The potential of rosmarinic acid, carnosic acid and carnosol as antioxidants to preserve sheep *(Ovis aries)* sperm viability and morphological integrity

by
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Dedicated to my Mom.

I owe my passion, dedication and determination to you Mom – you are ever present in my life.
I miss you and love you and I hope that wherever you are, I have made you proud.

Declaration

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Summary

The use of assisted reproductive techniques (ART's) such as artificial insemination and embryo production, which requires the use of good quality sperm, is hampered by the susceptibility of ram sperm to cold stress and heat stress. Short-term (cold) storage has a definite effect on the viability, morphological integrity, and subsequently the fertilizing ability of sperm, due to an increase in oxidative stress caused by an excessive build-up of reactive oxygen species (ROS). Heat stress has a number of deleterious effects on sperm production and maturation, with the excessive build-up of ROS resulting in a decrease in sperm viability and quality. The study therefore investigated whether diterpenes in common rosemary and wild rosemary varieties, which has a natural antioxidant activity, can minimize the deleterious changes caused by cold storage and heat stress (HS). The findings indicated that sperm viability decreased over time under both cold and heat stress conditions, but that there was no significant contribution of the antioxidants (AO's) on the changes in viability. The wild rosemary species had a higher level of morphological abnormalities than the common rosemary species for the cold storage and heat stress studies. However, when considering the two species individually, there were no significant differences for morphology. There appeared to be no protection from the AO's on maintaining sperm viability for both the cold storage and HS studies, and the AO's also appeared to have no protective effects on morphology during the HS study. Cold storage resulted in an increase in the percentage of abnormal sperm when considering the individual effects of the AO's. To conclude, supplementation of sperm media with both rosemary aqueous extracts did not show a significant level of protection against the deleterious effects of cold storage and HS on sperm quality. The extraction technique that was used for the present study was not sufficient in optimizing the AO yield to its maximum potential. Rosmarinic acid was extracted at a higher concentration than that of carnosic acid and carnosol due to the hydrophobic properties of both carnosic acid and carnosol. The wild species was difficult to filter after extraction, likely due to the structure of the leaves, and this too would have hindered the AO yield. Future studies need to determine the most suitable extraction methods for each AO in each plant species in order to optimize the effects of the AO's on improving sperm quality. It would also be beneficial to determine how each of the AO's interact with the sperm membrane, how they are able to enhance the effect of the natural AO defense mechanism, and how they can minimize the negative impact of heat stress.

Opsomming

Die gebruik van ondersteunende reproduksietegnieke (ART's) soos kunsmatige inseminasie en embrio produksie, wat die gebruik van goeie gehalte sperme benodig, word beperk deur die vatbaarheid van ramsperme vir beskadiging tydens berging by lae temperature asook hitte stres. Korttermyn (koue) berging het 'n besliste uitwerking op die lewensvatbaarheid, morfologiese integriteit en die bevrugtingsvermoë van sperme, as gevolg van 'n toename in oksidatiewe stres wat veroorsaak word deur 'n oormatige opbou van reaktiewe suurstofspesies (ROS). Hitte stres het 'n aantal nadelige gevolge vir spermproduksie en rypwording, met die oormatige opbou van ROS wat lei tot 'n afname in sperm lewensvatbaarheid en -kwaliteit. Die studie het ondersoek of die diterpeen verbindings in gewone roosmaryn- en wilde roosmaryn variëteite, wat 'n natuurlike antioksidant (AO) aktiwiteit het, die skadelike veranderinge wat onderskeidelik deur koue berging en hitte stres (HS) veroorsaak word, kan verminder. Die bevindings het getoon dat sperm lewensvatbaarheid afgeneem het onder beide koue en hitte stres toestande, maar dat daar geen beduidende bydrae van die AO was om die afname in lewensvatbaarheid te voorkom nie. Die waterekstrak van die wilde roosmaryn spesie het 'n hoër vlak van morfologiese abnormaliteite as dié van die gewone roosmaryn spesie vir die koue berging en hitte stres studies. By die oorweging van die twee individuele spesies was daar egter geen beduidende verskille vir morfologie nie. Daar was geen beskermingseffek van die AO op die handhawing van spermvatbaarheid vir beide die verkoeling van koue stoor en HS nie, en die AO het ook tydens die HS-studie geen beskermende effekte op morfologie gehad nie. Koue berging het gelei tot 'n toename in die persentasie abnormale sperme wanneer die individuele effekte van die AO oorweeg word. Ter afsluiting het aanvulling van sperm verdunningsmedium met die waterekstrak van beide plantspesies nie 'n beduidende mate van beskerming getoon teen die skadelike effekte van verkoeling en HS op spermkwaliteit nie. Die onttrekkingstegniek wat vir die huidige studie gebruik is, was nie voldoende om die AO-opbrengs te optimaliseer nie. Rosmariniese suur was in hoër konsentrasies as karnosiensuur en karnosol teenwoordig in die waterekstrakte, wat toegeskryf kan word aan die hidrofobiese eienskappe van beide karnosiensuur en karnosol. Die wilde roosmaryn spesie se waterekstrak was moeilik om te filter na die onttrekking, waarskynlik as gevolg van die struktuur van die blare, wat moontlik ook die AO-opbrengs beperk het. Toekomstige studies moet gedoen word om die mees geskikte ekstraksiemetodes vir elke AO in elke plantspesie te bepaal ten einde die effekte van die AO op die verbetering van sperme te optimaliseer. Dit sal ook voordelig wees om te bepaal wat die interaksie van elkeen van die AO in die spermmembraan is, hoe hulle die effekte van die natuurlike AO verdediging meganisme kan versterk en hoe die invloed van die hitte stres reaksie beperk kan word.

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Alphabetical list of abbreviations

°C	Degrees Celsius
µL	Microliter
µm	Micrometer
AB	Antibiotics
ABP	Androgen-binding Protein
ACTH	Adrenocorticotropic Hormone
ADG	Average Daily Gain
AI	Artificial Insemination
ALP	Alkaline Phosphatase
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
AO	Antioxidant
ART	Assisted Reproductive Techniques
AV	Artificial Vagina
C	Concentration
CAF	Central Analytical Facilities
CAT	Catalase
cm	Centimeter
CoQ10	Coenzyme Q10
CRH	Corticotrophin Releasing Hormone
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
E/N	Eosin-nigrosin
EE	Electro-ejaculation
ET	Embryo Transfer
FSH	Follicle Stimulating Hormone
g	Grams
g/mol	Grams per mole
GnRH	Gonadotrophin Releasing Hormone
GPX	Glutathione Peroxidase
GSR	Glutathione Reductase

GST	Glutathione S-transferase
h	Hours
hCG	Human Chorionic Gonadotropin
HMOX1	Heme Oxygenase 1
HPA axis	Hypothalamic-pituitary-adrenal axis
HPLC	High-performance Liquid Chromatography
HPT axis	Hypothalamic-pituitary-testes axis
HS	Heat Stress
HSP	Heat Shock Proteins
IPCC	Intergovernmental Panel on Climate Change
IVC	*In Vitro* Culture
IVEP	*In Vitro* Embryo Production
IVF	*In Vitro* Fertilization
IVM	*In Vitro* Maturation
JIVET	Juvenile *In Vitro* Embryo Transfer
kg	Kilogram
LDH	Lactate Dehydrogenase
LH	Luteinizing Hormone
m	Mass
M	Molecular Weight
mg/L	Milligrams per liter
mL	Milliliter
mm	Millimeter
mM	Millimolar
MOET	Multiple Ovulation and Embryo Transfer
MS	Mass Spectrometry
MYE	Milk-yolk Extender
PUFA	Polyunsaturated Fatty Acids
PVC	Polyvinylchloride
R	Common Rosemary
RD	RapiDiff
ROS	Reactive Oxygen Species

SAS	Statistical Analysis Software
SEM	Standard Error of the Mean
SOD	Superoxide Dismutase
UHT	Ultra-high Temperature
V	Volume
WR	Wild Rosemary

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Chapter 1

General introduction

It is estimated that livestock production accounts for 43% of the gross value of agricultural production worldwide, and that the livestock sector will be the most important agricultural sector by 2020 (Madan, 2005). With a predicted population increase of just under 3 billion people by 2050, it is clear that the global demand for livestock products will continue to increase at a rapid rate in the coming decades, thereby emphasizing the need for food security (Thornton *et al.*, 2009). Apart from population growth, social and economic drivers such as rapid urbanisation, lifestyle changes and dietary habits, as well as increasing disposable incomes, are considered as major contributors to the changes being faced by current livestock production systems, especially those found in developing countries (Madan, 2005; Thornton *et al.*, 2009).

Approximately 80% of the agricultural land in South Africa is unsuitable for crop production, and minimal land is available for dairy and beef production, making the small stock industry a crucial and important contributor to the South African economy as well as to food security (Schoeman *et al.*, 2010). Sheep farming is practiced throughout South Africa, with an estimated 24.3 million sheep distributed throughout the country's nine provinces. It is estimated that 85% of the total number of sheep in the country are concentrated in the more arid regions of the Northern Cape, Eastern Cape, Free State and the Western Cape Provinces, with the latter being home to approximately 11% of the national sheep population. According to the Department of Agriculture, Forestry and Fisheries, there are approximately 8000 commercial sheep farms that employ approximately 35000 workers, as well as approximately 5800 communal sheep farms in the country (DAFF, 2014).

Between January 2014 and January 2017, the price of mutton has increased between 20.8% and 24% depending on the class of meat (AMT, 2017). This increase can be ascribed mainly to inflation, change of consumer lifestyles and insufficient supply to meet the consumer demand. Although South Africa is considered a major producer of mutton worldwide, it remains a net importer of mutton in order to satisfy the local demand with approximately 1318 tons of mutton and lamb being imported from Namibia in March 2017, this is 32.9% more than what was imported in the same month in 2016 (AMT, 2017).

Sheep are seasonal breeding animals that have developed a breeding strategy to ensure that their reproductive activity is restricted to the best time of the year that will allow for maximum growth and survivability of the offspring, as well as allow for maximum lactation of the ewe (Wayne *et al.*, 1989; Rosa and Bryant, 2003). Reproduction in sheep is mainly influenced by photoperiod, with sheep considered as short-day breeders. This implies that sheep become sexually active as the duration of daylight hours gets shorter in the late summer to early autumn (Rosa and Bryant, 2003). In South Africa, lambing predominantly occurs in late winter/early spring and late summer/early autumn. This seasonal reproductive strategy is a major obstacle for the sheep industry as it reduces the effectiveness of accelerated lambing programs restricts the flexibility to integrate lambing into other farm activities, and it limits farmers' access into favourable seasonal markets (Notter, 2002).

The use of assisted reproductive techniques (ART's) has gained popularity over recent decades as a method to allow sheep farmers to overcome the obstacle of seasonal reproduction and to increase mutton production as cost-effectively as possible while also improving food security. Assisted reproductive techniques include artificial insemination (AI), *in vitro* fertilization (IVF), multiple ovulation and embryo transfer (MOET), and juvenile *in vitro* embryo transfer (JIVET) (Boshoff, 2014). When compared to the commercial application of the ART's in the cattle industry, the ART's applied in the sheep industry requires refinement before their commercial application in the sheep industry will be equally successful as a tool to optimise the reproduction efficiency of flocks (Amiridis and Cseh, 2012).

The application of ART programs makes use of sperm obtained by means of semen collection or epididymal aspiration. Upon collection, semen needs to be stored at cool temperatures in order to decrease sperm metabolism and minimise a reduction in sperm quality. The successful application of ART techniques is highly dependent on the viability and morphological integrity of sperm, which deteriorates during short-term and long-term storage (Varisli *et al.*, 2009). Semen can be stored short-term in a liquid state at a temperature lower than body temperature, or it can be stored long-term in a solid state at sub-zero temperatures by using a process of cryopreservation. The successful preservation of sperm is achieved when the preservation methods are able to reduce sperm metabolism without impacting negatively on the viability and morphological integrity of the sperm (Kasimanickam *et al.*, 2007). The use of cooled semen is

considered a practical alternative to the use of cryopreserved semen in farm animals, as the damage resulting from the cooling process are less deleterious than the damage caused by the cryopreservation and thawing processes (Moradi *et al.*, 2013).

In addition to the damages induced by cooling, freezing and thawing of samples, the processing and manipulation of gametes also contributes to the production of reactive oxygen species (ROS). Although these free radicals play an important role in many physiological functions within the cell, they can also cause defects in molecular functioning of cells when levels are too high (Agarwal *et al.*, 2006). Sheep sperm are particularly susceptible to damages caused by ROS due to the high concentration of polyunsaturated fatty acids (PUFA) present in their plasma membranes (Da Silva Maia *et al.*, 2009; Fang *et al.*, 2015; Peker Akalin *et al.*, 2016). Studies have shown that the addition of antioxidants to semen extenders can minimize the effect of the oxidative stress caused by ROS. Maintaining or improving motility and morphological integrity of sperm after processing and during storage will ultimately contribute to optimise the viability of semen samples for use in ART's (Da Silva Maia *et al.*, 2009; Fang *et al.*, 2015).

Heat stress in another important aspect that plays a major role in the success of reproductive performance. Sheep are homeotherms that regulate heat production and heat loss to maintain a constant internal body temperature. The thermoregulatory ability of sheep becomes difficult in situations where heat production substantially increases through exercise or when the environment limits or hampers the degree of metabolic heat loss from the animal (Hansen and Fuquay, 2011). According to the Intergovernmental Panel on Climate Change (IPCC), the effects of global warming are estimated to result in a global temperature increase of approximately 1.8-4°C between 2090 and 2099, relative to that experienced between 1980 and 1999 (Thornton *et al.*, 2009). Exposure of livestock to such elevated temperatures has been found to result in a decrease of body weight, average daily gain (ADG) and growth rate, thereby indirectly increasing the incidence of impaired reproductive performance (Marai *et al.*, 2007).

Thermoregulation in the testes is of utmost importance as slight increases in temperature can result in a disruption of spermatogenesis, resulting in a lower fertility. There are a number of complex mechanisms in place in the testes to counteract the effects of stress, and these include DNA repair, heat shock response, oxidative stress response as well as apoptosis and cell death (Paul *et al.*, 2009). The testes also produce a number of antioxidant proteins that work to protect

germ cells from oxidative damage (Paul *et al.*, 2009). In addition to this, there is also a concurrent heat exchange system in place in order to maintain the delicate balance of heat distribution between the scrotal and core body temperatures – disruption of this system has detrimental consequences to the correct functioning of spermatogenesis (Paul *et al.*, 2009).

Studies have found that exposing rams to a heat stress environment for 14 days caused a reduction in testes weight of up to 70% (Setchell, 1998). Less obvious effects that have a great influence on fertility have been recorded, and include a reduction in sperm concentration and motility, and an increase in sperm abnormal morphology (Hansen and Fuquay, 2011). Heat stress primarily causes disruptions during spermatogenesis, before the spermatozoa are fully developed and enter the ejaculate. However, this effect of heat stress is only observed a few days after the damage has already occurred and remains long after the source of the heat stress has been removed. This is due to the fact that the sperm quality will only improve at the end of the affected cycle of spermatogenesis when the damaged germ cells have been ejaculated (Hansen and Fuquay, 2011; Setchell, 1998). Although the exact reason for germ cell damage during heat stress is not fully understood, it is thought that increased oxidative stress in the testes is one of the main factors contributing to or causing the observed sperm damage (Paul *et al.*, 2009).

The body contains a number of endogenous antioxidants that act as part of the natural defence mechanism against the deleterious effects of oxidative damage. This is a group of low-molecular-weight antioxidants, such as bilirubin, thiols, uric acid, and coenzyme Q10, as well as larger molecular enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (Agarwal *et al.*, 2006). Although sperm also contain this endogenous defence mechanism, the effects are less apparent during preservation. Studies have shown that frozen-thawed bull sperm are more easily peroxidised than fresh sperm, and that this results in a decrease of the intracellular antioxidants in the sperm membrane (Chandra *et al.*, 2012). Thus, there is a need to develop a method to support or supplement the effect of the endogenous antioxidant mechanism *in vitro*. Various studies have proven that the addition of an external source of antioxidant into the cooling or freezing extender can help to minimize the effects of oxidative damage on the sperm, which will assist in the maintenance of sperm motility, viability, and higher sperm mitochondrial activity (Bucak *et al.*, 2012).

The use of plants containing herbal antioxidants for medicinal and nutritional purposes has been gaining popularity during recent years (Motlagh *et al.*, 2014). An example of such a plant is common rosemary (*Rosmarinus officinalis* L.), a popular herb used in cooking, health products and cosmetics (Prez-Fons *et al.*, 2006). The antioxidant ability of common rosemary can be ascribed to diterpene molecules that are present in the leaves, with these molecules thought to act as free radical scavengers. Diterpenes may also play a role in the regulation of activity of some key enzymatic systems of the biological organisms on which they act (Prez-Fons *et al.*, 2006; Ban *et al.*, 2016). The three major phenolic compounds present in rosemary aqueous extract, based on their appearance in HPLC analyses, are carnosic acid, carnosol, and rosmarinic acid. These phenolic compounds inhibit free radical penetration in biological membranes by increasing membrane lipid order (Prez-Fons *et al.*, 2006). Although several studies have been carried out on the effects of external antioxidants in semen extenders to improve sperm viability and morphological integrity, the addition of rosemary to extenders is less well documented (Malo *et al.*, 2011; Zanganeh *et al.*, 2013; Motlagh *et al.*, 2014).

The aim of this study was thus two-fold, i.e. to determine firstly whether common rosemary *(Rosmarinus officinalis L.)* and wild rosemary *(Eriocephalus africanus L.)* differed in terms of the concentration of the diterpenes carnosic acid, carnosol and rosmarinic acid, which have been indicated as to have potent AO activity. Secondly, the study investigated the potential of sperm storage media, when supplemented with diterpenes extracted from common rosemary and wild rosemary, respectively, to minimise the damage caused by short-term storage and heat stress, respectively, as quantified by assessing viability and morphological integrity. An improved understanding of the effects of natural antioxidants in the livestock industry, included as part of media used to process and store sheep sperm, can potentially assist to prolong the lifespan as well as preserve the viability and morphological integrity of sperm, thus contributing positively to refining ART techniques for application in the sheep industry.

Chapter 2

Literature Review

2.1 INTRODUCTION

It is predicted that the world population will reach nearly 9.2 billion by 2050 (Jones and Thornton, 2009; Thornton *et al.*, 2009), resulting in a significant increase in the global demand for livestock products (Thornton *et al.*, 2009; Nardone *et al.*, 2010). Livestock production is an important contributor to the socioeconomic development of developing countries as it has the power to augment income and employment and reduce the incidence of rural poverty (Madan, 2005). Livestock are believed to utilize close to one third of the worlds entire land surface, mostly as permanent pasture, but also including the arable land used to produce livestock feed (McMichael *et al.*, 2007). In South Africa, 70% of the agricultural land is used for livestock farming, which accounts for 80% of the country's land resources (Meissner *et al.*, 2013). There is an increase in pressure worldwide to improve productive efficiency and meet the increased global demands for food security. Cost-effective animal production practices are continuously being developed, not only to ensure food security, but also to preserve scarce resources such as land, water, air and soil quality (de Vries and de Boer, 2010). The hope is that these practices will guarantee sustainable methods of production for future generations.

Artificial reproductive technologies (ART's) such as artificial insemination (AI), multiple ovulation and embryo transfer (MOET), embryo transfer (ET), *in vitro* fertilization (IVF) and juvenile *in vitro* embryo transfer (JIVET) are important management strategies that are being continuously developed and implemented to increase animal production, reproductive efficiency and rates of genetic improvement (Madan, 2005). The success of these technologies is highly dependent on the quality of the gametes that are being used, thus highlighting the importance of the continuous improvement of sperm and ova collection methods in order to maintain gamete viability and integrity throughout the process.

The origin of sperm can be from either fresh ejaculates that are collected by means of electro-ejaculation (EE) or the artificial vagina (AV) method, or from the cauda epididymis from culled animals, and harvested by means of aspiration. Depending on the eventual purpose of the sperm,

samples can be stored in a liquid state at reduced temperatures, or in a solid state at sub-zero temperatures using cryopreservation. Cryopreservation facilitates sperm transport over distances, permits quarantine of sperm samples when required, and allows for the use and exchange of germplasm from genetically superior animals long after they are deceased (Bailey *et al.*, 2000). However, freezing and thawing of samples has been found to deleteriously affect sample quality and viability through negatively affecting sperm motility and altering the biochemical and structural compartments of the sperm, resulting in an increased loss in viable sperm post-thaw (Crespilho *et al.*, 2014). Preserving semen in the liquid state at low temperatures has been rendered a viable alternative to cryopreservation protocols. Refrigerated semen can be stored for longer than fresh semen and the resulting damage to sperm quality is less severe than what is observed with cryopreservation. One consequence of liquid storage is the excessive production of reactive oxygen species (ROS), which can impact negatively on sperm viability (Baldassarre and Karatzas, 2004; Crespilho *et al.*, 2014).

It is well known that ROS play an important role in the ability for sperm to gain fertilization capacity, however, sperm cells are highly susceptible to cellular damage when ROS generation is excessive (Malo *et al.*, 2011a; Chandra *et al.*, 2012;). Under normal conditions, the generation of ROS is opposed by a balanced system of antioxidant (AO) defenses consisting of AO proteins and enzymes (superoxide dismutase (SOD), glutathione reductase (GSR), glutathione S-transferase (GST), glutathione peroxidase (GPX) and heme oxygenase-1 (HMOX1) that scavenge and suppress the formation of ROS (Agarwal *et al.*, 2006; Chen *et al.*, 2007). However, storage conditions used in semen preservation cause the AO defense mechanism to become defective. Sperm cell membranes contain proportionately high levels of polyunsaturated fatty acids (PUFA) when compared to other cells. Lipid peroxidation occurs when the excess ROS interact with the highly lipid sperm membrane, preventing normal sperm functioning (Malo *et al.*, 2010; Fang *et al.*, 2015).

Recent studies have reported that the addition of one or more antioxidants to semen extenders has the potential to protect the sperm against oxidative stress during preservation and prevent lipid peroxidation (da Silva Maia *et al.*, 2009; Chandra *et al.*, 2012; Mata-Campuzano *et al.*, 2014; Motlagh *et al.*, 2014). The use of natural AO's, including Fenugreek (Belguith-Hadriche *et al.*, 2013), *Rhodiola scara* roots (Zhang *et al.,* 2012) and rosemary, for preservation purposes is increasing in popularity because of their low incidence of toxicity problems (Malo *et al.*, 2011a).

Common rosemary *(Rosmarinus officinalis L.)* has been successfully included in ram, boar and bull cryopreservation extenders. The strong AO capacity of rosemary aqueous extract is believed to be due to the presence of several phenolic diterpenes that scavenge free radicals and prevent lipid oxidation (Yang *et al.*, 2016).

Anthropogenic activities are contributing to the changes that are currently being experienced in the earth's climate. During the last century, global temperature has increased by 0.7°C, and sea level has risen by approximately 25cm. Biological systems affected by this increase in temperature have experienced a shift in species distribution as well as changes in species population size, migration patterns and reproductive seasons of animals worldwide. These statistics are expected to get worse over time with analysts predicting an overall increase in average temperature of 2.1°C by 2050, along with a marked decline in rainfall and an increase in climatic variability (Salem *et al.*, 2011).

The impact of climate change on food safety is becoming a major global concern, particularly in developing countries where the agricultural sector is the backbone of the economy (McMichael *et al.*, 2007; Tirado *et al.*, 2010). It is believed that livestock contribute 15% of total food energy and 25% of dietary protein of people worldwide (Salem *et al.*, 2011). On top of this, the agricultural industry accounts for 22% of the total greenhouse-gas emissions worldwide with 80% of this being directly attributed to livestock production, feed production and livestock and feed transport (McMichael *et al.*, 2007).

Although the direct effects (increased temperature-related illness and death) of climate change on animal health are undesirable and extreme, the indirect effects (heat stress (HS)) are considered to be more problematic and worrying because of their subtlety in appearance and long-lasting deleterious effects on the productive and reproductive efficiency of animals (Nardone *et al.*, 2010; Hansen, 2009). Several studies have found that there is an increased incidence of decreased sperm motility, an increased proportion of morphologically abnormal sperm, and an overall decrease in sperm output of ejaculated samples in animals that are under the influence of HS conditions (Hansen, 2009).

Thus, the purpose of this chapter is to provide a detailed overview of the reproductive system of the ram, with special attention given to sperm quality, and the factors, both environmental and

physiological, that influence it. The effects of HS and cold stress on sperm are included, as well as the potential of AO's to counteract these effects.

2.2 REPRODUCTION IN THE RAM

As is seen in most male domestic mammals, the reproductive system of the ram consists of the testes, the epididymides, the accessory organs and the penis. The sperm as well as the male sex hormones are produced in the testes, with the latter being housed inside the scrotum. Mature sperm are stored in the tail portion of the epididymis (cauda epididymis) after acquiring the capacity for motility and fertilization as they move through the epididymis. During sexual excitement, the penis becomes erect and the sperm moves from the epididymis into the ductus deference and then onto the pelvic urethra where it is mixed with secretions from the secretory glands to constitute the ejaculated semen. Follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone are all involved in the control of reproduction in the ram (Cupps, 1991; Lambrechts, 1996; Hafez and Hafez, 2008). Figure 2.1. illustrates the reproductive tract of the ram as seen in left lateral dissections.

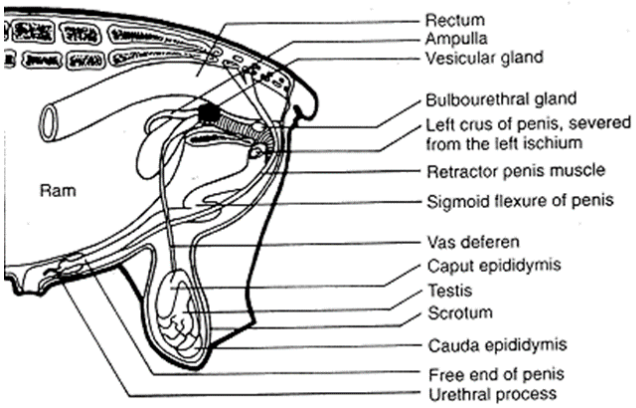

Figure 2.1. Diagram of the reproductive anatomy of the ram (Hafez and Hafez, 2008).

2.2.1 Anatomy and physiology of the ram reproductive system

2.2.1.1 Testes and Scrotum

The male gonads (testes) lie outside the abdomen within the scrotum. Each testis lies within the vaginal process, a separate extension of the peritoneum. Although the testes are held outside the abdominal cavity in a well-defined scrotum, they originate within the abdomen and undergo

a series of changes before descending through the inguinal canal to settle in the scrotum. Cryptorchidism is the condition that occurs when one or both of the testes fail to descend from the abdomen into the scrotum, this most often results in the male being sterile (Hafez and Hafez, 2008; Cupps, 1991).

The testes are the primary sex organs and are responsible for the production of the male gametes and the male sex hormones (Lambrechts, 1996). Testicular size in rams varies throughout the year, as with all seasonal breeders, and is larger than bulls, boars and stallions, as a percentage of body weight. This is an important consideration during the breeding season as the number of sperm per ejaculate is correlated with the size of the testis. The *rete testis*, a fibrous network of delicate tubules, is situated in the center of the testes and is the channel whereby the sperm move from the seminiferous tubules into the epididymis. A blood-testes barrier exists within the seminiferous tubules which maintains the difference in composition between tubular and *rete-testis* fluid versus blood plasma and interstitial fluid (Cupps, 1991; Lambrechts, 1996).

The scrotal sac consists of a peritoneum to which the testis and epididymis are attached by a mesorchium. The *tunica dartos* is the smooth muscle coat enclosing the two scrotal sacs. It is responsible for the variation in distance of the testes from the abdomen according to changes in temperature. Production of sperm needs to occur at 4-7°C below core body temperature (Cupps, 1991; Hafez and Hafez, 2008; Lambrechts, 1996).

2.2.1.2 Epididymis and Ductus Deference

After leaving the testis, sperm pass from the *rete testis*, though the efferent ducts and into the epididymis – a single narrow tube that performs vital functions such as transport, maturation and storage of sperm. The epididymis is separated into three regions; the head (*caput*), the body (*corpus*) and the tail (*cauda*). Fluid concentration and the first stages of cell maturation occur in the caput, cell maturation is completed in the corpus and the mature cells are stored in the cauda. The time needed for ram sperm to move through the epididymis and attain maturation is approximately 13 days and sperm remain fertile for several weeks. Non-ejaculated sperm are voided in the urine (Cupps, 1991).

The *ductus deference* is connected to the epididymis and transports sperm from the cauda to the urethra – the central duct of the penis (Lambrechts, 1996).

2.2.1.3 Accessory Sex Glands

There are four accessory sex glands in the male, i.e. the ampullae, the seminal vesicles, the prostate and the bulbo-urethral glands (Cowper's glands). The purpose of these glands is to produce secretions that contribute to ejaculated semen, forming the transport medium for the sperm. The accessory gland secretions are not essential for fertilization as sperm from the cauda epididymis are capable of fertilization before addition of these secretions (Cupps, 1991; Hafez and Hafez, 2008).

2.2.1.4 Penis

The penis is the external sex organ of the animal with the combined functions of depositing semen into the vagina of the female and excretion of urine. In the mammalian penis, there are three cavernous bodies aggregated around the penile urethra. Sexual stimulation results in dilation of the arteries supplying the cavernous bodies of the penis. In the ram, penile erection is controlled by the ischiocavernosus muscle that pumps blood from the dilated arteries in the cavernous spaces of the crura, into the rest of the *corpus cavernosum* (Hafez and Hafez, 2008).

2.2.2 Spermatogenesis

Spermatogenesis is the division and differentiation process that culminates in the production of mature spermatozoa in the seminiferous tubules of the testes (Cupps, 1991; Schlatt and Ehmcke, 2014). The seminiferous epithelium that lines the seminiferous tubules is composed of two basic cell types: the Sertoli cells and germ cells. Secretions produced by the Sertoli cells are influenced by the type of germ cells present in the seminiferous epithelium, and the two cell types are believed to be closely linked in both functional and morphological aspects of spermatozoa (Weinbauer and Wessels, 1999). The germ cells (spermatogonia) undergo continuous cellular divisions throughout the length of the tubule to produce spermatocytes. These cell divisions also ensure the continuation of the germ cell lineage as they allow the germ cells to continuously renew themselves. Primary spermatocytes undergo meiosis to produce secondary

spermatocytes and haploid spermatids – this is the first phase of spermatogenesis and is known as spermatocytogenesis (Hafez & Hafez, 2008).

Spermatids then undergo structural and differential changes during spermiogenesis to form highly specialized spermatozoa, with this last process completing the complicated, chronological and highly organized process of spermatogenesis (Hafez and Hafez, 2008; Chocu *et al.*, 2012).

The length of time it takes to complete a cycle of spermatogenesis (the duration between consecutive releases of spermatozoa) is dependent on the continuous influx of newly committed germ cells (Cupps, 1991). Although the process is uniform, the number of cellular stages and duration of each cycle differs between species. The duration of a cycle in the ram is approximately 10 days (Lambrechts, 1996; Hafez and Hafez, 2008).

2.2.2.1 Control of Spermatogenesis

The support and functional capacity of spermatogenesis is dependent on the combined function of FSH, LH and testosterone. Testes growth and functioning is controlled by the interaction of the pituitary and testicular hormones (Lambrechts, 1996). The hormones controlling spermatogenesis and testicular growth and function are controlled by both endocrine and paracrine mechanisms.

2.2.2.1.1 Endocrine Control

Testes function, androgen production and gamete maturation are regulated by the hypothalamus and the pituitary, while the interrelationship between the hypothalamo-hypophyseal system and the gonads are coordinated through feedback systems (Weinbauer and Wessels, 1999).

In order for normal testicular function to occur the hypothalamus secretes gonadotrophin-releasing hormone (GnRH) which stimulates the release of FSH and LH from the anterior pituitary (Cupps, 1991; Hafez and Hafez, 2008). Once these hormones are released they travel to the testes where they bind to receptors on their target cells and promote the synthesis and secretion of testicular products. These products can be used in the testes themselves under paracrine control,

or they can be directed away from the testes to be used for endocrine signaling (Lambrechts, 1996).

The Leydig cells are stimulated by LH to produce androgens. These androgens, mainly testosterone, are released into the bloodstream where they either induce or maintain a male phenotype and stimulate sexual organs, or act as negative feedback at the hypothalamic and pituitary level to block further LH secretion (Hafez and Hafez, 2008; Schlatt and Ehmcke, 2014). Follicle-stimulating hormone acts directly on the Sertoli cells to stimulate the production of androgen-binding protein (ABP) and inhibin. Androgen-binding protein forms a complex with androgen and is carried along with the sperm into the epididymis, the epithelial cells of the epididymis require high levels of androgen to carry out their normal functions. Inhibin acts on the pituitary and hypothalamus to block the secretion of FSH, while LH remains unaffected (Hafez and Hafez, 2008).

2.2.2.1.2 Paracrine Control

Inter-cellular testosterone is a good example of paracrine control and plays a vital role in the support of spermatogenesis (Lambrechts, 1996). The largest Leydig cells are found adjacent to the seminiferous tubules that contain the germ cells in their varying stages of differentiation and are dependent on androgens for development. The secretion of ABP and proteolytic enzyme from the Sertoli cells, in large amounts, inhibits the conversion of testosterone to estrogen, therefore allowing the testosterone to be used for paracrine signaling (Weinbauer and Wessels, 1999). Inhibin and activin are other examples of paracrine controls that modulate the production of steroids, other hormones and growth factors in the gonads. Activins also inhibit growth hormone and adrenocorticotrophin secretion (Hafez and Hafez, 2008).

2.3 THE SPERM CELL

2.3.1 Sperm morphology

A sperm consists of a head, a neck and a tail. Although sperm morphology is similar in most mammals, sperm length is species-specific and can range from around 50µm in man to 90µm in bulls (Boshoff, 2014). The entire sperm cell is surrounded by a plasma membrane (plasmalemma)

which is more firmly attached to the caudal margin of the head, the annulus and the longitudinal columns of the principle piece when compared to the rest of the sperm body (Brito, 2007). The basic morphology of a mammalian sperm cell can be seen in Figure 2.2.

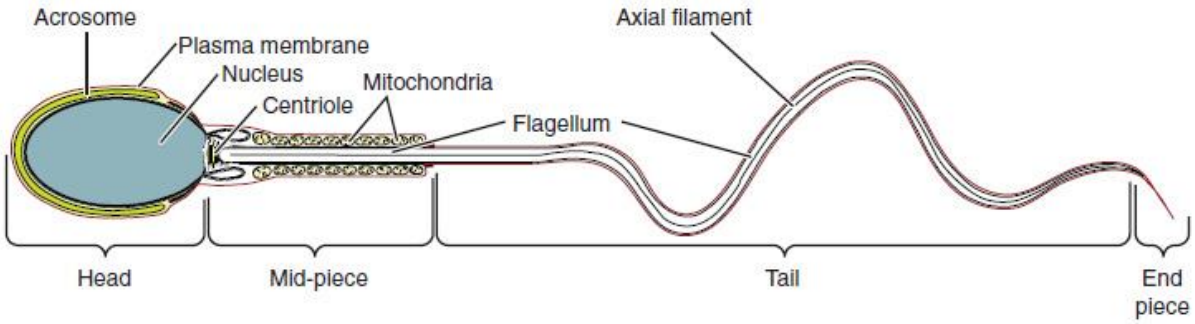

Figure 2.2. Diagram showing the basic morphology of a mammalian sperm cell. (Source: Anatomy & Physiology. OpenStax CNX. http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e-3ef2482e3e22@8.24).

The sperm head consists of the acrosome and the nucleus. The acrosome is a thin, double-layered membranous sac covering approximately two-thirds of the sperm head and forming a cap-like structure around it. The shape and size of the acrosome is also species-specific. The acrosome contains a number of hydrolytic enzymes, including acrosin and hyaluronidase that are involved in the fertilization process (Hafez and Hafez, 2008). These enzymes are released during the acrosome reaction, after which they play a crucial role in the lysis of the zona pellucida and the penetration of the corona radiata of the ovum (Boshoff, 2014). The oval flattened nucleus houses the highly compact chromatin comprising DNA that is complexed into basic proteins called protamines (Hafez and Hafez, 2008).

The neck is the connecting piece between the head and the flagellum (tail) and it varies in length and shape between species. There are single mitochondria with small projections present within the neck that act as an energy store for sperm (Boshoff, 2014).

The tail is comprised of the middle, principle and end pieces. The middle piece is characterized by its helical pattern of mitochondria that provide a source of energy for sperm motility and flexibility. The principle piece is surrounded by a fibrous sheath that provides stability for the contractile elements of the tail, such as proteins that are involved in signaling pathways. The end piece of the tail is comprised only of axoneme covered by plasmalemma, the axoneme is responsible for sperm motility (Boshoff, 2014; Hafez and Hafez, 2008).

2.3.2 Physiological alteration of the sperm surface

Sperm are not only transported via the epididymis into the vas deferens, they also undergo a maturation process during transit through the epididymis to gain the potential to fertilize the ova (Hafez & Hafez, 2008). These surface modifications are due to the sequential exposure of sperm to a highly dynamic protein environment that is created by the regionalized secretion and absorption of proteins across the epididymal epithelium (Leahy & Gadella, 2011). Sperm are also exposed to hormonal and enzymatically induced surface-modifying events from the seminal plasma after ejaculation, and while being transported through the female reproductive tract that enable them to gain fertilizing capacity upon reaching the ovum (Barrios *et al.*, 2000; Bernardini *et al.*, 2011).

2.3.2.1 Sperm maturation in the epididymis

Germ cells undergo morphological transformation into elongated spermatids through division, differentiation and meiosis in the testes during spermatogenesis. The sperm surface is further modified to transform the spermatids into fertile spermatozoa during their passage through the epididymis (Leahy & Gadella, 2011). As mentioned previously, the sperm plasma membrane has a high amount of PUFA that are known to contribute to membrane fluidity and flexibility. The definite lipid pattern of ejaculated sperm is acquired through the process of epididymal maturation. Studies carried out on ram sperm plasma membrane content have shown that the lipid and cholesterol content of whole ram sperm decreases during epididymal maturation. These changes are believed to explain the higher sensitivity of ejaculated sperm to cold shock when compared with testicular sperm under conditions of cold-storage (Mandal *et al.*, 2014).

Proteome, secretome and transcriptome are just a few of the important epididymal proteins that play a role in sperm maturation. These proteins can either form weak associations with the sperm surface or strong anchors. Proteins may also be removed or modified by proteolytic enzymes, and some have been reported in the surrounding epididymal fluid in their active enzymatic form. Potential mediators of sperm-egg communication are also acquired and modified in the epididymis, illustrating the importance of this organ in the attainment of successful fertilization (Leahy & Gadella, 2011).

The acquisition of sperm motility is arguably one of the most important changes that the sperm cell experiences during its progress through the epididymis. Without the acquisition of motility, the sperm would be unable to gain hyperactivity in the female reproductive tract and move towards the oocyte (Yeung & Cooper, 2002; Sullivan & Mieusset, 2016). Motility increases progressively as the sperm migrates from the corpus to the cauda epididymis, and is dependent on the generation of intracellular cAMP by adenylyl cyclase and their subsequent successive protein phosphorylations. Serine and tyrosine kinases and phosphatases generate a short cascade of phosphorylations and dephosphorylations that induce active bend propagation and regular flagella beating of sperm. The intracellular cAMP level increases progressively and simultaneously with ATP level and metabolic capacity from the corpus to the cauda regions of the epididymis (Dacheux & Dacheux, 2014).

The potential for the sperm to bind to the zona pellucida and oocyte membrane is also acquired during the sperms transit through the testes and epididymis and ending in the female reproductive tract after capacitation. It is believed that the redistribution of several testicular sperm surface proteins including tACE, basigin, TEX101 and several members of the ADAM protein family (ADAM1B, ADAM2, ADAM3, ADAM5 and ADAM32), are responsible for the potential acquisition of the sperm-egg interactions in the epididymis. Most of these surface changes are linked to local activation of proteolytic activities on the sperm surface or from luminal components. Sperm-egg interactions may also be induced by the binding of epididymal proteins to the sperm membrane, including ARSA, CD52, CRISP1, CRISP4, CRISP7, DCX, EPPIN, MFGE8, SPAM1 AND SPINK13 (Dacheux & Dacheux, 2014).

The controlled generation and recycling of ROS in the epididymis is important to ensure that sperm are protected from oxidative stress during their maturation process. Exposure of sperm to excessive levels of ROS in the epididymis can compromise fertilizing capacity and DNA integrity of the sperm cells. Reactive oxygen species play an important role in the process of signal transduction and the acquisition of hyperactivation, capacitation, acrosome reaction, zona pellucida binding and oocyte penetration in sperm. The controlled recycling and generation of ROS is maintained through the complex interactions between a number of enzymes present in the male reproductive tract including GPX, CAT, SOD and indolamine dioxygenase (Vernet *et al.*, 2004).

2.3.2.2 Sperm maturation in seminal plasma

Seminal plasma is a physiological secretion from the accessory glands of the male reproductive tract. It acts as a vehicle for ejaculated sperm, and a natural medium for maturation of the sperm through hormonal, enzymatic and surface-modifying events once the sperm reaches the female reproductive tract (Barrios *et al.*, 2000; Bernardini *et al.*, 2011). The function and chemical composition of the seminal plasma is very complicated and varies according to accessory gland contributions and fertility status of the male. Seminal plasma secretion is under endocrine control by FSH, LH and testosterone in the testes and the anterior pituitary gland (Lambrechts, 1996). Biological components such as choline, citric acid, fructose, inositol and ergothioneine, are present in abnormal amounts in seminal plasma when compared to other body fluids and are specific for the regulation of sperm function (Barrios *et al.*, 2000).

During mating, the sperm are diluted with seminal plasma in the male genital tract and carried through the urethra in suspension to be deposited in the female reproductive tract. It is believed that the seminal plasma acts as a coating for the sperm that is essential for the acquisition of motility and fertilizing ability. Studies have shown that some seminal plasma proteins are absorbed by the sperm after ejaculation, resulting in stabilization of the membrane until the appropriate time, while other proteins result in membrane exocytosis. The timing of membrane stabilization and exocytosis in a temporal-spatial framework conducive to and facilitative of fertilization is vital for the successful initiation of capacitation and acrosome reaction (Barrios *et al.*, 2000).

Seminal plasma contains many substances that support sperm cells in their transition from the epididymis into the female tract. Sodium and potassium establish and maintain osmotic balance, calcium stimulates steroidogenesis in the Leydig cells of the testes, lactate dehydrogenase (LDH) is important for sperm metabolism and the acquisition of capacitation and fertilization, and alkaline phosphatase (ALP) is essential for providing the sperm with energy for survival, motility and fertilization (Asadpour, 2012).

2.4 GENERAL FACTORS AFFECTING SPERM QUALITY

General evaluation of sperm samples involves the assessment of sperm quality parameters such as overall motility, sample volume, concentration, percent viability, normal morphology and acrosome integrity. These parameters are important when determining the quality of an ejaculate and whether or not it can be used for application in ART's.

There are a number of internal and external factors that influence these traits and reduce fertility which could impact negatively on the reproductive efficiency in males. These factors include the nutritional status of the animal, season, stress and disease.

2.4.1 Nutrition

The effects of nutritional status on reproduction are more prominent in the male than they are in the female. In the male, nutritional restrictions have the ability to delay the onset of puberty and depress the production and characteristics of semen. Nutrition does not have a direct effect on the spermatogenic functioning of the testes, but rather indirectly acts on the endocrine function of the testes to alter sperm quality. Young and growing animals are more susceptible to these changes than mature animals. The main effects of nutrition on sperm quality are under- and overfeeding, vitamin and mineral deficiencies, and toxic agents (Hafez & Hafez, 2008).

2.4.1.1 Effect of under- and overfeeding

It is generally believed that animals that are highly conditioned produce semen of a superior quality. This is not always the case as these animals are often fed liberally to improve their condition which increases their susceptibility to becoming overweight, thus depressing the spermatogenic process and resulting in increased morphological abnormalities and decreased fertility (Lambrechts, 1996).

Although mature animals are able to maintain sperm production and testosterone secretion during periods of underfeeding, young and growing animals require enough energy and protein resources to contribute to growth as well as reproductive functioning. A low level of nutrition in

young animals inhibits the endocrine activities within the testes resulting in a decrease in growth and a reduction in the secretory function of the reproductive organs of the male (Boshoff, 2014).

The secretion of LH and testosterone is also reduced when animals are fed at sub-maintenance levels. This reduces the trophic support of the testes which in turn causes a reduction in the number of sperm produced (Lambrechts, 1996). The effects of underfeeding are seen in the libido and testosterone production long before they are seen in the semen quality (Hafez & Hafez, 2008).

2.4.1.2 Vitamin deficiencies

The deficiencies of specific vitamins are generally connected to a decrease in the reproductive ability of most farm animals. A vitamin A or carotene deficiency leads to testicular degeneration in all farm animals. This effect is probably indirectly due to the suppression of the release of pituitary gonadotropins. In situations where the testicular damage is not permanent, spermatogenesis can be restored with injections of gonadotropic hormones or vitamin A (Hafez & Hafez, 2008).

Vitamin E is another vitamin that is important for normal reproduction. Its role in the fertility of males is not fully understood, but it is believed that its ability to scavenge for free radicals could protect sperm from ROS damage caused my lipid peroxidation (Boshoff, 2014).

2.4.1.3 Mineral deficiencies and possible toxic agents

There is little information available on the specific deficiencies of minerals on male reproduction. Studies have seen a decrease in the libido and semen characteristics of bulls experiencing iodine deficiencies. Improvements of sperm production and fertility have been seen when farm animals are supplemented with copper, cobalt, manganese and zinc (Hafez & Hafez, 2008).

Endocrine disrupting chemicals such as naturally occurring chemicals, ionizing radiations and earth salts have the potential to cause toxicity problems when animals are exposed to them during the early developmental stages. Plant estrogens are also believed to be a possible inhibitor of spermatogenesis (Boshoff, 2014).

2.4.2 Season

Sheep show an annual cycle of reproduction that is characterized by a breeding and a non-breeding season. Although rams can remain sexually active outside the breeding season, their sperm production decreases, the size and weight of the testes decreases and libido decreases (Gootwine, 2011). These variations are associated with a variation in sperm production and quality.

Photoperiod initiates the beginning of the breeding season and rams will start to become more sexually active when the days get shorter (i.e. autumn) (Boshoff, 2014). Melatonin is thought to be the mediator of the photoperiod effect by changing its pattern of secretion with the change in day length, however, this physiological mechanism is not yet fully understood (Gootwine, 2011). FSH and LH production is also stimulated with the change in daylight hours, which in turn stimulates an increase in testosterone production to stimulate the rams reproductive cycle (Boshoff, 2014).

2.4.3 Stress

There are many types of stress that an animal can experience including immobilization, electro-shock and physiological stress. The effects of stress on a particular animal are not only limited to the type and duration of the stress but also on the level of circulating sexual hormones present in the animal at the time of the stress (Boshoff, 2014).

There are many environmental and management stressors that an animal can be exposed to at any given time. These include nutrition, housing, handling and thermal stressors. Improper handling techniques cause both behavioural and physiological stress that may adversely affect reproduction. Studies have showed that rearing gilts in confined pens as compared to group pens resulted in delayed puberty because of the handling and isolation stress that the animals experienced (Etim & Oguike, 2014).

There are a variety of endocrine regulatory points whereby stress limits the efficiency of reproduction (Etim & Oguike, 2014). During periods of stress, the hypothalamic-pituitary-adrenal (HPA) axis secretes a number of hormones including vasopressin and corticotropin releasing

hormone (CRH). These hormones are able to affect the hypothalamic-pituitary-testes (HPT) axis, resulting in an increase in the activity of the HPA axis and a reduction in the activity of the HPT axis. Corticotrophin-releasing hormone RH acts together with β-endorphin to directly inhibit the release of GnRH from the hypothalamus, which in turn inhibits the hormonal cycle that regulates sperm production. Glucocorticoid circulation is increased by the CRH-mediated increase in adrenocorticotropic hormone (ACTH) secretions, inhibiting the secretion of hormones from the hypothalamus and pituitary, thus indirectly inhibiting the spermatogenic process and directly inhibiting testosterone production (Boshoff, 2014).

Heat stress has a marked deleterious effect on the production of abnormal sperm. The combination of a high ambient temperature and high humidity for sustained periods of time can render males sterile for up to six weeks (Boshoff, 2014). HS has also been shown show to delay the onset of puberty in dairy cattle when they experience prolonged periods of increased ambient temperatures (Etim & Oguike, 2014).

2.4.4 Disease

Male infertility can be affected by the presence of bacterial or viral infections that originate within the genital tract. Infection in the animal body has the potential to lead to a decrease in spermatogenic output, the destruction of sperm function or the obstruction of the seminal tract (Boshoff, 2014).

2.4.4.1 Fever as a symptom of disease

In order for the testes to function correctly, they have to remain within a small thermal range that is lower than that of the body. An increase in testicular temperature can have a marked effect on spermatogenesis and the quality and functionality of the sperm produced. Elevated temperatures caused by fever has the potential to decrease spermatogenic output and alter the functioning of the epididymis by causing protein degradation. It is important that these proteins are protected from elevated temperatures as they play a vital role in the processes of sperm maturation, motility and fertilization (Boshoff, 2014). Decapacitated sperm found in an ejaculated sample are an indication of a higher than normal body temperature (Lambrechts, 1996).

2.5 ASSISTED REPRODUCTIVE TECHNIQUES (ART's)

The application of ART's enables the rate of genetic progress to be increased. Techniques such as AI and ET increase the selection differential of the animal, while a technique such as JIVET accelerates the process of genetic progress by shortening the generation interval of the animal (Baldassarre & Karatzas, 2004).

As seasonal breeders, sheep breeding is limited to certain times of the year resulting in a reduction in the effectiveness of accelerated lambing programmes, a restriction in the flexibility to integrate lambing into other farm activities, and it limits farmers' access into favourable seasonal markets (Notter, 2002). The adaptation of ART's for use in sheep can potentially help to overcome these reproductive restrictions and improve genetic gain in the species (Amiridis & Cseh, 2012).

2.5.1 Artificial Insemination (AI)

Artificial insemination is regarded as a first generation ART and has made the greatest contribution to genetic improvement programs around the world (Baldassarre & Karatzas, 2004). The major advantages of AI include genetic improvement, control of venereal diseases, availability of accurate breeding records, economic service and safety through the elimination of having to deal with dangerous males (Hafez & Hafez, 2008). The progress being made in semen sexing for AI purposes also renders it an important technique for gender control, especially in the dairy industry where it allows for the selection of female-only sperm to improve productive efficiency (Foote & Parks, 2011).

Sperm can be collected using EE or the AV technique as mentioned previously. Sperm can either be used as fresh ejaculates, refrigerated samples or frozen-thawed samples. Fresh ejaculates are preferred when the male is present in the flock, especially during the breeding season as sperm production and quality is at its highest. The use of refrigerated semen is popular when the ram is part of a consortium, where more than one farmer will utilize the genetics of the animal for flock improvement purposes. Studies suggest that the use of refrigerated semen produces good insemination results if it is used within 24h of collection. Frozen-thawed semen allows for long-

term preservation so that samples can be used over a vast area, used throughout the year or to conserve genetic material of a superior animal if it dies (Baldassarre & Karatzas, 2004).

Artificial insemination in the ewe can be done using one of four different methods; vaginal insemination, cervical insemination, transcervical insemination and laparoscopic or intrauterine insemination (Hafez & Hafez, 2008). Vaginal insemination is considered to be successful for fresh semen, while cervical and transcervical insemination is successful for refrigerated semen and in some cases frozen semen. Laparoscopic or intrauterine insemination is the most successful when using frozen-thawed semen to achieve high pregnancy rates (>70%) (Baldassarre & Karatzas, 2004).

2.5.2 Embryo Transfer (ET)

Embryo transfer is considered to be for the ewe what AI is for the ram (Hafez & Hafez, 2008). This technique can be used to expand the population of particular breeds or strains of sheep that are in demand. Embryo transfer also allows the importing and exporting of sheep in the form of frozen embryos rather than animals 'on the hoof', and it can be used in the event of infectious diseases to avoid transmission from the infected donor to its offspring (Gordon, 2004).

The basic requirements for an ET program are a source of embryos (donor superovulated using FSH) or by *in vitro* maturation (IVM)/IVF and *in vitro* culture (IVC), a reliable method of transferring the embryos, and suitably synchronised recipients. Donor animals are superstimulated with FSH to increase the number of follicles that develop during one estrous cycle (Hafez & Hafez, 2008). In the ewe, this is most commonly done using a progesterone pessary for 12 days, followed by injecting a gonadotropin such as FSH one day before pessary removal (Amiridis & Cseh, 2012). This procedure stimulates additional follicular growth and spontaneous ovulation without the need for endogenous LH or human chorionic gonadotropin (hCG) (Hafez & Hafez, 2008).

Embryo collection in sheep is done using laparoscopy or through transcervical collection. Cervical insemination does not render satisfactory fertilization rates in ET techniques and so the laparoscopic technique is more desirable and can be used on estrous synchronised and super ovulated donors using fresh or frozen-thawed semen (Amiridis & Cseh, 2012).

In vitro embryo production (IVEP) can also be used as an artificial approach to ET by collecting the oocytes from superovulated donors and inducing IVM, IVF and IVC of the oocytes prior to transfer or vitrification (Hafez & Hafez, 2008). The use of ET is not popular in sheep as it is expensive, and the ET method is not nearly as effective as AI in ensuring genetic progress.

2.6 SEMEN COLLECTION

Semen can be collected from rams by using the artificial vagina method (AV) or by means of electro-ejaculation (EE). It is important to collect high quality sperm samples from superior rams in order for the successful application of ART's such as AI.

2.6.1 Electro-ejaculation

The EE technique is carried out by inserting a bipolar electrical probe into the ram's rectum. Low voltage stimulation is given for two seconds followed by a two second rest period, this sequence is then repeated with a 1V increase in voltage strength during the second stimulation. This process is repeated until an ejaculation occurs (Hafez & Hafez, 2008). Studies have found that there is a higher variability in sperm concentration and the incidence of abnormal sperm found in EE samples when compared to AV samples (Bopape *et al.,* 2015), urine contamination has also been found to be a problem (Hafez & Hafez, 2008). The EE technique is considered to be stressful to the ram as indicated by the vocalizing, struggling and strong muscular contractions often displayed by the animal during collection, and should only be used in extreme situations (Bopape *et al.,* 2015).

2.6.2 The artificial vagina method

The AV is the preferred method of collection by welfare standards, but requires rams to be trained before use. The AV consists of a polyvinylchloride (PVC) pipe of about 20-25cm long, with a softer latex inner lining. A glass collection tube is placed at the one end of the AV. To reduce cold shock to sperm when ejaculation occurs, care should be taken to warm the collection tube prior to collection (37°C). In order to mimic the female reproductive tract, the AV is filled with warm water (approximately 48°C) between the outer and inner layers, creating a warm pressure for the ram. A dummy ewe is restrained while the ram is brought to her to mount. Upon

mounting, the ram's penis is gently diverted into the AV. After ejaculation, the collection tube containing the sample is placed in a water bath (37°C) until further processing (Boshoff, 2014).

Although the EE technique results in a lower recovery efficiency of the rams (urine contamination and lack of response to electrical stimulation) as well as a higher variation in sperm quality of collected samples, it is often preferred over the AV technique for industry purposes due to its convenience. Although the training period for the AV is not long (approximately a week), some animals may still reject the procedure after training, and animals with leg injuries are often not able to mount. The utilization of each technique is dependent on the objectives and conditions for semen collection (Bopape *et al.,* 2015).

2.7 EVALUATION OF SPERM QUALITY

2.7.1 Macroscopic evaluation

Macroscopic evaluation of sperm is carried out with the naked eye and focuses on the appearance of the ejaculate with regards to volume, colour and mass motility. It is often the only method of sperm quality evaluation by a farmer using his own rams to inseminate ewes in the farm environment.

2.7.1.1 Semen volume

The ejaculate volume differs between farm animals according to species size, and within species according to age and maturity (Boshoff, 2014). Table 2.1 indicates the average ejaculate volume of different farm animal species.

Table 2.1. Average ejaculatory volume of different farm animals (Boshoff, 2014).

Species	Semen volume produced by single ejaculation (mL)
Stallion	50 – 100
Bull	5.0 (1.0 – 15)
Ram	0.5 – 2.0
Boar	200 – 250

The ejaculate volume is affected by animal age and condition, skill of collector and collection method, season, and frequency of collection (Hafez & Hafez, 2008).

2.7.1.2 Colour

Colour scoring is considered a subjective way to evaluate semen and is assessed on a numeric scale ranging from 0 – 5 (0 being almost clear and 5 being thick creamy white). Ram semen is milky-white or a pale creamy colour. The ejaculate colour should be relatively uniform, with a dense appearance being indicative of a high sperm cell concentration (Boshoff, 2014). Blood in the ejaculate is indicated by a pink colour, urine is indicated by a strong odour with a yellow colour, and dilute semen, or semen that is gray or brown in colour indicates contamination of an infection from the ram reproductive tract. Contaminated samples should be discarded (Hafez & Hafez, 2008).

2.7.1.3 Mass motility

Mass motility is usually referred to as the mass cloud movement of sperm in undiluted semen and can be seen using the naked eye, or under the light microscope when a drop of undiluted semen is placed on a microscope slide without a coverslip. The appearance of mass motility is dependent on sperm concentration, percentage of immotile sperm and the degree of activity expressed by the rest of the sperm in the sample. Normal, viable ram semen exhibits a prominent wave-like motion that is characterized by the continuous moving and swirling motions of the motile sperm. Abnormal sperm exhibit a swimming pattern that is impaired and usually non-progressive in nature and can also influence the amount of mass motility observed in a sample (Boshoff, 2014).

Mass motility is scored on a scale from 0 – 5, where 0 indicates no motile sperm and 5 indicates the highest percentage of motile sperm (90-100% motility). This scoring system is based on the vigorous movement and the formation of waves (Table 2.2).

Table 2.2. Score sheet used to determine the amount of vigour present in the mass wave motion of a sperm sample (Hafez & Hafez, 2008).

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

2.7.2 Microscopic evaluation

Microscopic evaluation entails the analysis of sperm samples to determine the percentage of viable sperm, sperm concentration, sperm morphology and acrosome integrity. It provides more detailed information than macroscopic evaluation, and usually follows after macroscopic evaluation.

2.7.2.1 Percentage of live sperm

The percentage of live sperm in a sample can be determined using one of several staining methods. One example of a staining technique that is used to determine sperm viability is the eosin-nigrosin (E/N) staining technique. When the staining material is added to an aliquot of semen and a smear made, viable/living cells will not absorb any of the staining material while non-viable/dead cells will absorb the stain and appear pink or partially pink when viewed under the microscope. At least 100 sperm should be examined and classified (Brito, 2007).

2.7.2.3 Sperm concentration

It is important to be able to accurately determine the number of sperm and volume of the ejaculate as it gives an indication of how many females can be inseminated using a particular semen sample. The normal concentration of the ram ejaculate ranges from 3.5×10^9 to 6.0×10^9 sperm/mL (Hafez & Hafez, 2008).

Concentration is measured using a hemacytometer, colorimeter or a spectrophotometer. Although counting using a hemacytometer is time consuming, it is also extremely accurate. This

method of measurement is done using a microscope slide with precisely marked chambers where the number of sperm per chamber are manually counted (Hafez & Hafez, 2008). The concentration of ejaculates differs between species (Table 2.3).

Table 2.3. Sperm concentrations of different farm animals (Hafez & Hafez, 2008).

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

2.7.2.4 Sperm morphology

The evaluation of sperm morphology is a major component of standard semen analysis as it is widely believed that normal morphology is a strong indicator of male fertility classification (Van Den Hoven *et al.*, 2015). Fertility generally declines when the level of abnormal morphology exceeds 20% (Boshoff, 2014). Typically, there are three categories of morphological abnormalities in sperm (Figure 2.3). Primary abnormalities refer to those associated with the head and acrosome, secondary abnormalities refer to those that have the presence of a cytoplasmic droplet on the midpiece, and tertiary abnormalities refer to all other possible tail defects, such as; looped, bent back or coiled tails (Boshoff, 2014).

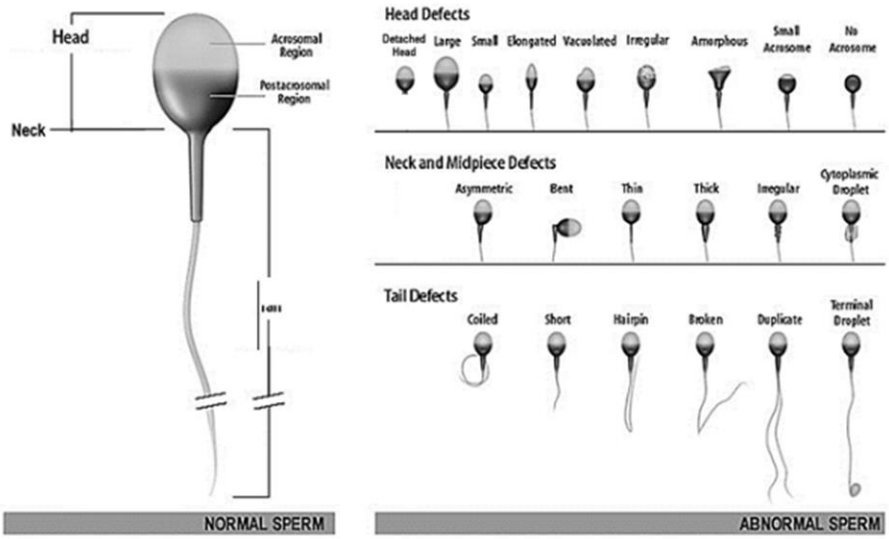

Figure 2.3. Different types of primary, secondary and tertiary morphological abnormalities that can be observed during the morphological evaluation of sperm samples (Source: www.fssc.com.au).

In rams, increased morphological abnormalities vary according to season with higher levels of morphological abnormalities occurring in spring and decreasing as the breeding season advances (Boshoff, 2014). There are a number of staining techniques used to evaluate sperm morphology, including the E/N staining technique mentioned previously, as well as the use of SpermBlue®. As with sperm viability, at least 100 sperm need to be examined and classified in order to be representative of the sample.

2.7.2.5 Acrosome integrity

In order for successful fertilization to occur, sperm must maintain an intact acrosome up until the point when the sperm binds with the zona pellucida of the oocyte and undergoes the acrosome reaction to release acrosome enzymes (Mehmood *et al.*, 2009). Acrosome integrity can be evaluated using a number of techniques including interference or phase contrast microscopy, or with specific staining techniques (Boshoff, 2014).

The use of the RapiDiff (RD) stain is one such staining technique. A slide is prepared with a smear of an aliquot of the semen sample and left to air-dry. Once dried, the slide is dipped into three reagents. The first reagent is a fixative containing Thiazine dye in methanol, the second reagent contains Eosin Y dye in a phosphate buffer and the final reagent contains Polychromed Methylene Blue in a phosphate buffer. This process allows a contrast between the sperm cell and the background of the slide so that intact acrosomes can be examined.

2.8 THERMAL STRESSORS AND SPERM QUALITY

2.8.1 Short-term (cold) storage

The use of cryopreservation in prolonging the storage time of semen indefinitely; has been studied extensively by many scientists. It is well known that cryopreservation has severe deleterious effects on the sperm structure, reducing viability, morphology, and subsequently the fertilizing ability of sperm (Crespilho *et al.*, 2014; Quan *et al.*, 2017). Deleterious effects result from cold shock, chemical toxicity from cryoprotectants, osmotic injury, oxidative injury and apoptosis (Quan *et al.*, 2017). The reduction in sperm quality and fertilizing potential displayed by cryopreserved sperm has motivated several studies to be carried out in order to find

alternative preservation protocols for semen in the liquid state at reduced temperatures (4°C to 5°C) (Crespilho *et al.*, 2014).

Cooled sperm samples are not subjected to the same degree of damage as cryopreserved samples, thus allowing a decrease in AI dose and optimizing the use of sires of high genetic merit (Crespilho *et al.*, 2014). For high success rates to be achieved with samples stored in a liquid form, sperm motility and metabolism needs to be reduced for extended periods in order to prevent premature changes in acrosome integrity and capacitation status, and this reduction needs to be reversible with a minimal amount of viable losses and morphological damage experienced by the sperm so that it is still able to retain fertilizing potential (O'Hara *et al.*, 2010; Crespilho *et al.*, 2014). Some authors have had high levels of success through the use of liquid storage over extended periods, however, refrigeration of semen still results in deleterious effects caused by cold shock. The general consensus is that although the damages are not as destructive as those experienced during cryopreservation, the quality of sperm deteriorates as the duration of storage increases (O'Hara *et al.*, 2010).

In an attempt to minimize the deleterious effects of cold storage on sperm quality parameters, hyperosmotic diluents such as trehalose as well as milk-egg yolk and external AO's have been added to semen extenders (Aisen *et al.*, 2002; Andrabi, 2009). Trehalose acts as an energy source for sperm (Gadea, 2003) and is also considered as a sperm membrane stabilizing component (Aisen *et al.*, 2002). The milk-egg yolk component of the extender contains lipoproteins that protect sperm against cold shock by stabilizing the sperm cellular membranes (Brinsko *et al.*, 2011). The external AO's act as free radical scavengers and also as an additional buffer for the natural AO defense system to prevent sperm membrane damage and a premature initiation of capacitation, hyperactivation and the acrosome reaction (Bucak *et al.*, 2012; Fang *et al.*, 2015; Amidi *et al.*, 2016).

2.8.2 Heat stress (HS)

Domestic livestock are considered to be homeotherms, this means that they are able to maintain a near constant body temperature despite being exposed to a wide range of environmental conditions (Silanikove, 2000; Al-Haidary, 2004). Animals utilize homeostatic responses such as raised respiration rate, panting, drooling, reduced heart rate and sweating, and behavioural

responses such as resting in the shade during the hottest part of the day and grazing before sunrise or during the evening (Silanikove, 2000).

The thermoneutral zone of the animal is the temperature range whereby the total heat production of the animal remains constant. This means that the total energy required to carry out essential physiological processes (digestion, excretion, metabolism of nutrients) is at a minimum, allowing the dietary energy obtained from food to be used for production purposes i.e. growth, egg and milk production. When the animal experiences environments that are too hot or too cold, heat production is increased in order to counter the effects of the environmental stressor. The increased energy loss results in a decrease in the remaining energy needed to carry out essential production processes, thereby decreasing the efficiency of energy utilization (Babinszky *et al.*, 2011).

Heat stress occurs when a combination of environmental conditions causes the effective temperature of the environment to be higher than the animal's thermo-neutral comfort zone. The most important environmental factors that influence effective temperature are increased air temperature, higher relative humidity, decreased air movement and higher solar radiation (Armstrong, 1994; Marai *et al.*, 2008; Salem *et al.*, 2011).

The biological and productive functions of the animal are compromised during periods of HS. The reason for this is that the physiological adjustments that the body must undergo in order to regulate the increase in body temperature have additional deleterious effects on the animal itself, hyperthermia has the ability to alter cell function if it is unable to be regulated (Hansen and Fuquay, 2011).

The drastic biological changes experienced by sheep under HS conditions include decreased feed intake efficiency and utilization, water metabolism disturbances, protein, water and mineral imbalances, as well as altered enzymatic reactions, hormonal secretions and blood metabolites (Marai *et al.*, 2008; Salem *et al.*, 2011). Reproductive performance is also affected by HS and a rise in body temperature above the comfort zone can compromise the function of germ cells, early developing embryos and other cells involved in reproduction (Hansen, 2009).

2.8.2.1 Mechanisms to counteract heat stress

Animals make use of convection, conduction, radiation and evaporation as physiological mechanisms to dissipate excessive metabolic heat into the environment under conditions of HS. The ability for the heat to be dissipated by the animal is dependent on the production status of the animal and coat colour. Environmental conditions, including the time of day and the environmental capacity for heat and vapour exchange, are also factors influencing the efficiency whereby animals can dissipate excessive metabolic heat (Armstrong, 1994; Marai *et al.*, 2008; Sevi and Caroprese, 2012).

In order to prevent an alteration in normal biological functioning, mammalian cells have a number of cellular mechanisms that are triggered under HS conditions, i.e. DNA repair, heat shock response, oxidative stress response and apoptosis (Paul *et al.*, 2009). The heat shock response is the synthesis of heat shock proteins (HSP) at the cellular level under conditions of HS. The expression and activity of HSP is under endocrine control and can act at the intracellular level (inhibition of cell proliferation and protein synthesis) or at the extracellular level (supporting innate immunity). Heat shock proteins are classified into different family groups according to their molecular weight and function. The most studied HSP have molecular weights of approximately 90, 70, and 27kDa and are referred to as HSP90, HSP70 and HSP27, respectively (Sevi and Caroprese, 2012). Recent studies have shown that HSP70 expression is increased in the blood mononuclear cells of sheep, the adipose tissue of feedlot steers and the muscles of pigs exposed to a variety of HS conditions. Due to the strong cryoprotective effects of HSP70, it is believed to assist in protein folding on exposure to hyperthermia and is generally associated with better thermotolerance. Unstressed cells have an abundance of HSP90 which is essential for normal cellular function and cellular adaptation to stress, and increased levels of HSP90 have been found in heat stressed mammalian cells supplemented with AO, indicating that HSP90 could also be involved in the cellular response against HS conditions (Chauhan *et al.*, 2014).

The oxidative stress response is another important mechanism that cells activate under HS conditions and is characterized by the synthesis of AO proteins that protect cells from oxidative damage caused by excessive ROS production. Non-enzymatic AO's (vitamin A, vitamin C, β-carotene, selenium, etc.) are supplemented through the diet, while enzymatic AO's are those synthesized within the affected cells (SOD, GPX, GSR, GST, HMOX1) (Paul *et al.*, 2009). This

mechanism is believed to be the main response mechanism in the testes against ROS and acts in conjunction with the DNA repair system in the testes and seminal plasma.

2.8.2.2 Consequences when heat stress cannot be counteracted

The mechanism of thermoregulation in the testes is vitally important to enable and maintain normal spermatogenesis. A slight increase in temperature caused by the environment, fever, cryptorchidism or inflammation has the potential to disrupt normal spermatogenesis and cause problems with fertility (Paul *et al.*, 2009; Alves *et al.*, 2016). There are a variety of mechanisms that the testes display when exposed to conditions of stress. These mechanisms include DNA repair, heat shock response, and apoptosis and cell death (Paul *et al.*, 2009).

Studies have shown that cell metabolism in the testes is increased when testicular temperature rises above a set point. Normally, there is a countercurrent heat exchange system between the pampiniform plexus and the testicular artery that regulates temperature, however, this mechanism does not allow for a sufficient and proportionate increase in blood flow that meets the increase in cell metabolism, thus creating a hypoxic environment in the testes (Paul *et al.*, 2009; Hansen, 2009; Alves *et al.*, 2016). Hypoxia is categorized by decreased oxygen tension below that which is required for normal cellular function in a particular tissue (Paul *et al.*, 2009), this causes an increase in oxidative stress, DNA damage and apoptosis (Alves *et al.*, 2016).

Testicular degeneration is another major concern regarding the effects of HS on the testes (Marai *et al.*, 2008; Alves *et al.*, 2016). Degeneration of the testes has a direct negative effect on sperm production and quality causing a decrease in testes weight (Setchell, 1998), a reduction in sperm motility and an increase in sperm defects, sperm oxidative stress and sperm DNA fragmentation (Alves *et al.*, 2016). Studies have shown that ram testes weight decreased by about 70% when exposed to a hot environment for 14 days, and that exposing the testes to a temperature of 42°C for 45 minutes caused up to a 50% reduction in testes weight 21 days after the heat stress conditions were applied. The reduction in testes size and subsequent deleterious effects on sperm production and sperm quality only return to normal 30 to 42 days later (Setchell, 1998; Alves *et al.*, 2016). Thus, the elimination of HS does not lead to an improvement in reproductive efficiency and sperm output until the germ cells have completed spermatogenesis. This highlights

the importance of maintaining a HS free environment in order to maintain a high level of reproductive efficiency in rams (Hansen & Fuquay, 2011).

The effects of HS are more prominently seen in the germ cells (spermatocytes and spermatids) than in the Leydig and Sertoli cells because of the high mitotic rates of the germ cells at the beginning stages of spermatogenesis (Hansen, 2009; Armengol *et al.*, 2015; Alves *et al.*, 2016). Heat stress is also known to decrease testosterone production and circulation, thus altering normal epididymal function and causing an increase in the number of dead sperm and a decrease in overall fertility. However, testosterone circulation returns to normal approximately two weeks after the initial reduction, even under continuous HS conditions (Hansen, 2009).

The effects of HS on sperm output, concentration, motility and morphological abnormalities has been investigated by a number of authors. Ejaculate volume and concentration were significantly lower in rams subjected to HS conditions compared to thermoneutral conditions (Marai *et al.*, 2008). A high percentage of live and progressively motile sperm are required to achieve high conception rates as immotile sperm are unable to efficiently migrate through the female reproductive tract and fertilize the ovum (Nakanishi *et al.*, 2004). High levels of ROS can be deleterious and cause oxidative damage to DNA, this includes the premature initiation of the acrosome reaction. Premature acrosome reaction coupled with high levels of immotile sperm has been linked to reduced fertility and inability to initiate zona pellucida binding with the sperm (Paul *et al.*, 2009).

2.8.3 Reactive Oxygen Species (ROS)

As mentioned previously, sperm require a small amount of ROS to achieve fertilizing capacity in the female reproductive tract. Hydrogen peroxide stimulates capacitation by initiating hyperactivation and acrosome reaction after ejaculation into the female. However, excessive levels of ROS in sperm damages the plasma membrane causing reduced motility, reduced ability to fuse with the oocyte and sperm cell DNA damage (Agarwal *et al.*, 2006; Bucak *et al.*, 2012).

Mammalian sperm are generally known to be more prone to oxidative stress due to the high amount of PUFA present in their plasma membrane. The ROS attack the PUFA causing a cascade of chemical reactions known as lipid peroxidation (Peker Akalin *et al.*, 2016; Chandra *et al.*, 2012).

Lipid peroxidation results in a loss of membrane fluidity which is essential for sperm motility and oocyte fusion. The acrosome reaction of sperm under oxidative stress is also defective as it is believed that excessive levels of hydrogen peroxide are responsible for inactivating several enzymes necessary to carry out the process (Agarwal *et al.*, 2006).

There are two mechanisms by which sperm DNA are protected from oxidative damage: the tight packing of DNA by protamines and the AO's of seminal plasma. However, ROS have the ability to counteract these mechanisms under conditions of serious oxidative stress causing DNA damage in the form of base modifications and deletions, mutations, chromosomal rearrangements and single and double-strand DNA breaks. Sperm are able to undergo self-repair and oocytes are able to aid in DNA damage repair of sperm if the damage is small, however, major damages can result in apoptosis and embryo fragmentation (Chandra *et al.*, 2012).

Oxidative stress of sperm under processing conditions induces an additional source of ROS attack on sperm. This is due to the fact that freezing and cooling cause the AO defense system activity to decrease allowing the membrane to become more susceptible to lipid peroxidation (Chandra *et al.*, 2012; Zanganeh *et al.*, 2013). The oxidative damages related to processing are also dependent on the structural stability of the plasma membrane. Domestic livestock such as rams and bulls that have higher levels of PUFA and lower levels of cholesterol present in the sperm plasma membrane are more sensitive to cooling and oxidative stress (Chandra *et al.*, 2012).

The body contains a number of mechanisms to minimize and repair the damages caused by free radical build-up, these include the use of AO protection systems. Antioxidants can be obtained through the diet or from endogenous AO sources such as the seminal vesicles. Normal sperm function is maintained by the prevention and removal of excess ROS formation by the seminal plasma AO system (Fang *et al.*, 2015). However, the seminal plasma AO system is altered during cooling and freezing thereby preventing the prolonged protection of sperm against oxidative damage during preservation (Zanganeh *et al.*, 2013). This has led to the discovery that oxidative damage can be minimized and sperm quality can be improved during preservation when AO's are included in the semen extender.

2.8.4 Antioxidants as mitigators of thermal stress

It is well known that the process of sperm storage and the effects of HS on sperm characteristics result in adverse changes in sperm membrane lipid composition, acrosome status, viability and motility, and sperm DNA damage (Amidi *et al.*, 2016). Ram sperm are known to have excessively high levels of PUFA and low levels of cholesterol and phospholipids in their plasma membranes when compared to other species (dos Santos Hamilton *et al.*, 2016), this makes ram sperm more vulnerable to oxidative stress caused by excessive ROS production, thus resulting in a loss of membrane and acrosome integrity and an increase in DNA fragmentation (da Silva Maia *et al.*, 2009; Mata-Campuzano *et al.*, 2014; Fang *et al.*, 2015; Akalin *et al.*, 2016; dos Santos Hamilton *et al.*, 2016).

Under normal conditions, ROS are required for fertility by initiating the acrosome reaction, sperm hyperactivation, sperm motility and sperm capacitation in the female reproductive tract (dos Santos Hamilton *et al.*, 2016). Naturally occurring ROS are generated by sperm metabolism and maintained by the sperm natural AO defense system (Bucak *et al.*, 2012) consisting of enzymatic AO's (SOD, CAT, GPX and GSR) and non-enzymatic AO's (synthetic AO's or dietary supplements) (Amidi *et al.*, 2016). To maintain normal sperm function, this AO defense system must act to continuously prevent the production of new ROS and scavenge existing ROS (Fang *et al.*, 2015). However, an alteration in the AO defense system brought about by sperm handling, cold shock and HS results in the excessive production of ROS, thus decreasing sperm quality and fertilizing potential.

Supplementation of sperm preservation media with an external source of AO's has been investigated as an effective way to improve the quality and fertility of ram sperm subjected to conditions of cold storage and HS. It is believed that the addition of these external AO's act as a buffer for the natural AO defense system by scavenging the excess ROS and preventing further ROS production (Mata-Campuzano *et al.*, 2014). Although the use of synthetic AO's has been favoured in the past, attention is being shifted to the use of natural plant derived AO's because of the toxicity problems experienced with the use of their synthetic counterparts.

Methionine, cysteamine, CAT and Trolox are a few examples of synthetic AO's that have been studied as possible additives for semen extenders. Methionine acts as a precursor amino acid for

glutathione and plays a vital role in detoxification. Bucak *et al*, (2012) showed that the addition of methionine to a Tris-based semen extender, maintained at 5°C, for up to 96h, improved sperm motility. A cryopreservation study by Maia *et al,* (2009) found that the addition of CAT to a Tris-based semen extender improved sperm viability but did not improve motility when compared to the control, similar results were found with the addition of Trolox to the extender. Fenugreek (*Trigonella foenum graecum L.*) and rosemary (*Rosmarinus officinalis L.*) extracts are two plants that have been studied as AO additives to semen extenders. The phenolic compounds contained in these plants are known for their strong AO, anti-inflammatory and anti-cancer properties (Belguith-Hadriche *et al.*, 2013; López-Jiménez *et al.*, 2013).

2.8.4.1 Common rosemary *(Rosmarinus officinalis L.)*

Common rosemary is an aromatic evergreen shrubby herb that is widely distributed in the Mediterranean region. It is well known and has been greatly valued for centuries as a medicinal herb for its powerful AO, antibacterial and antimutagenic properties (Sui *et al.*, 2012). The most important biological properties responsible for the potent antioxidant activity of rosemary are the numerous diterpene components that it contains, i.e. carnosol, carnosic acid and rosmarinic acid (Zanganeh *et al.*, 2013). Although there are few studies on the effects of rosemary aqueous extract on sperm quality under conditions of cold-storage and HS, it is believed that the addition of rosemary extract into semen extenders would be beneficial for use against the oxidative stress caused by these thermal stressors, thereby prolonging the lifespan as well as preserving the viability and integrity of the sperm.

Motlagh *et al.* (2014) showed that the addition of rosemary aqueous extract to a soybean lecithin-based semen extender improved motility and plasma membrane functionality in a dose dependent manner, but did not improve acrosome reaction or capacitation status following the freeze-thawing process of ram sperm. Malo *et al.* (2011) and Zanganeh *et al.* (2013) found similar results in boar and buck sperm preservation, respectively. Malo *et al.* (2010) also found that post-thaw viability and acrosome integrity of boar sperm were both positively affected by the combined addition of rosemary aqueous extract and cysteine to a lactose-egg yolk cryopreservation medium.

2.8.4.2. Wild rosemary *(Eriocephalus africanus* L.*)*

Wild rosemary *(Eriocephalus africanus* L.*)* is a perennial erect shrub that is endemic to South Africa. It has variable morphology depending on its growing conditions and can grow between 0.3 to 0.9m in height and 4m in diameter. The leaves are digitiform and mostly opposite, greyish in colour and can be covered in hairs (Heelemann *et al.*, 2015). Wild rosemary is a well-known medicinal plant that has been used to treat oedema, gastrointestinal disorders and skin infections (Merle *et al.*, 2007).

Catarino *et al.* (2015) found that wild rosemary is particularly enriched with mono- and dicaffeoylquinic acids. These account for approximately 90% in the stems, and 74% in the leaves of the total phenolic composition of that particular study. There were minor amounts of other phenolic acids and flavanones found in the hydroethanolic extracts done for that study. It was also found that both the stems and leaves of the wild rosemary plant showed potent AO properties (Catarino *et al.*, 2015).

2.8.4.3 Analysis of the diterpene components in rosemary aqueous extracts

Rosmarinic acid, carnosic acid and carnosol are some of the most important natural AO's found in the common rosemary plant. Apart from their powerful AO abilities, these bioactive compounds also have powerful anti-inflammatory, anti-microbial and anti-cancer properties (Erkan *et al.*, 2008; Wüst Zibetti *et al.*, 2013; Ribeiro-Santos *et al.*, 2015). The relative amounts of AO's present in the rosemary plant varies depending on the environmental stressors the plant experiences, i.e. water or high temperature stress can reduce CO_2 fixation in the plant resulting in an increase in ROS production. Under such conditions, the plant AO content will increase to offer protection against the increased oxidative stress (Almela *et al.*, 2006).

There are a number of extraction techniques that can be used to obtain the AO compounds from rosemary, including simple water and ethanol extraction. The amount of AO extracted from the plant during these processes is dependent on the collection, processing and storage of the plant, as well as the extraction process used and the chemical composition of the AO's themselves (Almela *et al.*, 2006; Herrero *et al.*, 2010). Figure 2.3 displays the chemical structures of rosmarinic acid, carnosic acid and carnosol found in rosemary. These AO compounds are

lipophilic in nature allowing them to target biological membranes effectively and attenuate oxidative stress (Prez-Fons *et al.*, 2006). Rosmarinic acid has an increased polarity when compared to carnosic acid and carnosol. This means that rosmarinic acid is more readily extracted when water is used as a solvent as oppose to the use of ethanol extraction. The extraction temperature also plays a role in the amount of AO extracted, carnosic acid and carnosol are extracted more efficiently at higher temperatures (200°C) in water than rosmarinic acid, which becomes more unstable at higher temperatures (Herrero *et al.*, 2010). The determination of the AO concentration in the extract can be carried out using mass spectrometry.

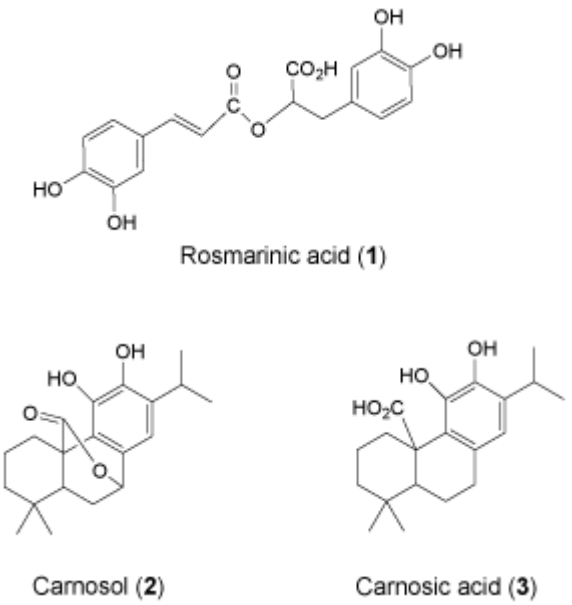

Figure 2.4. Chemical structures of rosmarinic acid, carnosic acid and carnosol present in rosemary (Adapted from: Prez-Fons *et al.*, 2006).

2.9 AIMS OF STUDY

In recent decades, considerable time and effort has gone into the research of ART's as a potential means to optimize production and reproductive efficiency in the ovine industry. Lamb and mutton has the potential to be more cost-effectively produced and sold if farmers are able to successfully implement breeding techniques such as AI, IVF and MOET. However, the current techniques that are being adapted to use in the sheep industry were taken from those respective ART's developed for the cattle industry, limiting the success of these techniques for sheep farmers.

Globally, the increasing demand for food security and radical climate change is resulting in the increased pressure to successfully adapt ART's for use in sheep so as to ensure better management of the reproductive efficiency of flocks at the same time as promoting genetic progress. The deleterious effects of semen preservation techniques and HS on sperm quality represent a major challenge in the successful implementation of ART's for improving reproductive efficiency in livestock.

Previous studies have investigated the use of AO's to counteract the deleterious effects of semen preservation protocols and HS on sperm viability and morphological integrity. However, the use of natural AO's has not been extensively investigated. The understanding of the effects of natural AO's included as part of preservation media can help to prolong the lifespan and preserve the viability and morphological integrity of sperm used for ART's in the sheep industry.

The aim of this study was thus two-fold, i.e. to determine firstly whether common rosemary *(Rosmarinus officinalis L.)* and wild rosemary *(Eriocephalus africanus L.)* differed in terms of diterpene (carnosic acid, carnosol and rosmarinic acid) content. Secondly, the study investigated the potential of sperm storage media, when supplemented with diterpenes extracted from common rosemary and wild rosemary, respectively, to minimise the damage caused by short-term storage and HS, respectively, as quantified by assessing viability and morphological integrity.

Chapter 3

Methods and Materials

Ethical clearance for the study was obtained from the Animal Ethics Committee of the University of Stellenbosch (Protocol number: SU-ACUD16-00069). All procedures and animal handling techniques were carried out in such a manner as to adhere to the guidelines set out by the South African National Standard for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008).

3.1 EXPERIMENTAL LOCATION

The samples used in the study were collected at the Sheep Research Section on the Mariendahl Experimental Farm of Stellenbosch University, and all analyses were carried out in the Animal Physiology laboratory of the Department of Animal Sciences, Stellenbosch University. The research area is characterized by a Mediterranean climate, with hot, dry summers (average temperature = 27°C) and cool, wet winters (average temperature = 15°C). The average annual rainfall is approximately 802mm, which is received mostly during the winter months (Climate-data, 2016).

3.2 EXPERIMENTAL ANIMALS AND HUSBANDRY

For the purpose of this study, a total of 30 adult Dohne Merino (*Ovis aries*) rams, aged 15 months, were used. A total of 10 rams were used for each collection session, and care was taken to ensure that different rams were used for each session. The rams form part of a stud flock established and maintained at the Mariendahl Research Farm. Rams were maintained under uniform nutritional conditions, and had *ad libitum* access to clean water. A standard sheep disease and parasite control programme is followed in the management of the trial animals.

3.3 EXPERIMENTAL DESIGN

This study consisted of a cold stress study (Experiment 1) and a heat stress study (Experiment 2).

3.3.1 Experiment 1: The potential of common rosemary and wild rosemary aqueous extract to preserve sheep sperm viability and morphological integrity during short-term cold storage at 5°C

The aim of this experiment was to investigate the potential of the diterpene components (carnosic acid, carnosol and rosmarinic acid) present in common and wild rosemary aqueous extract, to minimize the deleterious changes caused by short-term cold storage on the viability and morphological integrity of sheep sperm.

The experiment was carried out according to a 3X5 factorial design, with treatments including either antioxidant (AO) and/or trehalose. The respective treatments are indicated in Table 3.1. The experiment was replicated 5 times.

Table 3.1. The different inclusion and combination levels of common rosemary (R) and wild rosemary (WR) aqueous extract, respectively, in sheep sperm samples diluted with a milk-yolk-egg extender supplemented with different concentrations of trehalose, and maintained at 5 °C for a period of 72h.

Inclusion level of aqueous extract (g/100mL)	**Trehalose concentration**		
	0mM	**50mM**	**100mM**
0 (Control)	R0T0 / WR0T0	R0T50 / WR0T50	R0T100 / WR0T100
2.5	R2.5T0 / WR2.5T0	R2.5T50 / WR2.5T50	R2.5T100/WR2.5T100
5.0	R5T0 / WR5T0	R5T50 / WR5T50	R5T100 / WR5T100
7.5	R7.5T0 / WR7.5T0	R7.5T50 / WR7.5T50	R7.5T100 / WR7.5T100
10.0	R10T0 / WR10T0	R10T50 / WR10T50	R10T100 / WR10T100

The samples in this experiment were diluted with milk-yolk extender (MYE), prepared according to Paulenz *et al.* (2002), and maintained at 5°C for the duration of the experiment. Fresh extender was prepared for each replicate.

Sperm parameters (see Section 3.5) were recorded at 0h, 12h, 24h, 36h, 48h and 72h, respectively.

3.3.2 Experiment 2: The potential of common rosemary and wild rosemary aqueous extract to preserve sheep sperm viability and morphological integrity during heat stress conditions

The aim of Experiment 2 was to investigate the potential of the AO's present in the aqueous extract of common and wild rosemary, respectively, to minimize the deleterious effect of heat stress on the viability and morphological integrity of sheep sperm.

The experiment was carried according to a 3X5 factorial design, with treatments including the AO inclusion level and treatment temperatures. The MYE extender was supplemented with 50mM trehalose before addition of the AO extract. The respective treatments are indicated in Table 3.2. The experiment was replicated 5 times.

Table 3.2. The different inclusion levels of common rosemary (R) and wild rosemary (WR) aqueous extract, respectively, in sheep sperm samples diluted with a milk-egg yolk extender supplemented with 50mM trehalose, and maintained at 38.5 °C, 39.0 °C and 41.0 °C for up to 2.5h.

Inclusion level of aqueous extract (g/100 mL)	**Temperature**		
	38.5 °C	**39.0 °C**	**41.0 °C**
0 (Control)	R0T38.5 / WR0T38.5	R0T39 / WR0T39	R0T41 / WR0T41
2.5	R2.5T38.5 / WR2.5T38.5	R2.5T39 / WR2.5T39	R2.5T41 / WR2.5T41
5.0	R5T38.5 / WR5T38.5	R5T39 / WR5T39	R5T41 / WR5T41
7.5	R7.5T38.5 / WR7.5T38.5	R7.5T39 / WR7.5T39	R7.5T41 / WR7.5T41
10.0	R10T38.5 / WR10T38.5	R10T39 / WR10T39	R10T41 / WR10T41

The sperm samples were diluted with a MYE (Paulenz *et al.*, 2002) supplemented with 50mM trehalose. The sperm-MYE-trehalose samples were supplemented with the common rosemary/wild rosemary aqueous extract, as indicated in Table 3.2. The supplemented sperm-MYE samples were subsequently incubated at 38.5°C, 39.0°C and 41.0°C respectively, under controlled conditions.

Sperm parameters were recorded at 0h, 1h, 1.5h, 2h and 2.5h, respectively.

3.4 PREPARATION OF STOCK SOLUTIONS AND MEDIA

The eosin-nigrosin (E/N) stain was purchased from Kyron Laboratories, South Africa. All other chemicals and reagents were purchased from Sigma-Aldrich. All media, with the exception of the MYE, were prepared using autoclaved distilled water.

3.4.1 Preparation of the milk-yolk extender (MYE)

For the preparation of the MYE, fresh chicken eggs and fresh ultra-high temperature (UHT) fat-free milk was used (Paulenz *et al.*, 2002). The milk was stored at 5°C, and the eggs at room temperature (22°C) until being used. Two 50mL Falcon tubes (Lasec, South Africa) of MYE were prepared the afternoon before each collection took place, and stored at 5°C until use.

The extender was prepared by adding 1.25mL egg-yolk to 48.75mL milk, and each tube was gently agitated until a homogenous extender was achieved. Prior to analysis and dilution of sperm samples, on arrival at the laboratory after sample collection, the MYE was placed in a water bath maintained at 37°C to ensure that the sperm samples were not subjected to thermal stress during processing and evaluation.

3.4.2 Preparation of the trehalose stock solution

Two stock solutions of trehalose were prepared in 50mL Falcon tubes, namely 50mM and 100mM concentrations. To calculate the mass of the trehalose dehydrate powder to be used to prepare the different stock solutions, the following formula was used:

$$\boldsymbol{m = C \times V \times M}$$

Where:

$\boldsymbol{m}$ *is the mass of trehalose dihydrate to be weighed*

$\boldsymbol{C}$ *is the concentration to be prepared* $(i.e.\ 100mM\ and\ 200mM)$

$\boldsymbol{V}$ *is the volume to be prepared* $(50\ mL)$

$\boldsymbol{M}$ *is the molecular weight of trehalose* $(378.33\ g/mol)$

Care was taken to ensure that the calculations and units of measurement were correct before weighing out the dihydrate powder. The stock solutions were prepared by weighing out 1.892g and 3.780g of trehalose dihydrate for the 100mM and 200Mm, respectively, using a hypersensitive balance (Model PS750C/2, Lasec, South Africa), and dissolving the chemical in 50mL autoclaved distilled water in separate 50mL Falcon tubes. The solutions were mixed gently until all the powder was dissolved.

The stock solutions were maintained at 5°C until being used in the experiments. Prior to addition of the stock solutions to the samples, the trehalose was placed in a water bath maintained at 37°C to minimize any temperature shock to the sperm before being subjected to the respective treatments.

3.4.3 Preparation of the aqueous extracts

Two species of rosemary plant were used during this study, namely common rosemary *(Rosmarinus officinalis L.)* and wild rosemary *(Eriocephalus africanus L.)*. One kilogram of each species was donated by HERBS-APLENTY®, located in the Overberg Region of the Western Cape. Upon arrival at the laboratory, the plant material was left to air-dry for a week, after which the leaves were stripped from the stems. The leaves were stored in air-tight Zip lock bags, and protected from direct light until the extraction of the AO compounds.

Extracts of each species was prepared the evening before each experiment was carried out. All apparatus and containers were autoclaved before use in the extraction procedures. The extracts were prepared using the method described by Malo *et al.* (2011). Four quantities (2.5g, 5.0g, 7.5g and 10.0g) of each species was weighed using a hypersensitive scale, (Model PS750C/2, Lasec, South Africa), and ground by using a pestle and mortar to increase the surface area of the leaves for the extraction of the AO compounds to be carried out as effectively as possible. For extraction, Erlenmeyer flasks were filled with 100mL of autoclaved distilled water, and pre-heated to 100°C in a water bath. After the water temperature stabilized at 100 °C, the respective weight of common rosemary/wild rosemary samples was added to the respective marked flasks, aluminium foil placed over the opening to minimize evaporation, and weighed. The flasks were then returned to the water bath and maintained at 100°C for 10 minutes. After 10 minutes, the flasks were removed from the water bath, weighed again, and then left to cool to 25°C. Upon reaching 25°C, the extracts were filtered using 0.22µm syringe filters (Lasec, South Africa). The filtered extracts were stored at 5°C until being used.

3.4.4 Determination of diterpene components in common and wild rosemary

Based on the knowledge that there are three main diterpene components in common and wild rosemary, it was necessary to determine the concentration of each of those components in the

aqueous extract in order to know how each component contributes to the AO ability of these plants. In order to determine the quantities of these components, standards of each component were purchased from Sigma-Aldrich. These standards, as well as two samples of each rosemary species, were sent to the Central Analytical Facilities (CAF) department at Stellenbosch University where AO concentration was determined by means of mass spectrophotometry (MS).

The MS instrument that was used was the Waters Synapt G2 QTOF Mass Spectrometer linked to a Waters Acquity UPLC. The AO standards as well as the rosemary samples were diluted with a 50/50 methanol/water solution before being subjected to the MS procedure. The technique was carried out using a BEH C_{18} 100 x 2.1 mm 1.7um column, the gradient elution was 0.1% formic acid to acetonitrile, and the spectra was collected in ESI negative with a 15V cone voltage (CAF, Stellenbosch University).

3.5 COLLECTION AND PROCESSING OF SPERM SAMPLES

3.5.1 Period of collection

Sperm samples were collected from adult Dohne Merino rams that were maintained at the Mariendahl Experimental Farm of Stellenbosch University. The initial collections commenced at the beginning of June 2016 and were planned to run until the end of June 2016. However, due to unforeseen circumstances, after the initial collections were performed, further collections were postponed until August 2016. Table 3.3 indicates the dates of collections for each repetition of each experiment.

Table 3.3. Dates of sperm sample collection for each replicate of Experiment 1 and Experiment 2.

Collection Session	Date
Experiment 1, Collection 1	1 June 2016
Experiment 1, Collection 2	11 August 2016
Experiment 1, Collection 3	11 August 2016
Experiment 1, Collection 4	15 August 2016
Experiment 1, Collection 5	15 August 2016
Experiment 2, Collection 1	1 June 2016
Experiment 2, Collection 2	16 August 2016
Experiment 2, Collection 3	17 August 2016
Experiment 2, Collection 4	18 August 2016
Experiment 2, Collection 5	19 August 2016

3.5.2 Collection and transportation of samples

In the laboratory, prior to collection in the field, 50mL Falcon tubes were labelled with the date and the ram number (1 – 10), with the ram number corresponding to the same number on a score sheet where the volume, colour and viscosity were all recorded during the collection in the field. Water was boiled and adjusted to approximately 40°C, and then transferred to a thermos flask. After collection, sperm samples were sealed with parafilm (Lasec, South Africa), and placed in the thermos to minimise temperature fluctuations until later processing.

Semen samples were collected by means of electro-ejaculation (EE) using the method as described by Marco-Jimenez *et al*. (2005). The rams were restrained in a recumbent position, and the penis was gently everted from the sheath, and prevented from returning to the sheath by means of a piece of sterile gauze. The rectal probe was lubricated and inserted into the rectum with the electrodes positioned ventrally. Care was taken to ensure that the urethral process was suspended freely in each collection tube while the ram was stimulated. Stimulation consisted of a consecutive series of two second pulses of equal voltage, followed by a two second rest interval, where after the stimulation process was repeated, with an 1V increase in voltage strength during the second stimulation. The rams received no sedatives or anaesthetics prior to stimulation.

3.5.3 Evaluation of sperm samples

3.5.3.1 Macroscopic evaluation

Immediately after each sample was collected, the macroscopic parameters (volume, colour and viscosity) were recorded on a score sheet. The macroscopic evaluations were done by the same technician throughout the trial. The sample volume was determined using a calibrated collection tube. The tube was held perfectly vertical to ensure precise and correct readings were made and values were recorded for later analysis. Sample colour was assessed based on a numeric scale as described by Hafez & Hafez (2008). The scale ranges from 0 to 5 with 0 being almost clear, indicating a low sperm density, and 5 being thick creamy white and therefore indicative of a high sperm density. Viscosity of the samples was subjectively scored as either low, normal or high. Normal indicated a sample whose liquefied specimen could be poured from a graduated beaker, drop by drop, with no attaching agglutination between drops (Steinberg, 2015). After the caps of the sample tubes were placed back, tubes were then sealed with parafilm (Lasec, South Africa) to prevent water contamination, and transported to the laboratory in a thermos flask containing water (35 °C).

3.5.3.2 Microscopic evaluation

Upon arrival at the laboratory, all of the collected samples for each session were pooled, and microscopic evaluation was carried out.

Estimation of sperm concentration

Sperm concentration was determined using the haemocytometer method. A 10µL aliquot of raw semen was mixed gently with 990µL of distilled water to prepare a dilution with a 1:100 dilution factor. The haemocytometer chamber was loaded with 10µL of the semen-water diluent. The sperm concentration was determined as described by Matthews *et al.* (2003) and Hafez & Hafez (2008), by counting the number of sperm cells lying inside the four corner blocks as well as the centre block of the haemocytometer. A manual counter was used to count each sperm cell; the total number of sperm cells was multiplied by five to obtain the concentration, measured in million sperm per mL.

After concentration determination, each pooled sample from the specific collection day was then allocated to the respective treatments. The treatment samples were transferred to 2mL microcentrifuge tubes that were labelled according to a previously determined code. In each case, the MYE, trehalose and rosemary extract was added prior to the semen with the semen being the last component to be added to each tube. This was done to ensure that the sperm did not undergo more than one initial shock prior to evaluation.

3.5.3.3 Evaluation of viability

The E/N staining technique was used to evaluate sperm viability. One E/N smear was made for each sample at each time interval. The first smears, made at 0h, were done using freshly extended semen, while the later smears were done using semen that had been subjected to their respective treatments. Each slide was labelled according to a predetermined code prior to smears being made.

The E/N technique was carried out, with slight variation, as described by Cormier *et al.,* (1997). A 50µL aliquot of the extended sample was suspended in the centre of a clean microscopic slide using a pipette tip, two drops of modified E/N solution was mixed with the semen sample and a smear was prepared using this mixture. The smear was left to be air-dried and then stored in the slide box until further evaluation.

For the purpose of this study, sperm viability is defined as the percentage of live sperm per slide, and is calculated as per the equation below. A minimum of 100 sperm cells must be present in order to calculate sperm viability. Under the microscope, viable/living sperm cells appear uniform and white, while non-viable/dead sperm cells are either partially or fully stained in a pinkish colour (Figure 3.1).

$$\%\ \boldsymbol{live\ cells} = \frac{\boldsymbol{total\ live\ cells\ counted}}{\boldsymbol{total\ cells\ counted}} \times \mathbf{100}$$

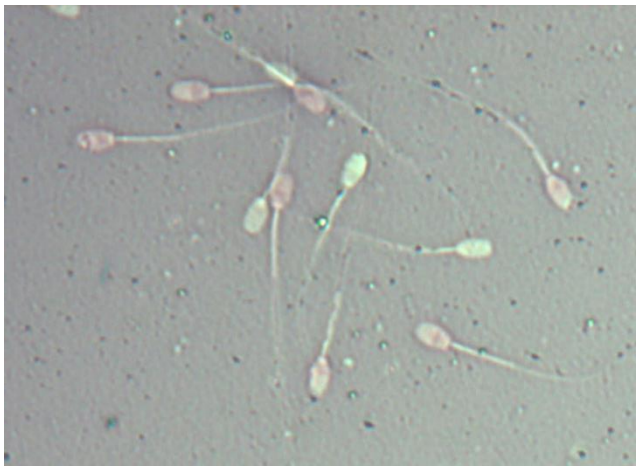

Figure 3.1 Eosin-nigrosin stain indicating the presence of live (white sperm) and dead (pink sperm) sperm

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3.5.3.4 Evaluation of morphology

The E/N staining technique was also used to evaluate sperm morphology. The E/N technique is explained in 3.5.3.3 above.

For the purpose of this study, the percentage of abnormal sperm cells present on each slide was calculated using the equation below. A minimum of 100 cells must be present in order to calculate the percentage of abnormal morphology. Abnormal sperm are characterised by the presence of any morphological effects that may be detrimental to sperm integrity. Typically, there are three categories of morphological abnormalities in sperm. Primary abnormalities refer to those associated with the head and acrosome, secondary abnormalities refer to those that have the presence of a cytoplasmic droplet on the midpiece, and tertiary abnormalities refer to all other possible tail defects, such as; looped, bent back or coiled tails (Boshoff, 2014). These categories can be seen in Figure 3.2 below.

$$\%\ \boldsymbol{normal\ morphology} = \frac{\boldsymbol{total\ morphologically\ normal\ cells\ counted}}{\boldsymbol{total\ cells\ counted}} \times \mathbf{100}$$

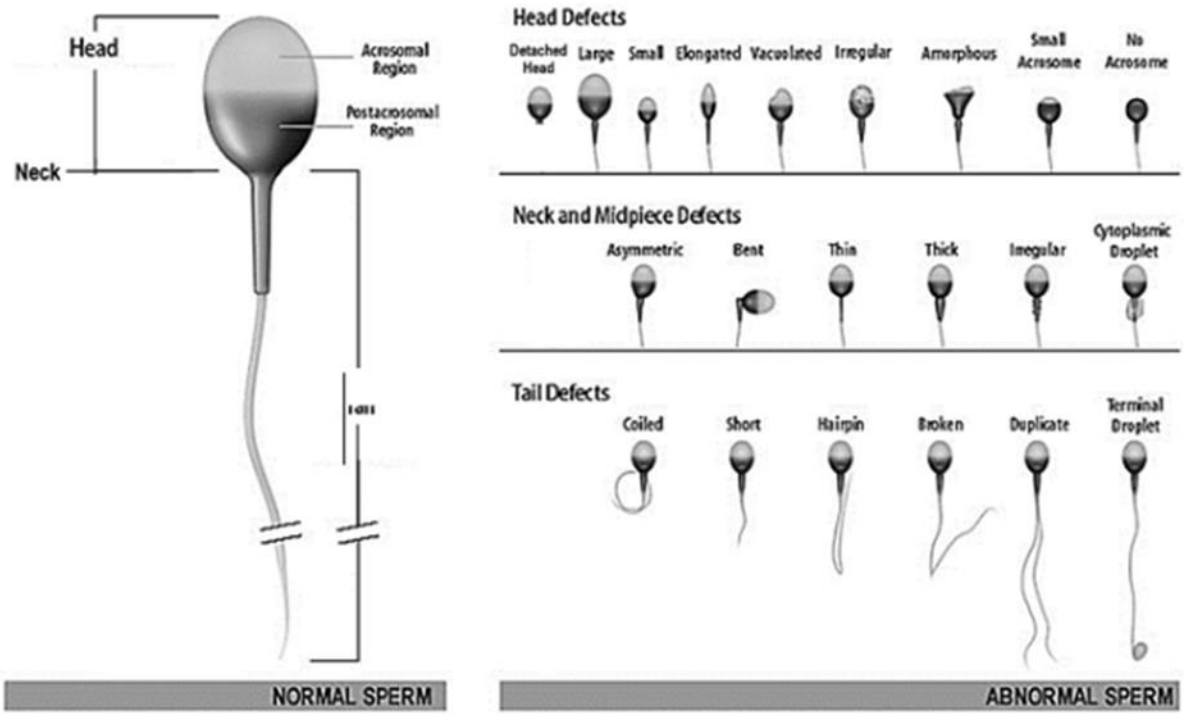

Figure 3.2 Different types of primary, secondary and tertiary morphological abnormalities that can be observed during the standard evaluation of sperm samples (Source: www.fssc.com.au).

3.5.3.5 Evaluation of acrosome integrity

The RapiDiff (RD) staining technique was used to evaluate acrosome integrity. One RD smear was made for each sample at each time interval. The first smears, made at 0h, were done using freshly extended semen, while the later smears were done using semen that had been subjected to their respective treatments. Each slide was labelled according to a predetermined code prior to smears being made.

The smears for RD staining were prepared by transferring 50µL of sample onto one end of a microscope slide and then dragging the drop across that slide using the tip of a different slide. Once the smear was made, the slide was left to air-dry before subsequent staining. The RD technique was carried out in accordance with the manufacturer's guidelines, and once the smears were completely air-dried, they were placed into the slide boxes until further evaluation.

For the purpose of this study, acrosome integrity was determined by calculating the percentage of sperm on each slide that had an intact acrosome. Intact acrosomes are characterised as having an even acrosomal membrane with no dissociation of the acrosomal cap at the side or damage visible to the front section of the acrosome.

The method for calculating the percentage of intact acrosomes was carried out using the equation below.

$$\%\ \boldsymbol{intact\ acrosome} = \frac{\boldsymbol{total\ cells\ with\ intact\ acrosome\ counted}}{\boldsymbol{total\ cells\ counted}} \times \mathbf{100}$$

Note: The RD slides were destined to be used to determine acrosome integrity. However, the sperm did not stain correctly during the RD procedure, consequently the slides were unable to be used. This could be due to an interaction between the rosemary extract and the RD solutions, but more research needs to be carried out in order to determine the actual cause. Therefore, the analysis of acrosome integrity was not performed.

3.7 STATISTICAL ANALYSIS

The generalized linear models of SAS Enterprise Guide 5.1, version 9.4 for Windows, as well as XLSTAT was used for data analysis. These analyses generate means adjusted for multiple comparisons using the Bonferroni t-test. The two-way analysis of variance (ANOVA) was used for the comparative analysis. Results were expressed as; the mean ± standard error of the mean (SEM) and were considered statistically significant when $p \leq 0.05$. All tables and graphs present in the results were prepared in Microsoft Excel.

Chapter 4

The diterpene concentration in common rosemary (*Rosmarinus officinalis* L.) and wild rosemary (*Eriocephalus africanus* L.) aqueous extract

Abstract

The aim of this part of the study was to determine the concentrations of the antioxidants (AO's) rosmarinic acid, carnosic acid and carnosol, found in common (*Rosmarinus officinalis* L.) and wild rosemary (*Eriocephalus africanus* L.), using aqueous extraction. During extraction it was found that wild rosemary was highly viscous when compared to common rosemary, making it difficult to filter. The concentrations of the AO's were analysed using mass spectrophotometry. The results of the analysis showed that the concentration determination for the common species was much higher than the wild species. There was also a much higher concentration of rosmarinic acid for both species due to the hydrophobic characteristics of carnosic acid and carnosol. Further studies need to be aimed at determining an effective extraction technique for all of the respective AO's to be extracted at their maximum yield.

4.1 INTRODUCTION

Common rosemary (*Rosmarinus officinalis* L.) is an aromatic evergreen shrubby herb that is widely distributed in the Mediterranean region. It is well known and has been greatly valued for centuries as a medicinal herb for its powerful antioxidant (AO), antibacterial and antimutagenic properties (Sui *et al.*, 2012). The most important biological properties responsible for the potent AO activity of rosemary are the numerous diterpene components that it contains, i.e. rosmarinic acid, carnosic acid and carnosol (Zanganeh *et al.*, 2013).

Wild rosemary *(Eriocephalus africanus* L.*)* is a perennial erect shrub that is endemic to South Africa. It has variable morphology depending on its growing conditions (Heelemann *et al.*, 2015). Wild rosemary is particularly enriched with mono- and dicaffeoylquinic acids with minor amounts of other phenolic acids and flavanones found in the plant (Catarino *et al.*, 2015).

4.2 METHODS AND MATERIALS

4.2.1 Preparation of the rosemary aqueous extracts

Two rosemary species were used during this study, namely common rosemary *(Rosmarinus officinalis* L.*)* and wild rosemary *(Eriocephalus africanus* L.*)*. One kilogram of each species was donated by HERBS-APLENTY®, located in the Overberg Region of the Western Cape. Upon arrival at the laboratory, the plant material was left to air-dry for a week, after which the leaves were stripped from the stems. The leaves were stored in air-tight Zip lock bags, and protected from direct light until being used. Extracts of each species were prepared the evening before each experiment was carried out.

Refer to Chapter 3 for further information regarding the processes used to carry out the aqueous extract preparations.

4.2.2 Determination of diterpene components in common and wild rosemary

Based on the knowledge that there are three main diterpene components in common and wild rosemary, it was necessary to determine the concentration of each of those components in the aqueous extract in order to know how each component contributes to the AO ability of these plants. In order to determine the quantities of these components, standards of each component were purchased from Sigma-Aldrich. These standards, as well as two samples of each rosemary species, were sent to the Central Analytical Facilities (CAF) department at Stellenbosch University where AO concentration was determined by means of mass spectrophotometry (MS).

The MS instrument that was used was the Waters Synapt G2 QTOF Mass Spectrometer linked to a Waters Acquity UPLC. The AO standards as well as the rosemary samples were diluted with a 50/50 methanol/water solution before being subjected to the MS procedure. The technique was carried out using a BEH C_{18} 100 x 2.1 mm 1.7um column, the gradient elution was 0.1% formic acid to acetonitrile, and the spectra was collected in ESI negative with a 15V cone voltage (CAF, Stellenbosch University).

The limit of detection (LOD) had to be used for concentrations that were <0.1mg/L, or not detected (n.d.) by the MS analysis in order to include them in the data. Due to certain limits in chemical analysis procedures, small concentrations cannot be precisely measured, these small concentrations are said to be below the LOD (Croghan & Egeghy, 2003). There are a number of methods to determine the LOD, the method used for this study was to divide the LOD (0.1mg/L) by the square root of 2, to yield a value of 0.071. See the equation below:

$$LOD = {}^{0.1}/_{\sqrt{2}}$$

4.3 RESULTS

The following tables indicate the concentrations of rosmarinic acid, carnosic acid and carnosol, respectively, that were analysed using mass spectrometry at the CAF lab at Stellenbosch University.

Concentrations that were quantified as being not detected (n.d.) or <0.1mg/L were calculated using the limit of detection method as described in section 4.2 above. This was done so that these concentrations had values that could be using during the statistical analysis for Chapter 5 and Chapter 6 below.

An average of the 2 concentrations for each rosemary inclusion level was calculated and used during statistical analysis in the following chapters.

4.3.1 Rosmarinic acid

The concentrations that were found for rosmarinic acid (Table 4.1.) were much higher for the common species when compared to the wild species. The wild species also showed an inclusion level where rosmarinic acid was not present, as well as 2 levels where rosmarinic acid was not detected.

Table 4.1. The concentration of rosmarinic acid found in 2 samples of each rosemary species at each extract inclusion level.

Rosemary Species	Rosemary Inclusion (g/100mL)	Antioxidant concentration (mg/L)
Common Rosemary	2.5 – a	146.48
Common Rosemary	2.5 – b	157.02
Common Rosemary	5.0 – a	141.94
Common Rosemary	5.0 – b	148.26
Common Rosemary	7.5 – a	176.06
Common Rosemary	7.5 – b	177.54
Common Rosemary	10.0 – a	194.90
Common Rosemary	10.0 – b	180.37
Wild Rosemary	2.5 – a	3.78
Wild Rosemary	2.5 – b	2.26
Wild Rosemary	5.0 – a	0.87
Wild Rosemary	5.0 – b	0.43
Wild Rosemary	7.5 – a	0.08
Wild Rosemary	7.5 – b	0.00
Wild Rosemary	10.0 – a	n.d
Wild Rosemary	10.0 – b	n.d

4.3.2 Carnosic acid

The concentrations found for carnosic acid in the common species were much lower than those found for rosmarinic acid. In addition to this, all of the wild species values were not detected for carnosic acid.

Table 4.2. The concentration of carnosic acid found in 2 samples of each rosemary species at each extract inclusion level.

Rosemary Species	Rosemary Inclusion (g/100mL)	Antioxidant concentration (mg/L)
Common Rosemary	2.5 – a	0.15
Common Rosemary	2.5 – b	0.25
Common Rosemary	5.0 – a	0.32
Common Rosemary	5.0 – b	2.35
Common Rosemary	7.5 – a	
Common Rosemary	7.5 – b	0.08
Common Rosemary	10.0 – a	1.09
Common Rosemary	10.0 – b	2.52
Wild Rosemary	2.5 – a	n.d.
Wild Rosemary	2.5 – b	n.d.
Wild Rosemary	5.0 – a	n.d.
Wild Rosemary	5.0 – b	n.d.
Wild Rosemary	7.5 – a	n.d.
Wild Rosemary	7.5 – b	n.d.
Wild Rosemary	10.0 – a	n.d.
Wild Rosemary	10.0 – b	n.d.

4.3.3 Carnosol

The common species concentrations were higher than those found for carnosic acid, but still much lower than those found for rosmarinic acid. All of the wild species concentrations for carnosol were measured as <0.1mg/L.

Table 4.3. The concentration of carnosic acid found in 2 samples of each rosemary species at each extract inclusion level.

Rosemary Species	Rosemary Inclusion (g/100mL)	Antioxidant concentration (mg/L)
Common Rosemary	2.5 – a	1.55
Common Rosemary	2.5 – b	4.77
Common Rosemary	5.0 – a	1.91
Common Rosemary	5.0 – b	4.64
Common Rosemary	7.5 – a	1.67
Common Rosemary	7.5 – b	6.86
Common Rosemary	10.0 – a	4.79
Common Rosemary	10.0 – b	6.72
Wild Rosemary	2.5 – a	<0.1
Wild Rosemary	2.5 – b	<0.1
Wild Rosemary	5.0 – a	<0.1
Wild Rosemary	5.0 – b	<0.1
Wild Rosemary	7.5 – a	<0.1
Wild Rosemary	7.5 – b	<0.1
Wild Rosemary	10.0 – a	<0.1
Wild Rosemary	10.0 – b	<0.1

4.4 DISCUSSION

The aim of this section of the study was to determine the concentrations of rosmarinic acid, carnosic acid and carnosol, present in common and wild rosemary aqueous extracts. This was done using mass spectrophotometry, and the results that were found varied between the respective AO's.

Firstly, it was found that the aqueous extracts for the wild species were highly viscous when compared to the common species extracts and were thus difficult to filter. This could be due to the difference in the plant species structure. Wild rosemary is a hardy and fibrous plant and could thus possibly have a higher structural carbohydrate content than that of common rosemary. This

could make it more difficult to filter the aqueous extract. The extraction method could also be insufficient to effectively breakdown the plant structure. The extraction method and filtrate viscosity could also be expected to yield differing concentrations of the respective AO's as was found in these results.

Studies have shown that the concentration and potency of each antioxidant component is dependent on factors such as the plant structure, time of year of harvesting, weather conditions and storage conditions post harvesting, as well as processing temperature and processing method (Almela *et al.*, 2006). Herrero *et al.* (2010) showed that the extraction level for carnosic acid and carnosol was lower than that of rosmarinic acid when using water extraction at 100°C, however, extraction levels of these two compounds increased at higher temperatures.

Another problem that was encountered during the AO analysis was that the carnosol standard could not be fully dissolved in the 50/50 methanol/water solution and DMSO had to be added to the standard to aid in dissolution. Further investigation into the reasoning of this showed that carnosic acid and carnosol are both hydrophobic and less polar when compared to rosmarinic acid, this means that it is generally more difficult for these standards to dissolve easily in water (Herrero *et al.*, 2005; Prez-Fons *et al.*, 2006). This could also be due to the extraction procedure that was used, as well as the plant structure.

As seen in the tables above, rosmarinic acid was higher in concentration than both carnosic acid and carnosol throughout the analysis. This again could be due to the extraction process, the plant structure or the storage methods that were used post collection.

4.5 CONCLUSIONS

There are a number of serious limitations that were encountered during this study that would need to be seriously reconsidered in future studies. It would firstly be important to carry out a pilot study in order to determine of the extraction techniques that are being used are effective, this was not carried out during this study. A better understanding of the species as well as their AO composition should be considered, wild rosemary does not have as high a phenolic content as that of common rosemary and should thus probably be considered separately. The extraction method should be refined so that the concentration yield of each of the AO's in each species

could be maximised. The stability of the AO's should be investigated to determine if they would be able to be stored at sub-zero temperatures for a prolonged period of time, thus making it possible to do less extractions and ensure that the concentration analysis is standardized throughout the study. Finally, the permissive function of each of the AO's should be determined, this is important as it gives an indication on how the AO's affect and are effected by each other in their bioactive properties.

Chapter 5

The potential of common rosemary and wild rosemary aqueous extract to preserve sheep sperm viability and morphological integrity during short-term storage at 5°C

Abstract

This study investigated the potential of common (*Rosmarinus officinalis* L.) and wild (*Eriocephalus africanus* L.) rosemary aqueous extracts to preserve the viability and morphological integrity of sheep sperm during short-term storage at 5°C, over a period of 72h. Sperm samples were diluted with a milk-yolk extender supplemented with trehalose at either 0mM, 50mM, or 100mM. Aqueous extracts for the different rosemary cultivars were prepared using different weights of the dry plant (0g (control), 2.5g, 5g, 7.5g and 10g) and used in the diluents at their respective treatment levels. There were no significant differences in viability for the common and wild cultivar types at the different AO inclusion levels. Sperm viability decreased significantly from 0h to 12h of cold storage and then remained stable for the remainder of the experiment. The percentage of morphological abnormalities was significantly higher for the wild type cultivar at all rosemary and trehalose inclusion levels when compared to the common type cultivar. However, there were no significant differences for morphology within each cultivar type.

5.1 INTRODUCTION

The use of assisted reproductive techniques (ART's) such as artificial insemination (AI) and embryo transfer (ET) to improve reproductive efficiency and thus contribute to food security has gained popularity over recent decades. Artificial insemination is considered as one of the most important tools regarding the advancement of animal production, as it allows for the use of the genetic material (ejaculate) from one genetically superior male to inseminate several females to ultimately ensure the effective propagation of the genetic material as far and wide as possible (Bailey *et al.*, 2000).

Despite the importance and widespread use of these technologies for genetic improvement, little advances are reported on the development of species-specific extenders and preservation protocols (Crespilho *et al.*, 2014). However, this generalization is not applicable to all species. Freeze-drying of mouse and rat sperm has been successfully carried out in recent studies and positive results have been obtained for freeze-drying for a number of wild species as a future method of conservation (Kaneko *et al.*, 2014).

Sperm, whether it be ejaculate or epididymal in origin, can be stored short-term in liquid form or long-tern in a cryopreserved state. The use of both short-term and long-term storage can have significant deleterious effects on sperm viability and morphological integrity, often rendering samples useless due to a loss in fertilizing ability and death of sperm in a particular sample (Bailey *et al.*, 2000; Kasimanickam *et al.*, 2007). Due to the considerably lower success rates achieved using cryopreserved sperm, short-term cooling is being investigated as a viable and more practical alternative to ensure the success of the application of ART's in sheep.

The aim of short-term storage is to prevent the deleterious changes that take place during normal metabolism of sperm post ejaculation, including cell death and the development of morphological abnormalities. Short-term cooling of sperm is considered favourable as the decrease in temperature acts to reduce the contribution of the metabolic pathways and degradative enzymes present in the sperm cell (Abaigar *et al.*, 2001). However, cooling still results in deleterious effects on sperm function, a consequence of the excessive production of reactive oxygen species (ROS) within the sperm membrane. Although a normal consequence of sperm metabolism, excessive accumulation of ROS in the sperm membrane results in a decrease in sperm quality because of the limited ability for sperm to resist oxidative stress (Crespilho *et al.*, 2014).

Semen extenders used in processing generally consist of an energy source (eg. trehalose), a membrane-stabilising component (typically egg yolk), and an osmotically balanced medium to prevent sperm dehydration (fat free long-life milk) (Andrabi, 2009; Aisen *et al.*, 2002). Antibiotics (AB) can also be added to prevent bacterial contamination, but have generally been found to have an adverse effect on sperm by damaging morphological integrity, initiating the natural pathways that lead to the acquisition of fertilizing potential and the production of toxins.

Sperm contain high concentrations of polyunsaturated fatty acids (PUFA) in their plasma membrane that increase the sperm's susceptibility to oxidative stress and lipid peroxidation caused by the excessive accumulation of ROS (Motlagh *et al.*, 2014). Antioxidants (AO's) prevent this accumulation by scavenging for free radicals and preventing further ROS production (Wang *et al.*, 2008). The addition of AO's is becoming more popular as recent studies have indicated that

AO addition minimizes the deleterious effects sperm experience during processing, as well as improving the post-thaw quality of samples (Andrabi, 2009).

The use of synthetic AO's such as Trolox and catalase (CAT) is unfavourable due to their possible toxic effects in the sample, and this has led to the investigation of the use of natural plants such as rosemary as an alternative AO source (Yang *et al.*, 2016). Common rosemary is considered to have powerful AO properties due to the presence of diterpenes and phenolic acids, and its aqueous extract has been used effectively for preservation and treatment against oxidative stress in semen samples (Motlagh *et al.*, 2014). Zanganeh *et al.* (2013), showed that the addition of rosemary aqueous extract to an egg-yolk Tris-based extender at 4% improved total and progressive motility, functional membrane and overall integrity of post-thawed buck semen. Malo *et al.* (2010) also found that motility was improved and lipid peroxidation was decreased in the presence of rosemary in the semen extender of boars.

Motlagh *et al.* (2014) showed that there was an overall increase in percentage of total and progressive motility and higher membrane functionality in ram sperm samples that were frozen in a lecithin-based semen extender supplemented with rosemary aqueous extract. The successes that have been experienced with the use of rosemary as a semen extender in a range of different production animals, combined with the fact that rosemary has such powerful AO properties, makes it a natural AO worth investigating to further develop ram reproductive efficiency for ART purposes. Thus, the aim of this study was to supplement sperm media with aqueous extracts from common rosemary *(Rosmarinus officinalis L.)* and wild rosemary *(Eriocephalus africanus L.)*, and to establish whether the diterpenes (carnosic acid and carnosol) and phenolic acid, rosmarinic acid, contained in these aqueous extracts enabled sperm to offer resistance to cold shock, as quantified by assessing sperm viability and morphological integrity.

5.2 MATERIALS AND METHODS

Ethical clearance for the study was obtained from the Animal Ethics Committee of the University of Stellenbosch (Protocol number: SU-ACUD16-00069). All procedures and animal handling techniques were carried out in such a way as to adhere to the guidelines set out by the South African National Standard for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008).

5.2.1 Experimental location

The samples used in the study were collected at the Sheep Research Section on the Mariendahl Experimental Farm of Stellenbosch University, and all analyses were carried out in the Animal Physiology laboratory of the Department of Animal Sciences, Stellenbosch University.

5.2.2 Experimental animals

A total of 30 adult Dohne Merino (*Ovis aries*) rams, aged 15 months, were used in this study. During each collection session, a total of 10 rams were used, and care was taken to ensure that different rams were used for each session. The rams form part of a research flock established and maintained at the Mariendahl Research Farm. Rams were maintained under uniform nutritional conditions, and had *ad libitum* access to clean water. A standard sheep disease and parasite control programme is followed in the management of the trial animals.

5.2.3 Experimental Design

The aim of this experiment was to investigate the potential of common and wild rosemary aqueous extracts, to minimize the effect of short-term cold storage on the viability and morphological integrity of sheep sperm.

The experiment designed involved a 3X5 factorial design, with treatments including either AO and/or trehalose. The respective treatments are indicated in Table 5.1. The experiment was replicated five times.

Table 5.1. The different inclusion levels of common rosemary (R) and wild rosemary (WR) aqueous extract, respectively, in sheep sperm samples diluted with a milk-egg yolk extender, supplemented with trehalose, and maintained at 5°C for a period of 72h.

Inclusion level of aqueous extract (g/100mL)	Trehalose concentration		
	0mM	50mM	100mM
0 (Control)	R0T0 / WR0T0	R0T50 / WR0T50	R0T100 / WR0T100
2.5	R2.5T0 / WR2.5T0	R2.5T50 / WR2.5T50	R2.5T100/WR2.5T100
5.0	R5T0 / WR5T0	R5T50 / WR5T50	R5T100 / WR5T100
7.5	R7.5T0 / WR7.5T0	R7.5T50 / WR7.5T50	R7.5T100 / WR7.5T100
10.0	R10T0 / WR10T0	R10T50 / WR10T50	R10T100 / WR10T100

The samples in this experiment were diluted with milk-yolk extender (MYE), prepared according to Paulenz *et al.* (2002), and maintained at 5°C for the duration of the experiment. Sperm parameters were recorded at 0h, 12h, 24h, 36h, 48h and 72h, respectively.

5.2.4 Preparation of the trehalose stock solutions and rosemary aqueous extracts

Two stock solutions of trehalose were prepared in 50mL Falcon tubes, namely 50mM and 100mM concentrations. The stock solutions were maintained at 5°C until being used in the experiments. Prior to addition of the stock solutions to the samples, the trehalose was placed in a water bath maintained at 37°C to minimize any temperature shock to the sperm before being subjected to the respective treatments.

Two rosemary species were used during this study, namely common rosemary *(Rosmarinus officinalis L.)* and wild rosemary *(Eriocephalus africanus L.)*. One kilogram of each species was donated by HERBS-APLENTY®, located in the Overberg Region of the Western Cape. Upon arrival at the laboratory, the plant material was left to air-dry for a week, after which the leaves were stripped from the stems. The leaves were stored in air-tight Zip lock bags, and protected from direct light until being used. Extracts of each plant species were prepared the evening before each experiment was carried out.

Refer to Chapter 3 for further information regarding the processes used to carry out trehalose and aqueous extract preparations.

5.2.5 Semen collection

Semen samples were collected by means of electro-ejaculation (EE) using the method as described by Marco-Jimenez *et al*. (2005). (Refer to Chapter 3 for a more detailed description of the EE technique). Semen samples were collected from 10 rams during each collection session. Care was taken to collect samples from different rams during each collection session to avoid exhausting rams, and risking a decrease in sperm quality prior to the treatments. All equipment and instruments during collection and processing were prewarmed (37°) to avoid cold shock to sperm. Immediately after collection and before returning to the lab, samples were subjected to macroscopic evaluation, including volume, colour and viscosity. Samples were sealed with parafilm (Lasec, South Africa) and maintained in a water bath at a constant temperature (37°) until further processing. Refer to Chapter 3 for more information on the macroscopic evaluation procedures.

5.2.6 Sample evaluation

5.2.6.1 Evaluation of viability and morphology

Sperm viability and normal morphology were determined using eosin-nigrosin (E/N) slides prepared for each sample at each time interval.

The E/N technique was carried out as described by Cormier *et al.,* (1997). A 50µL aliquot of the extended sample was transferred onto a clean microscopic slide, and two drops of modified E/N solution were mixed with the semen sample and a smear was prepared using this mixture. The smear was left to be air-dried and then stored in the slide box until further evaluation.

Sperm viability is defined as the percentage of live sperm per slide, with a minimum of 100 sperm cells need to be evaluated in order to calculate percentage viability. Viable/living sperm cells appear uniform and white, while non-viable/dead sperm cells are either partially or fully stained in a pinkish colour. Refer to Chapter 3 for more information regarding the calculation process for sperm viability.

Sperm morphology is calculated by determining the percentage of sperm on each slide with abnormal morphology with a minimum of 100 sperm cells needed for the calculation to be carried out. Refer to chapter 3 for more information regarding the calculation process for sperm morphology. Normal sperm are characterised by the absence of any detrimental morphological effects that may be detrimental to sperm integrity. Typically, there are three categories of morphological abnormalities in sperm. Primary abnormalities refer to those associated with the head and acrosome, secondary abnormalities refer to those that have the presence of a cytoplasmic droplet on the midpiece, and tertiary abnormalities refer to all other possible tail defects, such as; looped, bent back or coiled tails (Boshoff, 2014).

5.2.6.2 Evaluation of acrosome integrity

The initial trial design included assessment of acrosome integrity. However, the RapiDiff (RD) staining material reacted with the diluent containing the rosemary extracts, and therefore assessment of acrosome integrity was not possible. Future studies need to determine why the stain reacted with the rosemary extract supplemented diluents.

5.2.7 Statistical Analysis

The generalized linear models of SAS Enterprise Guide 5.1, version 9.4 for Windows, as well as XLSTAT was used for data analysis. These analyses generated means adjusted for multiple comparisons using the Bonferroni t-test. The two-way analysis of variance (ANOVA) was used for the comparative analysis. Results were expressed as the mean ± standard error of the mean (SEM), and were considered statistically significant when $p \leq 0.05$. All tables and graphs present in the results were prepared in Microsoft Excel.

5.3 RESULTS

5.3.1 The effect of storage time, rosemary species, AO inclusion level and trehalose concentration on sperm viability

The common rosemary species had lower ($p \leq 0.05$) levels of viability for the 100mM trehalose inclusion level over the entire 72h period. The difference in viability between the common and wild species was only significant ($p < 0.05$) at the 100mM trehalose inclusion level (Table 5.2).

Table 5.2. Sperm viability (LSMean±SEM) recorded for sperm samples diluted with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, supplemented with trehalose (0mM, 50mM, 100mM), maintained at 5°C, over a period of 72h.

	0mM	50mM	100mM
Common	16.263 ± 0.552	15.566 ± 0.492	14.332^{b} ± 0.455
Wild	17.679 ± 0.552	16.185 ± 0.492	17.629^{a} ± 0.455

^{a,b} Different subscripts within columns represent significant differences ($p \leq 0.05$)

Sperm viability decreased over time for both rosemary species, at all trehalose inclusion levels (Figure 5.1). At a trehalose inclusion level of 0mM and 50mM the highest viability was found at 0h for both rosemary species. Percentage viability declined significantly from 12h for both species at both trehalose inclusion levels (0mM, 50mM).

At a trehalose inclusion level of 100mM, viability was significantly higher for the wild species when compared to the common species at 0h. The common species also had significantly lower viability compared to all other samples at 36h, 48h and 72h, respectively (Figure 5.1).

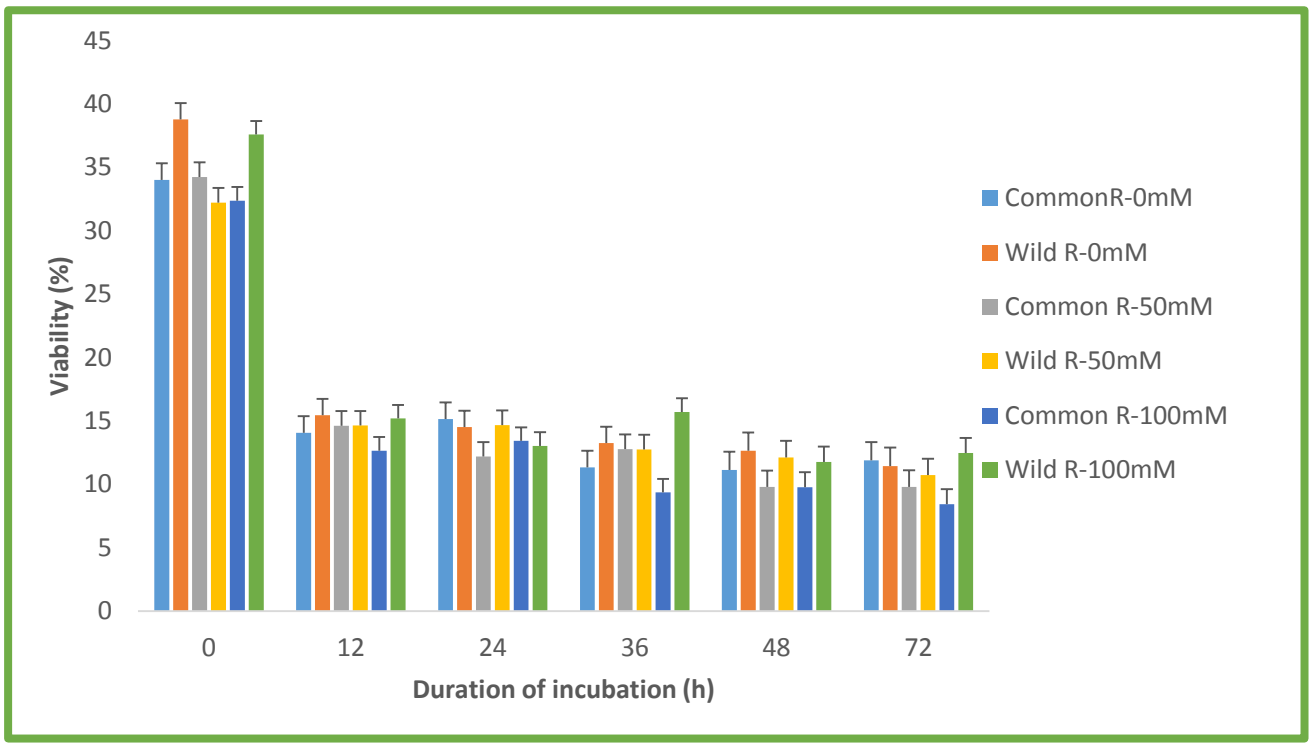

Figure 5.1. The potential of rosmarinic acid, carnosic acid and carnosol to maintain ram sperm viability during a 72h storage period at 5°C. Samples were subjected to 1 of 5 different AO inclusion levels and 1 of 3 different trehalose inclusion levels.

Figure 5.2 presents the effects of the rosemary species and AO inclusion level on sperm viability. There were no significant ($p>0.05$) differences for viability between the species and the AO inclusion level for the 0mM and 50mM trehalose inclusion levels. However, at a trehalose inclusion level of 100mM the wild species at an AO inclusion level of 2.5g, had a significantly higher viability than the common species at an AO inclusion level of 2.5g and 7.5g.

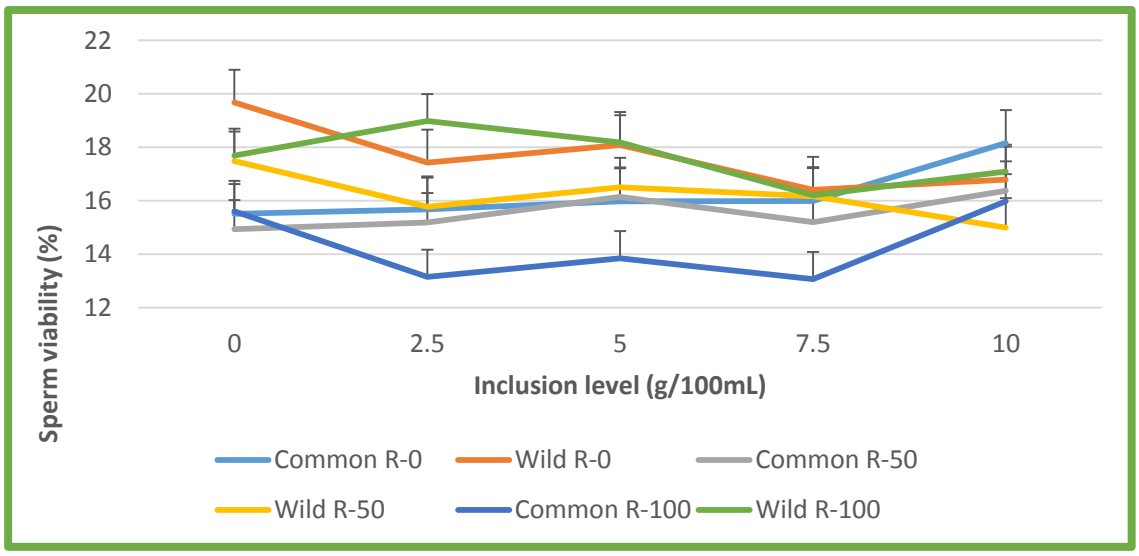

Figure 5.2. The influence of rosmarinic acid, carnosic acid and carnosol inclusion level and trehalose inclusion level on cold storage induced changes in ram sperm viability.

The viability for all AO inclusion levels and all trehalose inclusion levels had significantly ($p \leq 0.05$) higher viabilities at 0h when compared to all the other time intervals, as presented in Table 5.3.

Table 5.3. Sperm viability (LSMean±SEM) recorded for sperm samples diluted with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, at different antioxidant inclusion levels, supplemented with trehalose (0mM, 50mM, 100mM), maintained at 5°C, and determined for each time interval, over a period of 72h.

	0mM	50mM	100mM
0h*0.0g	$37.066^{a} \pm 2.045$	$34.086^{a} \pm 1.830$	$38.210^{a} \pm 1.695$
0h*2.5g	$30.776^{a} \pm 2.045$	$31.773^{a} \pm 1.830$	$34.388^{a} \pm 1.695$
0h*5.0g	$37.901^{a} \pm 2.045$	$34.700^{a} \pm 1.830$	$34.080^{a} \pm 1.695$
0h*7.5g	$36.814^{a} \pm 2.045$	$32.805^{a} \pm 1.830$	$29.759^{a} \pm 1.695$
0h*10.0g	$39.469^{a} \pm 2.045$	$32.814^{a} \pm 1.830$	$38.482^{a} \pm 1.695$
12h*0.0g	$16.868^{b} \pm 2.045$	$15.435^{b} \pm 1.830$	$13.724^{b} \pm 1.695$
12h*2.5g	$15.508^{b} \pm 2.045$	$15.620^{b} \pm 1.830$	$15.784^{b} \pm 1.695$
12h*5.0g	$14.203^{b} \pm 2.045$	$14.348^{b} \pm 1.830$	$13.043^{b} \pm 1.695$
12h*7.5g	$12.983^{b} \pm 2.045$	$14.629^{b} \pm 1.830$	$11.512^{b} \pm 1.695$
12h*10.0g	$14.215^{b} \pm 2.045$	$13.063^{b} \pm 1.830$	$15.561^{b} \pm 1.695$
24h*0.0g	$14.941^{b} \pm 2.045$	$15.720^{b} \pm 1.830$	$13.247^{b} \pm 1.695$
24h*2.5g	$15.782^{b} \pm 2.045$	$11.736^{b} \pm 1.830$	$11.080^{b} \pm 1.695$
24h*5.0g	$13.285^{b} \pm 2.045$	$12.736^{b} \pm 1.830$	$15.152^{b} \pm 1.695$
24h*7.5g	$15.428^{b} \pm 2.045$	$12.990^{b} \pm 1.830$	$14.044^{b} \pm 1.695$
24h*10.0g	$14.740^{b} \pm 2.045$	$13.922^{b} \pm 1.830$	$12.604^{b} \pm 1.695$
36h*0.0g	$10.315^{b} \pm 2.045$	$10.460^{b} \pm 1.830$	$13.683^{b} \pm 1.695$
36h*2.5g	$11.553^{b} \pm 2.045$	$12.527^{b} \pm 1.830$	$13.454^{b} \pm 1.695$
36h*5.0g	$13.474^{b} \pm 2.045$	$13.266^{b} \pm 1.830$	$13.532^{b} \pm 1.695$
36h*7.5g	$10.341^{b} \pm 2.045$	$14.056^{b} \pm 1.830$	$11.462^{b} \pm 1.695$
36h*10.0g	$15.788^{b} \pm 2.045$	$13.506^{b} \pm 1.830$	$10.542^{b} \pm 1.695$
48h*0.0g	$13.631^{b} \pm 2.297$	$11.628^{b} \pm 2.047$	$10.387^{b} \pm 1.895$
48h*2.5g	$11.723^{b} \pm 2.297$	$10.998^{b} \pm 2.047$	$10.468^{b} \pm 1.895$
48h*5.0g	$12.438^{b} \pm 2.297$	$10.865^{b} \pm 2.047$	$10.318^{b} \pm 1.895$
48h*7.5g	$11.728^{b} \pm 2.297$	$10.726^{b} \pm 2.047$	$10.099^{b} \pm 1.895$
48h*10.0g	$9.873^{b} \pm 2.297$	$10.557^{b} \pm 2.047$	$12.572^{b} \pm 1.895$
72h*0.0g	$12.736^{b} \pm 2.297$	$9.917^{b} \pm 2.047$	$10.633^{b} \pm 1.895$
72h*2.5g	$13.989^{b} \pm 2.297$	$10.211^{b} \pm 2.047$	$11.211^{b} \pm 1.895$
72h*5.0g	$10.887^{b} \pm 2.297$	$12.034^{b} \pm 2.047$	$9.973^{b} \pm 1.895$
72h*7.5g	$9.896^{b} \pm 2.297$	$8.872^{b} \pm 2.047$	$10.962^{b} \pm 1.895$
72h*10.0g	$10.786^{b} \pm 2.297$	$10.265^{b} \pm 2.047$	$9.446^{b} \pm 1.895$

[a,b] Different subscripts within columns represent significant differences ($p<0.05$)

5.3.2 The effect of storage time, rosemary species, AO inclusion level and trehalose concentration on sperm morphology

The common rosemary species had significantly ($p \leq 0.05$) lower levels of abnormal morphology than the wild species across all trehalose inclusion levels over the full 72h period (Table 5.4).

Table 5.4. Sperm abnormal morphology (LSMean±SEM) recorded for sperm samples diluted with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, supplemented with trehalose (0mM, 50mM, 100mM), maintained at 5°C, over a period of 72h.

	0mM	50mM	100mM
Common	$13.822^{b} \pm 0.488$	$14.915^{b} \pm 0.404$	$13.359^{b} \pm 0.525$
Wild	$21.825^{a} \pm 0.490$	$19.878^{a} \pm 0.404$	$22.132^{a} \pm 0.525$

^{a,b} Different subscripts within columns represent significant differences ($p \leq .05$)

The inclusion of the wild species resulted in a significantly higher percentage of abnormal morphology at 24h, when compared to the common species at 48h, at a trehalose inclusion level of 0mM (Figure 5.3). The common species also had the lowest percentage of morphological abnormalities at 48h for the 50mM trehalose inclusion level when compared to the wild species at 0h and 36h, respectively. For the 100mM trehalose inclusion level, the wild species at 0h, 12h, 24h and 36h had significantly higher morphological abnormalities when compared to the common species at 0h, 12h and 24h, respectively (Figure 5.3).

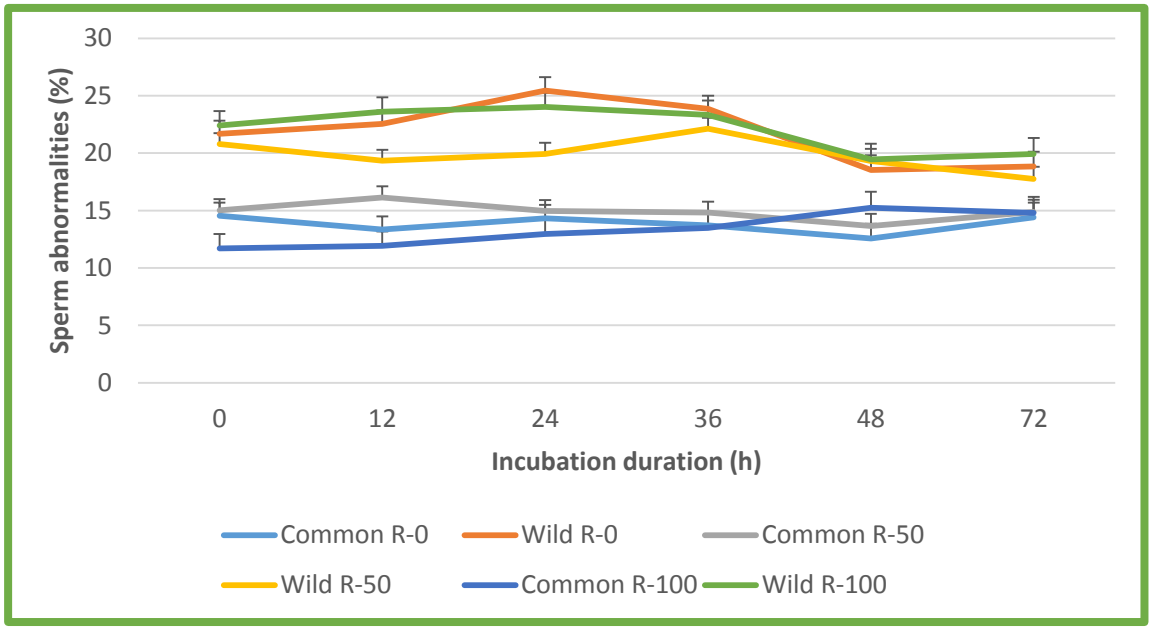

Figure 5.3. The potential of rosmarinic acid, carnosic acid and carnosol, as well as trehalose, to minimize morphological degradation in ram sperm during a 72h storage period.

The percentage of morphological abnormalities for both the 0mM and 100mM trehalose inclusion levels was significantly higher for the wild species when compared to the common species at all AO inclusion levels (Figure 5.4). At the 50mM trehalose inclusion level, the wild species at an AO inclusion level of 0g, 2.5g and 7.5g had significantly higher morphological abnormalities than the common species at 0g, 2.5g, 7.5g and 10g, respectively.

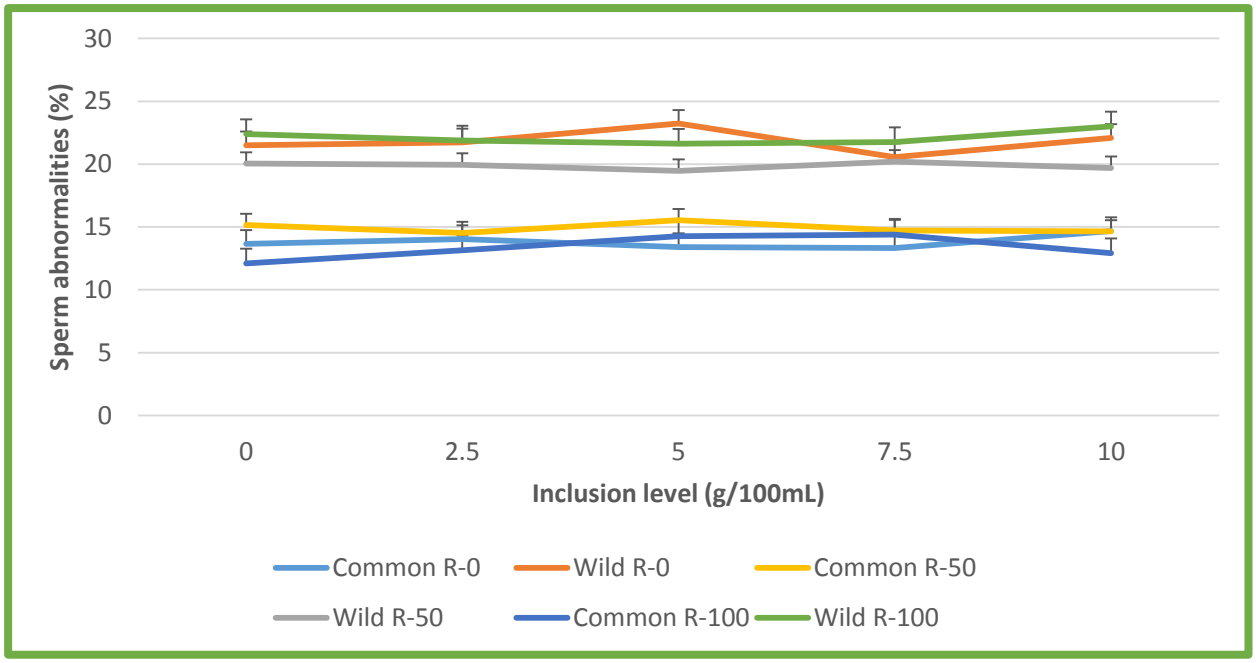

Figure 5.4. The influence of rosmarinic acid, carnosic acid and carnosol, as well as trehalose, on cold-storage induced changes in ram sperm morphology.

5.3.3 The potential of rosmarinic acid, carnosic acid and carnosol to preserve sheep sperm viability and morphological integrity during short-term cold storage

5.3.3.1 The effects of rosmarinic acid, carnosic acid and carnosol on sperm viability

When considering how the AO's (rosmarinic acid, carnosic acid and carnosol) contributed to the percentage of sperm viability, at all the trehalose inclusion levels, based on their concentrations, it was found that there were no significant differences in viability between the wild and common species for any of the AO concentrations. The results are presented in Table 5.5 for rosmarinic acid, Table 5.6 for carnosic acid and Table 5.7 for carnosol, respectively.

Table 5.5. Sperm viability (LS mean±SEM) recorded for the contribution of rosmarinic acid concentration on sperm samples diluted with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, supplemented with trehalose (0mM, 50mM, 100mM), maintained at 5°C, over a period of 72h.

Rosemary variety	Rosmarinic acid concentration (mg/L)	Trehalose inclusion level		
Common rosemary		**0mM**	**50mM**	**100mM**
Control	0	17.908 ± 1.497	16.596 ± 1.326	17.086 ± 1.395
2.5	151.75	15.914 ± 2.118	15.569 ± 1.876	13.512 ± 1.973
5.0	145.1	16.325 ± 2.118	16.528 ± 1.876	14.259 ± 1.973
7.5	176.8	16.343 ± 2.118	15.650 ± 1.876	13.354 ± 1.973
10.0	187.635	18.640 ± 2.118	16.790 ± 1.876	16.367 ± 1.973
Wild rosemary				
Control	0	17.908 ± 1.497	16.596 ± 1.326	17.086 ± 1.395
2.5	3.02	17.725 ± 2.118	16.082 ± 1.876	19.363 ± 1.973
5.0	0.65	18.505 ± 2.118	16.818 ± 1.876	18.613 ± 1.973
7.5	0.08	16.824 ± 2.118	16.549 ± 1.876	16.513 ± 1.973
10.0	0.07	17.338 ± 2.118	15.340 ± 1.876	17.491 ± 1.973

Table 5.6. Sperm viability (LS mean±SEM) recorded for the contribution of carnosic acid concentration on sperm samples diluted with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, supplemented with trehalose (0mM, 50mM, 100mM), maintained at 5°C, over a period of 72h.

Rosemary variety	Carnosic acid concentration (mg/L)	Trehalose inclusion level		
		0mM	**50mM**	**100mM**
Common rosemary				
Control	0	17.908 ± 1.490	16.596 ± 1.320	17.086 ± 1.390
2.5	0.2	15.914 ± 2.107	15.569 ± 1.867	13.512 ± 1.966
5.0	1.335	16.325 ± 2.107	16.528 ± 1.867	14.259 ± 1.966
7.5	0.08	16.343 ± 2.107	15.650 ± 1.867	13.354 ± 1.966
10.0	1.805	18.640 ± 2.107	16.790 ± 1.867	16.367 ± 1.966
Wild rosemary				
Control	0	17.908 ± 1.490	16.596 ± 1.320	17.086 ± 1.390
All treatments	0.07	17.598 ± 1.054	16.197 ± 0.933	17.995 ± 0.983

Table 5.7. Sperm viability (LS mean±SEM) recorded for the contribution of carnosol concentration on sperm samples diluted with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, supplemented with trehalose (0mM, 50mM, 100mM), maintained at 5°C, over a period of 72h.

Rosemary variety	Carnosol concentration (mg/L)	Trehalose inclusion level		
		0mM	50mM	100mM
Common rosemary				
Control	0	17.908 ± 1.490	16.596 ± 1.320	17.086 ± 1.390
2.5	3.16	15.914 ± 2.107	15.569 ± 1.867	13.512 ± 1.966
5.0	3.275	16.325 ± 2.107	16.528 ± 1.867	14.259 ± 1.966
7.5	4.265	16.343 ± 2.107	15.650 ± 1.867	13.354 ± 1.966
10.0	5.755	18.640 ± 2.107	16.790 ± 1.867	16.367 ± 1.966
Wild rosemary				
Control	0	17.908 ± 1.490	16.596 ± 1.320	17.086 ± 1.390
All treatments	0.07	17.598 ± 1.054	16.197 ± 0.933	17.995 ± 0.983

5.3.3.2 The effects of rosmarinic acid, carnosic acid and carnosol on sperm morphology

The percentage of abnormal morphology was significantly higher for the wild species at a rosmarinic acid concentration of 0.07mg/L when compared to the wild species at a rosmarinic concentration of 151.75mg/L, 145.1mg/L, 176.8mg/L and 187.635mg/L, respectively, for the 0mM and 100mM inclusion levels. At the 50mM trehalose inclusion level, the highest percentage of morphological abnormalities was for the wild species at a rosmarinic acid concentration of 0.08mg/L when compared to the common species at 151.75mg/L, 176.8mg/L and 187.635mg/L, respectively.

Table 5.8. Sperm abnormal morphology (LS mean±SEM) recorded for the contribution of rosmarinic acid concentration on sperm samples diluted with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, supplemented with trehalose (0mM, 50mM, 100mM), maintained at 5°C, over a period of 72h.

Rosemary variety	Rosmarinic acid concentration (mg/L)	Trehalose inclusion level		
Common rosemary		**0mM**	**50mM**	**100mM**
Control	0	$17.506^{bc} \pm 0.827$	$17.697^{abc} \pm 0.649$	$17.254^{bc} \pm 0.882$
2.5	151.75	$14.021^{c} \pm 1.159$	$14.546^{c} \pm 0.918$	$13.054^{c} \pm 1.247$
5.0	145.1	$13.529^{c} \pm 1.159$	$15.636^{bc} \pm 0.918$	$14.066^{c} \pm 1.247$
7.5	176.8	$13.338^{c} \pm 1.159$	$14.774^{c} \pm 0.918$	$14.345^{c} \pm 1.247$
10.0	187.635	$14.763^{c} \pm 1.159$	$14.627^{c} \pm 0.918$	$12.782^{c} \pm 1.247$
Wild rosemary				
Control	0	$17.506^{bc} \pm 0.827$	$17.697^{abc} \pm 0.649$	$17.254^{bc} \pm 0.882$
2.5	3.02	$21.910^{ab} \pm 1.159$	$20.029^{a} \pm 0.918$	$22.102^{ab} \pm 1.247$
5.0	0.65	$23.562^{a} \pm 1.159$	$19.629^{ab} \pm 0.918$	$21.716^{ab} \pm 1.247$
7.5	0.08	$20.784^{ab} \pm 1.159$	$20.300^{a} \pm 0.918$	$21.998^{ab} \pm 1.247$
10.0	0.07	$22.378^{a} \pm 1.159$	$19.743^{ab} \pm 0.918$	$23.160^{a} \pm 1.247$

[a,b] Different subscripts within columns represent significant differences ($p<0.05$)

The contribution of carnosic acid (Table 5.9) showed that all wild rosemary treatments had significantly higher levels of morphological abnormalities when compared to the controls and all other common rosemary treatments, at all trehalose inclusion levels. These results were the same for carnosol and can be seen in Table 5.10.

Table 5.9. Sperm abnormal morphology (LS mean±SEM) recorded for the contribution of carnosic acid concentration on sperm samples diluted with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, supplemented with trehalose (0mM, 50mM, 100mM), maintained at 5°C, over a period of 72h.

Rosemary variety	Carnosic acid concentration (mg/L)	Trehalose inclusion level		
		0mM	**50mM**	**100mM**
Common rosemary				
Control	0	$17.506^{b} \pm 0.827$	$17.697^{ab} \pm 0.646$	$17.254^{b} \pm 0.878$
2.5	0.2	$14.021^{b} \pm 1.159$	$14.546^{b} \pm 0.914$	$13.054^{b} \pm 1.242$
5.0	1.335	$13.529^{b} \pm 1.159$	$15.636^{b} \pm 0.914$	$14.066^{b} \pm 1.242$
7.5	0.08	$13.338^{b} \pm 1.159$	$14.774^{b} \pm 0.914$	$14.345^{b} \pm 1.242$
10.0	1.805	$14.763^{b} \pm 1.159$	$14.627^{b} \pm 0.914$	$12.782^{b} \pm 1.242$
Wild rosemary				
Control	0	$17.506^{b} \pm 0.827$	$17.697^{ab} \pm 0.646$	$17.254^{b} \pm 0.878$
All treatments	0.07	$22.159^{a} \pm 0.508$	$19.925^{a} \pm 0.457$	$22.244^{a} \pm 0.621$

[a,b] Different subscripts within columns represent significant differences ($p<0.05$)

Table 5.10. Sperm abnormal morphology (LS mean±SEM) recorded for the contribution of carnosol concentration on sperm samples diluted with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, supplemented with trehalose (0mM, 50mM, 100mM), maintained at 5°C, over a period of 72h.

Rosemary variety	Carnosol concentration (mg/L)	Trehalose inclusion level		
		0mM	50mM	100mM
Common rosemary				
Control	0	$17.506^{b} \pm 0.827$	$17.697^{ab} \pm 0.646$	$17.254^{b} \pm 0.878$
2.5	3.16	$14.021^{b} \pm 1.159$	$14.546^{b} \pm 0.914$	$13.054^{b} \pm 1.242$
5.0	3.275	$13.529^{b} \pm 1.159$	$15.636^{b} \pm 0.914$	$14.066^{b} \pm 1.242$
7.5	4.265	$13.338^{b} \pm 1.159$	$14.774^{b} \pm 0.914$	$14.345^{b} \pm 1.242$
10.0	5.755	$14.763^{b} \pm 1.159$	$14.627^{b} \pm 0.914$	$12.782^{b} \pm 1.242$
Wild rosemary				
Control	0	$17.506^{b} \pm 0.827$	$17.697^{ab} \pm 0.646$	$17.254^{b} \pm 0.878$
All treatments	0.07	$22.159^{a} \pm 0.508$	$19.925^{a} \pm 0.457$	$22.244^{a} \pm 0.621$

^{a,b} Different subscripts within columns represent significant differences ($p<0.05$)

5.4 DISCUSSION

This study investigated the potential of common and wild rosemary aqueous extracts, added to a sperm preservation medium containing MYE, and supplemented with different concentrations of trehalose, to minimize the deleterious effects of short-term cold storage (maintained at 5°C) on ram sperm viability and morphology, over a 72h period.

The percentage of sperm viability at a trehalose inclusion level of 0mM and 50mM decreased over the 72h period with an initial decline at 12h, viability then decreased very slowly and remained relatively stable between 12h and 72h post-collection. At a trehalose concentration of 100mM, there was a further steep decline in viability for the common rosemary species at 36h. This is in line with previous studies that reported a decrease in sperm viability with time, irrespective of the storage conditions (O'Hara *et al.*, 2010; Crespilho *et al.*, 2014; Mata-Campuzano *et al.*, 2014). Normal sperm metabolism generates a number of by-products, including ROS that lead to a decrease in sperm viability. Although storing sperm at cooled temperatures decreases sperm metabolism – thus decreasing oxygen consumption and ROS build-up and maintaining sperm survival – sperm quality still decreases over time (Crespilho *et al.*, 2014).

The addition of AO's into the semen extender to minimize the deleterious effects of cold storage on sperm viability has been extensively investigated. Antioxidants (methionine (Bucak *et al.*, 2012), royal jelly (Moradi *et al.*, 2013), lycopene and cysteamine (Akalin *et al.*, 2016)) supplemented into the preservation media have the ability to scavenge excess free radicals produced by ROS, thereby buffering the natural AO defense mechanism of the sperm cell (Motlagh *et al.*, 2014). The results of this study showed that there were no significant differences for viability when the preservation media was supplemented with varying levels of AO and supplemented with trehalose at 0mM and 50mM concentrations. However, the viability of the samples differed significantly at a trehalose inclusion level of 100mM for the different AO inclusion levels. There was also a marked decline in viability at all trehalose concentrations and all AO inclusion levels at 12h post-collection, as previously mentioned. This was to be expected, when compared to the studies where ram sperm was stored at 5° C for long periods. Soltanpour & Moghaddam (2013) found a decrease in ram sperm viability from 60.33% at 0h to 34.25% after 3 days, this result was irrespective of the extender used in the freezing process. A Hušová *et al.* (2013) also showed that over a 96h period there was a decrease in sperm viability for all semen extenders used.

The results seem to indicate that there is an effect of the trehalose inclusion level at 100mM on sperm viability (Table 5.2). There was a higher variability in sperm viability for the common rosemary species when compared to the wild rosemary species over the full 72h period and at the different AO inclusion levels. Previous studies on the effects of different trehalose concentrations added to semen extenders show variable results. In a cryopreservation study carried out by Aisen *et al.* 2002, the results showed that addition of trehalose at a higher inclusion level (100mM – 200mM) may have toxic effects on sperm quality, resulting in more variability of sperm quality parameters. Ahmad and Aksoy (2012) found that the inclusion of trehalose at 70mM and 100mM to a ram semen extender showed higher motility, membrane integrity and acrosome integrity, than samples supplemented with trehalose at concentrations lower than 50mM and higher than 200mM. These results should be further investigated to determine which concentration of trehalose is most beneficial for ram sperm supplemented with natural AO's, and different rosemary species specifically.

There was a distinct difference between the common and wild species for percentage morphological abnormalities. The wild species had significantly higher percentages of

abnormalities at all trehalose inclusion levels, and all AO inclusion levels, over the full 72h period. There are currently no studies, to my knowledge, comparing the effects of different rosemary species on sperm quality in terms of their AO capabilities. However, studies have shown that the potency, composition and yield of the AO compounds found in rosemary may vary according to the extraction process, the time of harvest, part of the plant being used, species of the plant, age of the plant and stage of plant development. Handling methods, storage conditions, drying method and the type of solvent used for extraction are also factors affecting rosemary AO composition (Ribeiro-Santos *et al.*, 2015).

The percentages of abnormal morphology showed significant differences when comparing the type of rosemary species, as mentioned in Table 5.4. above. However, there were no significant ($p>0.05$) differences within the wild or common species when considered separately over time or at different AO inclusion levels. This is in line with Rather *et al.* (2016) who demonstrated that there were no significant differences among the different AO incorporated groups (ascorbic acid, butylated hydroxytoluene (BHT) and taurine), and the control up to 48h of storage. However, the percentages of abnormal morphologies differed significantly when comparing the different AO groups to each other.

The contribution of the AO's basically mirrored the results for the cold storage study in terms of viability and morphology. The contribution of each separate AO could not be quantified as there was no ratio calculated for each of them and their individual effects. This is one of the limiting factors of the current study. More research and planning should have been carried out on determining an efficient way to extract each AO compound separately to determine how that specific compound contributes to the maintenance of sperm quality, and to determine if there is a permissive effect of the AO's when they act separately or in a group.

5.5 CONCLUSIONS

There was a significant decrease in sperm viability from 0h to 12h post-collection for both the common and wild rosemary species at trehalose concentrations of 0mM and 50mM. This decrease in viability was followed by a relatively stable level of viability for the remainder of the study. At a trehalose inclusion level of 100mM, there was a further sharp decrease in viability for the common species at 36h, after which the viability remained stable. There were no significant

differences in viability when considering the inclusion of AO's at different levels for trehalose inclusion levels of 0mM and 50mM. However, there was a significant change at 100mM trehalose. This seems to indicate that there is a destabilising effect of the higher concentration of trehalose on sperm viability.

The differences in morphological abnormalities was significant between the common and wild species throughout the study. However, there were no significant differences for abnormal morphology when comparing the species individually at any of the AO and trehalose inclusion levels. The reason for the marked differences between the species could be due to a number of factors including growth of the plant, handling and storage and the extraction process. However, further investigation into the exact reason for these differences should be carried out.

The individual contributions of the AO's could not be given as they were not extracted and analysed separately, thus the results of AO contribution basically mirrored the results seen for the rest of this chapter. This is a limiting factor of this study and needs to be addressed in further studies.

Chapter 6

The potential of the aqueous extract of common rosemary and wild rosemary to preserve sheep sperm viability and morphological integrity during heat stress

Abstract

This study investigated the potential of the aqueous extract of common and wild rosemary, respectively, to preserve the viability and morphological integrity of sheep sperm during conditions of heat stress at 38.5°C, 39°C and 41°C, over a period of 2.5h. Sperm samples were diluted with a milk-yolk extender supplemented with 50mM trehalose. The semen extender was supplemented with aqueous extracts from common or wild rosemary species at different inclusion levels (0g/100mL, 2.5g/100mL, 5g/100mL, 7.5g/100mL, 10g/100mL). Results indicated that there was a decrease in viability between 0h and 1h, followed by stable levels of viability for sperm treated with the common and wild species at 38.5°C. The percentage of morphological abnormalities was significantly higher for the wild species at all treatment levels when compared to the common species. There was no significant beneficial effect on morphology at any of the different treatment levels. However, further investigation into the effects of the antioxidant (AO) inclusion level at each time interval showed a steady (insignificant) increase in abnormal morphology for the common species.

6.1 INTRODUCTION

Mammals being homeothermic, need to regulate their heat production and heat loss in order to maintain a constant body temperature, and thus to ensure the optimal functioning of physiological processes. The ability of mammals to thermoregulate becomes difficult in situations where the heat production of the body is increased due to an increase in exercise, production or environmental ambient temperature (Hansen, 2009). Heat stress (HS) is caused by a combination of a high ambient temperature, high relative humidity, solar radiation and decreased air movement (Marai *et al.*, 2008). During times of HS there is an increase in core body temperature above the upper limits of the thermoneutral zone, this increase in temperature resulting from a reduction of heat flow from the body that is usually present as a normal heat loss mechanism (Al-Haidary, 2004; Armstrong, 1994).

Heat stress causes major changes in the biological functioning of an animal. These changes commonly manifest as a reduction in feed intake, efficiency and utilization and disturbances in

metabolism of water, protein, energy, mineral balances, enzymatic reactions, hormonal secretions and blood metabolites. These changes result in a low live body weight and impaired reproduction in the animal (Marai *et al.*, 2008). In females, HS has the ability to delay and sometimes prevent the onset of puberty as well as interfere with the normal reproductive cyclicity of the female. This may result in hypogonadism, which in turn affects all other reproductive processes. The alteration in endocrine function, which suppresses productive and reproductive functions, may also result in diminished follicular development and suppressed peripheral concentration of gonadotropins, ultimately inhibiting normal reproduction (Sejian *et al.*, 2012). In males, HS can inhibit libido and disrupt testicular function, resulting in testicular degradation. According to Hansen (2005), HS can result in a reduction in the number of normal and fertile sperm, with heat-stress affected sperm that can remain in the epididymis up to two months after the end of the HS period (Hansen, 2005). A reduction in endocrine function, particularly with regards to a reduction in testosterone production, is partly responsible for these deleterious effects (Marai *et al.*, 2008).

Thermoregulation in the testes is vitally important as even a very slight increase in scrotal temperature above normal can result in the disruption of spermatogenesis and a reduction in sperm fertilizing ability (Paul *et al.*, 2009). The importance of maintaining a stable thermal environment in the testes is the reason the testes are suspended in the scrotal sack outside the main body cavity in most mammals. The scrotal sack ensures that the intratesticular temperature is always slightly lower than core body temperature. The pampiniform plexus, an arterio-venous plexus in the testes, acts as a countercurrent heat exchange mechanism to keep the testes at a constant temperature using circulatory blood flow. Cooling of the testes is further controlled by the *tunica dartos* muscles that control the position of the testes relative to the body (Hansen, 2009; Paul *et al.*, 2009). It is believed that increased metabolism in the testes after HS may not be met by a sufficient increase in blood flow to and from the testes resulting in hypoxia which can cause cell cycle arrest and apoptosis (Paul *et al.*, 2009).

A rise in testicular temperature in response to HS can cause a reduction in sperm output, a decrease in sperm motility and damage to sperm DNA, thus resulting in germ cell damage and subfertility (Marai *et al.*, 2008; Hansen, 2009). The reason that germ cells in particular are affected by HS is not fully understood but it is speculated that oxidative stress experienced by the testes is part of the reason. Spermatocytes, spermatids and B spermatogonia are the germ

cells most sensitive to HS. As these cells are not yet fully mature, the damages caused by HS are delayed until the cells reach a stage whereby they can be ejaculated. In addition to this, even after the removal of HS, sperm production does not return to normal until the youngest damaged germ cells have been ejaculated (Hansen and Fuquay, 2011).

Although the production of reactive oxygen species (ROS) is beneficial and even necessary for the normal processes of hyperactivation, capacitation and acrosome reaction to occur, higher than normal levels of ROS can be deleterious and cause oxidative damage to sperm DNA. There are a number of antioxidant (AO) proteins within the testes that serve as free-radical scavengers to protect the germ cells from oxidative damage. These include, superoxide dismutase (SOD), glutathione reductase (GSR), glutathione peroxidase (GPX), glutathione S-transferase (GST), and heme oxygenase 1 (HMOX1) (Chandra *et al.*, 2012; Paul *et al.*, 2009). However, if the balance between ROS production and free-radical scavenging is disrupted, testicular function is also disrupted, ultimately compromising the processes involved in sperm production (Paul *et al.*, 2009).

The collection of sperm samples from animals that have experienced HS poses an additional problem during processing as the deleterious effects of both storage and HS on the sperm are compounded. It is believed that the addition of AO's to the sperm samples prior to storage may aid in minimizing the deleterious effects of HS on the sperm, resulting in improved viability and morphological integrity of samples. However, there have not been many studies carried out on the effects of different rosemary varieties on minimizing the effects of HS on ram sperm viability and morphology. Most studies involving the use of AO in semen extenders in ruminants focused on the potential of the compounds to minimize the changes caused by cold stress and cryopreservation (Zanganeh *et al.*, 2013; Daghigh-Kia *et al.*, 2014). Thus, the aim of this experiment was to establish whether common rosemary *(Rosmarinus officinalis L.)* and wild rosemary *(Eriocephalus africanus L.)* aqueous extracts, enabled sperm to offer resistance to HS, as quantified by assessing viability and morphological integrity.

6.2 MATERIALS AND METHODS

Ethical clearance for the study was obtained from the Animal Ethics Committee of the University of Stellenbosch (Protocol number: SU-ACUD16-00069). All procedures and animal handling

techniques were carried out in such a way as to adhere to the guidelines set out by the South African National Standard for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008).

6.2.1 Experimental location

The samples used in the study were collected at the Sheep Research Section on the Mariendahl Experimental Farm of Stellenbosch University, and all analyses were carried out in the Animal Physiology laboratory of the Department of Animal Sciences, Stellenbosch University.

6.2.2 Experimental animals

A total of 30 adult Dohne Merino (*Ovis aries*) rams, aged 15 months, were used in this study. During each collection session, a total of 10 rams were used, and care was taken to ensure that different rams were used for each session. The rams form part of a research flock established and maintained at the Mariendahl Research Farm. Rams were maintained under uniform nutritional conditions, and had *ad libitum* access to clean water. A standard sheep disease and parasite control programme was followed in the management of the trial animals.

6.2.3 Experimental Design

The experiment was carried according to a 3X5 factorial design, with treatments including the AO inclusion level and treatment temperatures. The MYE extender was supplemented with 50mM trehalose before addition of the AO extract. The respective treatments are indicated in Table 6.1. The experiment was replicated 5 times.

Table 6.1. The different inclusion levels of common rosemary (R) and wild rosemary (WR) aqueous extract, respectively, in sheep sperm samples diluted with a milk-yolk extender, supplemented with 50mM trehalose, and maintained at 38.5°C, 39.0°C and 41.0°C for up to 2.5h.

Inclusion level of aqueous extract (g/100mL)	Temperature		
	38.5°C	**39.0°C**	**41.0°C**
0 (Control)	R0T38.5 / WR0T38.5	R0T39 / WR0T39	R0T41 / WR0T41
2.5	R2.5T38.5 / WR2.5T38.5	R2.5T39 / WR2.5T39	R2.5T41 / WR2.5T41
5.0	R5T38.5 / WR5T38.5	R5T39 / WR5T39	R5T41 / WR5T41
7.5	R7.5T38.5 / WR7.5T38.5	R7.5T39 / WR7.5T39	R7.5T41 / WR7.5T41
10.0	R10T38.5 / WR10T38.5	R10T39 / WR10T39	R10T41 / WR10T41

The samples in this experiment were diluted with a milk-yolk extender (MYE), prepared according to Paulenz *et al.* (2002), and maintained at 5°C for the duration of the experiment. Sperm parameters were recorded at 0h, 1h, 1.5h, 2h and 2.5h, respectively.

6.2.4 Preparation of the trehalose stock solution and rosemary aqueous extracts

A 50mM trehalose stock solution was prepared in a 50mL Falcon tube and maintained at 5°C until used. Prior to addition of the stock solution to the samples, the trehalose was placed in a water bath maintained at 37°C to minimize any temperature shock to the sperm before being subjected to the respective treatments.

Two rosemary species were used during this study, namely common rosemary *(Rosmarinus officinalis L.)* and wild rosemary *(Eriocephalus africanus L.)* varieties. One kilogram of each variety was donated by HERBS-APLENTY®, located in the Overberg Region of the Western Cape. Upon arrival at the laboratory, the plant material was left to air-dry for a week, after which the leaves were stripped from the stems. The leaves were stored in air-tight Zip lock bags, and protected from direct light until being used. Extracts of each rosemary variety were prepared the evening before each experiment was carried out.

Refer to Chapter 3 for further information regarding the processes used to carry out trehalose and aqueous extract preparations.

6.2.5 Semen collection

Semen samples were collected by means of electro-ejaculation (EE) using the method as described by Marco-Jimenez *et al*. (2005). (Refer to Chapter 3 for a more detailed description of the EE technique).

Semen was collected from 10 rams for each collection session and rams were rotated between collections in order to avoid overusing them and risking a decrease in sperm quality prior to the treatments. Care was taken to ensure that all equipment and instruments during collection and processing were prewarmed (37°) to avoid cold shock to sperm. Immediately after collection and before returning to the lab, samples were subjected to macroscopic evaluation, including volume, colour and viscosity. Samples were sealed with parafilm (Lasec, South Africa) and maintained in a water bath at a constant temperature (37°) until further processing. Refer to Chapter 3 for more information on the macroscopic evaluation procedures.

6.2.6 Sample evaluation

6.2.6.1 Evaluation of viability and morphology

Sperm viability and normal morphology were determined using eosin-nigrosin (E/N) slides prepared for each sample at each time interval.

The E/N technique was carried out, with slight variation, as described by Cormier *et al.,* 1997. A 50µL aliquot of the extended sample was transferred to the centre of a clean microscopic slide using a pipette tip, two drops of modified E/N solution was mixed with the semen sample and a smear was prepared using this mixture. The smear was left to be air-dried, and then stored until evaluation.

Sperm viability is defined as the percentage of live sperm per slide with a minimum of 100 sperm cells needed in order for viability to be determined. Under the microscope, viable/living sperm cells appear uniform and white, while non-viable/dead sperm cells are either partially or fully stained in a pinkish colour. Refer to Chapter 3 for more information regarding the calculation process for sperm viability.

Normal sperm are characterised by the absence of any detrimental morphological effects that may be detrimental to sperm integrity. Typically, there are three categories of morphological abnormalities in sperm. Primary abnormalities refer to those associated with the head and acrosome, secondary abnormalities refer to those that have the presence of a cytoplasmic droplet on the midpiece, and tertiary abnormalities refer to all other possible tail defects, such as; looped, bent back or coiled tails (Boshoff, 2014). Sperm morphology is calculated by determining the percentage of sperm on each slide with normal morphology with a minimum of 100 sperm cells needed for the calculation to be carried out. Refer to Chapter 3 for more information regarding the calculation process for sperm morphology.

6.2.6.2 Evaluation of acrosome integrity

As mentioned, the initial trial design included assessment of acrosome integrity. However, the RapiDiff (RD) staining material reacted with the diluent containing the rosemary extracts, and therefore assessment of acrosome integrity was not possible.

6.2.7 Statistical Analysis

The generalized linear models of SAS Enterprise Guide 5.1, version 9.4 for Windows, as well as XLSTAT was used for data analysis. These analyses generated means adjusted for multiple comparisons using the Bonferroni t-test. The two-way analysis of variance (ANOVA) was used for the comparative analysis. Results were expressed as the mean ± standard error of the mean (SEM), and were considered statistically significant when $p \leq 0.05$. All tables and graphs present in the results were prepared in Microsoft Excel.

6.3 RESULTS

6.3.1 The effect of temperature, rosemary species and AO inclusion level on sperm viability

Sperm stored in extenders supplemented with the aqueous extract of the common rosemary species, exhibited a higher viability ($P \leq 0.05$) at 38.5°C and 41°C respectively over the 2.5h observation period (Table 6.2).

Table 6.2. Sperm viability (LSMean ± SEM) recorded in samples supplemented with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, and subjected to heat stress over a period of 2.5h.

	38.5°C	39°C	41°C
Common	54.565[a] ± 1.347	44.715 ± 1.333	52.003[a] ± 1.200
Wild	50.552[b] ± 1.347	47.433 ± 1.333	48.268[b] ± 1.200

[a,b] Different subscripts within columns represent significant differences (P≤0.05)

The percentage of sperm viability at 38.5°C was significantly higher for the common species between 0h and 1h post-collection, and the wild species between 0h and all the other time intervals (Figure 6.1). At a storage temperature of 39°C, the wild species exhibited a higher viability at 0h when compared to the wild species at 1.5h and the common species at 2h and 2.5h. The percentage viability for the common species at 0h was not considered statistically higher than any of the other values at 39°C, however, the biological difference between the viability for the common species control group compared to all the other samples (except the wild species control) would be considered high enough to take note of when considering the effects of HS on sperm viability.

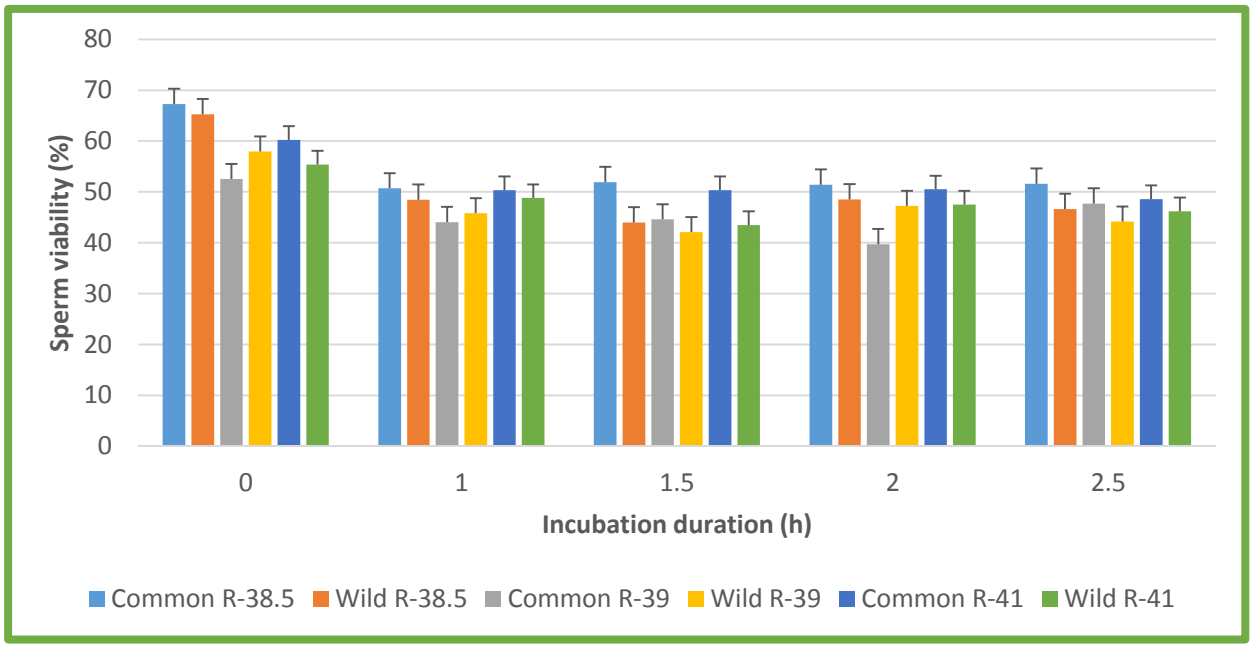

Figure 6.1. The potential of rosmarinic acid, carnosic acid and carnosol to minimise the effect of heat stress on ram sperm viability.

6.3.2 The effect of temperature, rosemary species and AO inclusion level on sperm morphology

Sperm stored in extenders supplemented with the aqueous extract of the common rosemary species exhibited significantly lower ($p \leq 0.05$) percentages of morphological abnormalities, at all the temperatures, over the 2.5h observation period (Table 6.3).

Table 6.3. Sperm abnormal morphology (LSMean ± SEM) recorded for samples supplemented with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, and subjected to heat stress, over a period of 2.5h.

	38.5°C	39°C	41°C
Common	$29.638^{b} \pm 0.684$	$32.579^{b} \pm 0.739$	$31.878^{b} \pm 0.824$
Wild	$49.208^{a} \pm 0.684$	$49.896^{a} \pm 0.739$	$49.469^{a} \pm 0.824$

^{a,b} Different subscripts within columns represent significant differences ($P<0.05$)

The wild species had significantly higher percentages of abnormal morphology than the common species for all the time intervals, at all the treatment temperatures (Figure 6.2).

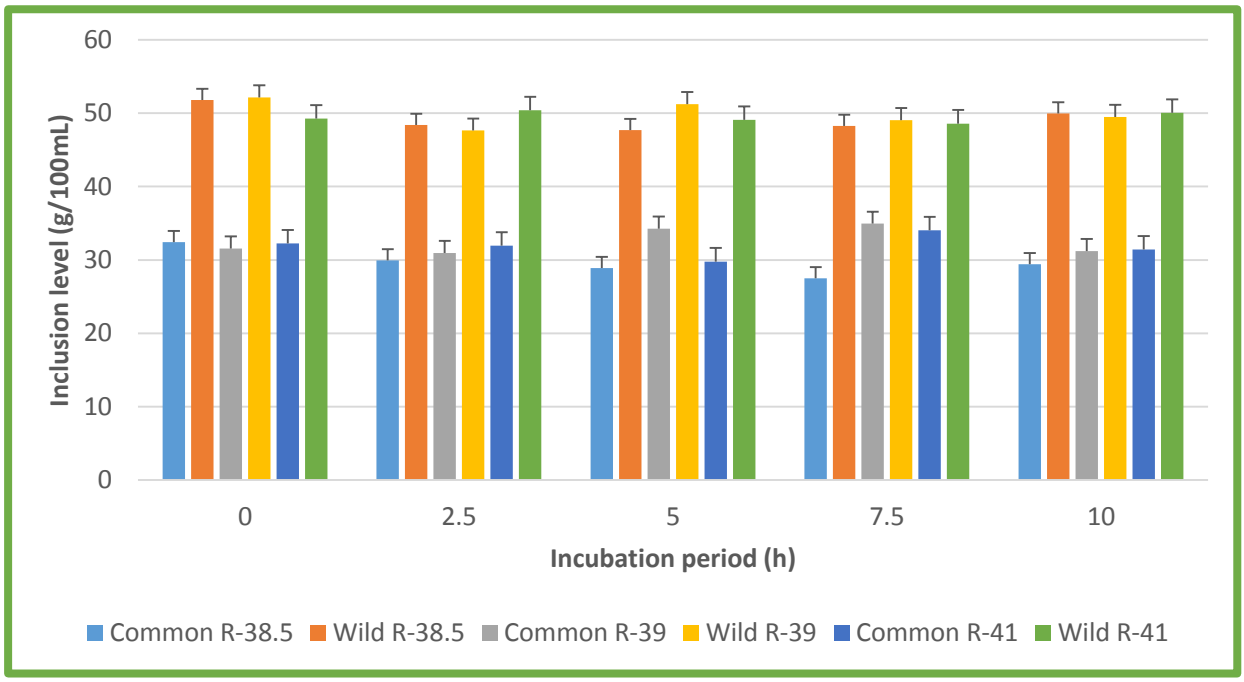

Figure 6.2. The potential of rosmarinic acid, carnosic acid and carnosol to minimise morphological changes in ram sperm subjected to heat stress.

The percentages of abnormal morphology were significantly higher for all AO inclusion levels of the wild species, at all temperatures (Table 6.4). These results indicate that the common species

had much lower morphological abnormalities than the wild species, this is similar to the results presented in Table 6.3.

Table 6.4. Sperm abnormal morphology (LS mean ±SEM) recorded for samples supplemented with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, at their different AO inclusion levels, and subjected to heat stress, over a period of 2.5h.

	38.5°C	**39°C**	**41°C**
Common*0.0g	$32.432^{b} \pm 1.529$	$31.555^{b} \pm 1.653$	$32.225^{b} \pm 1.843$
Common*2.5g	$29.949^{b} \pm 1.529$	$30.952^{b} \pm 1.653$	$31.923^{b} \pm 1.843$
Common*5.0g	$28.878^{b} \pm 1.529$	$34.255^{b} \pm 1.653$	$29.779^{b} \pm 1.843$
Common*7.5g	$27.510^{b} \pm 1.529$	$34.930^{b} \pm 1.653$	$34.041^{b} \pm 1.843$
Common*10.0g	$29.422^{b} \pm 1.529$	$31.202^{b} \pm 1.653$	$31.420^{b} \pm 1.843$
Wild*0.0g	$51.791^{a} \pm 1.529$	$52.131^{a} \pm 1.653$	$49.254^{a} \pm 1.843$
Wild*2.5g	$48.362^{a} \pm 1.529$	$47.623^{a} \pm 1.653$	$50.385^{a} \pm 1.843$
Wild*5.0g	$47.686^{a} \pm 1.529$	$51.230^{a} \pm 1.653$	$49.088^{a} \pm 1.843$
Wild*7.5g	$48.231^{a} \pm 1.529$	$49.025^{a} \pm 1.653$	$48.578^{a} \pm 1.843$
Wild*10.0g	$49.968^{a} \pm 1.529$	$49.472^{a} \pm 1.653$	$50.041^{a} \pm 1.843$

^{a,b} Different subscripts within columns represent significant differences ($P \leq 0.05$)

The results for the percentage of abnormal morphology when looking at the combined effects of the rosemary species and the AO inclusion level, within each time interval (Figure 6.2), show a distinct difference in the amount of morphological abnormalities i.e. the common species has lower percentages of abnormalities than the wild species for all the AO inclusion levels and within all the different time intervals.

In Table 6.5. the percentage of abnormal morphology for the wild species within all the time intervals stays relatively constant throughout. However, the percentage of abnormal morphology for the common species shows a general pattern of increasing abnormalities within each time interval after the 0h observation period.

Combined effects of cultivar type and AO inclusion level: 0h

Table 6.5. Sperm abnormal morphology (LS mean ±SEM) recorded for samples supplemented with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, at their different AO inclusion levels, and subjected to heat stress, at the 0h observation time.

	38.5°C	39°C	41°C
Common*0.0g	$27.773^{bc} \pm 2.863$	$31.090^{c} \pm 2.777$	$28.986^{bcd} \pm 4.242$
Common*2.5g	$27.347^{bc} \pm 2.863$	$32.616^{c} \pm 2.777$	$24.407^{cd} \pm 4.242$
Common*5.0g	$28.332^{bc} \pm 2.863$	$35.741^{bc} \pm 2.777$	$24.037^{d} \pm 4.242$
Common*7.5g	$26.534^{c} \pm 2.863$	$32.473^{c} \pm 2.777$	$27.762^{bcd} \pm 4.242$
Common*10.0g	$25.937^{c} \pm 2.863$	$32.665^{c} \pm 2.777$	$29.612^{bcd} \pm 4.242$
Wild*0.0g	$44.195^{a} \pm 2.863$	$47.580^{ab} \pm 2.777$	$51.086^{a} \pm 4.242$
Wild*2.5g	$44.180^{a} \pm 2.863$	$53.204^{a} \pm 2.777$	$46.904^{ab} \pm 4.242$
Wild*5.0g	$47.305^{a} \pm 2.863$	$49.922^{a} \pm 2.777$	$48.201^{ab} \pm 4.242$
Wild*7.5g	$40.868^{ab} \pm 2.863$	$49.480^{ab} \pm 2.777$	$48.258^{ab} \pm 4.242$
Wild*10.0g	$48.783^{a} \pm 2.863$	$47.838^{ab} \pm 2.777$	$45.311^{abc} \pm 4.242$

^{a,b,c,d} Different subscripts within columns represent significant differences (P≤0.05)

Combined effects of cultivar type and AO inclusion level: 1h

Table 6.6. Sperm abnormal morphology (LS mean ±SEM) recorded for samples supplemented with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, at their different AO inclusion levels, and subjected to heat stress, at the 1h observation time.

	38.5°C	39°C	41°C
Common*0.0g	$41.211^{abc} \pm 3.680$	$28.606^{c} \pm 4.337$	$35.874^{ab} \pm 4.617$
Common*2.5g	$29.427^{c} \pm 3.680$	$29.445^{c} \pm 4.337$	$36.046^{ab} \pm 4.617$
Common*5.0g	$33.228^{bc} \pm 3.680$	$35.150^{abc} \pm 4.337$	$34.786^{ab} \pm 4.617$
Common*7.5g	$23.840^{c} \pm 3.680$	$40.361^{abc} \pm 4.337$	$38.041^{ab} \pm 4.617$
Common*10.0g	$26.307^{c} \pm 3.680$	$31.835^{bc} \pm 4.337$	$29.332^{b} \pm 4.617$
Wild*0.0g	$48.794^{ab} \pm 3.680$	$56.294^{a} \pm 4.337$	$52.454^{a} \pm 4.617$
Wild*2.5g	$52.472^{a} \pm 3.680$	$47.948^{abc} \pm 4.337$	$48.877^{ab} \pm 4.617$
Wild*5.0g	$51.452^{ab} \pm 3.680$	$47.465^{abc} \pm 4.337$	$45.519^{ab} \pm 4.617$
Wild*7.5g	$47.990^{ab} \pm 3.680$	$51.307^{ab} \pm 4.337$	$52.587^{a} \pm 4.617$
Wild*10.0g	$52.230^{a} \pm 3.680$	$47.751^{abc} \pm 4.337$	$51.038^{ab} \pm 4.617$

^{a,b,c} Different subscripts within columns represent significant differences (P≤0.05)

Combined effects of cultivar type and AO inclusion level: 1.5h

Table 6.7. Sperm abnormal morphology (LSmean ±SEM) recorded for samples supplemented with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, at their different AO inclusion levels, and subjected to heat stress, at the 1.5h observation time.

	38.5°C	39°C	41°C
Common*0.0g	$30.852^{bcde} \pm 4.181$	$32.733^{b} \pm 4.156$	$33.433^{bcd} \pm 3.941$
Common*2.5g	$27.475^{de} \pm 4.181$	$34.951^{b} \pm 4.156$	$36.369^{abcd} \pm 3.941$
Common*5.0g	$29.490^{cde} \pm 4.181$	$38.045^{ab} \pm 4.156$	$28.345^{d} \pm 3.941$
Common*7.5g	$26.297^{e} \pm 4.181$	$35.089^{b} \pm 4.156$	$37.123^{abcd} \pm 3.941$
Common*10.0g	$31.460^{bcde} \pm 4.181$	$31.931^{b} \pm 4.156$	$29.519^{cd} \pm 3.941$
Wild*0.0g	$55.597^{a} \pm 4.181$	$50.282^{ab} \pm 4.156$	$53.400^{a} \pm 3.941$
Wild*2.5g	$48.556^{abc} \pm 4.181$	$45.007^{ab} \pm 4.156$	$54.209^{a} \pm 3.941$
Wild*5.0g	$49.045^{abc} \pm 4.181$	$56.538^{a} \pm 4.156$	$52.138^{ab} \pm 3.941$
Wild*7.5g	$48.144^{abcd} \pm 4.181$	$47.629^{ab} \pm 4.156$	$48.230^{abc} \pm 3.941$
Wild*10.0g	$50.429^{ab} \pm 4.181$	$47.875^{ab} \pm 4.156$	$51.868^{ab} \pm 3.941$

^{a,b,c,d,e} Different subscripts within columns represent significant differences (P≤0.05)

Combined effects of cultivar type and AO inclusion level: 2h

Table 6.8. Sperm abnormal morphology (LS mean ±SEM) recorded for samples supplemented with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, at their different AO inclusion levels, and subjected to heat stress, at the 2h observation time.

	38.5°C	39°C	41°C
Common*0.0g	$31.882^{d} \pm 3.187$	$34.077^{bcd} \pm 3.328$	$30.499^{b} \pm 3.885$
Common*2.5g	$32.588^{cd} \pm 3.187$	$27.168^{d} \pm 3.328$	$38.389^{ab} \pm 3.885$
Common*5.0g	$28.873^{d} \pm 3.187$	$30.152^{d} \pm 3.328$	$29.854^{b} \pm 3.885$
Common*7.5g	$30.482^{d} \pm 3.187$	$31.758^{cd} \pm 3.328$	$30.038^{b} \pm 3.885$
Common*10.0g	$34.541^{bcd} \pm 3.187$	$25.664^{d} \pm 3.328$	$31.750^{ab} \pm 3.885$
Wild*0.0g	$53.084^{a} \pm 3.187$	$50.395^{ab} \pm 3.328$	$45.971^{ab} \pm 3.885$
Wild*2.5g	$47.797^{abc} \pm 3.187$	$47.402^{abc} \pm 3.328$	$50.289^{a} \pm 3.885$
Wild*5.0g	$44.351^{abcd} \pm 3.187$	$55.819^{a} \pm 3.328$	$50.244^{a} \pm 3.885$
Wild*7.5g	$51.274^{a} \pm 3.187$	$47.518^{abc} \pm 3.328$	$48.070^{ab} \pm 3.885$
Wild*10.0g	$50.360^{ab} \pm 3.187$	$51.831^{a} \pm 3.328$	$50.165^{a} \pm 3.885$

^{a,b,c,d} Different subscripts within columns represent significant differences (P≤0.05)

Combined effects of cultivar type and AO inclusion level: 2.5h

Table 6.9. Sperm abnormal morphology (LS mean ±SEM) recorded for samples supplemented with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, at their different AO inclusion levels, and subjected to heat stress, at the 2.5h observation time.

	38.5°C	39°C	41°C
Common*0.0g	30.442^{c} ± 3.040	31.270^{c} ± 3.859	32.332^{ab} ± 4.109
Common*2.5g	32.910^{bc} ± 3.040	30.581^{c} ± 3.859	24.404^{b} ± 4.109
Common*5.0g	24.468^{c} ± 3.040	32.189^{c} ± 3.859	31.875^{ab} ± 4.109
Common*7.5g	30.399^{c} ± 3.040	34.969^{bc} ± 3.859	37.242^{ab} ± 4.109
Common*10.0g	28.867^{c} ± 3.040	33.916^{bc} ± 3.859	36.889^{ab} ± 4.109
Wild*0.0g	57.287^{a} ± 3.040	56.106^{a} ± 3.859	43.360^{ab} ± 4.109
Wild*2.5g	48.807^{a} ± 3.040	44.554^{abc} ± 3.859	51.644^{a} ± 4.109
Wild*5.0g	46.278^{ab} ± 3.040	46.405^{abc} ± 3.859	49.338^{a} ± 4.109
Wild*7.5g	52.880^{a} ± 3.040	49.194^{abc} ± 3.859	45.744^{a} ± 4.109
Wild*10.0g	48.039^{a} ± 3.040	52.066^{ab} ± 3.859	51.823^{a} ± 4.109

^{a,b,c} Different subscripts within columns represent significant differences (P≤.05)

6.3.3 The potential of rosmarinic acid, carnosic acid and carnosol to preserve sheep sperm viability and morphological integrity during heat stress

6.3.3.1 The effects of rosmarinic acid, carnosic acid and carnosol on sperm viability

The contribution of rosmarinic acid to the percentage of viability was not significantly different at any of the different treatment concentrations or at any of the different temperatures.

Table 6.10. Sperm viability (LS mean±SEM) recorded for the contribution of rosmarinic acid concentration on sperm samples supplemented with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, and subjected to heat stress over a period of 2.5h.

Rosemary variety	**Rosmarinic acid concentration (mg/L)**	**Trehalose inclusion level**		
Common rosemary		**38.5°C**	**39°C**	**41°C**
Control	0	54.325 ± 2.270	47.673 ± 2.184	54.273 ± 1.917
2.5	151.75	56.270 ± 3.210	46.807 ± 3.089	51.895 ± 2.711
5.0	145.1	54.698 ± 3.210	45.434 ± 3.089	51.135 ± 2.711
7.5	176.8	55.270 ± 3.210	45.479 ± 3.089	50.805 ± 2.711
10.0	187.635	51.361 ± 3.210	42.642 ± 3.089	51.144 ± 2.711
Wild rosemary				
Control	0	54.325 ± 2.270	47.673 ± 2.184	54.273 ± 1.917
2.5	3.02	54.124 ± 3.210	46.558 ± 3.089	51.243 ± 2.711
5.0	0.65	50.721 ± 3.210	48.808 ± 3.089	47.383 ± 2.711
7.5	0.08	46.580 ± 3.210	44.518 ± 3.089	45.018 ± 2.711
10.0	0.07	47.913 ± 3.210	45.153 ± 3.089	44.184 ± 2.711

The percentage of viability was highest for the control of both the wild and common species at 41°C when compared to all the treatments for the wild species as seen in Table 6.11. There were no significant differences in contribution at 38.5°C or 39°C. This was the same for carnosol, as seen in Table 6.12.

Table 6.11. Sperm viability (LS mean±SEM) recorded for the contribution of carnosic acid concentration on sperm samples supplemented with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, and subjected to heat stress over a period of 2.5h.

Rosemary variety	Carnosic acid concentration (mg/L)	Trehalose inclusion level		
		38.5°C	39°C	41°C
Common rosemary				
Control	0	54.325 ± 2.271	47.673 ± 2.176	54.273^{a} ± 1.921
2.5	0.2	56.270 ± 3.211	46.807 ± 3.077	51.895^{ab} ± 2.717
5.0	1.335	54.698 ± 3.211	45.434 ± 3.077	51.135^{ab} ± 2.717
7.5	0.08	55.270 ± 3.211	45.479 ± 3.077	50.805^{ab} ± 2.717
10.0	1.805	51.361 ± 3.211	42.642 ± 3.077	51.144^{ab} ± 2.717
Wild rosemary				
Control	0	54.325 ± 2.271	47.673 ± 2.176	54.273^{a} ± 1.921
All treatments	0.07	49.834 ± 1.606	46.259 ± 1.538	46.957^{b} ± 1.359

^{a,b} Different subscripts within columns represent significant differences ($p<0.05$)

Table 6.12. Sperm viability (LS mean±SEM) recorded for the contribution of carnosol concentration on sperm samples supplemented with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, and subjected to heat stress over a period of 2.5h.

Rosemary variety	Carnosol concentration (mg/L)	Trehalose inclusion level		
		38.5°C	39°C	41°C
Common rosemary				
Control	0	54.325 ± 2.271	47.673 ± 2.176	54.273^{a} ± 1.921
2.5	3.16	56.270 ± 3.211	46.807 ± 3.077	51.895^{ab} ± 2.717
5.0	3.275	54.698 ± 3.211	45.434 ± 3.077	51.135^{ab} ± 2.717
7.5	4.265	55.270 ± 3.211	45.479 ± 3.077	50.805^{ab} ± 2.717
10.0	5.755	51.361 ± 3.211	42.642 ± 3.077	51.144^{ab} ± 2.717
Wild rosemary				
Control	0	54.325 ± 2.271	47.673 ± 2.176	54.273^{a} ± 1.921
All treatments	0.07	49.834 ± 1.606	46.259 ± 1.538	46.957^{b} ± 1.359

^{a,b} Different subscripts within columns represent significant differences ($p<0.05$)

6.3.3.2 Effects of rosmarinic acid, carnosic acid and carnosol on sperm morphology

The wild rosemary species supplemented with a rosmarinic acid concentration of 0.07mg/L had the highest percentage of abnormalities when compared to the controls of both species at 38.5°C. The percentage of abnormalities at 39°C was highest at a rosmarinic acid concentration of 0.65mg/L and 0.07mg/L, these differences were significantly higher to those seen at a rosmarinic acid concentration of 151.75mg/L, 145.1mg/L and 187.635mg/L, respectively. At 41°C abnormalities were significantly higher for the wild species at a rosmarinic acid concentration of 3.02mg/L, 0.65mg/L and 0.07mg/L when compared to the common species at concentrations of 151.75mg/L, 145.1mg/L and 187.635mg/L, respectively.

Table 6.13. Sperm abnormal morphology (LS mean±SEM) recorded for the contribution of rosmarinic acid concentration on sperm samples supplemented with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, and subjected to heat stress over a period of 2.5h.

Rosemary variety	Rosmarinic acid concentration (mg/L)	Trehalose inclusion level		
Common rosemary		**38.5°C**	**39°C**	**41°C**
Control	0	$42.112^{b} \pm 1.258$	$41.843^{bc} \pm 1.328$	$40.739^{bc} \pm 1.411$
2.5	151.75	$29.949^{c} \pm 1.779$	$30.952^{d} \pm 1.879$	$31.923^{d} \pm 1.996$
5.0	145.1	$28.878^{c} \pm 1.779$	$34.255^{d} \pm 1.879$	$29.779^{d} \pm 1.996$
7.5	176.8	$27.510^{c} \pm 1.779$	$34.930^{cd} \pm 1.879$	$34.041^{cd} \pm 1.996$
10.0	187.635	$29.422^{c} \pm 1.779$	$31.202^{d} \pm 1.879$	$31.420^{d} \pm 1.996$
Wild rosemary				
Control	0	$42.112^{b} \pm 1.258$	$41.843^{bc} \pm 1.328$	$40.739^{bc} \pm 1.411$
2.5	3.02	$48.362^{ab} \pm 1.779$	$47.623^{ab} \pm 1.879$	$50.385^{a} \pm 1.996$
5.0	0.65	$47.686^{ab} \pm 1.779$	$51.230^{a} \pm 1.879$	$49.088^{a} \pm 1.996$
7.5	0.08	$48.231^{ab} \pm 1.779$	$49.025^{ab} \pm 1.879$	$48.578^{ab} \pm 1.996$
10.0	0.07	$49.968^{a} \pm 1.779$	$49.472^{a} \pm 1.879$	$50.041^{a} \pm 1.996$

^{a,b,c,d} Different subscripts within columns represent significant differences ($p<0.05$)

The contributions for carnosic acid and carnosol were the same in how they contributed to the samples at their different treatment levels. The abnormalities were significantly higher for all the wild species treatments at 38.5°C and 39°C when compared to the controls of both the wild and common species. The abnormalities for all of the common species treatments (except the control) were significantly lower than the rest of the treatments. At 41°C, all the wild species treatments were significantly higher for abnormalities when compared to the controls of both species, and all of the common species treatments, (except for a carnosic acid concentration of 0.08mg/L and a carnosol concentration of 4.265mg/L), were significantly lower for abnormalities

than the rest of the treatment groups. These results can be seen in Table 6.14. and Table 6.15. for carnosic acid and carnosol, respectively.

Table 6.14. Sperm abnormal morphology (LS mean±SEM) recorded for the contribution of carnosic acid concentration on sperm samples supplemented with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, and subjected to heat stress over a period of 2.5h.

Rosemary variety	Carnosic acid concentration (mg/L)	Trehalose inclusion level		
		38.5°C	39°C	41°C
Common rosemary				
Control	0	$42.112^{b} \pm 1.253$	$41.843^{b} \pm 1.325$	$40.739^{b} \pm 1.404$
2.5	0.2	$29.949^{c} \pm 1.771$	$30.952^{c} \pm 1.874$	$31.923^{c} \pm 1.986$
5.0	1.335	$28.878^{c} \pm 1.771$	$34.255^{c} \pm 1.874$	$29.779^{c} \pm 1.986$
7.5	0.08	$27.510^{c} \pm 1.771$	$34.930^{c} \pm 1.874$	$34.041^{bc} \pm 1.986$
10.0	1.805	$29.422^{c} \pm 1.771$	$31.202^{c} \pm 1.874$	$31.420^{c} \pm 1.986$
Wild rosemary				
Control	0	$42.112^{b} \pm 1.253$	$41.843^{b} \pm 1.325$	$40.739^{b} \pm 1.404$
All treatments	0.07	$48.562^{a} \pm 0.886$	$49.338^{a} \pm 0.973$	$49.523^{a} \pm 0.993$

^{a,b,c} Different subscripts within columns represent significant differences ($p<0.05$)

Table 6.15. Sperm abnormal morphology (LS mean±SEM) recorded for the contribution of carnosol concentration on sperm samples supplemented with antioxidant-rich aqueous extracts of common rosemary and wild rosemary, respectively, and subjected to heat stress over a period of 2.5h.

Rosemary variety	Carnosol concentration (mg/L)	Trehalose inclusion level		
		38.5°C	39°C	41°C
Common rosemary				
Control	0	$42.112^{b} \pm 1.253$	$41.843^{b} \pm 1.325$	$40.739^{b} \pm 1.404$
2.5	3.16	$29.949^{c} \pm 1.771$	$30.952^{c} \pm 1.874$	$31.923^{c} \pm 1.986$
5.0	3.275	$28.878^{c} \pm 1.771$	$34.255^{c} \pm 1.874$	$29.779^{c} \pm 1.986$
7.5	4.265	$27.510^{c} \pm 1.771$	$34.930^{c} \pm 1.874$	$34.041^{bc} \pm 1.986$
10.0	5.755	$29.422^{c} \pm 1.771$	$31.202^{c} \pm 1.874$	$31.420^{c} \pm 1.986$
Wild rosemary				
Control	0	$42.112^{b} \pm 1.253$	$41.843^{b} \pm 1.325$	$40.739^{b} \pm 1.404$
All treatments	0.07	$48.562^{a} \pm 0.886$	$49.338^{a} \pm 0.973$	$49.523^{a} \pm 0.993$

^{a,b,c} Different subscripts within columns represent significant differences ($p<0.05$)

6.4 DISCUSSION

The aim of this study was to investigate the potential of common and wild rosemary aqueous extracts, added to a sperm preservation medium, containing MYE and 50mM trehalose, to

minimize the deleterious effects of heat stress (HS) on ram sperm viability and morphology, over a 2.5h period.

The percentage viability was significantly higher for the common species at 38.5°C and 41°C, while the viability at 39°C was similar for both species over time (Table 6.2). Viability declined significantly between 0h and 1h post-collection for both the common and wild species at 38.5°C and then remained relatively stable until the end of the study. The samples at 39°C and 41°C also experienced an initial decline in viability after 0h before stabilising and remaining constant, however, these decreases in viability were not considered statistically significant (Figure 6.1). This is contradictory to a study showing no significant decrease in sperm viability of rabbits after exposure to increased temperatures over a 3h period (Sabés-Alsina *et al.*, 2016).

The percentage of morphological abnormalities was significantly higher for the wild rosemary species when compared to the common rosemary species at all temperatures over the full 2.5h period (Table 6.3), as well as at all temperatures and time intervals (Figure 6.2), and at all temperatures and AO inclusion levels (Table 6.4). This necessitated further investigation into the effects of the AO inclusion level on morphology within the different time intervals. These results showed that morphological abnormalities stayed relatively constant for the wild species within all the time intervals, however, percentage of morphological abnormalities for the common species showed a slow but progressive increase within the different time intervals. Although the statistical analysis indicates that not all of these differences are considered significant to each other, the differences in percentage of abnormalities between the common and wild species are vast when considering the biological implications. The maintained level of morphological abnormalities for the wild species could be an indication that although the wild species at the different AO inclusion levels shows higher levels of abnormal morphology, its effects on the samples also show a greater level of stability when compared to the effects of the common species.

There is little research available that investigates the immediate effects of HS on sperm cells, most studies investigate the effects of localised HS on the testes and how this has a detrimental effect on sperm quality parameters over time. However, these studies do generally demonstrate a loss in sperm viability and normal morphology over time, even after the source of HS has been removed (Júnior *et al.*, 2015). Rocha *et al.* (2015) reported that the percent of morphologically

normal sperm in ram decreased from 92 ± 2.7% on day 0 to 82 ± 6.9% and 39 ± 4.2% on days 4 and 8 post testicular insulation, respectively. This decrease continued until the animals became azoospermic and only returned to normal after day 106 post insulation.

In a study conducted to determine the role of ROS in rat testicular germ cell apoptosis induced by heat stress, it was found that cells under heat stressed conditions (37°C and 43°C) showed definite DNA fragmentation and therefore, apoptosis (Ikeda *et al.*, 1999). Cells exposed to HS conditions of 43°C showed an increase in apoptotic cells from <1% (at culture at 32.5°C) to 14% after 1h of exposure. Intracellular peroxide levels followed a temperature-dependent increase when exposed to HS conditions, these levels were significantly decreased following the addition of catalase. The increase in intracellular peroxide levels was seen as early as 5 minutes after exposure to HS at 43°C, and the protective effects of the AO's were seen after 24h of culture (Ikeda *et al.*, 1999). These results seem to correlate with the results of the current study; there was a decrease in viability (an increase in apoptosis) under conditions of HS, however, the effects of the AO's did not show a significant level of protection against a loss in viability and morphology. This may suggest that the effects of the AO's should be recorded after a longer period after the HS conditions are removed.

The results of the HS study showed that there was no significant contribution of rosmarinic acid on viability. The contributions of carnosic acid and carnosol showed a significant contribution under HS conditions at 41°C for all treatments of the wild rosemary species when compared to the controls, however, this decrease in viability would not really show any biological significance when compared to the contributions of these AO's at the lower temperatures. The effects of rosmarinic acid, carnosic acid and carnosol showed significant differences in the contributions to the percentage of morphological abnormalities. However, the levels of morphological abnormalities were similar to those found when investigating the effects of time, temperature and AO inclusion levels on morphology. Thus, it could be said that the significant differences in contribution were actually representative of the large differences in abnormalities when comparing the common and wild species, rather than being representative of the protective capacity of the AO's themselves. The reason for this is that there was no decrease in the percentage of abnormalities as would be expected if the AO's had a protective effect on morphology.

The lack of contribution from the AO's in the HS study can potentially be ascribed to the fact that the full effects were not given enough time to show their contribution before being investigated. This would correlate with previous studies that found a significant increase in GPX1 mRNA after testicular exposure to HS at 42°C (Paul *et al.*, 2009). Ikeda *et al.* (1999) found that CAT activity significantly increased after 24h of culture to protect against an increased level of apoptosis caused by oxidative stress.

6.5 CONCLUSIONS

There is little research available that investigates the direct and immediate effects of HS on sperm cells. Most studies look at the long-term effects of localised testes insulation on sperm quality parameters. However, these studies have found decreases in sperm viability and morphological integrity caused by HS conditions which is conducive to the current study.

This study found that viability was significantly higher for the common (*Rosmarinus officinalis* L.) species when compared to the wild (*Eriocephalus africanus* L.) species. The percentage of viability declined significantly between 0h and 1h at 38.5°C and then remained relatively constant throughout. Viability decreased for both rosemary species at 39°C and 41°C, however, these decreases were not considered significant.

The percentage of morphological abnormalities was significantly higher for the wild species when compared to the common species at all treatment levels over the full 2.5h period. Further investigation within the different time intervals showed that abnormal morphology for the wild species remained fairly constant throughout and that there was a slight and constant (not significant) increase in abnormal morphology for the common species. The stable level of abnormal morphology for the wild species could indicate that the AO's in the wild species have a longer lasting and more potent effect on sperm cell protection than the common species, irrespective of the higher level of abnormalities.

More research needs to be done on the direct effects of HS on sperm cell parameters and the ability for AO supplementation to limit these effects. The level of protection that AO's provide should be recorded over a longer period of time after the HS conditions have been removed in order to determine whether or not their effects are significantly relevant.

The HS study showed that there was no significant contribution of the respective AO's to viability, and that the contribution of the AO's to protecting against abnormal morphology, although statistically significant, showed no biological significance in terms of protecting the sperm from the deleterious changes caused by excessive ROS and lipid peroxidation. Future studies should be aimed at testing different extraction methods that maximize the extraction concentration of the different rosemary species and allow for better sperm preservation results. An extended observation period should also be considered to assess the ability of the AO's to buffer changes caused by ROS.

Chapter 7

General conclusions and recommendations

The major expected increase in the human population places emphasis on the need for food security, which will lead to an increased demand for livestock products. The use of assisted reproductive techniques (ART's) has gained popularity over recent decades as a method to allow sheep farmers to optimize the reproductive efficiency of sheep populations in such a way that they can satisfy the need for food safety on a global scale. The use of artificial insemination (AI) and embryo transfer (ET) are two such techniques that have the potential to contribute to the cost-efficient production of lamb and mutton. However, the potential of these techniques to contribute to optimized production of lamb and mutton is hampered by protocols that still require refinement before their commercial application in the sheep industry will be equally as successful as that in the cattle industry.

One of the major drawbacks of the application of ART's in sheep is the ability to preserve sperm viability and morphological integrity during short-term (cold) storage. The storage process is known to compromise sperm membrane integrity, thus affecting fertilizing ability of the sperm in the female reproductive tract. In addition to the deleterious effects of storage on sperm, the production of reactive oxygen species (ROS) during gamete manipulation further impacts negatively on sperm quality.

In light of the expected increases in global temperature due to climate change, the ability to minimize the effects of heat stress (HS) on sheep reproductive performance is also important. Heat stress has a number of deleterious effects on sperm production and maturation, resulting in a reduction in sperm viability and morphological integrity, even weeks after the exposure to heat stress is removed. The excessive build-up of ROS due to increased cell metabolism is the main factor contributing to the decrease in sperm quality parameters.

The sperm cell employs a natural AO defense mechanism that works to protect the cell against the excessive production of ROS. However, this mechanism may become defective under extreme levels of thermal stress (cold stress and HS). Thus, an investigation was warranted to

determine the potential of AO supplementation to semen extenders to minimize the detrimental effects of cold and heat stress, respectively, on sperm viability and morphological integrity.

The determination of the concentrations of rosmarinic acid, carnosic acid and carnosol in common (*Rosmarinus officinalis* L.) and wild (*Eriocephalus africanus* L.) rosemary aqueous extracts

The aim of this part of the study was to determine the concentration (using mass spectrometry (MS)) of each of the main diterpene components (rosmarinic acid, carnosic acid and carnosol) in rosemary aqueous extract, in order to understand how each component contributes to the AO ability of the rosemary varieties. The results of the MS analysis yielded interesting results. The concentration of the diterpene components was higher for the common species when compared to the wild species. Rosmarinic acid concentration in both the common and wild species was significantly higher than the concentrations of carnosic acid and carnosol. The concentrations of carnosic acid in the wild species were undetected at all levels, and the concentration for carnosol was <0.1mg/L at all levels for the wild species. A limit of detection (LOD) of 0.07mg/L was used for the undetected levels and the levels of <0.1mg/L.

A pilot study of the extraction technique was not carried out for the present study which was a major limitation of this study and should be considered in future. This would allow for a better extraction technique to be used in order to maximise the yield of the AO individually from the different plant species. The structural make-up of the plants should also be considered when selecting an appropriate extraction technique to ensure that the AO's can be sufficiently extracted from a more fibrous plant species that potentially has a higher structural carbohydrate fraction. Stability trials need to be carried out in order to determine if the extracts can be maintained at sub-zero temperatures for prolonged periods so that the concentration of each AO for each treatment can be controlled, thus giving more accurate data. Finally, it is important to understand the permissive function of each of the AO's and how they work together to elicit a response against the deleterious effects of ROS.

The potential of common rosemary and wild rosemary aqueous extract to preserve sheep sperm viability and morphological integrity during short-term cold storage at 5°C

This study investigated the potential of common and wild rosemary aqueous extracts, added to a sperm preservation medium, containing MYE and varying concentrations of trehalose (0mM, 50mM, 100mM), to minimize the deleterious effects of short-term cold storage (maintained at 5°C) on ram sperm viability and morphology, over a 72h period. The results showed that, irrespective of AO application, there was an initial significant decrease in sperm viability from 0h to 12h post-collection, followed by mostly stable levels of viability for the remainder of the trial.

The AO inclusion level only had a significant effect on viability at 100mM, suggesting the possibility that higher levels of trehalose have a toxic or destabilizing effect on sperm viability. However, previous studies on the effects of different concentrations of trehalose on sperm quality parameters have reported conflicting results. Thus, the effects of trehalose on ram sperm specifically supplemented with natural AO's such as rosemary, should be investigated at different inclusion levels to determine which concentration is most efficient. The effects and combinations of different sugars (i.e. fructose) and natural AO's can also be investigated to determine whether trehalose has the best protective ability on sperm quality overall.

Statistical analysis of the percentage of morphological abnormalities showed a significantly higher level of abnormalities for the wild rosemary species when compared to the common rosemary species at all treatment levels over the full 72h period. However, the two species did not differ in terms of inclusion levels.

Previous studies have demonstrated that the potency, composition and yield of the AO compounds found in rosemary species vary according to species, extraction process and storage conditions of rosemary samples. These factors could explain the differences in percentages between the two species. However, this could be a possible area of research needing further study. There are currently no studies comparing the differences in AO extraction and effect on sperm quality between different rosemary species. Further investigation should be carried out by comparing different extraction techniques, at different temperatures, and applied at different

inclusion levels to determine the optimum method that could be used to preserve sperm quality using the AO's available in different rosemary species.

For the cold storage study, there was no significant individual contributions of the AO's for maintaining sperm viability. However, the level of morphological abnormalities decreased considerably when considering the individual contributions of the AO's for the wild species, suggesting that the AO's had a greater protective effect on the sperm samples in the wild species. These findings were unexpected as the concentrations of the AO's in the common species were higher than that of the wild species (as previously mentioned). This could be attributed to the different rosemary species having different potency levels, biosynthetic pathways, metabolic routes and methods of adaptation to different environmental stressors. Rosmarinic acid, carnosic acid and carnosol are known to be effective membrane stabilizers by protecting against lipid peroxidation caused by excessive ROS production, however, this was not in line with the current study as none of the AO's showed a significant level of protection on sperm viability.

The intracellular interactions between the AO's present in rosemary are vast and extremely complicated. Future studies should aim to firstly find the extraction method for each cultivar type that yields the highest concentration of AO's from the plant. Secondly, the interactions of these AO's should also be further investigated to determine when protective action is at its highest based on inclusion levels of each AO and their effects on free radical scavenging, thus the permissive effects of the AO's should be considered. Furthermore, the fact that the wild species seemingly has a more sustained effect on maintaining morphological stability, should make the wild species a subject of further investigation to determine how to optimize its extraction levels and apply it to maintaining these stabilizing effects.

The potential of common rosemary and wild rosemary aqueous extract to preserve sheep sperm viability and morphological integrity during heat stress conditions

The aim of this study was to investigate the potential of common and wild rosemary aqueous extracts, added to a sperm preservation medium, containing MYE and 50mM trehalose, to minimize the deleterious effects of HS (38.5°C, 39°, 41°C) on ram sperm viability and morphology,

over a 2.5h period. These results showed that viability was higher for the common species when compared to the wild species, and that there was a decrease in viability between 0h and 1h followed by a relatively stable level of viability for the remainder of the study. The percentage of morphological abnormalities was significantly higher for the wild species, as seen in the previous study. However, levels of abnormal morphology did not show any significant differences among the different species. When investigating the morphological changes within the time intervals, it was found that the wild species remained relatively stable at all times, while the percentage of morphological abnormalities increased slightly and constantly (insignificantly) within the time intervals for the common species.

There is little research available that investigates the direct effects of HS on the sperm cell. Studies have shown that over time HS causes a disruption to sperm functioning, viability and morphology. However, these effects have only been investigated over long periods after the source of the HS has been removed, and only on sperm that have been insulated within the testes. More research should be carried out on the effects that HS has on the morphology of ejaculated sperm during HS, immediately after the HS conditions have been removed, and a few days after. This will give a better insight into these detrimental effects as the current effects of HS on morphology are based mainly on the effects of HS on epididymal maturation of sperm morphology. Future studies should also compare ejaculated and epididymal sperm in terms of HS resilience and also to determine the innate mechanisms of sperm to cope with HS.

The individual contributions of the AO's were not significant for viability. The contributions of the AO's to abnormal morphology showed significance between the different species, however, this was due to the significantly higher percentage of abnormalities for the wild species when compared to the common species, instead of being due to the AO's displaying high levels of protection to minimize the deleterious effects of HS on the sperm.

The exact reasons for this are unknown and have not been extensively studied. Some studies have shown that the protective effects of AO's against HS take longer to present themselves and thus should be investigated after a longer time period (i.e. 24h post HS exposure). Thus, the effects of the AO's should be investigated over a longer time period to determine if they display protective capabilities on sperm quality. It would also be interesting to determine whether the addition of an external AO source to sperm samples exposed to different HS conditions, i.e.

testicular insulation, *in vitro* exposure of sperm to different temperatures, will influence the heat shock response, and whether this will contribute to improve the resilience of ram sperm to heat stress.

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Appendix A

Figure A1: Image showing the process of rosemary extraction. Dried rosemary leaves were ground down and boiled at 100°C for 10 minutes.

Figure A2: Images of the ram being stimulated using electro-ejaculation (left) so that semen could be collected (right)

Appendix B

Preparation of smears for analysis

Throughout the current study, the line smear technique was used. Smears were prepared using 10µL of undiluted semen/sperm.

Starfrost® slides (Lasec, South Africa) are used, as the slide surface has been treated with a silane-coating. The coating creates an electrostatic attraction, then ensuring a better adhesion of the preferred sample to the slide.

Using a pipette, a drop of the sperm sample is placed near the frosted end of the slide, a spreader slide is used to spread the drop. Place the spreader slide into the drop of sperm at an angle of 45° and push the spreader along the sample slide surface (away from the frosted slide) to evenly spread the semen sample across the slide. Allow the slide to air-dry in a horizontal position. (Figure A4).

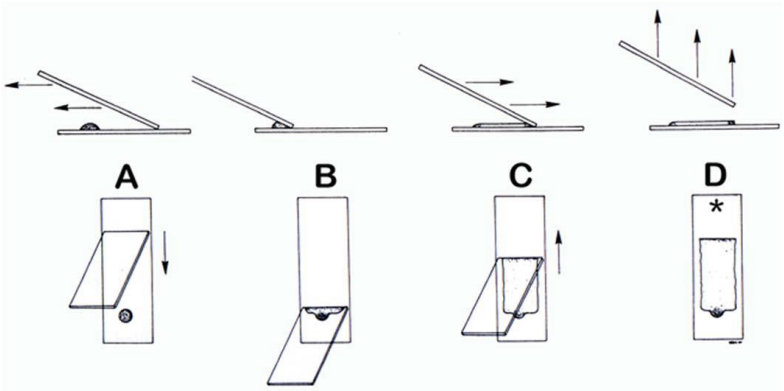

Figure B1: Representation of spreading a smear on a frosted end microscope slide (http://ahdc.vet.cornell.edu)

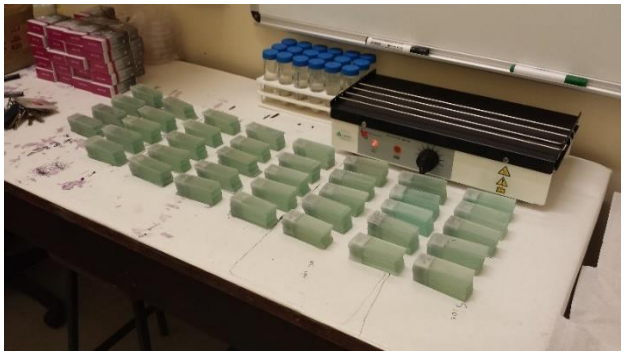

Figure B2: Image of slides being prepared for analysis

Appendix C

Score sheet template for semen collections

Time of collection: 08:30

Assistant: Rudolph Nieuwenhuis

Dr Helet Lambrechts

Ram	Sample Volume	Sample Colour	Viscosity	Comments
1		0/1/2/3/4/5	Low/normal/high	
2		0/1/2/3/4/5	Low/normal/high	
3		0/1/2/3/4/5	Low/normal/high	
4		0/1/2/3/4/5	Low/normal/high	
5		0/1/2/3/4/5	Low/normal/high	
6		0/1/2/3/4/5	Low/normal/high	
7		0/1/2/3/4/5	Low/normal/high	
8		0/1/2/3/4/5	Low/normal/high	
9		0/1/2/3/4/5	Low/normal/high	
10		0/1/2/3/4/5	Low/normal/high	