positive, (Table 4.4), compared to Week 0, which did not reveal all positive correlations (Table 4.3). Week 3 displayed similar correlations as Week 1 for rectal temperature and all the other variables (Table 4.5). Week 5 and 7 again displayed very low correlations for rectal temperature and all the other variables (Tables 4.6 and 4.7). Week 9 displayed drastically higher correlations for rectal temperature and all the other variables, specifically for temperatures of the scrotum, with correlations for all variables being positively correlated again, in comparison to correlation results of Week 5 and Week 7 (Table 4.8). Week 11 displayed even higher correlations for the rectal temperatures and the temperatures of the side of the ram than Week 9 (Table 4.9). The correlations for Week 11 for rectal temperature and all the other variables were greater than those of Week 0.

Table 4.5 presents the correlations calculated for the respective thermographic parameters and rectal temperature measurements at Week 3 of sampling in a scrotal insulation study.

Table 4.5 The correlation matrix calculated for the thermographic parameters and rectal temperature measurements at Week 3 of a scrotal insulation study using adult Dohne Merino rams.

Parameter	Side Middle (˚C)	Side Back (˚C)	Side Front (˚C)	Scrot Middle (˚C)	Scrot Left (˚C)	Scrot Right (˚C)	Scrot Top (˚C)	Scrot Bottom (˚C)	Rectal temperature (˚C)
Body									
Side Middle (˚C)	**1**	0.395	**0.810**	0.019	0.330	0.007	0.347	0.444	0.342
Side Back (˚C)	0.395	**1**	0.574	-0.409	-0.049	-0.104	-0.146	0.283	0.334
Side Front (˚C)	**0.810**	0.574	**1**	-0.105	0.454	-0.100	0.449	0.513	0.485
Scrotum									
Middle (˚C)	0.019	-0.409	-0.105	**1**	0.208	0.111	0.236	0.300	0.122
Left (˚C)	0.330	-0.049	0.454	0.208	**1**	-0.026	0.379	**0.624**	**0.698**
Right (˚C)	0.007	-0.104	-0.100	0.111	-0.026	**1**	-0.388	-0.181	0.003
Top (˚C)	0.347	-0.146	0.449	0.236	0.379	-0.388	**1**	0.432	0.276
Bottom (˚C)	0.444	0.283	0.513	0.300	**0.624**	-0.181	0.432	**1**	**0.617**
Rectal temperature (˚C)	0.342	0.334	0.485	0.122	**0.698**	0.003	0.276	**0.617**	**1**

Values in bold are different from 0 with a significance level alpha=0.05

Table 4.6 presents the correlations calculated for the respective thermographic parameters and rectal temperature measurements at Week 5 of sampling in a scrotal insulation study.

Table 4.6 The correlation matrix calculated for the thermographic parameters and rectal temperature measurements at Week 5 of a scrotal insulation study using adult Dohne Merino rams.

Parameters	Side Middle (˚C)	Side Back (˚C)	Side Front (˚C)	Scrot Middle (˚C)	Scrot Left (˚C)	Scrot Right (˚C)	Scrot Top (˚C)	Scrot Bottom (˚C)	Rectal temperature (˚C)
Body									
Side Middle (˚C)	**1**	**0.832**	**0.824**	0.210	0.069	-0.014	0.252	0.067	0.032
Side Back (˚C)	**0.832**	**1**	**0.791**	-0.238	0.056	-0.207	0.070	0.169	-0.216
Side Front (˚C)	**0.824**	**0.791**	**1**	0.226	0.081	-0.237	0.371	0.253	0.031
Scrotum									
Middle (˚C)	0.210	-0.238	0.226	**1**	0.109	0.047	0.327	-0.145	0.542
Left (˚C)	0.069	0.056	0.081	0.109	**1**	0.500	0.115	0.352	0.116
Right (˚C)	-0.014	-0.207	-0.237	0.047	0.500	**1**	0.120	-0.070	0.434
Top (˚C)	0.252	0.070	0.371	0.327	0.115	0.120	**1**	0.557	0.204
Bottom (˚C)	0.067	0.169	0.253	-0.145	0.352	-0.070	0.557	**1**	**-0.579**
Rectal temperature (˚C)	0.032	-0.216	0.031	0.542	0.116	0.434	0.204	**-0.579**	**1**

Values in bold are different from 0 with a significance level alpha=0.05

Table 4.7 presents the correlations calculated for the respective thermographic parameters and rectal temperature measurements at Week 7 of sampling in a scrotal insulation study.

Table 4.7 The correlation matrix calculated for the thermographic parameters and rectal temperature measurements at Week 7 of a scrotal insulation study using adult Dohne Merino rams.

Parameters	Side Middle (˚C)	Side Back (˚C)	Side Front (˚C)	Scrot Middle (˚C)	Scrot Left (˚C)	Scrot Right (˚C)	Scrot Top (˚C)	Scrot Bottom (˚C)	Rectal temperature (˚C)
Body									
Side Middle (˚C)	**1**	**0.656**	**0.783**	0.492	0.410	0.147	**0.625**	0.283	0.169
Side Back (˚C)	**0.656**	**1**	**0.616**	0.351	**0.639**	0.306	**0.582**	0.175	-0.092
Side Front (˚C)	**0.783**	**0.616**	**1**	0.241	0.366	0.345	**0.596**	0.529	-0.177
Scrotum									
Middle (˚C)	0.492	0.351	0.241	**1**	0.513	0.347	0.430	0.049	0.049
Left (˚C)	0.410	**0.639**	0.366	0.513	**1**	**0.617**	**0.628**	0.180	0.255
Right (˚C)	0.147	0.306	0.345	0.347	**0.617**	**1**	0.280	0.530	0.179
Top (˚C)	**0.625**	**0.582**	**0.596**	0.430	**0.628**	0.280	**1**	0.387	-0.153
Bottom (˚C)	0.283	0.175	0.529	0.049	0.180	0.530	0.387	**1**	-0.232
Rectal temperature (˚C)	0.169	-0.092	-0.177	0.049	0.255	0.179	-0.153	-0.232	**1**

Values in bold are different from 0 with a significance level alpha=0.05

Table 4.8 presents the correlations calculated for the respective thermographic parameters and rectal temperature measurements at Week 9 of sampling in a scrotal insulation study.

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Table 4.8 The correlation matrix calculated for the thermographic parameters and rectal temperature measurements at Week 9 of a scrotal insulation study using adult Dohne Merino rams.

Parameters	Side Middle (˚C)	Side Back (˚C)	Side Front (˚C)	Scrot Middle (˚C)	Scrot Left (˚C)	Scrot Right (˚C)	Scrot Top (˚C)	Scrot Bottom (˚C)	Rectal temperature (˚C)
Body									
Side Middle (˚C)	**1**	**0.712**	**0.692**	0.527	0.009	0.028	0.130	0.260	0.430
Side Back (˚C)	**0.712**	**1**	**0.779**	0.280	-0.168	-0.071	0.140	0.001	0.062
Side Front (˚C)	**0.692**	**0.779**	**1**	0.428	0.185	-0.095	0.222	-0.240	0.123
Scrotum									
Middle (˚C)	0.527	0.280	0.428	**1**	0.494	0.480	**0.731**	0.519	**0.770**
Left (˚C)	0.009	-0.168	0.185	0.494	**1**	0.519	**0.791**	0.240	0.516
Right (˚C)	0.028	-0.071	-0.095	0.480	0.519	**1**	**0.656**	0.565	0.561
Top (˚C)	0.130	0.140	0.222	**0.731**	**0.791**	**0.656**	**1**	0.555	**0.643**
Bottom (˚C)	0.260	0.001	-0.240	0.519	0.240	0.565	0.555	**1**	**0.783**
Rectal temperature (˚C)	0.430	0.062	0.123	**0.770**	0.516	0.561	**0.643**	**0.783**	**1**

Values in bold are different from 0 with a significance level alpha=0.05

Table 4.9 presents the correlations calculated for the respective thermographic parameters and rectal temperature measurements at Week 11 of sampling in a scrotal insulation study.

Table 4.9 The correlation matrix calculated for the thermographic parameters and rectal temperature measurements at Week 11 of a scrotal insulation study using adult Dohne Merino rams.

Parameter	Side Middle (˚C)	Side Back (˚C)	Side Front (˚C)	Scrot Middle (˚C)	Scrot Left (˚C)	Scrot Right (˚C)	Scrot Top (˚C)	Scrot Bottom (˚C)	Rectal temperature (˚C)
Body									
Side Middle (˚C)	**1**	**0.770**	**0.716**	0.394	-0.072	-0.108	0.362	0.428	0.567
Side Back (˚C)	**0.770**	**1**	**0.687**	0.455	0.360	0.236	0.356	0.394	0.424
Side Front (˚C)	**0.716**	**0.687**	**1**	0.562	0.172	0.303	0.369	0.358	**0.679**
Scrotum									
Middle (˚C)	0.394	0.455	0.562	**1**	0.489	0.466	**0.611**	0.565	**0.755**
Left (˚C)	-0.072	0.360	0.172	0.489	**1**	0.484	0.420	0.478	0.107
Right (˚C)	-0.108	0.236	0.303	0.466	0.484	**1**	0.340	0.440	0.505
Top (˚C)	0.362	0.356	0.369	**0.611**	0.420	0.340	**1**	**0.622**	0.396
Bottom (˚C)	0.428	0.394	0.358	0.565	0.478	0.440	**0.622**	**1**	0.467
Rectal temperature (˚C)	0.567	0.424	**0.679**	**0.755**	0.107	0.505	0.396	0.467	**1**

Values in bold are different from 0 with a significance level alpha=0.05

Figure 4.1 presents the correlations calculated for the respective thermographic parameters and rectal temperature measurements on the day before Dohne Merino rams were fitted with scrotal insulation devices (Week 0) and on the last day of scrotal insulation (Week 1), in the scrotal insulation study.
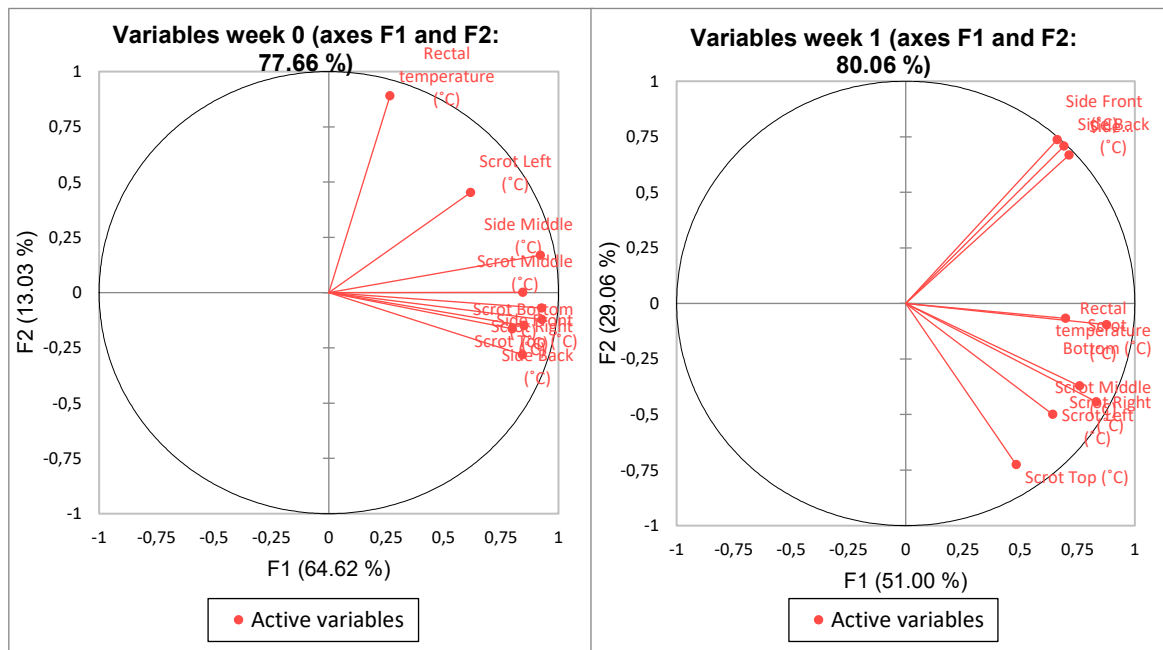

Figure 4.1 PCA plots depicting the relationship between the thermographic parameters and the rectal temperature of Dohne Merino rams before the fitting of the scrotal insulation devices (Week 0) and on the last day of scrotal insulation (Week 1), during a scrotal insulation study.

Figure 4.2 presents the correlations calculated for the respective thermographic parameters and rectal temperature measurements of Dohne Merino rams at Week 3 and Week 9 of sampling in the scrotal insulation study.
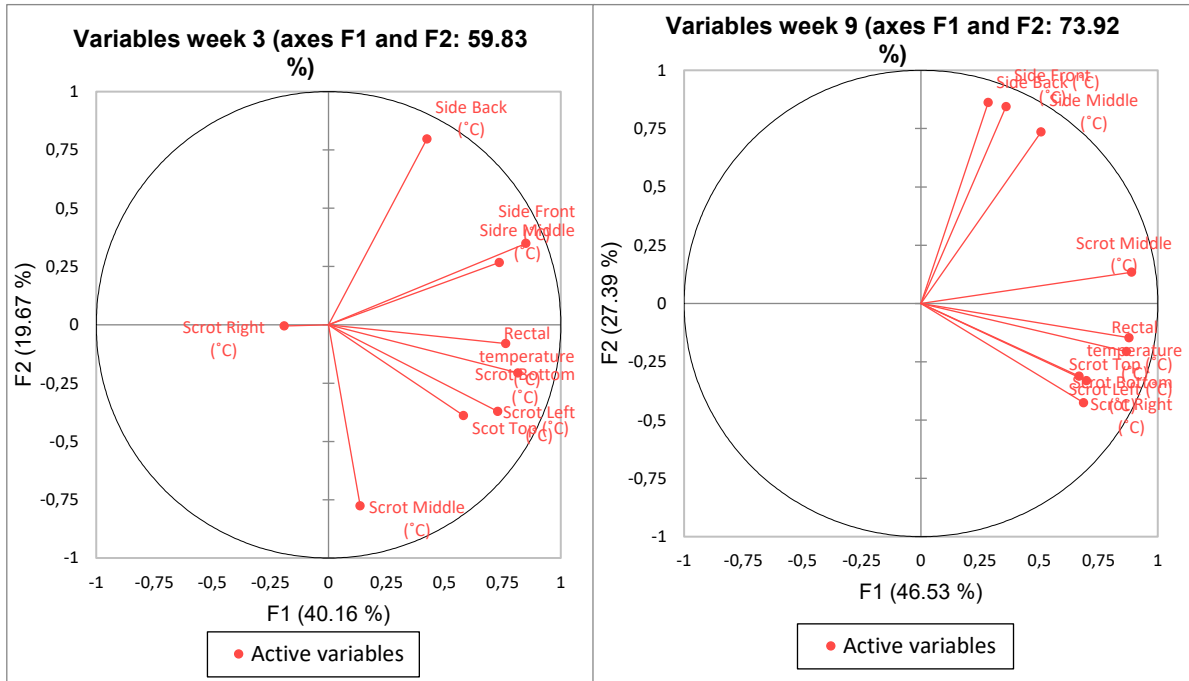
Figure 4.2 PCA plots depicting the relationship between the thermographic parameters and the rectal temperature of Dohne Merino rams at Week 3 and Week 9 of sampling during the scrotal insulation study.

Figure 4.3 presents the correlations calculated for the respective thermographic parameters and rectal temperature measurements of Dohne Merino rams at Week 11 of sampling in the scrotal insulation study.
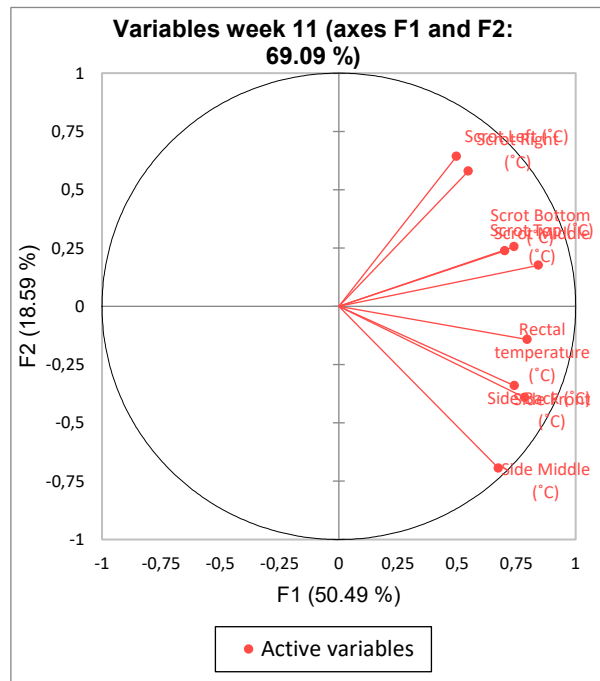

Figure 4.3 A PCA plot depicting the relationship between the thermographic parameters and the rectal temperature of Dohne Merino rams at Week 11 of sampling during the scrotal insulation study.

4.3.4 iButton and weather data

Figure 4.4 presents the relationship between the iButton data and the ambient temperatures recording every hour during the scrotal insulation period of seven days. As expected, the iButton temperatures were generally higher during the day- and night-time periods, when compared to the ambient temperatures recorded during the same time intervals (Figure 4.4).
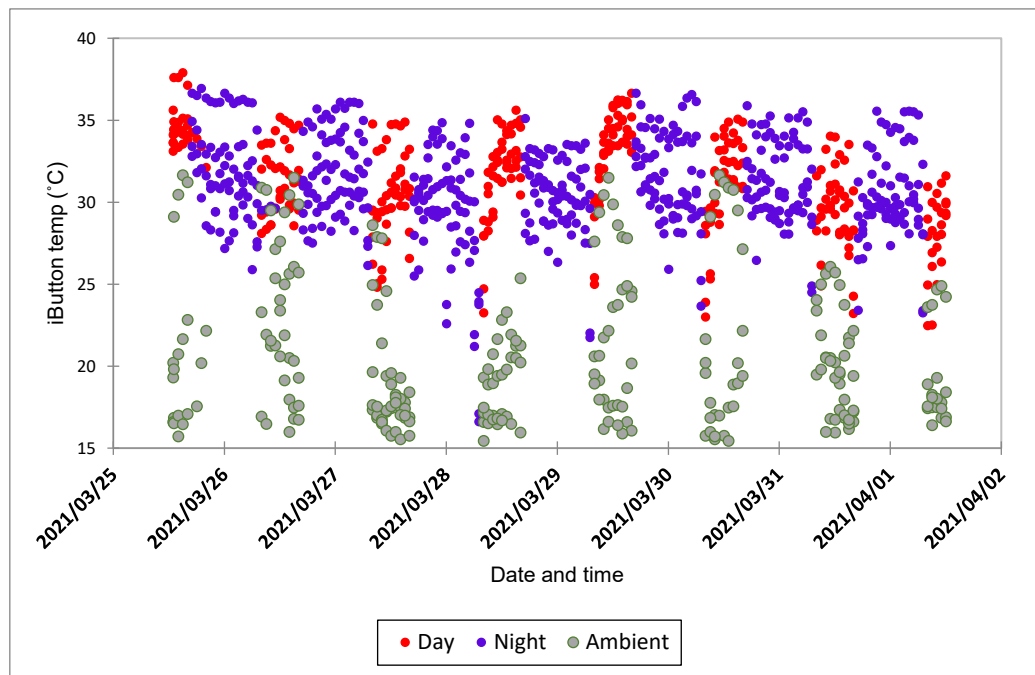

Figure 4.4 A comparison of the day- and night-time temperatures recorded by respectively, iButton temperature loggers placed inside the scrotal insulation devices, and a CR800 Series weather station, at hourly intervals.

According to the correlation matrixes (Pearson), the correlation between the iButton temperature and the ambient temperature for the individual rams was the highest for Ram 7 (0.760), and the lowest for Ram 10 (0.423) (Table 4.10).

Table 4.10 The correlation matrix calculated for Ram 7 and Ram 10 for the iButton temperature and ambient temperature over 7 days of scrotal insulation in a scrotal insulation study on Dohne Merino rams.

Variables	iButton temp. (˚C)	Ambient temp. (˚C)
Ram 7		
iButton temp. (˚C)	**1**	0.760
Ambient temp. (˚C)	0.760	**1**
Ram 10		
iButton temp. (˚C)	**1**	0.423
Ambient temp. (˚C)	0.423	**1**

Figure 4.5 presents the relationship between the ambient temperature and temperatures measured by iButton temperature loggers placed inside the scrotal insulation devices of 5 Dohne Merino rams over 7 days of scrotal insulation during a scrotal insulation study. Ram 6 (LR), 7 (HR), 10 (HR), 13 (LR), and 15 (HR), had iButtons placed inside the scrotal insulation devices.
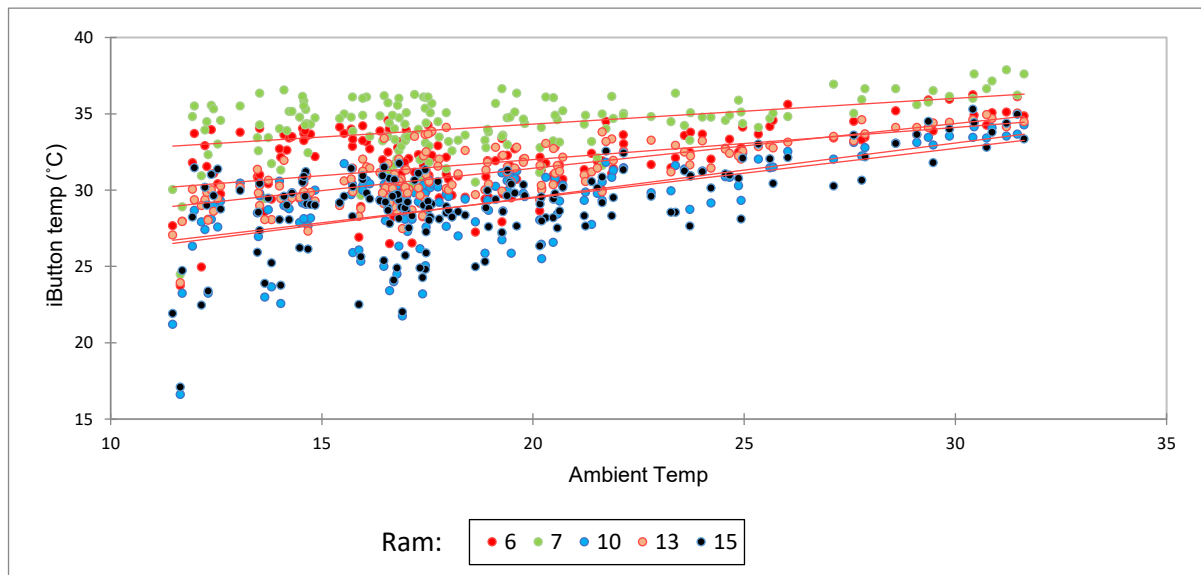

Figure 4.5 Relationship between the ambient temperature and temperatures measured by iButton temperature loggers placed inside the scrotal insulation devices of 5 rams over 7 days of scrotal insulation during a scrotal insulation study.

According to the correlation matrixes (Pearson), the correlation between the iButton temperature and the ambient temperature for the groups, HR and LR, when considering all the data, were 0.438 and 0.461, respectively (Table 4.12). These correlation values indicate that both groups display a poor correlation between the iButton temperature and ambient temperature. However, when considering the time of the day, the correlation (Pearson) between the iButton temperature and the ambient temperature for Day and Night, were 0.728 and 0.25, respectively (Table 4.12). These correlation values indicate that the ambient temperature for the Day displays a high correlation between the iButton temperature and ambient temperature, whereas the correlation for ambient temperature for the Night was poor.

Table 4.12 A correlation matrix calculated for the high resilience (HR) group, low resilience (LR) group, Day, and Night, for the iButton temperature and ambient temperature over 7 days of scrotal insulation in the scrotal insulation study on Dohne Merino rams.

Variables	iButton temp. (˚C)	Ambient temp. (˚C)
HR group		
iButton temp. (˚C)	**1**	0.438
Ambient temp. (˚C)	0.438	**1**
LR group		
iButton temp. (˚C)	**1**	0.461
Ambient temp. (˚C)	0.461	**1**
Day		
iButton temp. (˚C)	**1**	0.728
Ambient temp. (˚C)	0.728	**1**
Night		
iButton temp. (˚C)	**1**	0.250
Ambient temp. (˚C)	0.250	**1**

4.3.5 The recovery rate of spermatogenesis

Figure 4.6 presents the relationship between percentage abnormal spermatozoa for each ram and each sampling week (1, 2 and 3) during Phase 1 of the scrotal insulation study.

Figure 4.6 The relationship between the percentage abnormal spermatozoa for each ram and each sampling week of Phase 1 of the scrotal insulation study.

Figure 4.7 presents the relationship between percentage abnormal spermatozoa for each ram and each sampling week (0, 1, 3, 5, 7, 9 and 11) of the scrotal insulation study.

Figure 4.7 The relationship between the percentage abnormal spermatozoa for each ram and each sampling week of the scrotal insulation study.

Figure 4.8 presents the relationship between percentage live spermatozoa for each ram and each sampling week (1, 2 and 3) during Phase 1 of the scrotal insulation study.
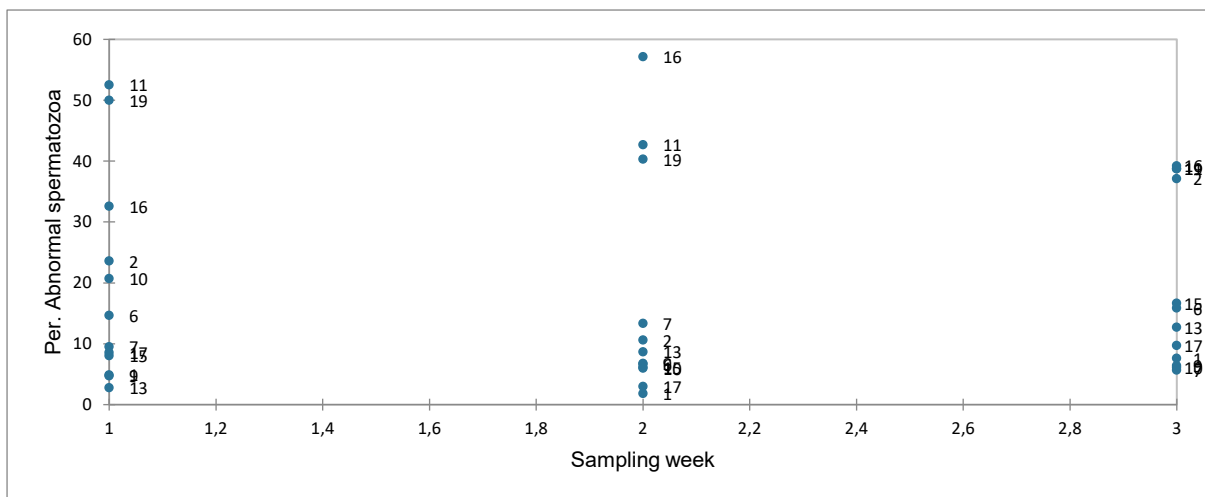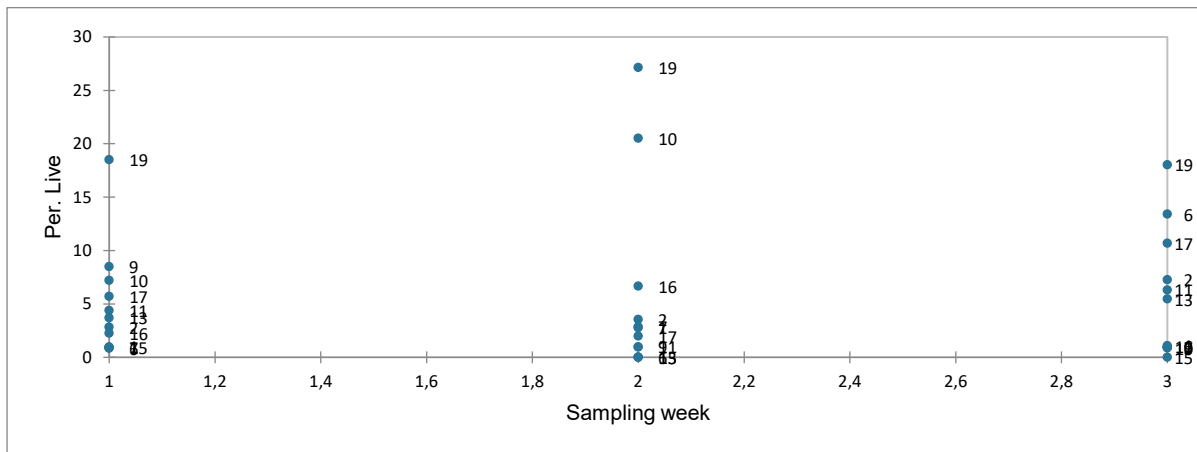

Figure 4.8 The relationship between the percentage live spermatozoa for each ram and each sampling week of Phase 1 of the scrotal insulation study.

Figure 4.9 presents the relationship between percentage live spermatozoa for each ram and each sampling week (0, 1, 3, 5, 7, 9 and 11) of the scrotal insulation study.
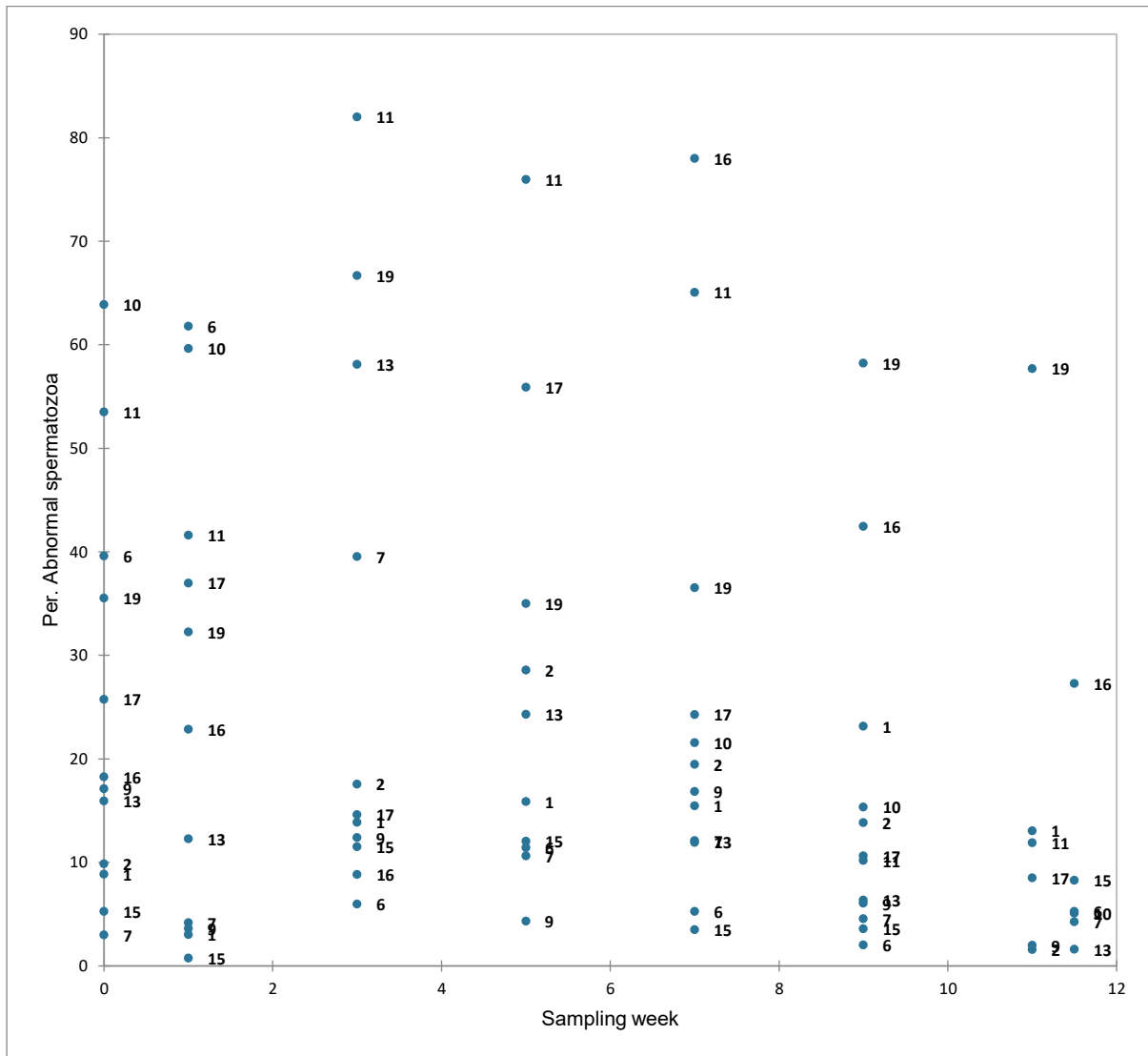

Figure 4.9 The relationship between the percentage live spermatozoa for each ram and each sampling week of the scrotal insulation study.

Figure 4.10 presents the relationship between the rectal temperatures for each ram and each sampling week (0, 1, 3, 5, 7, 9 and 11) of the scrotal insulation study.

Figure 4.10 The relationship between the rectal temperatures for each ram and each sampling week of the scrotal insulation study.

Figure 4.11 presents the relationship between the average scrotal temperatures for each ram and each sampling week (0, 1, 3, 5, 7, 9 and 11) of the scrotal insulation study.
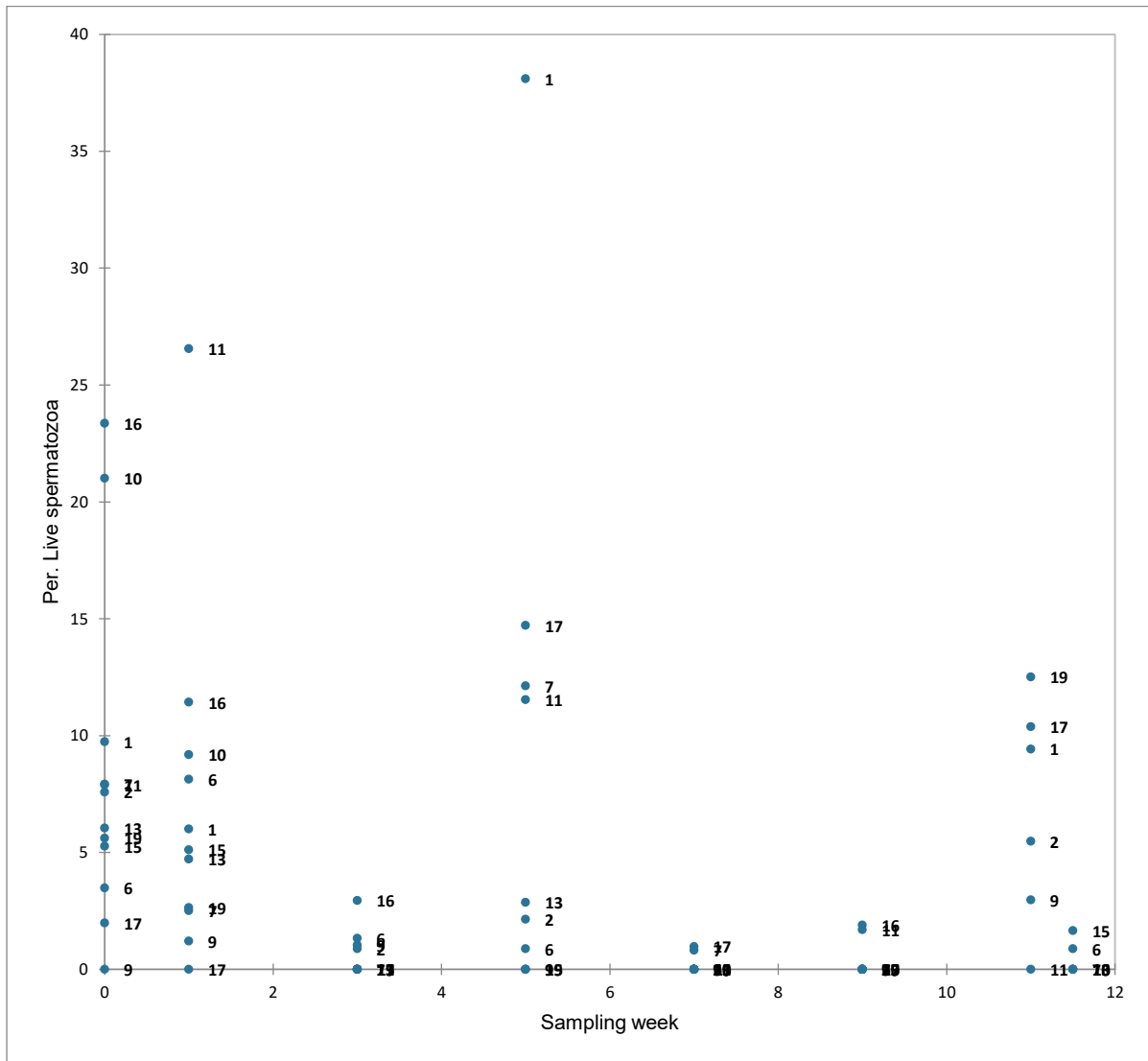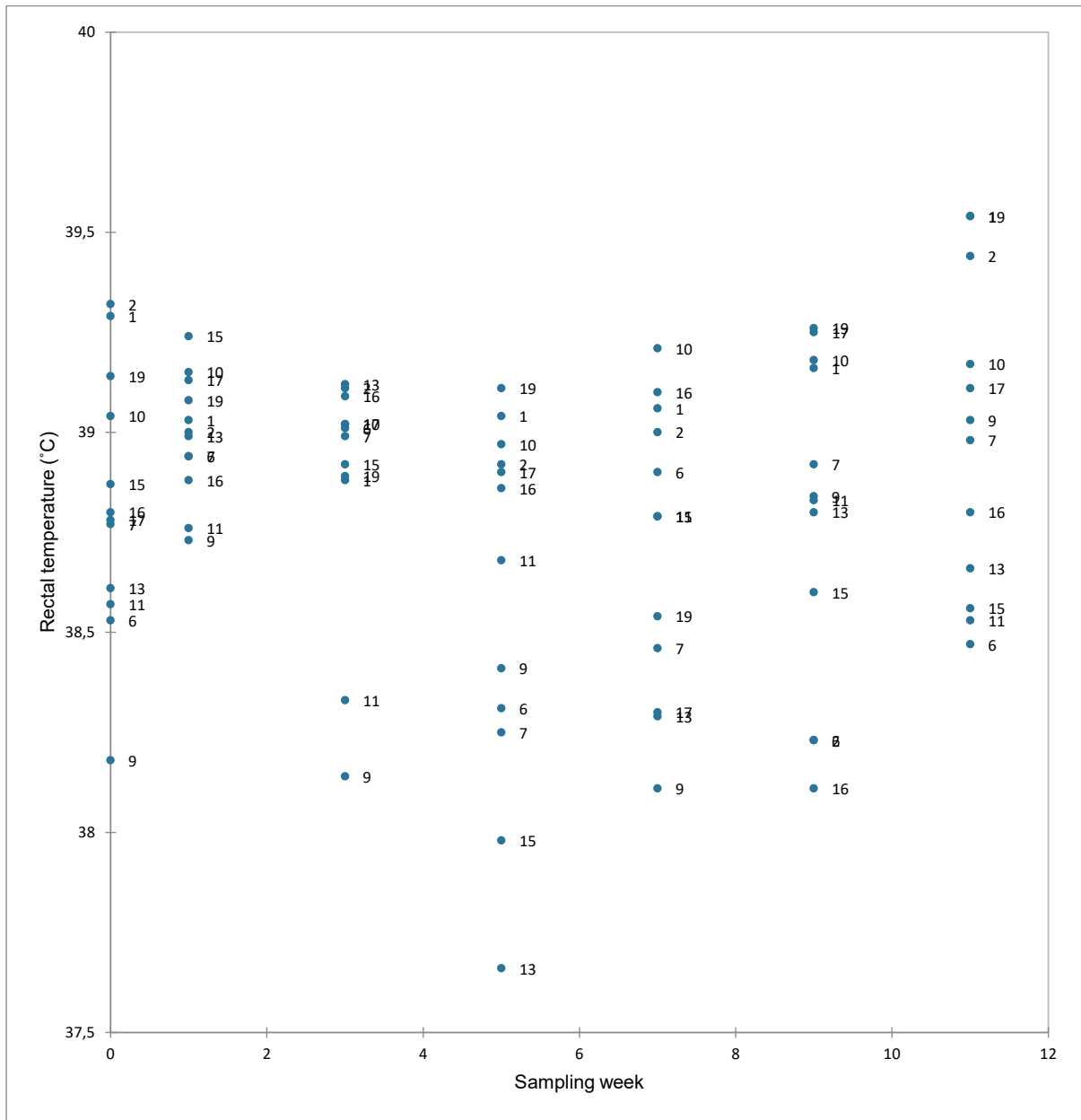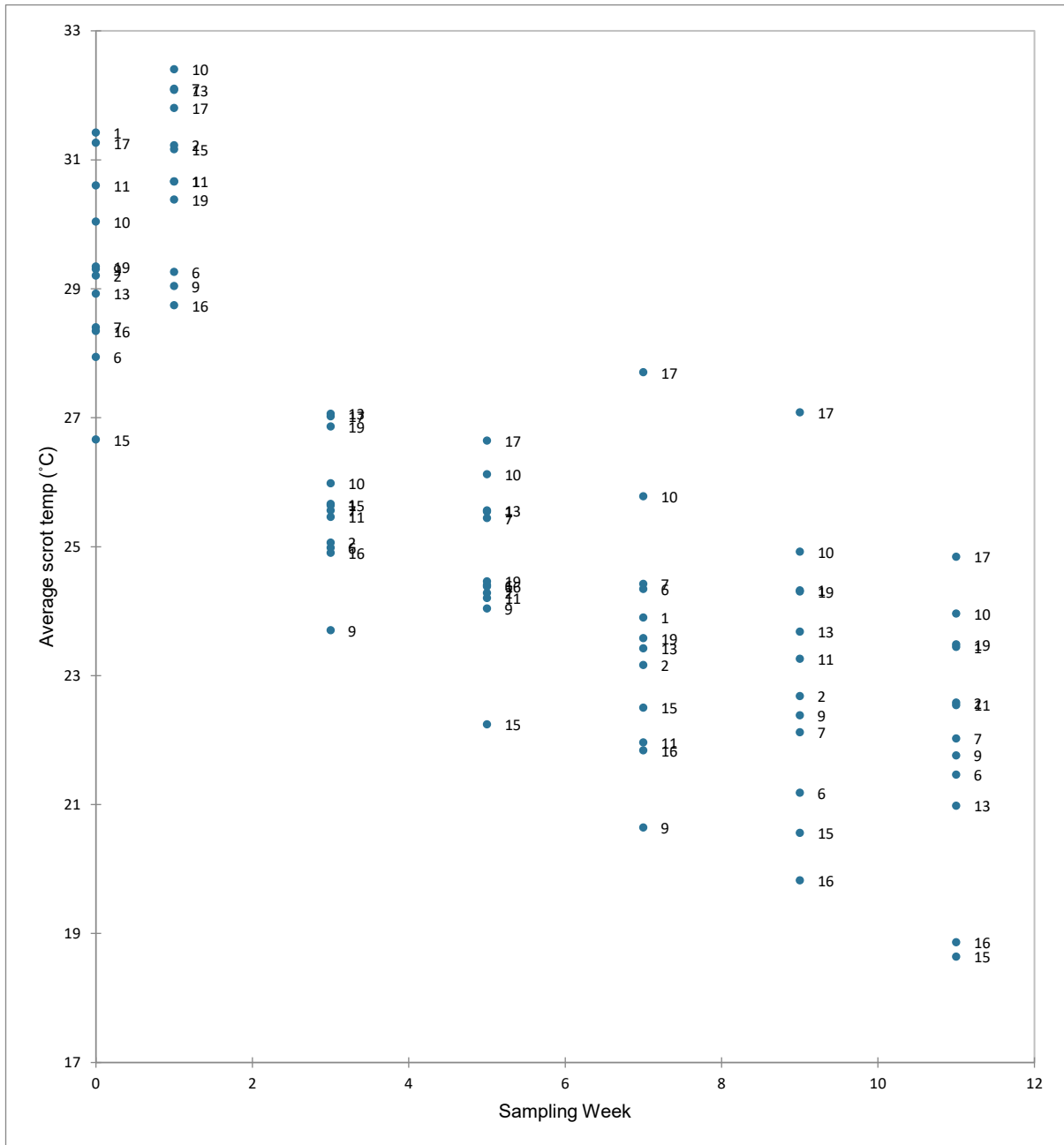

Figure 4.11 The relationship between the average scrotal temperatures for each ram and each sampling week of the scrotal insulation study.

Figure 4.12 presents the change in ambient temperature over the seven days of scrotal insulation during a scrotal insulation study.
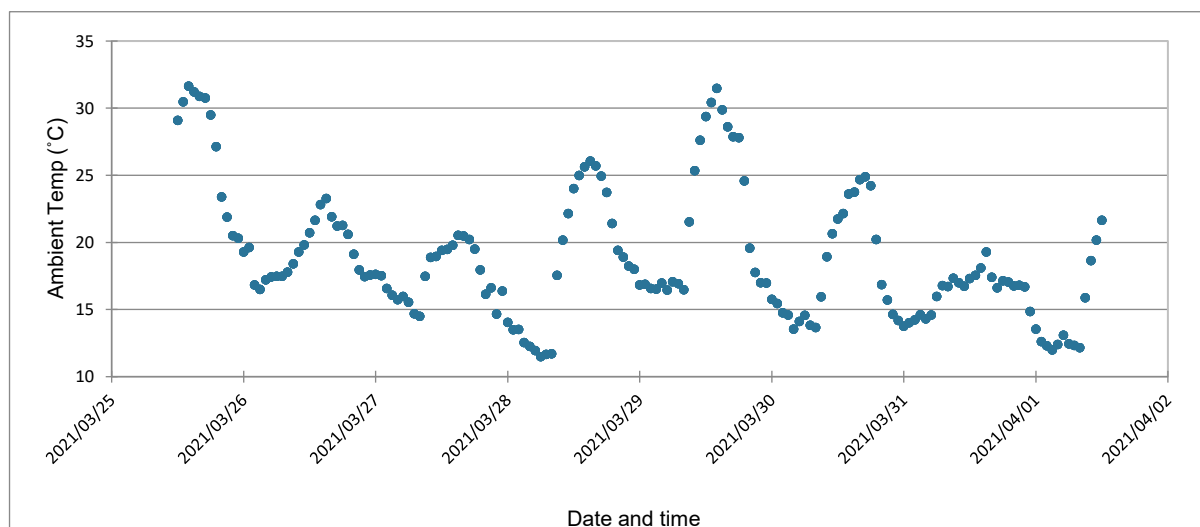
Figure 4.12 The change in the ambient temperature over the duration of scrotal insulation during the week of scrotal insulation.

4.4 Discussion

4.4.1 Acrosome integrity, sperm viability, and sperm morphology during liquid storage of spermatozoa

The microscopic data from Phase 2 for the storage of the semen samples in a MYE extender at refrigerator temperature (4-5°C) over time (0-48h), indicates that there was no change over time when acrosomal abnormality is considered. The absence of a change in acrosomal integrity may potentially be ascribed to the high percentage of acrosomal abnormalities that were recorded in this study, i.e., in most cases 100%.

A similar observation was made when sperm survivability was investigated, which was close to 0 in this phase. The analysis of these two sperm quality parameters requires a staining method that stains the diluted semen with the same, high contrast and opacity. Importantly, the colour of the spermatozoa, live or dead, should show clear contrast between one another, as well as to the background. This was not the case in this study. High variability was observed in the contrast and opacity on the microscopic photos. A factor that potentially could have influenced the staining, is the composition of the seminal plasma from the different rams.

The low percentage of live spermatozoa and high percentage of abnormal spermatozoa observed for the control group may have been caused by various factors. The rams were not specifically fed for

optimal reproductive performance and grazed on kikuyu grass during the day. A contributing factor to these low percentages may be that the rams were slightly overweight. The rams have not bred with ewes during the entire study and did therefore not have a high ejaculation frequency as rams would have in a herd under normal mating circumstances, which would also result in poor quality spermatozoa.

The study of Rocha *et al*. (2015) found that scrotal insulation negatively affects the proteins found in seminal plasma. These proteins are possibly involved in protecting the spermatozoa, aiding in their maturation, and fertilization. Soleilhavoup *et al.* (2014) also found individual differences in rams for the preservation ability of spermatozoa by liquid storage following scrotal insulation and attributed this to differences in the protein composition of the seminal plasma. Junquera and Pe (2009) proposed that the structure of proteins surrounding spermatozoa is supported by seminal plasma proteins in a similar way to fibronectin, which acts to stabilize the membrane and cytoskeleton of the spermatozoa. The differences among rams in the protein composition of seminal plasma, may thus influence the level of interaction of spermatozoa with Eosin-Nigrosin, as these proteins will determine the strength of the membrane structure.

The HR, LR, and control groups did not differ significantly for the percentages of abnormal and live spermatozoa over time (0-48h) during liquid storage. The HR group displayed the highest increase of abnormal spermatozoa over the 48h period, followed by the control group and the LR group, respectively. As expected, the LR group displayed the highest reduction in live spermatozoa over the 48h period, followed by the HR group, and then the control group.

When considering the resilience groupings in the study, it can be expected that the HR group would have displayed a lower increase compared to the other groups in percentage abnormal spermatozoa over the time for refrigerated storage. The spermatozoa in Phase 2 however, displayed an opposite trend in that the HR rams display a faster reduction in quality at refrigerated temperatures than the other groups. This difference between the two groups is attributed to the high level of variation within the groups (R-squared = 0.039). A much larger starting group would potentially allow for a better selection of the highest and lowest resilient rams, which may increase the difference measured between the groups.

4.4.2 Recovery of spermatogenesis by assessing the recovery of body (side and rectal) and scrotal temperatures.

The scrotal surface temperatures can potentially be used as a reflection of the temperatures of the testes and epididymis within the scrotum (Coulter *et al*., 1988). Infrared thermography was used in the present study to measure scrotal surface temperatures as well as temperatures on the side of the rams. Prior to scrotal insulation, there was no correlation between the rectal temperature and the temperatures of scrotum and side of the body of the rams. The mammalian testes function effectively at 2–8˚C lower than normal body temperature, which explains the poor correlation between the rectal temperature and scrotal temperatures (Fleming *et al.*, 2004; Hafez and Hafez, 2008).

In Week 0, there was a high correlation (0.887) between the scrotal temperature measured at the top of the scrotum and the one measured at the middle of the scrotum. In Week 1, there was however a medium correlation (0.584) between these two measurements. The spermatic cord enters the scrotum where the top measurements of the scrotum was taken. This is the region where the heat exchange between the body and testis occurs, as the spermatic cord encapsulates the blood vessels that aid in thermoregulation of the testes. A single thermographic measurement at the middle of the testes is therefore a good representative of the scrotal temperature for studies assessing the effects of heat on the testes. Week 1 and Week 3 displayed much higher correlations for rectal temperature to the scrotal and side temperatures of the rams. This was expected and highlights the warming effect of the scrotal insulation devices on the scrotum. Low rectal correlations were again observed for Week 5 and 7 for all other variables. These values increase again from Week 9, specifically for scrotal temperatures. Week 11 again displays improved correlations for rectal and side temperatures.

When rectal temperatures, which represent as closely as possible an animal's core body temperature, are plotted for each ram at every sampling of the scrotal insulation study, the variation in the rectal temperatures between the 12 rams were much smaller in Week 1 compared to Week 0 (Marai *et al*., 2007). This reduced level of variation in the rectal temperature between the 12 rams persisted into Week 3. From Week 5 to 11, the variation between rectal temperatures for the 12 rams increased again. The condensing of the rectal temperatures at day 7 of scrotal insulation indicates that the body temperatures of the rams increased to compensate for the increase in testicular temperature caused by scrotal insulation. The body temperature of the rams however only increased up to a specific level [between 38.73 ˚C (ram 15) and 39.24˚C (ram 11) for Week 1]. The midpoint between these two values was 38.96 ˚C, which is the normal body temperature for sheep (39˚C). The rectal temperatures of the

12 rams increased from Week 7 (range: 38.29˚C – 39.21˚C) to Week 11 (range: 38.47˚C – 39.54˚C), but they were still maintained within the physiologically normal range for rectal temperatures of sheep.

By averaging the scrotal surface temperatures, it is possible to see the change in scrotal temperatures as measured by infrared thermography for the individual rams from the week before scrotal insulation, until 10 weeks after scrotal insulation. The temperature measurements on the left, middle and the right side of the testes were moderately correlated to the temperatures on the side of the body. The temperature measurements at the top and bottom of the scrotum were however highly correlated to the temperatures on the side of the body. As soon as the scrotal insulation devices were removed, a poor correlation was observed for scrotal temperatures and side temperatures. This is understandable as the scrotal temperatures have increased due to scrotal insulation. This caused the difference between the scrotal temperatures to be much higher compared to the side temperatures of the rams. The scrotal temperatures took until week 7 (42 days) to return to normal when looking at the correlations between side temperatures.

The variation between the average scrotal temperatures of the 12 rams decreased from Week 1 to 5, increasing again from week 7 (42 days). This is the same time it takes for the production of spermatozoa from spermatogonia during normal spermatogenesis (42-53 days)(Cruz Júnior *et al.*, 2015). The observation in the current study agrees with the time that it took for semen quality to recover to normal levels (35-63 days) after scrotal insulation in the study of Alves *et al.* (2016). Their study however showed that testicular temperatures returned to normal levels as soon as 24h after scrotal insulation, which contradicts the findings in our study.

The scrotal temperatures of the 12 rams decreased as soon as the scrotal insulation devices were removed from the testes at Week 1 (range: 29.26˚C – 32.4˚C) until the end of the study at Week 11 (18.64˚C – 24.84˚C). The lower scrotal temperatures observed from Week 3 to Week 11 in comparison with Week 1 can be explained by the change in ambient temperature, which followed the same trend. The scrotal surface temperature is thus highly affected by the ambient temperature. Marai *et al*. 2007 also states that the scrotal surface temperature is significantly affected by the ambient temperature. In the current study, the ambient temperature before scrotal insulation was not consistent with the ambient temperatures after scrotal insulation. Given this environmental change, it can therefore not be conclusively determined how long testicular temperature took to recover to normal levels when assessing infrared thermography alone. These temperatures are expected to be lower than those of Week 0 due to the reduction in ambient temperatures at this time of the study, as well as due to the

difference in the times of these measurements at Week 0 (in the afternoon when devices were fitted) and Week 11 (in the morning before semen sampling). The decreasing scrotal temperatures since the end of insulation and the re-establishment of the variation between these values over the study period, together with the increasing rectal temperatures from Week 7 to Week 11 and the re-establishment of the variation between these values over the study period, confirms the recovery of the difference between body temperature and testicular temperature to normal levels (2-8˚C) during this period (Fleming *et al.*, 2004; Hafez and Hafez, 2008).

The average scrotal temperatures indicate that rams have differences in their ability to keep the testes at specific temperatures at the same ambient temperature. Ram 10, 17, and 19 for example, consistently were in the top half of highest scrotal temperatures on most sampling days, whereas ram 6, 9, and 15, were in the bottom half on most sampling days.

4.4.3 The relationship between testicular temperatures (iButton temperature) and ambient temperatures during scrotal insulation

The use of an iButton inside a scrotal insulation bag can be used as a way of measuring testicular temperature. Individual differences were observed between rams for the correlations of the iButton temperatures of these rams and the ambient temperatures. The less correlated the iButton temperatures and the ambient temperatures were for these rams, the more capable a ram was of keeping the testicular temperature lower than the rams with higher correlations at the same ambient temperature.

Thermoregulation of the testes of these rams with poorer correlations are thus better than those with higher correlations. According to Moule and Waites (1963), individual differences also exist for rams with regards to their ability to prevent seminal degeneration during exposure to high ambient temperatures.

The high correlation between the iButton temperatures and ambient day temperatures recorded, indicates that the temperatures inside the scrotal insulation devices increased or decreased as the ambient day temperatures increased or decreased. The poor correlation for the iButton temperatures and the ambient night temperatures indicates that the temperatures inside the devices during the night did not decrease in relation to the decrease in the ambient night temperatures. Firstly, it indicates that even during the night, the devices warmed the scrotums to high levels. In general, night iButton temperatures recorded slightly below that of the iButton temperatures during the day. On

some days during the night, iButton temperatures were higher than the iButton temperatures of the day.

These findings can be explained by the difference in the behaviour of sheep during the day and night, causing iButton temperatures of the night to be higher than they would potentially be if rams behaved the same during the day and night. The rams were brought into the shed during the night and kept as a group in a single pen. Rams also lay down, and close to one another as part of normal herding behaviour when they sleep, which would reduce heat loss by means of convection. This keeps the body temperatures of the group warmer compared to what it would be if a ram was penned individually. There would also be less air movement around the scrotal insulation devices as the rams lay down. The scrotal insulation devices would also press against the rams in this position, reducing the heat loss from the outside surface area of the devices from air movement. The devices were thus successful in increasing the temperatures around the scrotum of the rams to levels much higher than the ambient temperatures during both, the day and night.

4.4.4 Recovery of spermatogenesis by assessing sperm viability and sperm morphology

No difference was observed for the percentage abnormal spermatozoa between Week 0 and Week 1. Spermatozoa that were stored in the epididymis at Week 0, were still in the epididymis at Week 1 of sampling. Epididymal transit of spermatozoa takes between 13-15 days (Cruz Júnior *et al*., 2015). This suggests that the heat stress induced by the scrotal insulation bag, was not carried through or "absorbed" on epididymal level. This was also the case for the percentage live spermatozoa reported for Week 0 and Week 1, respectively. The study of Howarth, (1969) on elevated ambient temperatures on rams indicates that epididymal spermatozoa are resistant to heat stress, which could be attributed to the low metabolic rate of these spermatozoa, or due to the cell division not occurring in the epididymis. Rocha *et al.* (2015), however, reported that semen sample parameters can be negatively affected due to disrupted sperm maturation and epididymal function. Kastelic *et al*. (2017) also applied scrotal insulation on rams and saw a delay in the appearance of abnormal spermatozoa, which indicates that epididymal sperm were not affected, as in our study.

According to Alves *et al*. (2016), heat stress affects spermatozoa differently in the epididymis than in the testes, with DNA fragmentation occurring in the seminiferous tubules and epididymis, and sperm morphology that is affected in the testes. The pachytene spermatocytes and spermatids are the cells in the testes most easily affected by heat, as germ cells have a high mitotic activity, making them highly susceptible to heat stress (Setchell, 1998; Lue *et al*., 1999; Shiraishi *et al*., 2012). Cruz Júnior *et al*.

(2015) also found that heat stress has the greatest influence on the early stages of spermatogenesis, which would thus impact the multiplication of spermatogonia, and early meiosis the most. This was evident in the current study when Week 3 of sampling is considered. In Week 3, there is considerable change in the variation between the individual rams, as several rams clearly display a very high percentage of abnormal spermatozoa in comparison with previous samplings. The percentages of abnormal spermatozoa from the rams became less from Week 3 to Week 11. Ram 19 was however consistently one of the highest producers of abnormal spermatozoa. Ram 11 for example, had very high percentages of abnormal spermatozoa from Week 3 to 7, but recovered to levels well within the range of the rest of the rams from Week 9 to 11. This indicates that there are individual differences between rams in the ability of spermatozoa to handle heat stress.

Sampling Week 11 of Phase 2 can be compared with sampling Week 3 of Phase 1, as this sampling was 14 days from the first sampling, which would have allowed the spermatozoa from the time of first sampling to move through the epididymis. Most rams displayed a percentage of abnormal spermatozoa of less than 40% at Week 11 of Phase 2 and Week 3 of Phase 1. Ram 19 and 16, respectively displayed the same high values in both weeks of the respective phases. Ram 2 was the only other ram that had a high percentage of abnormal spermatozoa in Week 3 of Phase 1, but it had a low percentage in Week 11 of Phase 2. This gives a good indication of the recovery time of percentages of abnormal spermatozoa in Phase 2 due to heat stress. It is clear from data of the current study that the morphology of spermatozoa took until Week 11 (70 days) to recover to normal levels (percentages abnormal spermatozoa for most rams below 40%). This period coincides with the time it takes for spermatozoa to be produced and passed through the epididymis (53 + 15 days) (Cruz Júnior *et al.*, 2015). This finding agrees with the study of Cruz Júnior *et al.* (2015) where a period of 10 to 11 weeks was required to restore spermatogenesis after scrotal insulation, as measured using percentage normal spermatozoa. This also indicates that the early stage of spermatogenesis was affected the most, which is when spermatogonia multiply and meiosis occurs, as also found by Cruz Júnior *et al.* (2015).

A decline was observed in the percentage of live sperm from Week 0 to Week 1 for most of the rams from the group. At Week 3, two scrotal insulated rams displayed less than 0.8% and 1.32% live spermatozoa. Seven rams had 0% live sperm at this point, with one ram not giving a good enough sample for use. Week 5 displays the percentages of live sperm for seven of the ten scrotal insulated rams as the other 3 scrotal insulated rams exhibited 0% live spermatozoa. Eight of the ten scrotal insulated rams again displayed 0% live spermatozoa at Week 7, which is 42 days since the end of

scrotal insulation. This period coincides with the time it takes for spermatozoa to be produced from spermatogonia (Cruz Júnior *et al.*, 2015). Nine of these rams displayed 0% at Week 9, with percentages seeming to improve from week 11. The percentage live spermatozoa from Week 11 of Phase 2 and Week 3 of Phase 1 can be compared due to the time required for spermatozoa to be transported through the epididymis. These last samplings from each phase displayed similar values for live spermatozoa (ranging from 0 – 15% for most rams), indicating the recovery of the number of live sperm per sample.

4.5 Conclusion

There were no significant differences between the HR, LR, and Control groups for sperm viability and morphologically abnormal spermatozoa over time during liquid storage (0-48h). Individual differences were observed between rams for both scrotal temperatures and iButton temperatures, indicating that certain rams were more capable to thermoregulate the testes. The body temperature of rams increased in response to the increase in testicular temperature caused by scrotal insulation. Scrotal temperatures took approximately 42 days to recover to normal levels, which coincides with the time it takes for spermatozoa to be produced from spermatogonia during normal spermatogenesis (42-53 days). Individual differences were observed between rams in the ability of spermatozoa to tolerate heat stress, as found by assessing the change in percentages of viability and morphologically abnormal spermatozoa over the entire phase of the scrotal insulation study. The morphology of spermatozoa took 70 days to recover to normal levels, which indicates that early spermatogenesis was most affected by heat stress. Infrared thermography proved to be an effective tool in determining temperature-related changes in spermatozoa production. Based on these findings, the scrotal insulation devices designed in the present study were effective in heating the testes and causing a reduction in sperm quality.

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Chapter 5

The influence of heat stress on the hyperactivation potential of ram spermatozoa

Abstract

This study investigated the influence of heat stress on the hyperactivation potential of ram spermatozoa. Twelve adult Dohne Merino rams classified according to fresh and post-thaw sperm morphology as high resilience (HR) or low resilience (LR), were used in the study. Five semen samples were collected from each ram over a period of two weeks. These samples were processed in the laboratory where they were first subjected to macroscopic evaluation. The concentration was also determined, after which the semen samples were diluted with a milk-yolk extender (MYE). After dilution, the semen samples were subjected to a sperm hyperactivation functional test using procaine hydrochloride. Semen samples were incubated at 38.5°C for 30 minutes after the addition of 10mM procaine hydrochloride. Samples were evaluated at 0min, 15min, and 30min of incubation, and sperm viability, sperm morphology, and sperm acrosome integrity were quantified. The HR and LR groups did not differ significantly for percentage live and abnormal acrosomes at all time intervals, respectively. The HR and LR groups displayed significant ($p \leq 0.05$) differences for percentage abnormal spermatozoa at all time intervals, respectively. Significant ($p \leq 0.05$) individual differences were observed between the rams for sperm viability and morphology, but not for acrosome integrity when considering the entire 30 minutes of incubation. The LR group showed a faster rate in the reduction of quality in terms of abnormal spermatozoa than the HR group. The subpopulation structure of ejaculates should be considered when using induced hyperactivation as a sperm functional test, especially when ejaculation frequency is high. Simulations of the *in-vivo* environment of the female reproductive tract in an *in-vivo* environment may assist to obtain a more precise determination of fertilizing ability from heat stressed spermatozoa.

5.1 Introduction

With the current trend in the global human population growth, it is predicted that the demand for animal production will increase significantly in the coming years (Nardone *et al.*, 2010). The increased demand for animal products will occur simultaneously in the time period in which the world will experience changes in the global climate, that will ultimately affect agricultural production (Nardone *et al.*, 2010).

Many African countries for example, live in extreme poverty and underdevelopment for various reasons, which is largely influenced by the variability in climate causing the regular occurrence of floods, droughts, high temperatures, and the degradation of land (Washington *et al.*, 2006). Such countries need to drastically improve their level of agricultural production as a method to alleviate poverty. One way is to improve animal production by making use of assisted reproductive techniques (ART's), that allow genetically superior animals to produce more offspring than would be possible through a natural mating system (Baldassarre and Karatzas, 2004). In areas prone to such climatic conditions, and with the future in mind, sheep farming allows for sustainable production where other forms of animal production or crop production is difficult (Cloete and Olivier, 2010).

It is therefore important to assess the quality of spermatozoa from sheep in these regions to achieve optimal results by using ARTs such as artificial insemination (AI). This includes the determination if specific rams are better adapted for their spermatozoa to handle the effects of heat stress on the testes, and ultimately, the fertilizing ability of the spermatozoa on the oocyte. Spermatozoa are subjected to a range of changes before they can achieve full fertilization capacity (Mortimer and Maxwell, 1999). For this reason, several *in-vitro* sperm functional tests exist that aid in making more accurate predictions with regards to the effect of abnormalities on the fertilizing ability of spermatozoa (Mortimer and Maxwell, 1999). One of these tests is known as a hyperactivation functional test.

Hyperactivation is characterized as a state of vigorous flagellar beating of the mammalian spermatozoa within the oviduct that occurs during the process of sperm capacitation (Kay and Robertson, 1998; Chang and Suarez, 2011). Hyperactivation is used by spermatozoa in three steps to ultimately participate in fertilization of the oocyte, i.e., spermatozoa firstly have to detach themselves from the eptihelium of the oviduct, thus breaking free from the oviductal strorage reservoir. Spermatozoa also need a stronger mobility to get through the mucus of the oviduct, and to penetrate the visco-elastic cumulus matrix that surrounds the oocyte (Chang and Suarez, 2011). Lastly,

spermatozoa use hyperactviation to pierce through the viteline membrane and zona pellucida of the oocyte to ultimately fuse with the plasma membrane.

A sperm functional test that makes use of deliberate hyperactivation to determine sperm viability and fertilizing ability, is potentially a valuable tool to predict the fertilizing ability of spermatozoa that were subjected to heat stress. The use of this functional test thus aids in determing if high resilient spermatozoa, in terms of their ability to handle the stress associated with the processes involved in cryopreservation and thawing, are also more resilient in terms of their recovery after heat stress, to ultimately fertilize the oocyte. The aim of this study was therefore to determine whether a sperm hyperactivation functional test is effective in discriminating between Dohne Merino rams in the resilience of their spermatozoa to heat stress.

5.2 Materials and methods

Ethical clearance for this study was obtained from the Animal Care and Use Ethics Committee of the University of Stellenbosch (#ACU-2019-9835). The animal care and husbandry followed in this study, adhered to the guidelines stipulated in the South African National Standards document 10386:2008.

5.2.1 Experimental location

The rams that were used in this study were kept on the Welgevallen Experimental farm, situated in Stellenbosch, South Africa (33°56'33"S, 18°51'56"E). Stellenbosch has an average temperature of 16.2˚C, and an average annual rainfall of 787 mm.

5.2.2 Experimental animals and husbandry

A total of 12 adult Dohne Merino rams (*Ovies aries*) were used in the study. The rams grazed in kikuyu grass paddocks during the day and stayed inside a shed during the evenings. Water was available *ad libitum*.

The rams were carefully monitored during the study at each sampling session for any health- or injury related problems. Attention was paid to any abnormal behaviour before or after sampling, as well as in the paddocks. If rams were to show any signs of extreme discomfort or pain, a veterinarian was contacted for assistance. Rams were sheared in 6-month cycles, being at the end of January and at the beginning of August. The hooves of the rams were examined regularly and trimmed when necessary.

5.2.3 Experimental design

The post-insulation sperm viability and potential fertilizing ability of the HR and LR rams used in Phase 2 was assessed by using a sperm functional test in Phase 3. Five semen samples were collected from each ram during this phase over a two-week period. The collection of semen samples in Phase 3 started on 5 August 2021 and finished on 19 August 2021. The post-insulation sperm viability, sperm morphology, and sperm acrosome integrity, were determined by a sperm functional test involving the use of procaine hydrochloride to induce hyperactivation (Van der Horst *et al.*, 2020).

5.2.4 Experimental procedures

5.2.4.1 Semen collection

Electro-ejaculation (EE) was used to collect the semen samples - for more details on the EE method, please refer to Chapter 3. Each ram was sampled every second day over a period of two weeks.

5.2.4.2 Sample processing

Semen samples were transported to the laboratory directly after collection in a thermos containing water that was pre-heated to 37˚C. In the lab, the samples were placed in a Memmert water bath (Lasec, South Africa) pre-set to 37˚C. The samples were then subjected to macroscopic evaluation, which entails the assessment of the sample volume, colour, and mass motility. The concentration of the samples was determined with a Neubauer Haemocytometer (Prathalingam, 2006) (Lasec, South Africa).

5.2.4.3 Dilution and hyperactivation functional test

Samples were diluted with a milk-yolk extender (MYE) to achieve a final concentration of 600×10^6 spermatozoa/mL. The samples were gently mixed with procaine hydrochloride (Sigma-Aldrich, United States of America, PCode: 102370538) in a 1:1 ratio to the MYE to hyperactivate the spermatozoa (Van der Horst *et al.*, 2020). The amount of procaine hydrochloride needed to make 50mL of a 10mM stock solution was calculated with the following formula:

m = c x V x M

c = 10mM, V = 50mL, and the molecular weight (M) of Procaine hydrochloride is 272.77g/mol.

Therefore, m = 0.01 x 0.05 x 272.77 = 0.136g procaine hydrochloride was required for preparation of the stock solution.

These samples were placed in a HERACell 150i Thermo Fisher CO_2 incubator (SepSci, South Africa) set to 38.5˚C with a CO^2 level of 5% for a total time of 30 minutes. Microscope slides (Lasec, South Africa) were pre-warmed on an electrothermal slide drying bench (Lasec, South Africa) set to 35˚C. Microscope slides were prepared from the incubated samples at intervals, 0min, 15min, and 30min, since the addition of procaine hydrochloride. The 0min interval is the period directly after the procaine hydrochloride was added to the semen samples when the '0min' microscope slides were prepared. These slides thus display the spermatozoa before they would have been able to undergo hyperactivation. Eosin-Nigrosine (Kyron Laboratories, South Africa) was used to stain the incubated samples. The microscope slides were allowed to air dry after being prepared. These microscope slides were subjected to microscopic evaluation that involved the assessment of sperm viability, - morphology, and acrosome integrity (please refer to Chapter 3 for more details).

5.3 Statistical analysis

The data in this study were analysed using Microsoft XLStat Annual Version 2021.4.1. A one-way analysis of variance (ANOVA) was used for the percentage of abnormal spermatozoa, percentage of abnormal acrosomes, and percentage live spermatozoa for the resilience groups over all time intervals combined. The one-way analysis of variance (ANOVA) approach was used for the percentage of abnormal spermatozoa, percentage of abnormal acrosome, and percentage live spermatozoa for the resilience groups at every time interval, 0min, 15min, and 30min, respectively. Significant differences were noted at a significance level of $p \leq 0.05$ (95%).

5.4 Results

5.4.1 Descriptive statistics

Table 5.1 presents the descriptive statistics for sperm viability, sperm morphology, and sperm acrosome integrity measured for the resilience groups, and control group, of 12 Dohne Merino rams over 30 minutes of incubation at 38.5˚C.

Table 5.1 Descriptive statistics for sperm viability, sperm morphology, and sperm acrosome integrity measured for resilience groups, and control group, of Dohne Merino rams for 30 minutes of incubation at 38.5˚C.

Parameter	Mean ± SE	Range	Coefficient of variation
Control group			
Sperm viability (% live)	1.697 ± 0.492	0.000 – 10.938	1.560
Sperm morphology (% abnormal)	43.048 ± 3.717	8.000 – 75.833	0.465
Acrosome integrity (% abnormal)	99.933 ± 0.046	99.000 – 100.000	0.002
High-resilience group			
Sperm viability (% live)	1.986 ± 0.316	0.000 – 10.744	1.370
Sperm morphology (% abnormal)	26.683 ± 2.076	5.172 – 73.874	0.669
Acrosome integrity (% abnormal)	99.962 ± 0.028	98.131 – 100.000	0.002
Low-resilience group			
Sperm viability (% live)	1.722 ± 0.340	0.000 – 16.529	1.699
Sperm morphology (% abnormal)	46.604 ± 2.803	5.233 – 90.909	0.517
Acrosome integrity (% abnormal)	99.976 ± 0.017	99.065 – 100.000	0.001

5.4.2 Sperm viability, sperm morphology, and acrosome integrity during incubation at 38.5˚C, following induced hyperactivation

There were no significant differences between the HR, LR, and control groups for percentage live and percentage abnormal acrosome over the 30 minutes of incubation (Table 5.2). Percentage abnormal differed significantly between the HR and LR group ($p ≤ 0.05$), as well as for the HR and Control group ($p ≤ 0.05$) (Table 5.2). The LR and control group did not differ significantly (Table 5.2). The significant difference between the HR and control group in comparison with the non-significant difference between the LR and control group is due to the low percentages of abnormal spermatozoa from ram 16 (control) with regards to its original grouping as an LR ram, and the high percentages from ram 9 (control) with regards to its original grouping as an HR ram.

Table 5.2 The influence of incubation at 38.5°C for 30min on the sperm parameters (mean ± SE) recorded for groups of Dohne Merino rams, after induced hyperactivation.

Parameter	Group		
	HR	LR	Control
% Live	1.986 ± 0.326	1.722 ± 0.326	1.697 ± 0.515
% Abnormal	26.683[b] ± 3.870	46.604[a] ± 2.448	43.048[a] ± 2.448
% Abnormal Acrosome	99.962 ± 0.024	99.976 ± 0.024	99.933 ± 0.038

[a-b] Different superscripts in columns denote significant differences ($p ≤ 0.05$)

Figure 5.1 presents the influence of artificial hyperactivation on sperm viability, sperm morphology, and acrosome integrity of ejaculated spermatozoa obtained from Dohne Merino rams for 30min of incubation at 38.5°C.

Figure 5.1 The influence of artificial hyperactivation on sperm viability, sperm morphology, and acrosome integrity of ejaculated spermatozoa obtained from Dohne Merino rams for 30min of incubation at 38.5°C.
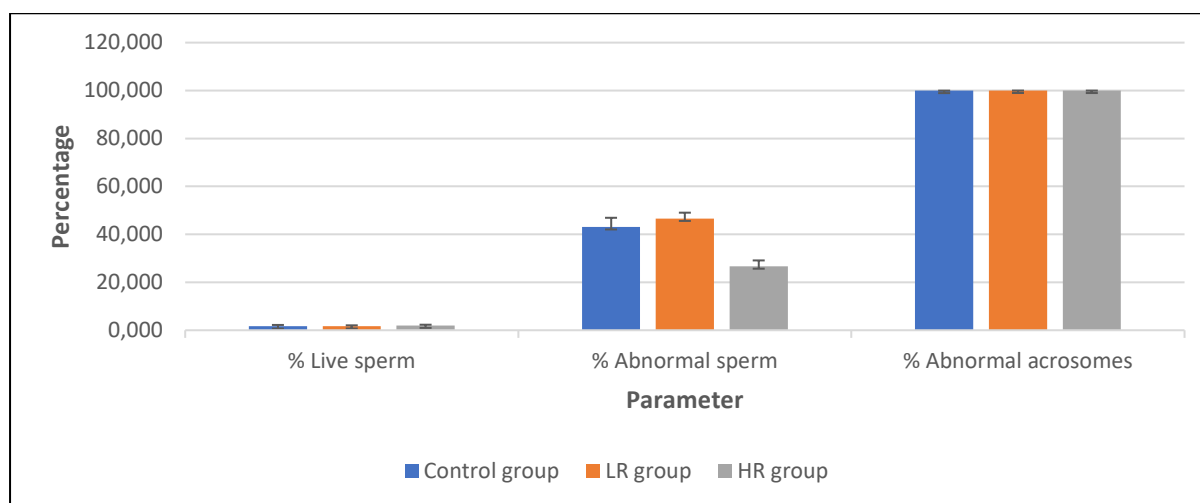
There were no significant differences between the HR, LR, and control groups for percentage live and abnormal acrosome at the 0min-interval (Table 5.3). Percentage abnormal differed significantly between the HR and LR group ($p \leq 0.05$), as well as the HR and Control group ($p \leq 0.05$). Considering all parameters, the LR and control group did not differ significantly (Table 5.3). The significant difference between the HR and control group in comparison with the non-significant difference between the LR and control group is due to the low percentages of abnormal spermatozoa from ram 16 (control) with regards to its original grouping as an LR ram, and the high percentages from ram 9 (control) with regards to its original grouping as an HR ram.

Table 5.3 The influence of incubation at 38.5°C at the 0min-interval on the sperm parameters (mean ± SE) recorded for Dohne Merino rams, after induced hyperactivation.

Parameter	Group		
	HR	LR	Control
% Live	2.736 ± 0.700	2.727 ± 0.700	2.243 ± 1.106
% Abnormal	27.674[b] ± 4.217	45.785[a] ± 6.667	46.841[a] ± 4.217
% Abnormal Acrosome	99.961 ± 0.048	99.963 ± 0.048	99.800 ± 0.077

[a-b] Different superscripts in columns denote significant differences ($p \leq 0.05$)

There were no significant differences between the HR, LR, and control groups for percentage live and percentage abnormal acrosome at the 15min-interval (Table 5.4). Percentage abnormal differed significantly between the HR and LR group ($p \leq 0.05$), as well as the HR and Control group ($p \leq 0.05$). Considering all parameters, the LR and control group did not differ significantly from one another

(Table 5.4). The significant difference between the HR and control group in comparison with the non-significant difference between the LR and control group is due to the low percentages of abnormal spermatozoa from ram 16 (control) with regards to its original grouping as an LR ram, and the high percentages from ram 9 (control) with regards to its original grouping as an HR ram.

Table 5.4 The influence of incubation at 38.5°C at the 15min-interval on the sperm parameters (mean ± SE) recorded for Dohne Merino rams, after induced hyperactivation.

	Group		
Parameter	**HR**	**LR**	**Control**
% Live	2.224 ± 0.553	1.013 ± 0.553	1.553 ± 0.874
% Abnormal	26.321[b] ± 4.371	45.472[a] ± 4.371	40.191[ab] ± 6.912
% Abnormal Acrosome	100.000 ± 0.023	99.965 ± 0.023	100.000 ± 0.036

[a-b] Different superscripts in columns denote significant differences (p ≤ 0.05)

There were no significant differences between the HR, LR, and control groups for percentage live and percentage abnormal acrosome at the 30min-interval (Table 5.5). Percentage abnormal differed significantly between the HR and LR group (p ≤ 0.05). The HR and Control group, as well as the LR and control group, respectively, did not differ significantly from one another (Table 5.5).

Table 5.5 The influence of incubation at 38.5°C at the 30min-interval on the sperm parameters (mean ± SE) recorded for Dohne Merino rams, after induced hyperactivation.

	Group		
Parameter	**HR**	**LR**	**Control**
% Live	0.998 ± 0.365	1.428 ± 0.365	1.296 ± 0.577
% Abnormal	26.054[b] ± 4.317	48.555[a] ± 4.317	42.112[ab] ± 6.826
% Abnormal Acrosome	99.925 ± 0.049	100.000 ± 0.049	100.000 ± 0.077

[a-b] Different superscripts in columns denote significant differences (p ≤ 0.05)

Table 5.6 presents the least squares means of the percentage live, percentage abnormal, and percentage abnormal acrosome for semen samples from 12 Dohne Merino rams incubated at 38.5˚C, over 30 minutes, following induced hyperactivation. The least squares means for the 12 rams for percentage live, percentage abnormal, and percentage abnormal acrosome over the 30 minutes of incubation presents significant (p ≤ 0.05) differences between the individual rams for percentage live and percentage abnormal, but not for percentage abnormal acrosome (Table 5.6). The percentages for abnormal acrosomes were consistently very close to 100.

Table 5.6 The influence of incubation at 38.5°C for 30 minutes on the sperm parameters (mean ± SE) recorded for individual Dohne Merino rams, after induced hyperactivation.

Ram ID	% Live	% Abnormal	% Abnormal acrosome
2 (LR)	4.137[a] ± 0.663	64.535[ab] ± 3.132	100.000 ± 0.055
19 (LR)	0.879[bc] ± 0.663	74.759[a] ± 3.132	100.000 ± 0.055
1 (HR)	3.716[ab] ± 0.663	54.224[bc] ± 3.132	99.935 ± 0.055
7 (HR)	3.604[ab] ± 0.663	27.956[ef] ± 3.132	100.000 ± 0.055
13 (LR)	1.108[abc] ± 0.663	47.606[cd] ± 3.132	99.938 ± 0.055
11 (LR)	2.159[abc] ± 0.663	34.850[de] ± 3.132	99.941 ± 0.055
10 (HR)	0.944[abc] ± 0.663	14.096[fg] ± 3.132	100.000 ± 0.055
16 (Control)	2.563[abc] ± 0.663	38.501[de] ± 3.132	99.933 ± 0.055
15 (HR)	0.860[bc] ± 0.663	13.053[fg] ± 3.132	100.000 ± 0.055
9 (Control)	0.832[bc] ± 0.663	47.596[cd] ± 3.132	99.933 ± 0.055
6 (LR)	0.330[c] ± 0.663	11.269[g] ± 3.132	100.000 ± 0.055
17 (HR)	0.805[bc] ± 0.663	24.085[efg] ± 3.132	99.875 ± 0.055

[a-g] Different superscripts in columns denote significant differences (p ≤ 0.05)

5.5 Discussion

5.5.1 Acrosome integrity, sperm viability, and sperm morphology following a sperm functional test

When analysing sperm morphology of the semen samples before induced hyperactivation and after incubation of these hyperactivated semen samples at 38.5°C, the spermatozoa from the LR group exhibited a faster decline in quality (45.79% to 48.56%) in comparison with spermatozoa from the HR group (27.67% to 26.05%). This faster reduction in quality of sperm morphology, indicates that the spermatozoa produced by the HR rams were more resilient to tolerate incubation at 38.5°C, when compared to both the LR and Control groups. As expected, the sperm viability for both the HR and LR groups decreased over time, as evident in the viability recorded for post-incubation samples.

When analysing the percentage live, percentage abnormal, and percentage abnormal acrosome for the individual rams (irrespective of group) over the 30 minutes of incubation, significant (p ≤ 0.05) differences between the individual rams for percentage live and abnormal spermatozoa were observed. No significant differences were however observed for abnormal acrosome values.

5.5.2 Seminal plasma proteins

The proteins found in seminal plasma are negatively affected by scrotal insulation (Rocha *et al*., 2015). These proteins are potentially involved in the maturation and protection of spermatozoa. Individual differences because of differences in the protein composition of the seminal plasma have also been found in rams for the preservation ability of spermatozoa by liquid storage following scrotal insulation (Soleilhavoup *et al.*, 2014). It has been proposed by Junquera and Pe (2009) that the proteins that surround spermatozoa are supported by seminal plasma proteins in a similar way to fibronectin, which aid in the stabilization of the membrane and cytoskeleton of the spermatozoa. The protein composition of seminal plasma that differs between individual rams may thus potentially contribute to minimising the deleterious effects of processing on spermatozoa, as these proteins play a role in the strength of the membrane structure. This difference in strength may have caused spermatozoa from different rams to differ in the amount of Eosin-Nigrosin that could enter the sperm head due to membrane damage.

5.5.3 Sperm subpopulations

It is commonly known that mammalian ejaculates contain subpopulations of spermatozoa (Holt and van Look, 2004). The sizes of these subpopulations have been found to differ between individual rams (Maroto-Morales *et al.*, 2012). The ability of spermatozoa to undergo the acrosome reaction varies depending on the specific subpopulation that the spermatozoa belong to (Maroto-Morales *et al.*, 2012). For spermatozoa to fertilize an egg, both cells must have completed their respective maturation processes before meeting in the ampulla of the oviduct (Curry, 2000). Due to the variation in times of insemination and ovulation, spermatozoa should be able to survive within the female reproductive tract for a variable amount of time, and should show full capability of fertilization in the oviduct at the time of ovulation (Curry, 2000). The more heterogenous the ejaculate is, the higher the fertilizing ability would be at the unpredictable time of fertilization (Curry, 2000). In the current study, it may be that some spermatozoa appear to have undergone the acrosome reaction following induced hyperactivation. The presence of subpopulations in ejaculates do however present the possibility that a certain percentage of the analysed spermatozoa, have simply degraded in quality and exhibit a damaged acrosome because of ageing. The rams in this phase of the study had a higher ejaculation frequency than in the previous phases, which may result in a larger degree of heterogeneity in the sperm subpopulations than in the previous two phases of this study. The higher ejaculation frequency would give the spermatozoa less time to mature in the cauda epididymis, causing that these

spermatozoa would only be able to undergo hyperactivation at a later stage in comparison with more mature spermatozoa.

When making use of ARTs, it is important to consider the presence of subpopulations of spermatozoa in ejaculates, the level of heterogeneity among them, and the fertilizing ability of these subpopulations. The fertilizing ability of these subpopulations can however be affected by factors such as heat stress. Heat stress on spermatozoa may include heat stress in the testes of rams, as well as heat stress in the female reproductive tract. Given the ability of spermatozoa from the HR rams in this study to better handle the heat stress from incubation, following previous exposure to heat stress during scrotal insulation, spermatozoa from HR rams may also display a greater ability to handle heat stress in the reproductive tract of the ewe, potentially caused by for example a mild fever. The spermatozoa that are more resilient to heat stress in the female reproductive tract would have greater fertilizing ability than spermatozoa with lower resilience to heat stress. Simulations of the *in vivo* environment of the female reproductive tract can be mimicked in an *in vitro* environment, allowing for more precise analyses of the potential fertilizing ability of spermatozoa from heat stressed rams.

5.6 Conclusion

The HR and LR groups did not differ in terms of percentage live and abnormal acrosomes at all time intervals. The HR and LR groups differed significantly for percentage abnormal spermatozoa at all time intervals. There was a faster rate of reduction in the quality of sperm morphology for the LR group compared to the HR group over the 30 minutes of incubation. Significant differences were observed between the individual rams for percentage live and abnormal spermatozoa, but not for percentage abnormal acrosomes, when considering incubation over the entire 30 minutes. It is important to consider the subpopulation structure of ejaculates when using induced hyperactivation as a sperm functional test, especially when ejaculation frequency is high. Simulating potential heat stress experienced by spermatozoa in the female reproductive tract *in vitro* may assist in understanding how the fertilizing ability of heat-stressed spermatozoa may be affected.

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Chapter 6

General conclusions and recommendations

6.1 General conclusion

It is well known that the world is experiencing global warming, which is coincidently occurring over the same period that the growing global human population demands a significant increase in the production of food. It is important that farmers understand the effects of heat stress on animals to ensure that assisted reproductive techniques (ART's) are applied effectively for optimal production, specifically in the regions that will be most severely affected by global warming. A key focus point is the selection of animals that are genetically superior in their ability to handle heat stress.

One of the most popular and commonly used ARTs in the world is artificial insemination (AI), and its success rate is highly dependent on the quality of semen used for insemination. The quality of semen is negatively affected by all processes involved from the time of semen collection up to the time of AI. However, the most important aspect is the quality of the semen sample at the exact time of ejaculation. Various factors may reduce sperm quality even before ejaculation, of which heat stress is a major concern.

This study investigated the effects of heat stress on the quality and functionality of ovine spermatozoa. After the first phase of the study, a group of 13 Dohne Merino rams was divided into a high resilience (HR) and low resilience group (LR) in terms of the ability of their spermatozoa to handle the stress associated with the processes involved in cryopreservation and thawing. Prior to Phase 2, the group size was reduced to have two groups of 6 rams each. Ten of the 12 rams were subjected to scrotal insulation in Phase 2. The other two rams not subjected to scrotal insulation, were named the Control group, and consisted of one ram from the HR group and one ram from the LR group. These 12 rams were randomly divided into two groups of 6 rams each for the sampling days in Phase 2 and 3, and not according to the HR or LR groups. Scrotal insulation allowed for the assessment of heat stress on sperm quality and recovery over an 11-week period post-insulation. This made it possible to investigate if there were differences between the two resilience groups in the ability of their spermatozoa to handle stress, based on the ability of their spermatozoa to show resilience towards the processes involved in cryopreservation and thawing. The potential of spermatozoa to fertilize an ovum cannot be determined solely by the assessment of morphological sperm quality parameters, but it requires functional tests to assess fertilizing ability. In Phase 3, a relatively new sperm functional

test was used to evaluate the potential fertilizing ability of spermatozoa after scrotal insulation, and at least one spermatogenic cycle was completed.

6.1.1 Effect of scrotal insulation on the viability, morphology, and acrosome integrity of ram spermatozoa

The aim of this part of the study was to determine the influence of scrotal insulation on sperm sample quality, and the resumption of normal spermatogenic activity in the testes of adult Dohne Merino rams, to potentially classify rams in terms of resilience to heat stress and its related effect on physiological processes such as spermatogenesis. In the present study the designed scrotal insulation devices proved to be effective in insulating the testes, as was evident from the influence on spermatogenic activity. The percentages of live spermatozoa and abnormal acrosomes were respectively, very low and very high, which may be attributed to the Eosin-Nigrosin (NE) stain used in the study. The NE stain is a common vitality stain used in sperm studies, however, interaction of the stain components with the MYE diluent components complicated evaluation of the viability and acrosomal integrity of the spermatozoa.

During liquid storage, the HR, LR, and Control group semen did not differ in the percentage of abnormal and live spermatozoa. By assessing the quality and recovery of spermatozoa, as well as the changes in scrotal temperatures, individual rams did however display differences in the ability of their spermatozoa to offer resilience to heat stress over the 11-week period of the scrotal insulation study. Sperm morphology took approximately 70 days to recover to pre-insulation levels (below 40% for most rams), indicating that early spermatogenesis was most affected by the heat stress. The body temperature of the rams increased due to scrotal insulation to compensate for the increase in testicular temperature so that the difference between body temperature and testicular temperature (2 – 8°C) could be maintained. Scrotal temperatures took 42 days to recover to normal (18.64°C – 24.84°C), which coincides with the time it takes for spermatozoa to be produced from spermatogonia. The changes in scrotal and iButton temperatures both showed individual differences between the rams. These individual differences indicate differences in the testicular thermoregulatory ability of rams.

6.1.2 Assessment of the post-insulation viability and potential fertilizing ability of ejaculated spermatozoa using a sperm functional test

The aim of this phase of the study was to determine whether a sperm hyperactivation functional test is effective in discriminating between Dohne Merino rams in terms of the resilience of their

spermatozoa to heat stress. Similar as to Phase 2, it proved to be difficult to analyse the viability and acrosome integrity of spermatozoa in this phase where the NE stain was used.

No significant differences were observed between the HR and LR groups for percentage live and abnormal acrosome at all time intervals (i.e., 0min, 15min, and 30min, respectively). A significant difference was however observed for percentage abnormal spermatozoa at all time intervals. The LR group also showed a faster rate in the reduction of quality for sperm abnormalities than the HR group. Rams displayed significant individual differences for sperm viability and sperm morphology, but not for acrosome integrity when considering the entire 30 minutes of incubation. The presence of sperm subpopulations in ejaculates may potentially interfere with the accuracy of this method in determining fertilizing ability. The results obtained for fertilizing ability using this method may potentially be affected by ejaculation frequencies. The *in vivo* environment of the female reproductive tract should be simulated in an *in vitro* environment which may assist in more precise determination of fertilizing ability from heat stressed spermatozoa.

6.2 Recommendations

It is recommended that future studies selecting HR and LR rams according to their ability to handle the stress associated with processing, cryopreservation, and thawing, should make the selections from a much larger number of rams in the starting group. This would potentially allow for the selection of groups with a more distinct difference in the resilience of their spermatozoa to processing and cryopreservation.

Future studies inducing heat stress on the testes of rams by means of scrotal insulation should assess the potential influence of season of temperature regulation in the testes, and how this may potentially affect sperm quality and viability. Sheep are considered as seasonal breeders, and it is important to understand the plasticity of spermatozoa during and outside the normal breeding period, to maintain their integrity and function and thus ensure successful fertilization.

Future studies using scrotal insulation should measure rectal temperatures at different times of the day, specifically during the warmest part of the day. These measurements are important for a better insight to how the ram thermoregulates its body during high ambient temperatures in combination with heat stress on the testes, and how these temperatures affect spermatogenesis.

Infrared thermography might only be meaningful up to a certain temperature when assessing scrotal temperatures as an indication of testicular temperature. Too low ambient temperatures would affect the surface temperatures of the scrotum, which would not be a good indication of testicular temperature. It is recommended that future studies assess at what minimum temperature scrotal temperatures are no longer a good indication of testicular temperature. It would also be advantageous for future studies to assess the scrotal surface temperatures over several times of the day for the duration of the entire scrotal insulation study, while assessing the ambient temperatures at the same times. Such scrotal surface temperature measurements taken over several times of the day during scrotal insulation studies would provide a better indication of how ambient temperatures, radiation, and air movement, affects testicular temperature, and ultimately spermatogenesis.

Due to the difficulty of clearly inspecting sperm viability and acrosome integrity of the spermatozoa when staining with Eosin-Nigrosin, it is recommended that a different staining technique is used if this new method of hyperactivation as functional test is followed. The analysis of these two sperm quality parameters requires a staining method that stains the diluted semen with the same, high contrast and opacity. The colour of the spermatozoa, live or dead, should be clearly distinguishable and should clearly stand out from the background. The use of a fluorescence staining method may improve the ease of assessing the acrosomal status of spermatozoa in comparison with the NE staining method. Flow cytometry should be considered as a potential alternative method for the evaluation of sperm viability and acrosome integrity of semen samples. The use of synthetic semen extenders instead of extenders involving egg-yolk may provide better results of how the stained microscope slides are displayed.

Due to the presence of subpopulations of spermatozoa in ejaculates, it is also recommended that the time intervals are shortened when preparing microscope slides for the assessment of acrosomal status. This will minimize the number of spermatozoa that are counted as acrosome reacted spermatozoa, that are in fact, old and degraded, therefore giving a more accurate fraction of spermatozoa functionally capable of fertilization.

Future scrotal insulation studies should also include an investigation of the influence of the protein composition of seminal plasma from rams. The protein composition of seminal plasma may possibly determine the way in which semen samples from different rams are stained. Studies should assess the differences in the protein composition between individual rams, as well as between HR and LR rams, subjected to testicular heat stress by means of scrotal insulation. This would allow studies to

determine how the proteins in the seminal plasma are involved in assisting spermatozoa to handle heat stress, and to identify and quantify the proteins involved.

Future studies assessing fertilizing ability by means of a hyperactivation functional test as used in this study where sperm viability, -morphology, and acrosome integrity was used as assessment criteria, should include the assessment of subpopulations of spermatozoa in ejaculates. The sizes of the subpopulations present should be quantified, which would provide better insight to the fertilizing potential of semen from a specific ejaculate. Studies quantifying the subpopulations present in semen samples should also assess the effect of ejaculation frequency on the sizes of the subpopulations, as well as the fertilizing ability of the spermatozoa from these subpopulations at different ejaculations frequencies.

Studies using scrotal insulation to induce heat stress on spermatozoa should simulate the *in vivo* environment of the reproductive tract of the ewe in an *in vitro* environment under various conditions, for example a mild fever, to achieve more precise results on the fertilizing ability of spermatozoa. These simulations should mimic the female reproductive tract as closely as possible and should include different temperatures to induce heat stress in the female reproductive tract. These studies should include groups of HR and LR rams to identify if spermatozoa from these rams are more resilient to heat stress on the testes, and also exhibit a greater ability to offer resilience to heat stress in the female reproductive tract. The protocols for identifying and quantifying these subpopulations of spermatozoa in ejaculates should be standardized for future studies.

In conclusion, findings from this study contribute to the knowledge available on the effects of heat stress on the testes induced by scrotal insulation, and the effect thereof on spermatogenesis. Future studies to refine the protocols involved in the assessment of spermatozoa from different resilience groups of sheep subjected to heat stress, in the testes, as well as under *in vitro* conditions simulating the *in vivo* environment of the female reproductive tract, will improve our understanding on ability of spermatozoa to offer resilience to heat stress.

Appendix 1

Scrotal insulation device

① Flannel Fabric
Polyester batting (Loomtex Heatex)
Nylon (Waterproof)

② Flannel Fabric
Polysterene balls
Nylon (Waterproof)

Sheep.

① Velcro

② Velcro