QUANTIFICATION OF BULL SPERM TRAITS AS MEASURED BY CASA AND THE RELATIONSHIP TO PREGNANCY RATE FOLLOWING CONTROLLED BREEDING

by

MASINDI LOTTUS MPHAPHATHI

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae Agriculturae

to the

Department of Animal, Wildlife and Grassland Sciences

Faculty of Natural and Agricultural Sciences

University of the Free State, Bloemfontein

Study leader: Prof. T.L. Nedambale (Tshwane University of Technology, Pretoria)

Co-study leader: Prof. J.P.C. Greyling (University of the Free State, Bloemfontein)

May, 2017

Acknowledgements

I would like to express my sincere gratitude and appreciation to the following people and institutions. My skillful supervisors Prof J.P.C. Greyling and Prof T.L. Nedambale for their support, competent guidance, advice, mentorship, encouragement, constructive criticism during the trials and writing of the current dissertation and above all, their friendship. They are world renowned and have provided both an excellent education and a priceless experience in the field of Agriculture - Animal Science (Reproduction, Physiology and Cryobiology). Above all, I am grateful for their long hours of tuition and assistance that allowed me to complete a successful study. My thanks is also extended to the Agricultural Research Council (ARC), the Department of Agriculture, Forestry and Fisheries (DAFF) and Technology Innovation Agency (TIA) for their funding and the staff of the Germplasm Conservation and Reproductive Biotechnologies (GCRB) Programme for their support. The Limpopo Department of Agriculture (LDA) and Mara Research Station are also thanked for their support. My gratitude to M.M. Seshoka for dedicating herself on helping with my studies. Special thanks to Mrs M.E. Mphaphathi for her immense support throughout my research.

Dedications

- To God, for blessing me with this opportunity and guidance to start and finish this research work.
- This study is dedicated to my parents (Mr A.A. Mphaphathi and Mrs M.J. Mphaphathi) my sisters (Ms Pembelani Mphaphathi and Adivhaho Mphaphathi), my brother (Mr Thendo Mphaphathi) and my daughters (Nakisani Mphaphathi and Munaka Mphaphathi) for their love, endless support, motivation, understanding, courage and patience throughout my studies.
- To my aunties (Ms Flora Mavhandu, Matodzi Mavhandu, Mercy Mavhandu, Doris Chabalala and Khodani Mulaudzi) for their support throughout my studies.
- To my siblings (Mr Elvis Mudau, Mrs Alvina Mudau, Mr Fhumulani Mudau, Mr Vhutshilo Mudau, Ms Fhulufhelo Mudau and Sheila Mphaphathi).

Declaration

"I hereby declare that this dissertation which is submitted for a Magister Scientiae Agriculture degree to the Department of Animal, Wildlife and Grassland Sciences at the University of the Free State, is my own original work and has not previously been submitted to any other institution of higher education. I also declare that all sources cited or quoted are indicated and acknowledged by means of a comprehensive list of references."

____

Mr Masindi Lottus Mphaphathi

Bloemfontein

May 2017

Copyright© University of the Free State 2017

Table of Contents

		Page
Acknowledgements		i
Dedications		ii
Declaration		iii
Table of Contents		iv
List of Tables		viii
List of Figures		x
List of Plates		xi
List of Abbreviations		xiii
Chapter 1	**General Introduction**	1
1.1	Research problems	5
1.2	Objectives	5
1.3	Hypothesis	5
Chapter 2	**Literature review**	7
2.1	Nguni cattle breed	7
2.2	Bonsmara cattle breed	8
2.3	Semen and sperm motility evaluation	8
2.4	Sperm morphology evaluation	9
2.5	Semen extender and dilution	9
2.6	Semen cryoprotectants	10
2.7	Freezing of semen	11
2.8	The *in vitro* maturation, fertilization and culture of bovine oocytes	13

2.8.1 Collection of bovine ovaries and oocytes recovery 13

2.8.2 The *in vitro* maturation of bovine oocytes 14

2.8.3 The *in vitro* fertilization of bovine oocytes 14

2.8.4 The *in vitro* culturing of bovine embryo 15

2.9 Synchronization and timed artificial insemination in cows 15

Chapter 3 General materials and methods 18

3.1 Chemicals and reagents 18

3.2 Animal ethics 18

3.3 Study sites 18

3.3.1 GameteTek Cryo-Mobile laboratory 18

3.3.1.1 Detailed description of the CASA-SCA® technology 19

3.3.2 ARC Loskop farm (study site for the Nguni cattle breed) 21

3.3.3 Mara Research Station (study site for the Bonsmara cattle breed) 22

3.3.4 The KZN and Limpopo provinces (study sites for the emerging cattle farmers) 23

3.4 Bonsmara and Nguni bull semen donors 23

3.5 Semen collection from Bonsmara and Nguni bulls 24

3.6 Semen and sperm cell evaluation of Bonsmara and Nguni bulls 25

3.6.1 Macroscopic semen evaluation 25

3.6.2 Microscopic semen evaluation 25

3.6.3 Sperm motility and velocity trait evaluations 26

3.6.4 Sperm morphology evaluation 27

3.7 Cryopreservation of ARC Loskop Nguni bulls semen 27

3.7.1 Nguni bull semen dilution, equilibration and cryopreservation 27

3.7.2 Semen thawing, sperm motility and velocity traits evaluation before artificial insemination of cows 28

3.8 Selection of recipient Bonsmara, Nguni and Nguni type cows for oestrous synchronization and artificial insemination 29

3.8.1 Selection of Bonsmara cow recipients at the Mara Research Station 29

3.8.2 Selection of Nguni recipient cows at the Loskop farm 30

3.8.3 Selection of the Nguni type recipient cows of the emerging cattle farmers of KwaZulu Natal and Limpopo provinces 30

3.8.4 Selection of recipient Bonsmara cows of the emerging cattle farmers of Limpopo province 31

3.9 Oestrous synchronization of Bonsmara, Nguni and Nguni type cows 31

3.10 Timed artificial insemination in Bonsmara, Nguni and Nguni type cows 33

3.11 Pregnancy diagnosis in the Bonsmara, Nguni and Nguni type cows 34

3.12 The *in vitro* fertility assessment on cow oocytes with thawed semen of Bonsmara and Nguni bulls 35

3.12.1 Penetration of oocytes through *in vitro* fertilization 35

3.12.2 Semen thawing and *in vitro* fertilization 36

3.13 Data analysis 37

Chapter 4 Results 38

4.1 Characterization of body measurements and semen traits of Nguni bulls 38

4.2 Characterization of body measurements and semen traits of Bonsmara bulls 44

4.3 Cryopreservation of bull semen 48

4.3.1 Characterization of frozen-thawed sperm traits of the individual Bonsmara and Nguni bulls, using the CASA-SCA® system 48
4.4 Synchronization and artificial insemination of cows 52
4.4.1 Cow synchronization and artificial insemination at the ARC Loskop farm 52
4.4.2 All cows synchronized and artificial inseminated in the study sites 55
4.5 The *in vitro* fertilization of cattle oocytes to test sperm fertility 57

Chapter 5 Discussion 59
5.1 Characterization of body measurements and semen traits of Bonsmara and Nguni bulls 59
5.2 Cryopreservation of bull semen 62
5.3 Synchronization and oestrous response in cows 63
5.4 Sperm fertility test *in vivo* by artificial insemination of cows following oestrous synchronization 65
5.5 Sperm fertility test during *in vitro* fertilization of slaughter house cow oocytes 66

Chapter 6 General conclusions and recommendations 69
6.1 General conclusions 69
6.2 General recommendations 71
6.3 Implications 72

Abstract 73
References 76

List of Tables

Page

Table 3.1 The CASA - Sperm Class Analyzer® (V.5.2.0.1) settings used in this study to analyse both Bonsmara and Nguni bull sperm motility traits (semen characteristics) 21

Table 4.1 Body measurements of Nguni bulls 39

Table 4.2 Morphological characteristics of Nguni bulls sperm cells 38

Table 4.3 Characterization of ejaculated semen volume, pH and sperm concentration in Nguni bull 40

Table 4.4 Pearson correlation coefficients for body measurements and semen traits in Nguni bulls 41

Table 4.5 Individual Nguni bulls sperm motility traits in fresh semen 42

Table 4.6 Nguni fresh semen velocity sperm traits 43

Table 4.7 Characterization of ejaculated Bonsmara semen volume, pH and sperm cell concentration 44

Table 4.8 Morphological characteristics of Bonsmara bull sperm cells 45

Table 4.9 Individual fresh sperm motility traits for Bonsmara bulls 46

Table 4.10 Individual fresh sperm velocity traits for Bonsmara bulls 47

Table 4.11 The mean (±S.D) sperm traits (%) of the frozen-thawed bull sperm of individual semen from Bonsmara and Nguni bulls analyzed by the CASA-SCA® system 49

Table 4.12 The mean (±S.D) sperm velocity traits of individual Bonsmara and Nguni frozen-thawed semen analyzed by the CASA-SCA® system 50

Table 4.13 Pearson correlation coefficients of Nguni cow body weight and pregnancy 52

Table 4.14 The overall Pearson correlation coefficients of Bonsmara, Nguni and Nguni type cows oestrous response and pregnancy 55

Table 4.15 *In vitro* fertilization rate of matured oocytes following insemination by sperm cell from Bonsmara or Nguni semen 57

Table 4.16 Pearson correlation coefficient for bull sperm traits (sperm motility and velocity rate) and the oocyte fertilization (cleavage) rate 58

List of Figures

Page

Figure 3.1 Flow diagram of the oestrous synchronization, FTAI and PD in the recipient cows 35

Figure 4.1 Nguni and Bonsmara bull variation regarding sperm motility and velocity traits following thawing 51

List of Plates

Page

Plate 3.1 (A) GameTek Cryo-mobile laboratory (Nedambale, 2014), (B) liquid nitrogen tank with the freezer and (C) dilution (extender) preparation of raw semen in ARC laboratory 19

Plate 3.2 (A) Pouring of sheath wash medium and (B) washing of the sheath of the Nguni bull 24

Plate 3.3 (A) Nguni semen donors and (B) semen collection from Nguni bull 24

Plate 3.4 (A) Measuring of the collected semen volume and (B) pH of the bull 25

Plate 3.5 (A) Illustration showing different categories of bull sperm motion traits by CASA terminology and (B) illustration showing individual sperm linearity% 26

Plate 3.6 (A) Bull semen dilution with extenders, (B) freezing of semen straws with controlled freezer and (C) frozen semen straws stored in the liquid nitrogen tanks 28

Plate 3.7 (A) Liquid nitrogen tanks with frozen bull semen straws, (B) electronic thawing unit and (C) the CASA - SCA® system 29

Plate 3.8 (A) Nguni cows on natural grazing pastures and (B) synchronized Nguni cows in ARC Loskop farm 30

Plate 3.9 (A) Selection of recipient cows in Limpopo province and (B) pregnancy diagnosis with the aid of a portable ultrasound scanner 31

Plate 3.10 (A) Bonsmara and (B) Nguni type cows during oestrous synchronization 32

Plate 3.11 (A) Insertion of the CIDR®, (B) Nguni cows showing sign of heat and (C) heat mount detector on cow turned red following cow being mounted by another cow 33

Plate 3.12 (A) Evaluation of frozen/thawed semen before AI in an ARC Gametek Cryo-mobile laboratory on the field and (B) conducting AI in synchronized Nguni type cow following semen thawing 34

Plate 3.13 (A) The ARC portable ultrasound scanner for pregnancy diagnosis and (B) diagnosed foetus in the pregnant cow as observed on the scanner following AI 35

Plate 3.14 (A) Collected cow's ovaries, (B) matured cow's oocytes (C) CO_2 incubator for culturing of presumptive zygotes 37

Plate 4.1 Nguni calves born at the ARC Loskop farm following oestrous synchronization and fixed timed artificial insemination in Nguni cows 53

Plate 4.2 (A) Limpopo province Bonsmara calves born following oestrous synchronization and fixed timed artificial insemination 54

List of Abbreviations

AI	Artificial insemination
ARC	Agricultural Research Council
ALH	Amplitude of lateral head displacement
ANOVA	Analysis of variance
ART	Assisted reproductive technologies
BCF	Beat cross frequency
BO	Brackett and Oliphant
CASA	Computer aided sperm analyzer
CIDR®	Controlled intravaginal drug release®
COCs	Cumulus oocyte complexes
DPBS	Dulbecco's phosphate buffered saline
EYC	Egg yolk citrate
FTAI	Fixed timed artificial insemination
GLY	Glycerol
HPA	Hyperactive
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
i.m	intramuscular
LIN	Linearity
LN_2	Liquid nitrogen

MED	Medium
NPM	Non progressive motility
PD	Pregnancy diagnosis
PM	Progressive motility
RAP	Rapid
SCA®	Sperm Class Analyzer®
SLW	Slow
SOF	Synthetic oviduct fluid
STC	Static
STR	Straightness
TAI	Timed artificial insemination
TM	Total motility
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight line velocity
WOB	Wobble
HP	Hyperactive

CHAPTER 1

GENERAL INTRODUCTION

The biological and economic importance of a bull's contribution through natural breeding or artificial insemination (AI) to reproductive efficiency and production of meat or milk, or both is of great importance because each bull or its semen represents half of the genetic composition of its progeny. Therefore, semen evaluation can be an alternative and complementary method of estimating reproductive capacity of bulls (Coulter & Foote, 1979). Moreover, it is essential to find relationship between ejaculated semen and conception rate following insemination in cattle (Karunakaran & Devanathan, 2017). Semen evaluation is generally an accepted way to predict the potential fertility of a breeding bull (Kealey *et al*., 2006). However, the most definite indication of fertility from raw (fresh) or frozen-thawed semen is made on the basis of the pregnancy rate achieved from the recipient cows inseminated. Semen evaluation thus offers predictive information on the expected performance of the bull (Sharma *et al*., 2012) and also has the ultimate objective of checking the potential fertility of the sire (Bissonnette *et al*., 2009). The mammalian sperm as such is a highly specialized cell, with distinct features such as motility, generated by the elongated flagellum (Maroto-Morales *et al*., 2016). The motility of sperm is essential to transport the genetic material to the site of fertilization in the fallopian tube (Hung *et al*., 2008; García-Vázquez *et al*., 2016).

Generally visual traits are not being sufficient for a thorough objective evaluation of the sperm fertility potential or sperm motility rates evaluation (Dearing *et al.*, 2014; Gączarzewicz, 2015). Several systems with computer aided sperm analyses (CASA) are commercially available, such as ISASTM by Proiser, Hobson Sperm Tracker by Sound and Vision or CEROSTM by

Hamilton Thorne, (Tejerina *et al*., 2009), IVOS Version 10.7s; Hamilton Thorne Research, Bedford, MA (Mocé & Graham, 2006), SpermVision; Minitube (Somi *et al*., 2006) or the Sperm Class Analyzer® (SCA®), Microptic S.L., Barcelona, Spain (Berlinguer *et al*., 2009).

The CASA system suggests that, the greatest sperm motility or sperm velocity or straightest of movement in sperm is the best determinant of sperm cell quality (Mortimer *et al.*, 2015). It is thus important to the AI industry to obtain standardized or consistent comparable results (Tekin & Daşkin, 2016), when assessors make important sperm quality control decisions (Lenz *et al*., 2011). It is generally thought that if the setup and the operational procedure of the CASA are correctly defined, the reliability of CASA values are superior than that of visual estimation and the addition of detailed motion analyses unquantifiable by visual evaluation (Krause & Viethen, 1999).

Hyperactivity, a form of sperm motility characterized by extreme vigorous flagellar movement, has been proposed as essential for fertilization in mammal species (Schmidt & Kamp, 2004). The semen quality however only superficially reflects the fertility outcome. Not only is the interaction of the sperm with the oocyte important, but also the interaction between the female genital tract and the sperm and the embryo is important and this is often more difficult to evaluate (Vyt *et al*., 2008).

Different CASA systems generally yield different results, which is far from satisfactory (Holt *et al*., 1994). Obtaining comparable results when evaluators make semen quality control decisions is critical to the AI industry and for the cattle breeders. This is critical, especially when quality control is based on semen post-thaw variables. In recent years there has been an increase in the use of CASA systems to evaluate sperm motility traits (Holt *et al*., 1997),

resulting in high correlations between several CASA sperm motility traits and the actual *in vivo* fertility results of sperm from different species [horses: (Wilhelm *et al*., 1996); boar: (Holt *et al*., 1997); bulls: (Farrell *et al*., 1998)].

The CASA system has evolved over approximately 40 years through advances in devices to capture the image from a microscope with huge increases in computational power, concurrent with a reduction in size of computers and updated/expanded software programs (Amann & Waberski, 2014). Following the introduction of the CASA system, a wide variety of sperm traits have been found to be associated with IVF rates. Those selected as the best predictors generally include: linearity of sperm and percentage of sperm in IVF medium with velocities ranging from 10 to 20 µm/sec (Liu *et al*., 1991). Also the percentage of progressively motile sperm in semen or amplitude of lateral head displacement (ALH) and average path velocity (VAP) in medium (zona free hamster oocytes; (Aitken, 1994), the percentage of sperm with rapid motility; the percentage of sperm motile and (VCL) all in prepared sperm VCL and percentage of progressive motile sperm (Ford *et al*., 2001). It should be considered that many of the sperm motility measurements derived from CASA analysis are significantly correlated to each other (Liu *et al*., 1991) and there are significant variations in IVF and measurement techniques between laboratories (Berlinguer *et al*., 2009). In a study with boar sperm, hyperactivity was defined as a condition characterized by VCL > 97µm/s and ALH > 3.5 µm (Schmidt & Kamp, 2004).

The timing of AI is very critical for successful breeding of cows especially in a fixed timed artificial insemination (FTAI) program. One aspect which then requires special attention, is the oestrous synchronization technique, which can help to fix the time for AI and thus minimize cost, time and labor required for oestrous detection in cows (Schafer *et al*., 2007). Malik *et al*.

(2012) reported the percentage of oestrous response as 76.6 %, 75.0 % and 77.5 %, following the removal of controlled intravaginal drug release (CIDR®) devices from Brangus cows in different groups. In addition, a pregnancy rate of 23.3 %, 26.6 % and 37.5 % was recorded. Furthermore, a calving rate in heifers of 29.6 % and 57.8 % was reported, while in cows 22.1 % and 23.4 % gave birth to calves (Bodmer *et al*., 2005).

Nguni cattle, is a South African indigenous breed. The breed was selected on functional efficiency and breed characteristics, while maintaining its inherent traits. It is well adapted to prevailing environmental conditions and low maintenance costs (Collins-Lusweti, 2000). This breed will ensure sustainable, economic beef production for the South African population, in the face of climatic changes (Scholtz & Theunissen, 2010). The Bonsmara breed is a composite breed, also resistant to harsh conditions, which is why the majority of farmers prefer to farm with this breed. The typical African climate, parasite-related illnesses and diseases are risks to all cattle inherent farmers. The Bonsmara was then bred to excel even under these harsh conditions and rough climates (Van der Westhuizen *et al*., 2001). The performance of Bonsmara and Nguni cattle breeds of Southern Africa are adapted to prevailing conditions and play a most important role in livestock sector (Lusweti, 2000). Furthermore, these two cattle breeds are one of the most preferable by livestock farmers in South Africa.

Therefore, the objectives of this study were to evaluate the wide range of bull sperm motility and velocity traits using the CASA-Sperm Class Analyzer® (SCA®) technology. This apparatus were used to determine the Bonsmara and Nguni bull sperm motility traits prior to timed artificial insemination (TAI) and *in vitro* fertilization (IVF) with different traits belonging to various sources of semen (individuals). Moreover, a CASA technology could be a tool for predicting sperm fertility both *in vivo* and *in vitro* of the beef cattle. Also, to assess oestrous

response and pregnancy rates of both breeds (Bonsmara and Nguni) to CIDR® synchronization program using TAI.

1.1 Research problems

- Since the development of the CASA system, sperm cells assessment motility rate have not been correlated with the pregnancy and calving rate in cattle following insemination.
- The ability of the CASA system to predict fertility of the ejaculated semen remains a challenging obstacle and not widely exploited especially in the indigenous cattle of South Africa.

1.2 Objectives

The objectives of the study were:

- To characterise, compare and evaluate fresh (raw) semen of Bonsmara and Nguni breeds using computer aided sperm analysis (CASA) technology.
- To compare the oestrous synchronization response and conception rate of Bonsmara, Nguni and Nguni type cows following fixed timed artificial insemination (FTAI) with Bonsmara or Nguni semen.
- To find the relationship between cows conception rate (*in vivo* and *in vitro* fertilization) and bull sperm motility rate (sperm traits) assessed by CASA technology following insemination.

1.3 Hypothesis

- The sperm traits of Bonsmara and Nguni breed will be similar following assessment by CASA technology.

- Bonsmara cows will respond to oestrous synchronization better than Nguni cows.
- There will be a relationship between sperm traits and pregnancy rate of synchronized Bonsmara and Nguni cows following timed artificial insemination (TAI).

CHAPTER 2

LITERATURE REVIEW

2.1 Nguni cattle breed

Nguni cattle, is a South African indigenous breed. These Sanga cattle (*Bos taurus africanis*) originally found along the east coast of Southern Africa, are known as the Nguni and were found wherever the original African Nguni tribes settled (Swaziland, Zululand, Mozambique and Zimbabwe). The Nguni are generally small to medium framed cattle, with a wide range of colours patterns. Different ecotypes developed in the different agro-ecological areas and this diversity has been maintained within the breed. Nguni cattle are fertile with a long productive life, are resistant to ticks and tolerant to tick borne diseases (Mapiye *et al*., 2007). The Nguni cow is an excellent dam line for crossbreeding, with little occurrence of dystocia. It has quality meat, characteristics similar or exceeding that of exotic breeds. The breed was selected for functional efficiency and breed characteristics, while maintaining its inherent traits. It is also well adapted to prevailing South African environmental conditions and generally has low maintenance costs (Mapiye *et al*., 2007). This breed will help ensure sustainable, economic beef production for the South African population in the face of climatic changes (Macaskill, 2016). The Nguni was recognized as a developing cattle breed in 1983 under the Livestock Improvement Act (1977) and a Nguni Cattle Breeders' Society was established in 1986. It is currently numerically the second largest seed stock beef breed in South Africa (Collins-Lusweti, 2000).

2.2 Bonsmara cattle breed

According to Agricultural Research Council of South Africa, the Bonsmara breed is similar to most of the established cattle breeds of today. It has been scientifically bred (crossbreeding) and strictly selected for economical meat production in the extensive cattle grazing regions of South Africa. Ultimately three-quarter Afrikaner were mated to half-breeds to obtain progeny with 5/8 Afrikaner and 3/8 exotic (Shorthorn/Hereford) genetics (Schoeman, 1989).

The Department of Agriculture consequently decided to test the performance of various cross-breds between the indigenous and exotic breeds on its experimental farms, Mara and Messina Experimental Stations. After pilot trials it was decided to continue only with the better performing Hereford and Shorthorn cross-breds. According to Livestock production - man must measure (2007), the name "Bonsmara" was derived from "Bonsma" - the researcher (Prof. Jan C Bonsma) who played a significant role in the development of this cattle breed and "Mara" the farm on which the animals were bred, between 1937 and 1963.

2.3 Semen and sperm motility evaluation

The primary goal of semen evaluation is to assess the quality of ejaculated semen, but it also has the ultimate objective of checking the potential fertility of the sire. To confirm a bull's breeding soundness fertility, breeders begin with a physical assessment of the animal (e.g. testicular volume), as well as a summary evaluation of the semen (e.g. semen volume, mass progressive motility and sperm concentration of the ejaculate, sperm morphology) (Bissonnette *et al*., 2009).

Quality sperm motility assessed by CASA constitutes a powerful tool to evaluate the fertility potential of males in several domestic species such as the ram (Spalekov *et al*., 2011), buck

(Sundararaman & Edwin., 2008), boar (Vyt *et al*., 2008), cock (Mphaphathi *et al*., 2012), stallion (Katila, 2001) and bull (Veznik *et al*., 2001; Sundararaman *et al*., 2012). The CASA system has been recognized and used usually as a routine semen examination tool in human clinical laboratories worldwide (Lu *et al*., 2014).

The CASA system is commonly used for the standard evaluation of sperm motility rate in different farm species and the assessment of sperm kinetic traits that otherwise are indeterminable (Castellini *et al*., 2011). Amann (2004) was the first to discover the benefit of CASA systems to detect environmental influences on the quality of semen production and sperm function. The CASA system was recognized as one of the simplest and most reliable methods for assessing sperm motility traits (Alessandra *et al*., 2010).

2.4 Sperm morphology evaluation

One of the semen quality traits used to determine fertility is the percentage of sperm that express live and normal morphology. Sperm head morphometric was reported to correlate with fertility and can be used to predict a male's semen quality (Phetudomsinsuk *et al*., 2008). Good quality semen was reported to be a prerequisite for a successful and profitable AI in cattle (Attia *et al.*, 2016). The abnormal bull sperm characterized one of the more important effects on bull fertility test (Freneau *et al*., 2010). The most common bull sperm defects reported was found on distal mid-piece (6.1 %) and bent tail (1.0 %), using 6 beef cattle breeds. Bulls that had live normal sperm morphology of 83.0 % were found acceptable potential breeders among the beef breeds (Menon *et al*., 2011).

2.5 Semen extender and dilution

The semen extender is a buffered salt solution used to increase the semen volume (mL) related to the required dose and also protect sperm cells during storage (Gadea, 2003). Semen extender can be divided into two categories: those designed for short-term preservation (< 3 days) and extenders for long term semen preservation (> 4 days) *in vitro* (Gadea, 2003). In addition, the source of energy most commonly used in semen diluents is glucose, although other sugars have been tested (galactose, fructose, ribose or trehalose) (Gadea, 2003).

Egg yolk is commonly accepted to be an effective ingredient in semen extenders for protection of sperm against cold shock and the lipid-phase transition effect (Aboagla & Terada, 2004). The possibility of dilution and storage of sperm would make the work of breeders much easier, enabling the transport of semen even to distant farms, to inseminate large groups of females and to improve the utilization of sperm from superior males (Siudzin´ska & Łukaszewicz, 2008). An appropriate semen extender has to provide an energy source for sperm and maintain pH and osmolarity levels identical to those of the seminal plasma, the natural medium for sperm (Siudzin´ska & Łukaszewicz, 2008).

2.6 Semen cryoprotectants

Cryoprotectants (CPAs) are compounds that are used to achieve the required intracellular dehydration during cryopreservation of sperm and embryos. They do so either by entering the cell and displacing the water molecules out of the cell (permeating cryoprotectants), or by remaining largely out of the cell but drawing out the intracellular water by osmosis (non-permeating cryoprotectants). Usually, combinations of these compounds are used (Orief *et al.*, 2005). The combination of permeating cryoprotectant and non-permeating osmoprotectant protects cryopreserved cells by different mechanisms.

The penetrating CPAs increase membrane fluidity through rearrangement of membrane lipid and protein, resulting in greater dehydration at lower temperatures and minimized intracellular ice formation (Holt, 2000). However, the osmoprotectants have lower molecular weight, hydrophilic, non-toxic molecules that aid a cell stabilize its concentration of internal solutes under osmotic stress (Cleland *et al*., 2004). These non-permeating CPAs create an osmotic pressure that drops the freezing temperature of the medium and decreases extracellular ice formation (Aisen *et al*., 2002), thereby providing an additive protective effect.

The most commonly used cryoprotective agents are Dimethylsulfoxide and Glycerol, although many other additives have been used for specific purposes. Additionally, maintaining frozen cells at the proper storage temperature and using an appropriate warming rate, also contribute to minimizing damage to frozen cells and tissues (Blanco *et al*., 2011).

2.7 Freezing of semen

Cryopreservation refers to the technique of storing gametes at extremely low temperatures (-196 ºC) in suspended animation, for a longer duration of time until used (as compared to liquid preservation) so that it may be revived and restored to the same living state (Bakhach, 2009; Acharya & Devireddy, 2010). During cryopreservation, mammalian cells or tissues undergo cooling to sub-zero temperatures at which biological action is slowed down or completely stopped. In addition, at this low temperature of -196 ºC, no biological activity can occur, producing a state of “suspended animation” of tissue that can be maintained indefinitively. At the end of the cryopreservation process, biopreserved cells are thawed or warmed and ideally resume biological activity (Zhang *et al*., 2011).

Freezing and thawing of bull semen leads to a decrease in percentage of intact sperm, reducing the percentage of viable sperm to approximately 50 or 60 % (Woelders *et al*., 1997). All in all, three-time higher sperm dosages are needed for frozen semen to achieve a pregnancy rate comparable to that obtained with fresh semen. Fertility following cryopreservation is a vital branch of reproductive science and involves the biopreservation of gametes (sperm, oocytes), embryos and reproductive tissues (ovarian and testicular tissues) for use in assisted reproduction techniques. However, the complex process of cryopreservation usually leads to a loss in sperm motility, swelling and the damage of the acrosomal membrane and disruption or increased permeability of the sperm's plasma membrane (Watson, 1976).

The first attempts on cryopreservation of sperm were performed during the 1940's (Polge *et al*., 1949). The methodology developed during the 1950's is still used today in certain cryobiology laboratories. In thawed semen, the sperm motility normally decreases to approximately 50 % of the initial value. In bulls, thawed sperm motility of 56 % has been recorded for Holstein Friesland bulls (Mocé & Graham, 2006). In other species, thawed sperm motility ranged from 0 to 18 % for turkey, 20 to 39 % in crane sperm (Blanco *et al*., 2011) and an averaged sperm motility of 43.0 % was recorded in cocks (Mphaphathi *et al*., 2012).

Mammalian cell injury and death during the cryopreservation process is related to the formation of a large number of ice crystals within the cells (Orief *et al*., 2005). In turn, also compromising cell longevity and fertility, compared with fresh sperm (Mocé & Graham, 2006). Semen can be cryopreserved using the conventional slow freezing (Acharya & Devireddy, 2010), liquid nitrogen vapour (El-Sheshtawy *et al*., 2015) or by using the vitrification method (Isachenko *et al*., 2012). Cryopreservation of semen has also become a valuable tool for the preservation of genetic material of endangered species or sires with superior breeding values (Somi *et al*.,

2006). Cryopreserved cells and tissues can endure storage for centuries with almost no change in functionality or genetic information, making this storage a highly attractive method. However, fertilization results after insemination with frozen-thawed semen is still variable (Linde-Forsberg *et al*., 1999). On the other hand, vitrification requires a high percentage of permeable cryoprotectants in the medium (30 - 50 %, as compared to 5 - 7 % with the slow freezing method) and is unsuitable for the vitrification of sperm due to a lethal osmotic effect (Orief *et al*., 2005).

2.8 The *in vitro* maturation, fertilization and culture of bovine oocytes

2.8.1 Collection of bovine ovaries and oocyte recovery

The interest in bovine oocyte recovery in genetic selection programs is increasing, according to the report of Pieterse *et al*. (1988). Bovine oocytes are recovered from the collected ovarian follicles by either aspiration (Tavares *et al*., 2011), slicing (Machatkova *et al*., 2008) and/or with the ovum-pick up (OPU) method in live cows (Bage *et al*., 2003; Viana *et al*., 2010). Slaughterhouse bovine cow/heifer ovaries are transported to the laboratory in thermal recipients containing a 0.9% NaCl saline solution, at 37ºC. The cumulus-oocyte complexes (COCs) are obtained by aspirating the follicles with a 20-gauge needle, coupled to a 10 mL syringe (Tavares *et al*., 2011). The follicular fluid is then pooled into conical tubes and the sediment allowed to settle for approximately 10 to15 minutes (Song & Lee, 2007).

The average number of oocytes retrieved and recorded from non-synchronized Czech Simmental donors (n = 31.1 and usable oocytes of n = 10.7); Holstein dairy (n = 61.5 and usable oocytes of 13.0); beef cattle (n = 31.0 and usable oocytes of n = 8.3) by slicing method have been documented (Machatkova *et al*., 2008). The repeated OPU average number of

oocytes harvested from Nelore cows per session, ranged from 18 to 25 recovered oocytes (Silva-Santosa *et al*., 2011).

2.8.2 The *in vitro* maturation of bovine oocytes

For many years now scientists, embryologists and researchers have tried to understand and to excel on the female reproductive system by manipulating follicular development in the hope of producing more than one developmentally competent oocyte. The *in vitro* technology offers the possibility to circumvent these limits, but success rates have been variable (Blondin *et al*., 2002). The *in vitro* maturation (IVM) seems to be the limiting factor, as even after careful selection of a homogenous population of cumulus oocytes complexes (COCs), only 35 % will attain full cytoplasmic maturation and possess the competence to produce a viable, transferable blastocyst (Blondin & Sirard, 1995).

In fact, if IVM is bypassed and COCs are matured, *in vivo* and then fertilized and developed *in vitro*, the developmental potential of the COCs is increased, doubling the percentage of blastocysts produced after 11 days of *in vitro* culture (*in vitro*, 26.4 % blastocysts; *in vivo* 49.3 % blastocysts) (Van de Leemput *et al*., 1999). Cattle oocytes are generally matured *in vitro* for 24 hours (Seneda *et al*., 2001) before subjected to IVF technique.

2.8.3 The *in vitro* fertilization of bovine oocytes

Like sperm-sorting, technology for *in vitro* embryo production (IVEP) of bovine embryos has also encountered many challenges on the way toward widespread commercial application. However, IVEP technology may be more useful when combined with sperm-sorting technology (Wilson *et al*., 2006). This would allow the embryologist to predetermine the sex of offspring before IVF process (Morrell & Humblot, 2016). There have been many significant

developments in animal biotechnologies for the last few years, on semen cryopreservation, IVEP, sperm sexing, etc. (Morrell & Humblot, 2016).

2.8.4 The *in vitro* culturing of bovine embryos

The IVEP is a reproductive biotechnology that has great potential for speeding up genetic improvement in cattle industry (Camargo *et al*., 2006). The IVEP reproductive technology presents some of the following benefits: (i) a significant increase in embryos produced from high genetic value females, as oocytes can be recovered from pre-pubertal, pregnant and even dead or slaughtered donors, (ii) provides an excellent source of low cost embryos for basic research, embryo biotechnology studies (nuclear transfer, transgenesis, embryo sexing and stem cell research) and all kinds of embryo research which need a high number of embryos for manipulation and (iii) used as a strategy for the rescue of certain endangered animal species by interspecies embryo transfer (Paramio, 2010). Pontes *et al.* (2009) recorded the pregnancy rates following embryo transfer of IVEP (33.5 %), versus *in vivo* derived embryos (41.5 %) in recipient crossbred heifers.

2.9 Synchronization and timed artificial insemination in cows

Approaches that allow hormonal administrations, TAI and pregnancy diagnosis to be scheduled on the same day each week make synchronization protocols easier to manage and may facilitate protocol compliance (Fricke *et al*., 2003). Artificial insemination has been documented to have an enormous influence on cattle genetics globally. However, success of an AI program is closely related with the efficiency of oestrous detection in cows and heifers (Carvalho *et al*., 2008). Artificial insemination is an animal reproductive technology that has made it possible to increase the effective use of superior breeding bulls (Sharma *et al*., 2012), thus greatly improving the genetic quality of breeding in sheep (Kubovičová *et al*., 2011) and cattle herds

(Taponen, 2009). For the past few decades, the animal biotechnology of AI has permitted quick genetic improvement, a driving force for competitiveness in domesticated animal breeding (Gatti *et al*., 2004).

The best time for AI to be implemented occurs in the last part of standing heat. It is therefore recommended that cows observed to be in standing heat in the morning are to be inseminated in the afternoon. Cows observed on heat (oestrous) in the afternoon are to be inseminated in the morning of the following day (Fenton & Martinez, 1980). Under extensive beef cattle farming enterprises natural breeding is most frequently used (Scheepers *et al*., 2010). In general, oestrous detection efficiency in beef cattle is low, as the expression of oestrus is often compromised (Ambrose *et al*., 2010). It is estimated that less than 5 % of beef cows in the United States of America are inseminated per annum. The problem being accurate detection of oestrous (Geary *et al*., 1998, Vishwanath *et al*., 2004; Hansar *et al*., 2014).

Fortunately, several hormonal protocols have been developed to synchronize oestrous in order to facilitate and reduce the time needed for oestrous detection in cows/heifers. This specific protocol consists of three hormonal treatments: the first one, GnRH, is intended to synchronize follicular waves, the second one, PGF2α, given 7 days later, induces luteolysis and the third one, GnRH, given 36 to 48 hours after the PGF2α administration, induces ovulation at a predetermined time. Artificial insemination is then performed 16 to 24 hours after the second GnRH administration (Taponen, 2009).

Taponen (2009) recorded an average pregnancy rate of 51.5 % in a Charolais beef herd. In the same study, during summer and winter seasons, the pregnancy rates were 53.3 % and 49.1 %, respectively. In beef heifers and cows (Brahman × Hereford F_1) pregnancy rates of 54.7 % and

45.1 % were recorded, respectively (Williams *et al*., 2002). Pregnancy rates of 42.0 % after TAI in dairy cows have also been recorded by Ambrose *et al*. (2010).

In this study, the literature was reviewed on the significance of oestrous synchronization in cows, use of CASA technology, artificial insemination, *in vitro* fertilization, pregnancy diagnosis and calving rate in livestock. A fundamental biological question was studied on the efficiency of the CASA technology to assess sperm characteristics and its relationship with fertilization and pregnancy rate.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Chemicals and reagents

Chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated. All chemicals were of analytical grade. Oestrous synchronization hormones, straws and the freezing consumables were purchased from Embryo Plus® and Lion Bridge, Republic of South Africa.

3.2 Animal ethics

All experimental cattle used in this study were approved and cared according to the guidelines of the Agricultural Research Council (ARC), Animal Production Institute ethics committee (APIEC16/011).

3.3 Study sites

The following were the study sites: (i) GameteTek Cryo-Mobile laboratory, (ii) ARC Loskop farm (Nguni breed), (iii) Mara Research Station (Bonsmara breed), (iv) KwaZulu-Natal (KZN) and Limpopo province emerging cattle farms (Bonsmara and Nguni type cows).

3.3.1 GameteTek Cryo-Mobile laboratory

The idea of developing a GameteTek Cryo-Mobile laboratory was to provide (livestock reproduction) services to livestock farmers in the field and it is vital for rapid field work (Nedambale, 2014). In this study, collected semen from both Bonsmara and Nguni bulls were taken immediately to GameteTek Cryo-Mobile laboratory and analyzed for semen parameters

(sperm traits). In addition, purchased frozen semen straws of Nguni and Bonsmara breed were thawed inside the GameteTek Cryo-Mobile laboratory on the field and immediately analyzed through CASA technology (refer to Plate 3.1), before AI of synchronized cows.

Plate 3.1 **(A)** GameTek Cryo-mobile laboratory (Nedambale, 2014), **(B)** liquid nitrogen tank with the freezer and **(C)** dilution (extender) preparation of raw semen in ARC laboratory

3.3.1.1 Detailed description of the CASA-SCA® technology

- Curvilinear velocity (µm/s): The instantaneously recorded sequential progression along the whole trajectory of the sperm per unit of time (Somi *et al*., 2006).
- Average path velocity (µm/s): The mean trajectory of the sperm per unit of time (Somi *et al*., 2006).
- Straight line velocity (µm/s): The straight trajectory of the sperm per unit of time (= straight line distance from beginning to end of track divided by the time taken) (Somi *et al*., 2006).
- Linearity (%): The ratio of the straight displacement in the sum of elementary displacements during the time of the measurement. It is defined as (VSL/VCL) X 100 (Somi *et al*., 2006).
- Straightness (%): This indicates the linearity of the mean sperm trajectory and is defined as (VSL/VAP) X 100 (Somi *et al*., 2006).

- Beat cross frequency (Hz): The number of lateral oscillatory movements of the sperm head around the mean trajectory (Somi *et al*., 2006).
- Amplitude of lateral head displacement (μm): This is the mean width of the sperm head oscillation (Somi *et al*., 2006).
- Wobble (%): Which indicates the oscillation of the curvilinear sperm trajectory upon the mean trajectory and is defined as (VAP/VCL) X 100 (Somi *et al*., 2006).
- Static (%): The percentage static sperm (not moving during the analysis) (Vyt *et al*., 2008).
- Progressive motility (%): The percentage progressively moving sperm (Vyt *et al*., 2008).
- Non-progressive motility (%): The percentage of sperm not moving forward in a straight path (Vyt *et al*., 2008).
- Slow (%): The percentage of sperm moving at 1-10 μm/second (Vyt *et al*., 2008).
- Medium (%): The percentage of sperm moving at 11-25 μm/second (Vyt *et al*., 2008).
- Rapid (%): The percentage rapidly moving sperm (Vyt *et al*., 2008).
- Total motility rate: The ratio of motile sperm to the total cell concentration expressed as a percentage (Kathiravan *et al*., 2008).

The settings for the CASA system known as Sperm Class Analyzer® (SCA®) system used to assess the bull sperm motility and velocity traits is set out in Table 3.1. During the trial (fresh or frozen thawed semen sample) a live semen video signal was sent from the camera (Basler®, Germany) to the attached MacBook Pro (A1278, California) laptop and the images were recorded with the aid of commercially available SCA® software.

Table 3.1 The CASA - Sperm Class Analyzer® (V.5.2.0.1) settings used in this study to analyse both Bonsmara and Nguni bull sperm motility traits (semen characteristics)

Parameters	Settings
Brightness	300
Image per second	50
Optics	Ph-
Chamber	Cover slide
Frame rate (Hz)	60
Scale	10 X
Particle area (μm^2)	5 < 70
Slow (µm per second)	< 10
Medium (µm per second)	< 25
Rapid (µm per second)	< 100
Progressivity %	> 70 of straightness
Circular %	< 50 of linearity
Connectivity	12
Number of images	50
Image type	Phase contrast
Video source	Basler camera®
Video duration	One second

3.3.2 ARC Loskop farm (study site for the Nguni cattle breed)

The Nguni cows and bulls used in this study were stationed at the ARC-Loskop farm. The ARC Loskop farm is located (25°18′ south, 29° 20′ east) and situated in a Bushveld region in the eastern part of South Africa. Acocks (1988) classified the veld type as tree Savannah,

consisting of fairly dense bush with sour grass types as the main grazing component. Rainfall varies between 350 and 650 mm per year. Nguni cows and bulls were kept strictly under an extensive production environment, feeding solely on natural pasture, without supplementary feeding.

The Nguni cattle used in this trial were born from the pure bred Nguni breed cattle herd, kept at the Animal Production Institute-Loskop farm of the Agricultural Research Council. Cattle were kept in the Loskop camps throughout the trial. Veld condition of the grazing camps was monitored and rotation was done, based on the amount of forage available. All general cattle farm management and husbandry procedures were practiced. The bulls were weighed and body measurements taken, including scrotal circumference (SC). All data were recorded during the trial. Cows were weighed, body condition score (BCS) was taken and recorded at the beginning and end of the trial.

3.3.3 Mara Research Station (study site for the Bonsmara cattle breed)

The Bonsmara bulls and cows used were stationed at Mara Research Station, located about 54 km west of Makhado (23º05 'south, 29º25 'east) in the arid sweet bushveld area. The mean annual rainfall is 452 mm and the mean daily maximum temperature varies from 23 °C in June to 30 °C in January (Maiwashe *et al*., 2013). Cows were weighed at the beginning and end of the trial and were recorded during the trial. Bonsmara cows were kept strictly under an extensive production environment, feeding solely on natural pasture, without supplementary feeding.

The Bonsmara cattle used in this trial were born from the pure Bonsmara breed cattle herd, kept at the Mara Research Station farm in the Limpopo province, Makhado area. Animals were

kept in camps throughout the trial. Veld condition of grazing camps was monitored and rotation was done, based on the amount of forage available. All general cattle farm management and husbandry procedures were practiced.

3.3.4 The KZN and Limpopo provinces (study sites for the emerging cattle farmers)

According to Nedambale (2014), livestock farming is a mainstay of South African agriculture, but it is difficult for emerging farmers to participate into the mainstream market. Inseminations of emerging farmers's cows were conducted in the KZN and Limpopo provinces during the natural breeding season. The breeds were classified as Bonsmara and Nguni. For the duration of the experiment, cows were kept strictly under an extensive production environment, feeding solely on natural pasture without supplementary feeding. The recipients cows used in this trial were kept in camps and grazed on natural pasture. Veld condition of grazing camps was monitored and rotation was done based on the amount of forage available. All general cattle farm management and husbandry procedures were practiced.

3.4 Bonsmara and Nguni bull semen donors

The source of ejaculated raw semen was from ARC and Mara Research stations. The frozen bull semen straws were purchased from the commercial AI center. Raw semen was collected from total of four Bonsmara (n = 4) and Nguni (n = 4) bulls semen donors of known proven fertility (purebred from the ARC and Mara Research Station). Bosmara and Nguni bulls were aged 6 to 7 years, respectively. Before semen collection, Bonsmara and Nguni bulls sheath were washed for confirmation of the health status of Campylobacter and Trichomonas foetus diseases. A Dulbecco's phosphate-buffered saline was used during the sheath wash process. Collected samples of the sheath washing were kept at 5 ºC during transportation to the ARC-

Onderstepoort veterinarian laboratories, for further *in vitro* tests. All the semen donors were declared free of the Campylobacter and Trichomonas foetus diseases.

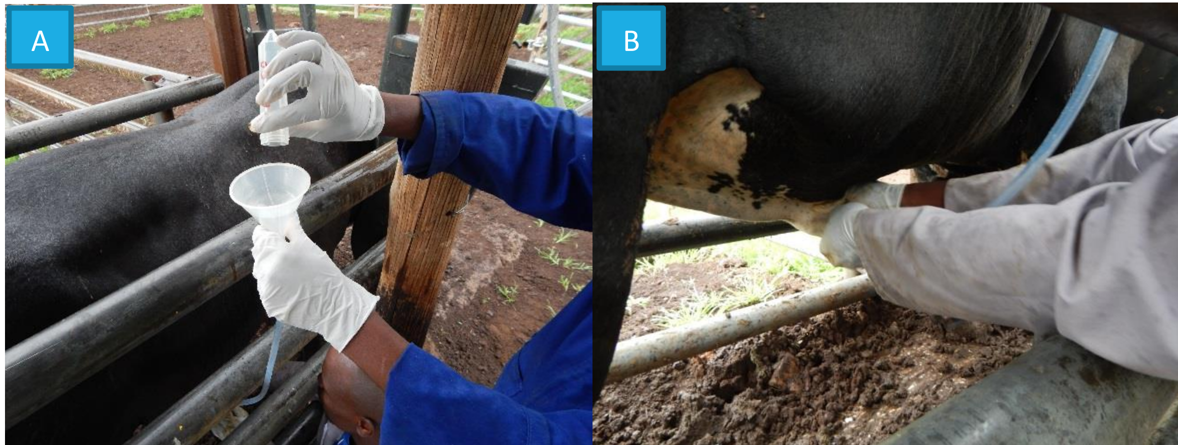

Plate 3.2 (A) Pouring of sheath wash medium and **(B)** washing of the sheath of the Nguni bull

3.5 Semen collection from Bonsmara and Nguni bulls

The sheath of each bull was properly cleaned with 70% alcohol prior to semen collection. The hygienic measures were practiced to avoid semen contamination. The electro ejaculator was used for semen collection. After collection, the semen samples were immediately transferred to a thermo-flask and maintained at 37 ℃, for further evaluation.

Plate 3.3 (A) Nguni semen donors and **(B)** semen collection from Nguni bull

3.6 Semen and sperm cell evaluation of Bonsmara and Nguni bulls

3.6.1 Macroscopic semen evaluation

- **Semen volume**: The raw ejaculated semen was directly measured (mL) using a graduated Falcon® collection tube (352099, USA).

- **Semen pH:** Semen pH was measured with the aid of a calibrated pH meter (OAKON®, pH 11 Series, Singapore).

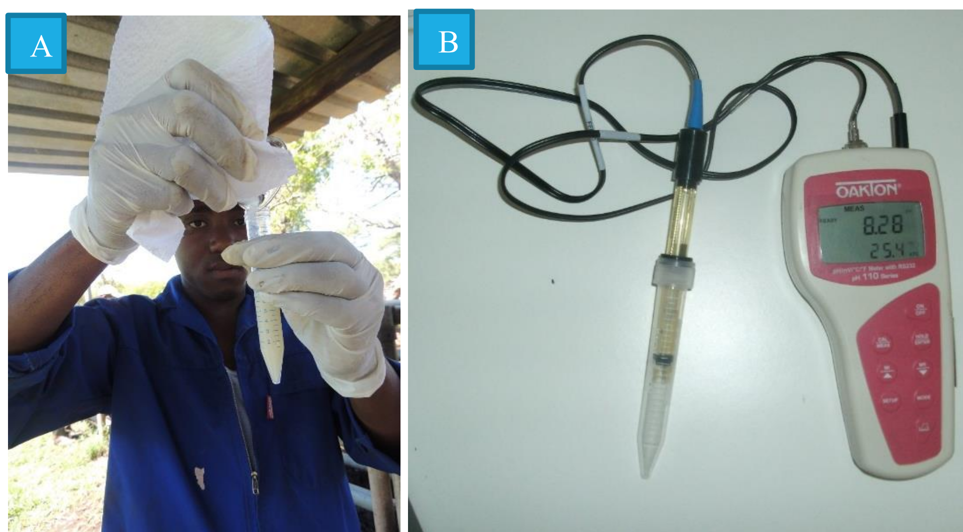

Plate 3.4 (A) Measuring of the collected semen volume and **(B)** pH of the bull

3.6.2 Microscopic semen evaluation

In brief, a Spectrophotometer® (JENWAY®, 6310) was used to estimate the sperm concentration of the collected bull semen. The bull semen sperm concentration (x 10^9/mL) was determined by a spectrophotometer. In brief, the instrument measures the amount of light absorbed by a semen sample and the more sperm are in the sample, the more light is being absorbed (Kumar *et al*., 2013). A sodium citrate solution was used for the sperm concentration evaluation. A volume of 2.9g of a sodium citrate was mixed with 100 mL of distilled water. A

3 mL of the 2.9 % sodium citrate was placed in a microcuvette (HemoCue AB®, Angelholm, Sweden) and calibrated for bull sperm concentration determination. Following the calibration, the microcuvette was removed from the spectrophotometer and 15 µL of raw semen added and then placed back into the spectrophotometer to obtain the readings. Thereafter, a formula (dilution factor × 34.43 × absorbance -0.22) was used to convert the readings recorded, in order to determine the final sperm concentration of the samples.

3.6.3 Sperm motility and velocity trait evaluations

The following CASA-SCA® sperm traits were evaluated: Total sperm motility was further divided into rapid, medium or slow and progressive and/or non-progressive motility rate. Sperm velocity characteristics measured were curvilinear, straight line and average path velocity, linearity, straightness, wobble, amplitude of lateral head displacement, beat cross frequency and hyperactive on raw or fresh semen (before freezing), or frozen/thawed at 0 min or a minute before FTAI or IVF.

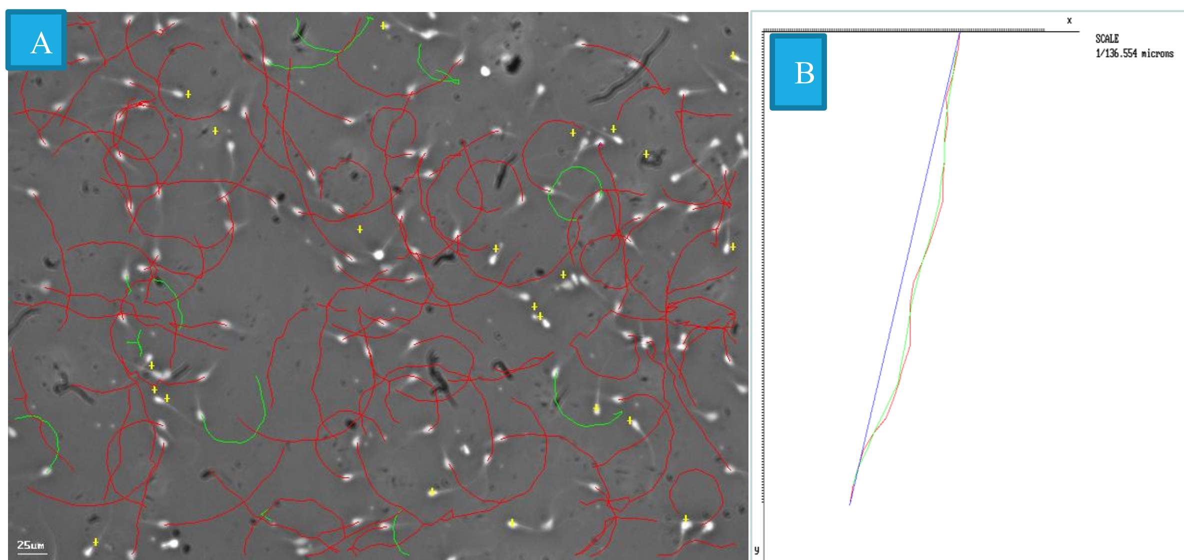

Plate 3.5 (A) Illustration showing different categories of bull sperm motion traits by CASA terminology and **(B)** illustration showing individual sperm linearity%

A 5 µL sample of diluted raw or frozen thawed semen were transferred to a warmed microscope slide (~76 x 26 x 1mm-Wadmar-Knittel, Germany) and covered with a warm cover slip (22 x 22 mm-Wadmar-Knittel, Germany) on the microscope warm plate, adjusted to 37 °C (Omron®, Japan). Two or three fields microscopic were captured (approximately 300 sperm) of each donor semen sample under the microscope glass slide (Mphaphathi *et al*., 2012). Thus, the focus knob was used to focus on the visible sperm found in microscope glass slide - coverslip. The track of individual sperm was identified and captured at 10 X magnification (Nikon®, Japan), before analysis and recorded on a Microsoft Excel® sheet. The field was visually assessed to remove possible debris and to reduce the risk of unclear tracks.

3.6.4 Sperm morphology evaluation

A 7 µL semen sample was mixed with 20 µL of a Nigrosin-Eosin staining solution into an Eppendorf (Simport, Canada) tube. Smears were prepared by taking a 5 µL drop of the raw (fresh) or diluted semen, smearing it across a glass slide and air-drying at room temperature. Sperm cells were evaluated for live normal, dead, live sperm with abnormalities on the head or mid-piece or tail abnormalities (percentages) (Brito *et al*., 2002; Oliveira *et al*., 2012). The sperm morphology was evaluated with the aid of a fluorescent microscope (BX51 TF, Olympus®, Japan). Total of 200 sperm were counted per bull/replicate.

3.7 Cryopreservation of ARC Loskop Nguni bulls semen

3.7.1 Nguni bull semen dilution, equilibration and cryopreservation

Following semen and sperm motility traits analyses, the samples were diluted with an egg yolk citrate (EYC) extender and diluted semen samples were equilibrated for 2 hours at 5 °C. At the end of the equilibration period, the semen sample was diluted further with EYC-fraction B

(containing 12 % Glycerol cryoprotectant). Diluted semen samples were further equilibrated for additional 2 hours at 5 ºC.

The semen samples were either loaded into 0.25 or 0.5 mL French straws and sealed with a polyvalent powder and frozen using a programmable freezer (CBS, USA), attached to the control rate freezer controller (Nobilis, China). The controlled rate freezer started from 5 to 4 ºC at a cooling rate of 0.08 (ºC/min) with holding period of 5 min, then from 4 to -130 ºC at the cooling rate of 6.0 (ºC/min), with a holding period of 10 min at -130 ºC. After freezing, the frozen semen straws were plunged directly into a liquid nitrogen (-196 °C) tank (CBS, XC 47/11) for storage until, used (AI or IVF).

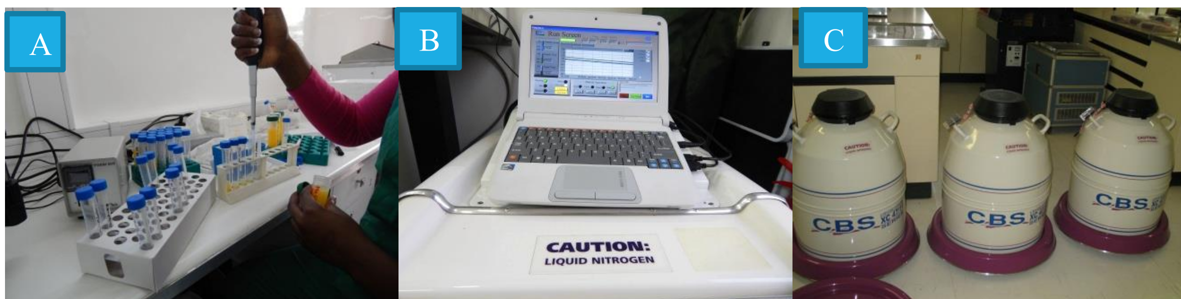

Plate 3.6 (A) Bull semen dilution with extenders, **(B)** freezing of semen straws with controlled freezer and **(C)** frozen semen straws stored in the liquid nitrogen tanks

3.7.2 Semen thawing, sperm motility and velocity traits evaluation before artificial insemination of cows

The frozen semen straws were removed from the liquid nitrogen tank (-196 °C) and exposed for 10 seconds in air then plunged into the electronic temperature control thawing unit (MiniTube®, Slovakia) central, built-in thawing chamber with a lift. The straws were exposed for 1 minute and the temperature was adjusted and maintained at 37 °C during the thawing process. The semen straw was dried of water, with disposable soft tissue. In brief, the semen

straw was cut at both ends and emptied into a 0.6 mL micro-centrifuge tube (Simport, Canada). The volume of 5 μL semen was aspirated using a hand pipette (Rainin, USA), fitted with a pipette tip. The SCA® was used for sperm motility and velocity traits evaluation, as described before (3.2.2). The sperm motility and velocity traits were evaluated immediately.

Plate 3.7 (A) Liquid nitrogen tanks with frozen bull semen straws, **(B)** electronic thawing unit and **(C)** the CASA - SCA® system

3.8 Selection of the recipient Bonsmara, Nguni and Nguni type cows for oestrous synchronization and artificial insemination

3.8.1 Selection of Bonsmara cow recipients at the Mara Research Station

Non-lactating multiparous Bonsmara cows (n = 22), ranging in age from 5 to 7 years, with a BCS ranging from 3.0 to 4.5 (scale, 1 to 5), were used during the 2015 breeding season. Non-pregnant status in these cows was confirmed based on the records, rectal palpation and use of a portable scanner. The cows were also selected based on a mean lactation (2 to 3), clinically healthy and production of live calves during the previous breeding season. All cows were free from reproductive abnormalities, during the experimental period (February to March, 2015) and cows were maintained on natural pastures.

3.8.2 Selection of Nguni recipient cows at the Loskop farm

Non-lactating multiparous cows, ranging from 5 to 7 years of age, with a BCS ranging from 3 to 4 (scale, 1 to 5), were used in the year, 2010 (n = 146), 2012 (n = 292) and 2015 (n = 44). Non-pregnant status in these cows was confirmed based on the records, rectal palpation and use of a portable scanner. The cows were also selected based on a mean lactation (2 to 3), clinically healthy and the production of live calves during the previous breeding season. All cows were free from reproductive abnormalities, during the experimental period (January to March) and all cows were maintained on natural pastures.

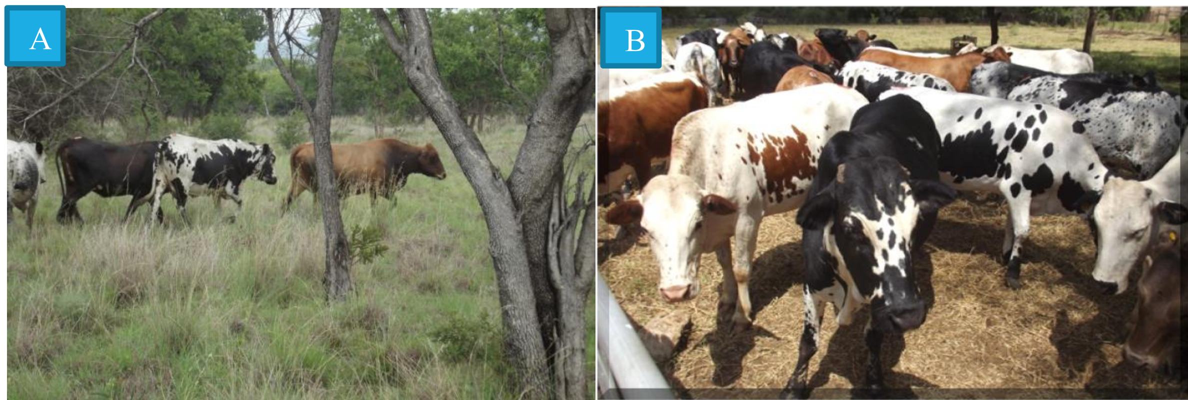

Plate 3.8 (A) Nguni cows on natural grazing pastures and **(B)** synchronized Nguni cows in ARC Loskop farm

3.8.3 Selection of Nguni type recipient cows of the emerging cattle farmers of KwaZulu Natal and Limpopo provinces

Lactating (at least three months postpartum) or dry cows ranging from 3 to 7 years of age, with a BCS ranging from 2 to 4 (scale of 1 to 5), were used in the years 2014 and 2015. Non-pregnant status in these cows was confirmed following rectal palpation and the use of a portable ultrasound scanner. The cows were also selected based on their health status and production of live calves during previous years. All cows were free from reproductive abnormalities, during

the experimental period and were maintained on natural pastures. The total number of cows selected and inseminated in the two provinces were, KZN (n = 68) and Limpopo province (n = 26).

Plate 3.9 (A) Selection of recipient cows in Limpopo province and **(B)** pregnancy diagnosis with the aid of a portable ultrasound scanner

3.8.4 Selection of recipient Bonsmara cows of the emerging cattle farmers of Limpopo province

In brief, lactating (at least three months postpartum) or dry cows, ranging from 4 to 6 years of age, with a BCS ranging between 2 to 4 (scale of 1 to 5), were recipients during the year 2011 (n = 52), 2012 (n = 29), 2014 (n = 13) and 2015 (n = 06), in the Limpopo province. The non-pregnant status in these cows was confirmed following rectal palpation and the use of a portable ultrasound scanner. The cows were also selected based on their health status and the production of live calves during previous years. All cows were free from reproductive abnormalities, during the experimental period and were maintained on natural pastures.

3.9 Oestrous synchronization of Bonsmara, Nguni and Nguni type cows

The oestrous cycles of the Nguni, Bonsmara and Nguni type cows were synchronized. All selected cows were subjected to a 9 day Ovsynch + controlled intravaginal drug release

(CIDR®) protocol. The oestrous synchronization protocol was as follows: Day – 16, supplementation of minerals and vitamins (optional); Day 0, insertion of the CIDR® devices (Pfizer, Animal Health, New Zealand) containing 1.9 g progesterone and estradiol benzoate (i.m); Day 5, GnRH (i.m. 2.5 mL); Day 8, removal of CIDR® and injection of prostaglandin ($PGF_{2\alpha}$) (i.m. 2 mL); Day 9, estradiol benzoate (i.m. 1 mL) and placing of heat mount detector and oestrous observation (signs of oestrus); Day 10, oestrous observation and AI (05h00, PM) and Day 11, repeat AI (06h00, AM).

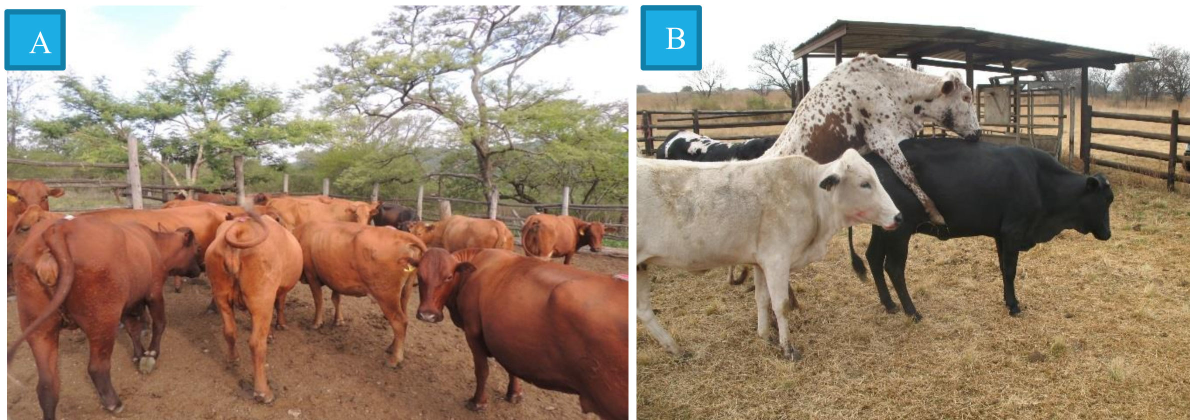

Plate 3.10 (A) Bonsmara and **(B)** Nguni type cows during oestrous synchronization

In brief, oestrous cycle synchronization was performed by inserting the CIDR® inside the reproductive tract of the recipient for 8 days. On the day of CIDR® removal, cows were administered 2 mL of $PGF_{2}\alpha$ (Estrumate®), oestrous signs were observed (visual observation of standing oestrus), done with the aid of heat mount (Kamar®) detectors (to reflect the response of the recipients). The detectors were placed on the tail head of the cows prior the commencement of oestrous activity. The percentage of oestrous response in cows was calculated as follows: Oestrous response = Number of cows in oestrous/Total number of cows synchronized X 100.

Plate 3.11 **(A)** Insertion of the CIDR®, **(B)** Nguni cows showing sign of heat and **(C)** heat mount detector on cow turned red following cow being mounted by another cow

3.10 Timed artificial insemination in Bonsmara, Nguni and Nguni type cows

The GameteTek Cryo-Mobile laboratory was used during thawing of semen straws and semen was immediately evaluated by CASA technology before each individual cow was inseminated. Bonsmara, Nguni and Nguni type cows were kept in the holding pen and inseminated at Day 10 following the removal of the CIDR® device by one experienced ARC technician. In brief, the hand was inserted into the rectum gently following wearing and applying lubricating gel and removal of the dung in cow's rectum.

All cows were artificially inseminated (AI) using frozen-thawed semen as follows. The semen straw was removed from the liquid nitrogen tank (-196 ºC) and thawed as described previously (3.6.3). A thawed semen straw was then loaded into the AI pistolette. The loaded AI pistolette was covered with a sanitary sheath and wrapped in a sanitized sleeve. The vulva of the cow was cleaned with a disposable paper towel. The insemination pistolette was introduced gently into the vulva, vagina, cervix and into the body of uterus, while the other hand manipulated the cervix through the rectum. Semen was then deposited inside the uterus body and the pistolette was removed slowly. The body of uterus was massaged and the vulva (clitoris) was also stimulated. The insemination pistolette was then removed, the sanitary sheath and the straws

were checked if the semen had been expelled successfully (the cotton wool should have moved to the other end of the semen straw).

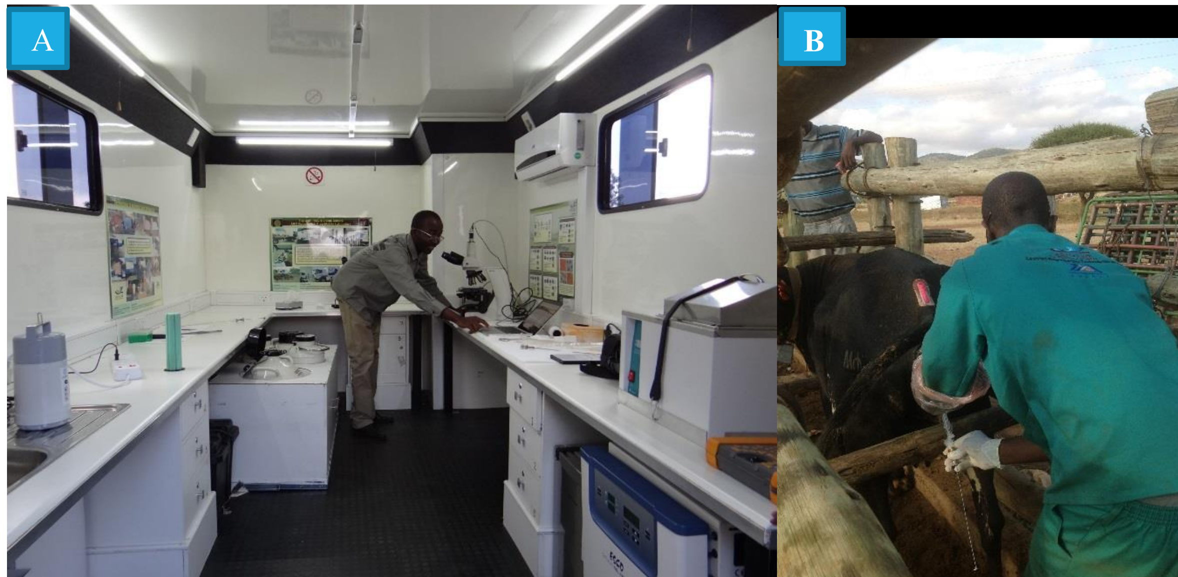

Plate 3.12 **(A)** Evaluation of frozen/thawed semen before AI in an ARC GameteTek Cryo-mobile laboratory on the field and **(B)** conducting AI in synchronized Nguni type cow following semen thawing

3.11 Pregnancy diagnosis in the Bonsmara, Nguni and Nguni type cows

After 90 days of TAI, pregnancy diagnosis (PD) was performed on the cows by the rectal palpation (traditional method) and also with the aid of linear probe attached to a portable ultrasound scanner (Ibex pro^{TM}, E.I. Medical Imaging, USA) to visualize foetal viability. The ultrasound scanner gel was smeared on the scanner probe. The probe was then inserted gently into the rectum and placed over the horn, in search of the foetus. The detection of an embryonic vesicle with a viable embryo (presence of heartbeat or skeletal bones) was used as an indicator of pregnancy. The scanner provided a black and white image. The pregnant and non-pregnant cows were recorded. Pregnant cows were closely monitored during the entire gestation period.

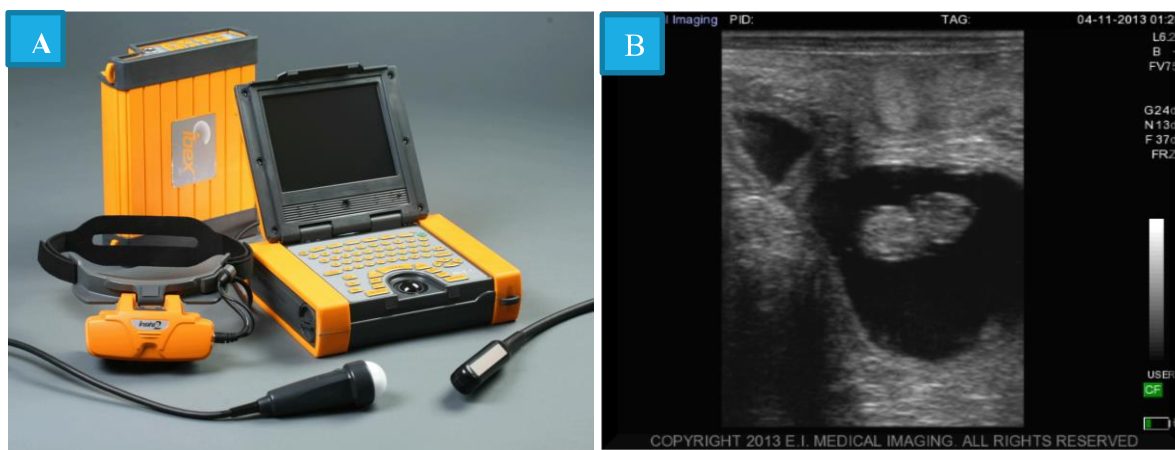

Plate 3.13 (A) The ARC portable ultrasound scanner for pregnancy diagnosis and **(B)** diagnosed foetus in the pregnant cow as observed on the scanner following AI

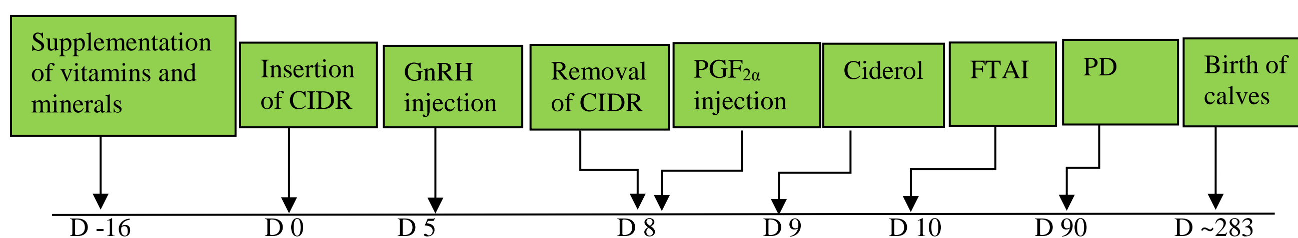

Figure 3.1 Flow diagram of the oestrus synchronization, FTAI and PD in the recipient cows. CIDR®= controlled intravaginal drug release; GnRH= Gonadotropin releasing hormone; $PGF_{2\alpha}$= prostaglandin; FTAI= fixed timed artificial insemination; PD= pregnancy diagnosis.

3.12 The *in vitro* fertility assessment on cow oocytes with thawed semen of Bonsmara and Nguni bulls

3.12.1 Penetration of oocytes through *in vitro* fertilization

In vitro fertilization technique was used as *in vitro* condition to measure the semen fertility from both Bonsmara and Nguni breed. Heterogeneous ovaries of unknown reproductive status cows were collected from the local slaughter house and transported to the laboratory in a normal saline solution in a thermos-flask at 37 °C, within 2 hours of slaughter. The retrieved

COCs were subsequently submitted to routine *in vitro* maturation (IVM) and IVF techniques. The COCs were matured in TCM 199 (Sigma) in a petri dish. Oocyte maturation was performed in Epidermal Growth Factor (EGF) medium (composed of M199 + 10 % FBS and Sodium pyruvate) for 22 hours in an atmosphere of 20 % O_2 and 5 % CO_2 at 39 ºC and a relative humidity of 100 % (Walters *et al*., 2004).

3.12.2 Semen thawing and *in vitro* fertilization

The temperature was adjusted and maintained at 37 °C during the semen thawing process, as described previously (3.6.3). In brief, frozen semen straws were removed from the liquid nitrogen tank (-196 °C) and exposed for 10 seconds in air then plunged into the electronic temperature control thawing unit. The straws were exposed for 1 minute and the temperature was adjusted and maintained at 37 °C during the thawing process. The semen straws (Nguni or Bonsmara bulls) were cut at the both ends and emptied into the 15 mL tube and 5 µL of semen was aspirated by a hand pipette, fitted with a pipette tip. The CASA-SCA® system was used for sperm motility and velocity traits evaluation, as described previously (3.2.2).

Oocytes were fertilized (1 x 10^6 sperm/mL) in 100 µL droplets (final volume) of BO-IVF medium. The individual semen donor (Bonsmara and Nguni) frozen/thawed semen straws were used under the same IVF conditions. Following fertilization (18 hours), presumptive zygotes were freed of the cumulus cells by vigorous pipetting and of excessive sperm and transferred to a culture medium [(synthetic oviductal fluid (SOF)-bovine serum albumin (BSA) and foetal bovine serum (SOF-BSA/SOF-FBS)].

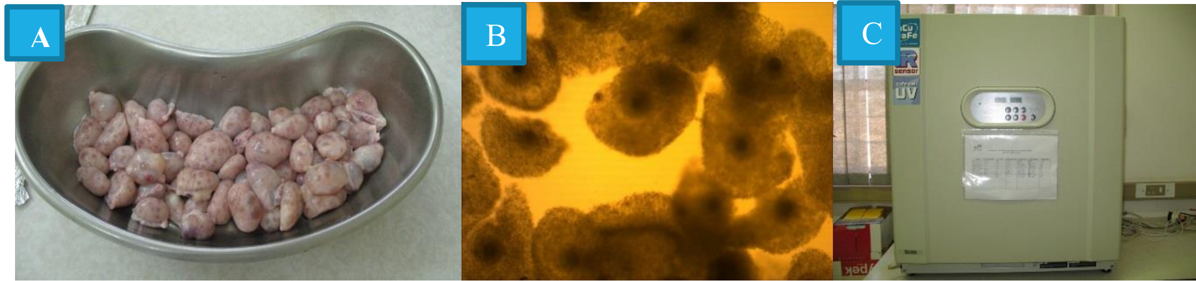

Plate 3.14 **(A)** Collected cow's ovaries, **(B)** matured cow's oocytes **(C)** CO_2 incubator for culturing of presumptive zygotes

3.13 Data analysis

Data was analyzed using GenStat® statistical programme. A significance level of $P < 0.05$ was used. Treatment means were compared using the Fisher's protected t-test least significant difference. The data was presented as mean±standard deviation (S.D).

CHAPTER 4

RESULTS

4.1 Characterization of body measurements and semen traits of Nguni bulls

The body measurements of the Nguni bulls results are set out in Table 4.1. The age of the bulls ranged from 6 to 7 years, scrotal circumference from 32 to 38 cm, with an average of 35.4 cm. Bull IDI 06-11 recorded a slightly bigger scrotal circumference (38.0 cm), compared to the other bulls ($P > 0.05$). The average BCS of 4 and a rectal body temperature of 38.5 °C was recorded for all the bulls. There was a significant difference on body weight, length and skin thickness among the bulls ($P < 0.05$).

More than 90 % of the Nguni bull sperm motility was recorded to be alive and normal (Table 4.2), with an overall average sperm motility of 92.1 %. The Nguni bull number IDI 08-48, recorded a slightly higher percentage of viable sperm (94.9 %) than bull IDI 06-88 (92.3 %), IDI 06-11 (91.1 %) and IDI 08-71 (90.1 %). The percentage of live sperm with mid-piece abnormalities differed significantly between the bulls ($P < 0.05$). There was no significant difference in live sperm with tail abnormalities.

Table 4.1 Body measurements of Nguni bulls

Bulls no	Age (years)	Body condition score (1 to 5)	Body weight (kg)	Shoulder height (mm)	Body length (mm)	Skin thickness (mm)	Scrotal circumference (cm)	Rectal temperature (°C)
IDI 06-11	5	4	535.3±2.8^{a}	1.3±0.1	1.6±0.0^{a}	1.1±0.1^{d}	38.0±1.3	38.6±0.4
IDI 06-88	5	4	506.2±3.0^{b}	1.3±0.1	1.5±0.0^{b}	1.7±0.1^{b}	35.8±1.0	38.5±0.4
IDI 08-48	7	4	445.0±3.3^{c}	1.2±0.0	1.3±0.0^{d}	1.2±0.1^{c}	35.0±1.3	38.4±0.6
IDI 08-171	7	4	443.8±3.6^{c}	1.3±0.0	1.4±0.0^{c}	1.7±0.1^{a}	32.6±1.3	38.5±0.5
Average	6	4	482.6±3.2	1.0±0.0	1.5±0.0	1.4±0.1	35.4±1.2	38.5±0.0

$^{a-d}$ Values with different superscripts in a column differ significantly ($P < 0.05$).

Table 4.2 Morphological characteristics of Nguni bull sperm cells

Bulls No	Live normal (%)	Dead (%)	Live sperm abnormalities (%)	
			Mid-piece	Tail
IDI 06-11	91.1±5.7	8.6±5.1	0.2±0.4^{b}	0.2±0.2
IDI 06-88	92.3±5.3	7.0±5.2	0.7±0.5^{b}	0.1±0.2
IDI 08-48	94.9±2.3	4.6±2.4	0.5±0.4^{b}	0.0±0.0
IDI 08-171	90.1±4.4	8.0±4.3	1.8±1.1^{a}	0.1±0.2
Average	92.1±4.4	7.1±4.3	0.8±0.6	0.1±0.1

^{a,b} Values with different superscripts in a column differ significantly ($P < 0.05$).

The average Nguni bull ejaculated semen volume ranged from 3.3 to 4.4 mL, while the semen pH ranged from 6.9 to 7.2 and the sperm cell concentration varied between 37.9 and 59.2 x 10^9 sperm/mL (Table 4.3). Numerically, Bull IDI 08-171 recorded a slightly higher semen volume (4.4 mL) than the other donors. Furthermore, Bull IDI 06-11 had slightly higher sperm concentration (59.2 x 10^9 sperm/mL) than others, but no significant difference were found ($P > 0.05$).

Table 4.3 Characterization of ejaculated semen volume, pH and sperm concentration in Nguni bulls

Bull No	Semen volume (mL)	Semen pH	Sperm concentration (x 10^9 sperm/mL)
IDI 06-11	3.4±0.8	7.1±0.2	59.2±24.9
IDI 06-88	3.6±2.0	7.2±0.3	37.9±12.5
IDI 08-48	3.3±1.7	6.9±0.2	57.0±23.9
IDI 08-171	4.4±2.5	7.0±0.3	43.8±14.1
Average	3.7±1.8	7.1±0.3	49.5±18.9

No significant differences ($P > 0.05$).

The Pearson correlation coefficients between semen traits and body measurements of Nguni bulls are set out in Table 4.4. The coefficients were lower to higher with a negative or positive correlation values ranging from r = - 0.5 to 0.9. The highest relationship was between body length and body weight (r = 0.9), followed by scrotal circumference and body weight (r = 0.8). Rectal temperature and semen pH showed a medium positive correlation (r = 0.4) and also body length and scrotal circumference (r = 0.4). A negative correlation was observed between semen volume and sperm concentration (r = - 0.0) also between body weight and BCS (r = - 0.1).

Table 4.4 Pearson correlation coefficients for body measurements and semen traits in Nguni bulls

	Body weight	Semen volume	Semen pH	Sperm concentration	Scrotal circumference	Shoulder Height	Body length	Skin thickness	Rectal temperature	BCS
Body weight	1.00									
Semen volume	0.3	1.00								
Semen pH	0.3	0.3	1.00							
Sperm concentration	0.1	-0.0	0.2	1.00						
Scrotal circumference	0.8	0.3	0.2	0.2	1.00					
Shoulder height	0.4	0.2	-0.1	0.1	0.3	1.00				
Body length	0.9	0.2	0.2	-0.1	0.4	0.4	1.00			
Skin thickness	-0.3	-0.2	-0.1	-0.4	-0.5	-0.2	0.1	1.00		
Rectal temperature	0.1	-0.2	0.4	0.3	-0.1	0.0	0.2	-0.0	1.00	
BCS	-0.1	0.1	-0.1	-0.2	0.1	-0.2	-0.3	-0.2	-0.5	1.00

BCS= body condition score.

There was an individual bull sperm motility variation; however, it was not statistically different. The individual bull sperm motility variation ranged from 89.5 to 95 % with an average of 93.0 % as set-out in Table 4.5. There was a higher numerical total sperm motility (95.6 %) for bull IDI 08-171. There were no significant differences in sperm PM, NPM, RAP, MED, SLW and STC percentages.

Table 4.5 Individual Nguni bulls sperm motility traits in fresh semen

Bull No	TM (%)	PM (%)	NPM (%)	RAP (%)	MED (%)	SLW (%)	STC (%)
IDI 06-11	93.3±1.7	45.9±12.6	47.5±13.5	83.7±6.4	8.8±5.0	1.1±1.1	6.7±1.7
IDI 06-88	89.5±8.5	45.1±12.4	44.4±11.4	81.3±12.5	7.2±5.1	1.1±1.3	10.5±8.5
IDI 08-48	93.4±3.5	50.5±16.1	42.9±16.0	84.8±4.2	8.2±5.0	0.5±0.6	6.6±3.5
IDI 08-171	95.6±3.5	52.9±8.4	41.3±7.4	88.5±5.4	5.3±4.8	1.8±3.2	4.4±3.5
Average	93.0±4.3	48.6±12.4	44.0±12.1	84.6±7.1	7.4±5.0	1.1±1.6	7.1±4.3

No significant differences ($P > 0.05$).

TM= total motility; PM= progressive motility; NPM= non progressive motility; RAP= rapid; MED = medium; SLW = slow; STC = static.

There was no significant difference on sperm VSL (µm/sec), VAP (µm/sec), LIN (%), STR (%) and BCF (Hz) (Table 4.6). The sperm trajectories were regular and linear. Individual bull sperm had significant difference on WOB and HPA among the bulls ($P < 0.05$). The average sperm VCL, LIN, STR and BCF was 168.7 (µm/sec), 45.5 %, 66.8 % and 22.1 %, respectively.

Table 4.6 Nguni fresh semen velocity sperm traits

Bull No	VCL (µm/sec)	VSL (µm/sec)	VAP (µm/sec)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)	HPA (%)
IDI 06-11	150.2±17.4^{b}	70.2±14.8	111.9±15.3	46.7±7.9	62.4±7.2	74.5±4.2^{a}	2.7±0.2^{c}	21.5±2.5	9.9±4.1^{b}
IDI 06-88	188.4±23.7^{a}	79.0±19.9	112.1±20.0	41.9±8.7	69.8±5.5	59.6±8.3^{b}	4.4±1.2^{a}	21.5±5.4	17.5±7.5^{a,b}
IDI 08-48	167.5±22.8^{a,b}	73.6±20.8	106.7±16.2	43.9±10.1	67.9±8.8	64.0±7.8^{b}	3.7±0.7^{a,b}	22.0±4.4	24.2±13.0^{a}
IDI 08-171	168.6±17.7^{ab}	83.4±10.6	124.1±7.2	49.4±3.3	67.1±6.5	74.0±6.6^{a}	3.1±0.6^{b,c}	23.5±4.2	16.3±8.3^{a,b}
Average	168.7±20.4	76.6±16.5	113.7±14.7	45.5±7.5	66.8±7.0	68.0±6.7	3.5±0.7	22.1±4.1	17.0±8.2

$^{a-c}$ Values with different superscripts within the column differ statistically.

VCL= curvilinear velocity; VSL= straight line velocity; VAP= average path velocity; LIN= linearity; STR= straightness; WOB= wobble; ALH= amplitude of lateral head displacement; BCF= beat cross frequency; HPA= hyperactive.

4.2 Characterization of body measurements and semen traits of Bonsmara bulls

The body measurements of the Bonsmara bulls are set out in Table 4.7. The body weight and scrotal circumference length of the bulls ranged from 607.3 to 871 kg and 41.0 to 43.3 cm, respectively. However, bull BM 2 had a slightly bigger scrotal circumference (43.3 cm) as compared to the other bulls ($P > 0.05$). The average Bonsmara bull ejaculated volume ranged from 3.8 to 4.8 mL, while the averaged semen pH and sperm concentration was 7.4 and 48.8 x 10^9 sperm/mL, respectively. Numerically, bull BM 3 recorded a slightly higher semen volume (4.8 mL) than the other donors ($P > 0.05$). Furthermore, Bull BM 2 recorded a slightly higher sperm concentration (53 x 10^9 sperm/mL), when compared to the other ($P < 0.05$) bulls.

Table 4.7 Characterization of ejaculated Bonsmara semen volume, pH and sperm cell concentration

Bull No	Body weight (kg)	Scrotal circumference (cm)	Semen volume (mL)	Semen pH	Sperm concentration (x 10^9 sperm/mL)
BM 1	668.3±3.5^b	41.8±0.7	4.7±0.9	7.2±0.3^b	45.9±1.6^{a,b}
BM 2	871.0±3.6^a	43.3±1.5	4.8±0.7	7.2±0.4^b	53.6±6.9^a
BM 3	607.3±5.0^d	41.0±1.0	3.8±0.9	7.2±0.2^b	52.3±4.6^a
BM 4	648.3±4.5^c	42.7±1.5	4.6±0.6	7.8±0.1^a	43.4±3.8^b
Average	698.7±4.2	42.2±1.2	4.5±0.8	7.4±0.3	48.8±4.2

$^{a-c}$ Values with different superscripts in a column differ significantly ($P < 0.05$). BM= Bonsmara.

Above 85 % of the Bonsmara bull sperm morphology were recorded to be alive and normal ($P < 0.05$), with an overall average of 89.4 % (Table 4.8). The Bonsmara bull number BN 3, had a numerical higher percentage of viable sperm morphology (93.0 %) compared to BN 2 (90.3 %), BN 1 (89.0 %) and BN 4 (85.3 %) ($P > 0.05$). The percentage of live sperm with mid-

piece abnormalities differed significantly between the bulls ($P < 0.05$). There was no recorded significant difference in live sperm with tail abnormalities.

Table 4.8 Morphological characteristics of Bonsmara bull sperm cells

Bulls No	Live normal (%)	Dead (%)	Live sperm abnormalities (%)	
			Mid-piece	Tail
BM 1	89.0 ± 2.6^{b}	8.0 ± 1.7^{b}	0.5 ± 0.5^{b}	2.7±1.5
BM 2	$90.3\pm1.5^{a,b}$	$6.0\pm1.0^{b,c}$	1.6 ± 0.5^{a}	2.0±1.0
BM 3	93.0 ± 1.0^{a}	4.0 ± 1.0^{c}	$1.3\pm0.6^{a,b}$	1.7±0.6
BM 4	85.3 ± 1.5^{c}	11.3 ± 2.1^{a}	$1.3\pm0.6^{a,b}$	2.0±1.0
Average	89.4±1.7	7.3±1.5	1.2±0.6	2.1±1.0

$^{a-c}$ Values with different superscripts in a column differ significantly ($P < 0.05$). BM= Bonsmara.

The individual bull's total fresh sperm motility ranged from 86.5 to 93.9 % and recorded an overall average of 91.3 % (Table 4.9). Higher total sperm motility of 93.9 % was recorded on bull BM 3 ($P > 0.05$). There were no significant differences in the sperm TM, PM, NPM, RAP, MED and SLW rate as set out in Table 4.9. The average sperm TM recorded was 91.3 % on Bonsmara fresh semen. Numerically, bull BM 3 recorded a slightly higher sperm TM (93.9 %) and rapid (67.2 %) than the other bulls ($P > 0.05$).

Table 4.9 Individual fresh sperm motility traits for Bonsmara bulls

Bull No	TM (%)	PM (%)	NPM (%)	RAP (%)	MED (%)	SLW (%)
BM 1	92.9±2.5	30.3±24.2	62.6±22.6	61.5±19.1	25.3±9.4	6.1±9.8
BM 2	86.5±2.0	44.4±16.4	42.0±15.4	48.4±39.0	31.0±31.4	7.1±9.6
BM 3	93.9±3.7	35.7±7.6	58.1±7.4	67.2±16.3	17.3±9.1	9.5±15.6
BM 4	91.7±8.4	30.6±13.9	60.9±14.8	41.5±14.4	26.3±8.2	23.9±11.6
Average	91.3±4.2	35.3±15.5	55.9±15.1	54.7±22.2	25.0±14.5	11.7±11.6

Values did not differ statistically within a column ($P > 0.05$).

BM= Bonsmara; TM= total motility; PM= progressive motility; NPM= non progressive motility; RAP= rapid; MED = medium; SLW = slow.

There was no significant difference in sperm velocity (VSL, LIN, STR and BCF) as indicated in Table 4.10. The sperm trajectories had no variation, as they were recorded to be regular and linear. Individual bull sperm VCL (µm/sec) and VAP (µm/sec) differed among the bulls ($P < 0.05$). The bulls had an average sperm VCL, LIN and STR of 128.3 (µm/sec), 45.5 %, 52.7 % and 60.7 %, respectively.

Table 4.10 Individual fresh sperm velocity traits for Bonsmara bulls

Bull No	VCL (µm/sec)	VSL (µm/sec)	VAP (µm/sec)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
BM 1	132.8±21.1	59.4±35.1	48.9±39.9^{a,b}	43.5±19.9	49.8±21.9	86.9±2.0	3.1±0.2^{a,b}	5.6±1.7
BM 2	123.6±46.7	73.7±24.3	45.1±10.8^{b}	61.4±10.1	69.7±16.1	88.8±6.3	2.6±0.2^{b}	6.9±3.0
BM 3	142.9±26.7	76.1±8.2	59.0±31.2^{a,b}	54.2±8.9	62.1±11.7	87.7±2.7	3.6±0.5^{a}	6.9±0.9
BM 4	113.8±16.6	57.7±4.7	59.1±13.5^{a,b}	51.8±11.4	59.3±12.8	87.2±3.9	3.3±0.6^{a,b}	6.7±0.4
Average	128.3±27.8	66.7±18.1	53.0±23.8	52.7±12.6	60.2±15.6	87.7±3.7	3.2±0.4	7.9±1.5

$^{a-b}$ Values with different superscripts within the column differ statistically.

BM= Bonsmara; VCL= curvilinear velocity; VSL= straight line velocity; VAP= average path velocity; LIN= linearity; STR= straightness; WOB= wobble; ALH= amplitude of lateral head displacement; BCF= beat cross frequency.

4.3 Cryopreservation of bull semen

4.3.1 Characterization of frozen-thawed sperm traits of the individual Bonsmara and Nguni bulls, using the CASA-SCA® system

The average total sperm motility (TM) rate of frozen-thawed semen from ARC Nguni bulls was 92.0 %, ARC Bonsmara 81.0 %, Nguni commercial-artificial insemination (Co- AI) 79.5 % and Bonsmara Co-AI 82.8 % (Table 4.11). Interestingly, individual Nguni bull IDI 06-11 (51.7 %) and Bonsmara bull JGC 94-320 (48.0 %) had the highest post-thaw sperm cell moving in a rapid rate compared to Nguni AG 03-177 (40.6 %) and Bonsmara HJB 05-045 (30.4 %) ($P < 0.05$). The PM%, NPM% and STC% sperm differed significantly between the breeds and among the bulls ($P < 0.05$).

The sperm trajectories were regular and linear ($P<0.05$) and showed a moderate ALH (µm) post-thaw. Individual bull sperm had no significant difference on thawed sperm traits [(VCL (µm/sec), VSL (µm/sec) and VAP (µm/sec)] among the bulls ($P<0.05$) (Table 4.12). The WOB (%) was similar for the ARC Nguni bulls ($P>0.05$). The STR (%), BCF (Hz) and HPA (%) differed significantly between the breeds and among the bulls ($P<0.05$) regarding the thawed semen.

Table 4.11 The mean (±S.D.) sperm traits (%) of the frozen-thawed bull sperm of individual semen from Bonsmara and Nguni bulls analyzed by the CASA-SCA® system

Breed/ bull no.	TM (%)	PM (%)	NPM (%)	RAP (%)	MED (%)	SLW (%)	STC (%)
Nguni (ARC)							
IDI 06-11	94.6±1.6[a-c]	38.8±8.0[a-c]	55.8±8.4[a-c]	51.7±14.8[a]	39.6±12.7[c,d]	3.2±2.6[c,d]	5.4±1.6[d-f]
IDI 06-88	85.1±13.4[c-e]	21.3±3.0[e-g]	63.8±15.7[a,b]	18.8±12.4[c,d]	57.7±11.7[a]	8.5±7.9[b-d]	14.9±13.4[b-d]
IDI 08-48	90.8±4.7[a-d]	34.4±6.8[b-d]	56.4±8.1[a-c]	20.2±9.1[c,d]	62.4±6.8[a]	8.1±5.1[b-d]	9.1±4.7[c-f]
IDI 08-171	97.6±3.3[a]	44.3±6.6[a,b]	53.4±6.4[a-c]	22.3±8.2[c,d]	62.1±6.3[a]	13.2±9.9[b]	2.3±3.3[e-f]
Average	92.0±11.5	34.7±6.1	57.3±9.7	28.2±11.1	55.4±9.4	8.2±6.4	7.9±5.7
Bonsmara (ARC)							
AG 03-205	83.5±10.1[c-e]	25.6±10.7[d-f]	57.9±13.5[a-c]	18.7±9.2[c,d]	41.8±11.7[c,d]	23.0±6.4[a]	16.5±10.1[b-d]
AG 07-418	57.2±6.9[f]	33.5±2.1[b-d]	23.7±4.8[d]	16.0±0.9[c,d]	36.3±10.2[d]	4.9±2.2[b-d]	43.3±7.6[a]
HJB 05-045	97.8±2.3[a]	49.4±0.8[a]	48.4±1.4[c]	30.4±4.2[b,c]	55.4±7.0[a,b]	12.3±3.5[b,c]	2.0±2.5[f]
AG 03-177	85.6±5.1[b-e]	30.9±11.2[c-e]	54.7±13.6[a-c]	40.6±7.4[a,b]	41.1±6.8[c,d]	3.9±1.7[b-d]	14.4±5.1[b-e]
Average	81.0±6.1	34.8±6.2	46.2±8.3	26.4±5.4	43.6±8.9	11.0±3.4	19.0±6.3
Nguni (Co-AI)							
GJR 07-014	74.6±11.6[e]	47.5±10.1[a]	27.0±12.5[d]	29.1±13.2[b-d]	42.6±5.0[c,d]	2.9±1.8[c,d]	25.4±11.6[b]
GJR 03-046	84.4±7.3[c-e]	31.7±9.7[c-e]	52.7±9.6[b,c]	26.4±9.4[b-d]	51.3±9.9[a-c]	6.7±3.0[b-d]	15.6±7.3[b-d]
Average	79.5±9.4	39.6±9.9	39.8±11.0	27.7±11.3	46.9±7.4	4.8±2.4	20.5±9.4
Bonsmara (Co-AI)							
JGC 94-194	79.6±16.4[d,e]	11.6±6.2[g]	68.0±15.9[a]	14.3±7.4[d]	61.5±11.7[a]	3.8±3.1[b-d]	20.4±16.4[b,c]
JGC 94-320	86.1±8.7[a-e]	18.2±5.2[f,g]	67.9±9.3[a,b]	48.0±11.9[a]	36.3±6.6[d]	1.7±1.4[d]	13.9±8.7[b-f]
Average	82.8±12.5	14.9±5.7	67.9±12.6	31.1±9.6	48.9±9.1	2.7±2.2	17.1±12.5

[a-f] Values with different superscripts in a column differ significantly.

TM= total motility; PM= progressive motility; NPM= non progressive motility; RAP= rapid; MED = medium; SLW = slow; STC = static; ARC= Agricultural Research Council; Co-AI= commercial artificial insemination center.

Table 4.12 The mean (±S.D) sperm velocity traits of individual Bonsmara and Nguni frozen-thawed semen analyzed by the CASA-SCA® system

Breed/ bull no.	VCL (µm/sec)	VSL (µm/sec)	VAP (µm/sec)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)	HPA (%)
Nguni (ARC)									
IDI 06-11	109.0±17.6^{a,b}	43.2±3.6^{a}	65.2±5.7^{a}	40.5±7.0^{a,b}	66.5±6.4^{a-d}	60.4±5.4^{a-c}	3.0±0.6^{a-c}	18.4±1.0^{a}	12.9±4.0^{a}
IDI 06-88	72.0±18.5^{d}	24.0±5.7^{c,d}	43.0±10.6^{b,c}	33.6±4.4^{b,c}	56.0±6.3^{d-f}	60.0±3.0^{a-d}	2.2±0.2^{e,f}	10.3±2.8^{f}	2.0±1.4^{d,e}
IDI 08-48	71.1±17.2^{d}	27.8±7.0^{c,d}	42.7±10.6^{b,c}	39.1±3.7^{a,b}	65.1±3.4^{a-d}	60.0±2.7^{a-d}	2.3±0.3^{d-f}	14.8±2.0^{a-d}	5.6±2.5^{b-d}
IDI 08-171	71.0±13.6^{d}	33.0±5.7^{b,c}	46.2±8.2^{b,c}	47.0±5.2^{a}	71.2±4.2^{a-c}	65.4±3.5^{a}	2.2±0.3^{e,f}	16.4±1.0^{a-c}	7.6±2.4^{b,c}
Average	80.8±16.7	32.0±5.5	49.3±8.8	40.0±5.1	64.7±5.1	14.6±3.6	2.4±0.3	15.0±1.7	7.0±2.3
Bonsmara (ARC)									
AG 03-205	64.0±13.7^{d}	24.5±7.3^{c,d}	37.4±8.5^{c}	38.3±8.1^{a,b}	65.0±7.8^{a-d}	58.6±5.7^{b-d}	2.6±0.6^{b-e}	14.1±6.4^{b-e}	4.8±3.6^{c,e}
AG 07-418	73.3±2.0^{d}	33.6±0.3^{a-c}	44.0±0.2^{b,c}	46.0±0.7^{a}	76.6±0.2^{a}	59.9±1.1^{a-d}	2.4±0.1^{c-f}	15.2±1.1^{a-d}	1.6±0.5^{d,e}
HJB 05-045	75.4±8.6^{d}	25.6±3.2^{c,d}	39.5±9.5^{c}	18.9±6.6^{d}	34.4±8.7^{g}	42.7±1.9^{e}	3.3±0.1^{a}	17.4±1.3^{a,b}	2.3±0.7^{d,e}
AG 03-177	101.1±9.3^{a-c}	34.0±6.9^{a-c}	54.6±5.8^{a,b}	33.5±5.1^{b,c}	61.8±7.1^{c-e}	54.0±2.4^{c,d}	3.3±0.2^{a}	14.8±1.8^{a-d}	7.9±4.4^{b,c}
Average	78.4±8.4	29.4±4.4	43.9±6.0	34.2±5.1	59.4±5.9	53.8±2.8	2.9±0.2	15.4±2.6	4.1±2.3
Nguni (Co-AI)									
GJR 07-014	88.2±10.6^{b-d}	40.1±4.4^{a,b}	53.3±2.9^{a,b}	46.2±8.3^{a}	75.2±5.9^{a,b}	61.1±7.9^{a,b}	2.8±0.3^{a-e}	15.6±4.1^{a-d}	9.7±5.7^{a,b}
GJR 03-046	81.8±11.6^{c,d}	29.2±4.6^{c}	45.6±4.5^{b,c}	35.8±3.2^{b,c}	64.0±5.8^{b-d}	56.0±2.9^{b-d}	2.8±0.5^{a-d}	13.0±1.8^{c-e}	3.5±2.5^{b-d}
Average	85.0±11.1	34.6±4.5	49.4±3.7	41.0±5.7	69.6±5.8	86.5±5.4	2.8±0.4	14.3±2.9	6.6±4.1
Bonsmara (Co-AI)									
JGC 94-194	67.8±8.4^{d}	18.8±2.2^{d}	42.1±5.2^{b,c}	27.9±2.3^{c}	45.0±4.6^{f,g}	62.3±4.5^{a,b}	2.0±1.0^{f}	8.9±1.8^{f}	0.5±0.5^{e}
JGC 94-320	117.7±16.3^{a}	31.7±4.5^{b,c}	62.6±6.6^{a}	27.0±2.5^{c,d}	50.7±5.3^{e,f}	53.5±3.1^{d}	3.2±0.7^{a,b}	12.3±1.8^{e-f}	2.5±1.8^{d,e}
Average	92.7±12.3	25.2±3.3	52.3±5.9	27.4±2.4	47.8±4.9	57.9±3.8	10.6±0.8	10.6±1.8	1.5±1.1

$^{a-f}$Values with different superscripts in a column differ significantly ($P < 0.05$). VCL= curvilinear velocity; VSL= straight line velocity; VAP= average path velocity; LIN= linearity; STR= straightness; WOB= wobble; ALH= amplitude of lateral head displacement; BCF= beat cross frequency; HPA= hyperactive.

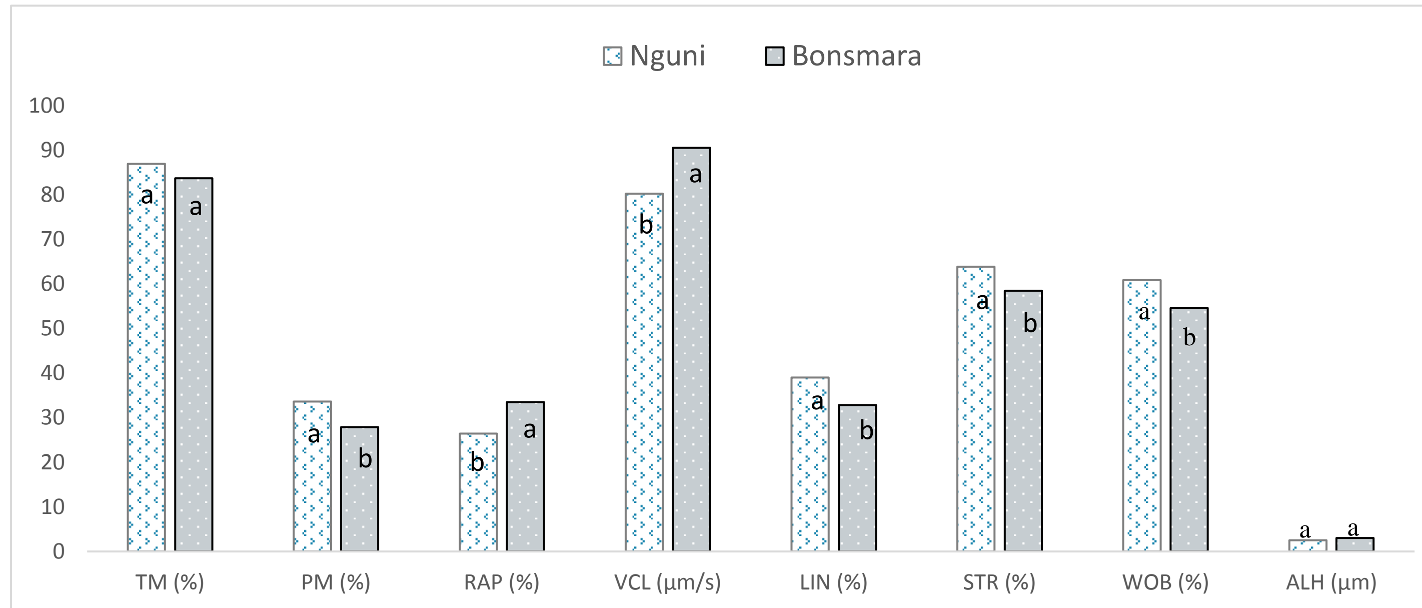

Figure 4.1 Nguni and Bonsmara bull variation regarding sperm motility and velocity traits following thawing

[a,b] Different superscript across the bars differ statistically. TM= total motility; PM= progressive motility; RAP= rapid; VCL= curvilinear velocity; LIN= linearity; STR= straightness; WOB= wobble; ALH= amplitude of lateral head displacement.

The Nguni and Bonsmara bulls recorded an average sperm total motility (TM%) of 87.0 % and 83.7 % after thawing ($P > 0.05$), respectively (Figure 4.1). The rapid sperm recorded was 26.4 % and 33.5 % for the Nguni and Bonsmara bulls, respectively ($P < 0.05$). A significant difference ($P < 0.05$) was recorded between the Nguni and Bonsmara bulls regarding sperm VCL (µm/s), LIN%, STR%, WOB% and ALH (µm).

4.4 Synchronization and artificial insemination of cows

4.4.1 Cow synchronization and artificial insemination at the ARC Loskop farm

The ARC Nguni cows that responded to the oestrous synchronization (93.9 %) were inseminated. Consequently, a pregnancy rate of 45.3 % was archived. However, more born calves were female (61.6 %). There was a positive correlation (r = 0.54) between age and the cows body weight at the initial start of oestrous synchronization protocol (Table 4.13). Moreover, there was a relationship between body weight and age of the cows at the final stage of oestrous synchronization protocol (r = 0.46). Interestingly, the gestation length and age of cows was negatively affected (r = -0.20).

Table 4.13 Pearson correlation coefficients of Nguni cow body weight and pregnancy

	Cow age	Cows initial BW	Cows final BW	Calf BW	Gestation length
Cow age	1.00				
Cows initial BW	0.54	1.00			
Cows final BW	0.46	0.68	1.00		
Calf BW	-0.01	0.03	0.08	1.00	
Gestation Length	-0.20	-0.47	-0.18	0.24	1.00

BW = body weight.

Plate 4.1 Nguni calves born at the ARC Loskop farm following oestrous synchronization and fixed timed artificial insemination in Nguni cows.

Plate 4.2 (A) Limpopo province Bonsmara calves born following oestrous synchronization and fixed timed artificial insemination.

4.4.2 All cows synchronized and artificial inseminated in the study sites

The oestrous response ($P<0.05$) was 83.0 %, 87.7 % and 84.0 % for the Nguni, Nguni type and Bonsmara cows, respectively. The Nguni type cows recorded a significant higher pregnant rate (65.7 %), compared to the Bonsmara (59.0 %) and Nguni (37.0 %) breeds ($P<0.05$). The majority of the recipients had an average BCS of 3, and a BCS of 4 was only recorded in the Bonsmara cows.

Table 4.14, indicate the sperm traits results evaluated by the computer assisted sperm analysis (CASA) and correlation with pregnancy rate. It was found that there was a relationship ($r = 0.52$) between oestrous response and pregnancy rate. Interestingly, rectal temperature during AI was positively correlated with oestrous response ($r = 0.04$). Moreover, there was a positively correlation ($r = 0.72$) between cow body weight (BW) and pregnancy rate. Surprisingly, there was a negative correlation ($r = -0.08$) between the BCS and oestrous response rate.

Table 4.14 The overall Pearson correlation coefficients of Bonsmara, Nguni and Nguni type cows oestrous response and pregnancy

	Pregnancy rate	Oestrous response	Cow BW	BCS	Rectal T at AI	TM	PM	RAP
Pregnancy rate	1.00							
Oestrous response	0.52	1.00						
Cow BW	0.72	-0.08	1.00					
BCS	0.01	-0.08	-0.31	1.00				
Rectal T at AI	-0.05	0.04	0.19	-0.15	1.00			
TM	0.06	0.08	0.24	-0.26	-0.14	1.00		
PM	0.03	0.06	0.11	-0.24	0.01	0.43	1.00	
RAP	0.08	0.07	0.20	0.06	-0.08	0.64	0.59	1.00

BW= body weight; BCS= body condition score; T= temperature; AI= artificial insemination; TM= total motility; PM= progressive motility; RAP= rapid.

4.5 The *in vitro* fertilization of cattle oocytes to test sperm fertility

A total of 368 oocytes were subjected to IVF (Table 4.15). The sperm TM% used ranged from 85 to 96 % for Bonsmara and Nguni bulls. There was no difference in cleavage rate between the breeds of semen donors used ($P > 0.05$). There was no significant difference on total cleavage rate which ranged from 15 to 19 %. There were positive correlations ($r = 0.30$) recorded between sperm TM% and total cleaved oocytes (Table 4.16). The sperm PM% and the 8 cell stage was negatively correlated ($r = -0.09$).

Table 4.15 *In vitro* fertilization rate of matured oocytes following insemination by sperm cell from Bonsmara or Nguni semen

Breed	TM (%)	Number of oocytes (IVF)	Cleavage (%)				Total cleaved (%)
			Lys	1 cell	2-4 cells	≥8 cells	
Nguni ARC	96.1±2.9[a]	88	10.3±3.9	11.4±4.4	9.4±3.9	6.4±3.5	15.8±7.4
Nguni CO	85.2±5.6[b]	105	13.4±2.3	10.0±3.4	12.0±3.5	7.8±3.1	19.8±6.6
Bonsmara ARC	92.9±4.7[a,b]	81	10.3±2.1	10.3±6.1	9.7±3.2	9.3±7.5	19.0±10.7
Bonsmara CO	87.7±2.9[b]	94	12.5±5.5	9.5±5.0	10.5±3.9	6.2±4.8	16.7±8-7
Total/average	90.5±4.0	368	11.6±3.5	10.3±4.7	10.4±3.6	7.2±4.7	17.6±8.3

[a,b] Values with different superscripts in the column differed significantly ($P < 0.05$).

TM= total motility; IVF= *in vitro* fertilization; ARC= Agricultural Research Council; Co= commercial.

Table 4.16 Pearson correlation coefficient for bull sperm traits (sperm motility and velocity rate) and the oocyte fertilization (cleavage) rate

Parameter	TM	PM	NPM	RAP	MED	SLW	STC	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF	HPA	8 cell	2-4 cell	Lys	TC
TM	1.00																			
PM	0.83	1.00																		
NPM	-0.68	-0.97	1.00																	
RAP	0.87	0.81	-0.70	1.00																
MED	-0.73	-.073	0.65	-0.97	1.00															
SLW	-0.86	-0.70	0.56	-0.72	0.53	1.00														
STC	-0.99	-0.83	0.68	-0.87	0.72	0.86	1.00													
VCL	0.73	0.53	-0.39	0.91	-0.92	-0.59	-0.72	1.00												
VSL	0.89	0.82	-0.71	0.96	-0.91	-0.73	-0.89	0.89	1.00											
VAP	0.83	0.71	-0.59	0.96	-0.94	-0.64	-0.83	0.95	0.97	1.00										
LIN	0.73	0.90	-0.89	0.55	-0.43	-0.62	-0.73	0.25	0.65	0.49	1.00									
STR	0.82	0.86	-0.79	0.68	-0.53	-0.79	-0.82	0.48	0.77	0.61	0.89	1.00								
WOB	0.46	0.72	-0.76	0.29	-0.21	-0.28	-0.46	-0.03	0.37	0.26	0.86	0.53	1.00							
ALH	0.59	0.28	-0.11	0.71	-0.69	-0.52	-0.59	0.85	0.65	0.70	-0.01	0.37	-0.39	1.00						
BCF	0.70	0.64	-0.54	0.80	-0.77	-0.62	-0.69	0.80	0.89	0.88	0.57	0.70	0.28	0.52	1.00					
HPA	0.91	0.89	-0.79	0.96	-0.89	-0.76	-0.91	0.82	0.97	0.93	0.73	0.83	0.44	0.61	0.61	1.00				
8 cells	0.37	-0.09	0.29	0.38	-0.31	-0.52	-0.37	0.60	0.34	0.40	-0.25	0.12	-0.58	0.78	0.38	0.28	1.00			
2-4 cells	0.22	0.01	0.08	0.42	-0.43	-0.40	-0.23	0.64	0.44	0.47	-0.13	0.27	-0.55	0.71	0.36	0.38	0.78	1.00		
Lys	0.60	0.36	-0.34	0.56	-0.57	-0.48	-0.31	0.60	0.50	0.48	0.08	0.40	-0.31	0.66	0.41	0.48	0.42	0.68	1.00	
TC	0.30	-0.03	0.17	0.43	-0.40	-0.47	-0.29	0.66	0.42	0.47	-0.19	0.22	-0.59	0.78	0.55	0.36	0.91	0.96	0.61	1.00

TM = total motility; PM = progressive motility; NPM = non progressive motility; RAP = rapid; MED = medium; SLW = slow; STC = static; VCL= curvilinear velocity; VSL= straight line velocity; VAP= average path velocity; LIN= linearity; STR= straightness; WOB= wobble; ALH= amplitude of lateral head displacement; BCF= beat cross frequency; HPA= hyperactive; TC = total cleaved.

CHAPTER 5

DISCUSSION

5.1 Characterization of body measurements and semen traits of Bonsmara and Nguni bulls

For the first time this study provided the detailed information on the characterization of Bonsmara and Nguni bull sperm cell evaluated by the CASA technology and their relationship with pregnancy rate. The baseline information consists of macroscopic (semen volume (mL), semen pH and sperm concentration ($x10^9$/mL) traits evaluated. The microscopic sperm traits evaluated included sperm morphology, total sperm motility (rapid, medium and slow) progressive and non-progressive sperm motility. The sperm velocity characteristics included curvilinear and straight-line velocity, average path velocity, linearity, straightness, wobble, amplitude of lateral head displacement, beat cross frequency and hyperactive data.

The average ejaculated semen volume of Bonsmara and Nguni bulls was 4.5 mL and 3.7 mL, respectively. The ejaculated semen volume from Bonsmara and Nguni breeds were comparable in the present study. However, it was similar to the overall ejaculated semen volume obtained from Cholistani breeding bulls (4.45 mL) as recorded by Farooq *et al*. (2013). The semen volume values of other breeds of bulls of 9.3, 11.5, 9.8, 12.9, 7.4, 12.8 and 9.9 mL was in contrast with the reported Local, Friesian, Sahiwal, Shahiwa × Friesian, Local × Friesian, two times Local × Friesian and three times Local × Friesian breeds, respectively (Hossain *et al*., 2012). The breed may also influence variation on the semen volume of bulls. Interestingly, Nguni bull semen volume was similar to the crosses of Local × Sahiwal bulls where the semen

volume averaged 3.7 mL (Latif *et al*., 2009). Moreover, dairy bulls (Holstein bulls) have been reported to have high average semen volume of 6.8 mL (Masoumi *et al*., 2011).

The Bonsmara semen pH ranged from 7.2 to 7.8, with an average value of 7.4 pH recorded in the present study. The semen pH from both breeds were found neutral. The Nguni semen pH ranged from 6.9 to 7.2, with an average value of 7.1 pH recorded in the present study. Rahman *et al*. (2014) reported a lower mean semen pH of 6.4 in four different cross bred bulls (Holstein-Friesian × Zebu, Sahiwal × Zebu, Sindhi × Zebu and Red Chittagong Bull), this might be the breed effect. Moreover, similarly to the report by Akhter *et al*. (2013) in Sahiwal x Friesian bulls. The 7.2 pH semen of Local x Friesian x Friesian breed was comparable with Nguni semen pH on our study. An average semen pH of 6.9 was also recorded in Kankrej bulls (Patel & Siddiquee, 2013). The semen pH is generally correlated to sperm motility traits and the metabolic rate in bulls. Bull sperm were found to tolerate a semen pH of 6.0 to 8.0 in the present study with an acceptable sperm TM in Bonsmara and Nguni bulls semen (fresh semen).

Sperm cell concentration differed significantly among individual Bonsmara bulls averaged 48.8×10^9 sperm/mL. However, sperm concentration did not differ significantly among individual Nguni bulls (average sperm concentration 49.5×10^9 sperm/mL), indicating homogeneity within the Nguni breed. Interesting, dairy bulls have been reported to have higher semen volume but Nasrin *et al*. (2008) reported low sperm concentration of 1.0×10^9 sperm/mL in Holstein Friesian × Local, Local x Holstein Friesian, Sahiwal x Holstein, Sindhi x Local, Red Chittagong and Red Chittagong.

The Nigrosin/Eosin staining solution was used to stain sperm of Bonsmara and Nguni bulls to determine the live and dead sperm cell. Most of the ejaculates with live and normal sperm were

above 85.3 % in the Bonsmara bulls, with an average mean of 89.4 %. Nguni bulls ejaculates with live and normal sperm were above 90.1 % with an average mean of 92.1 %. There were individual variations in the proportion of their live and normal sperm morphology in Bonsmara bulls in the present study, indicating heterogeneous within the breed. However, no individual variation was recorded in the Nguni bulls in the present study. A lower percentage (72.8 %) was reported in Friesland bulls by Vilakazi and Webb (2004) and by Sosa *et al*. (2002) of ≥ 50 % in Wagyu bulls. Higher sperm defects were found to be higher on Corriente than European-breed bulls on the study done by Quezada-Casada *et al*. (2016). The breed may also influence variation on the live and normal sperm percentages of bulls.

Individual variation ejaculates (raw semen) of the Bonsmara and Nguni bulls sperm TM traits (ranged from 86.5 to 93.9 % and 89.5 to 95 %, respectively) were recorded. With an overall average of 91.3 % and 93.0 % of sperm TM for Bonsmara and Nguni bulls were recorded in the present study, respectively. Chacur *et al*. (2012) reported the total sperm motility of 70 to 80 % in individual Nelore bulls which was comparable with the results of the present study. With the use of CASA system, several sperm motility traits describing the specific movements of sperm can be obtained in greater detail than what is possible in subjective evaluations (Vincent *et al*., 2012). No significant difference was recorded between bulls regarding the sperm PM, NMP and RAP traits studied in Bonsmara and Nguni breed on fresh (raw) semen. High repeatability of measurements was observed for most of the sperm traits assessed. There was a trend observed on higher sperm motility percentages in older (79.49 %), compared to younger bulls (77.12 %) of the Sahiwal breed (Galmessa *et al*., 2014).

The bulls must be physically sound, achieve a minimum scrotal circumference and have good semen quality to be considered satisfactory potential breeders (Palmer & Barth, 2003). In this

study the average scrotal circumference of 42.2 and 35.4 cm was recorded in Bonsmara and Nguni bulls, respectively. A scrotal circumference of 33.5 cm was reported in Nelore bulls by Martins *et al*. (2013) and was comparable with the ones of the Nguni bulls used in the present study. Furthermore, Sosa *et al*. (2002) reported the scrotal circumferences of 32 to 35 in Hereford bulls.

5.2 Cryopreservation of bull semen

The present study was conducted to quantify bull sperm traits measured by the CASA technology in an attempt to find the relationship between pregnancy rate and cryopreserved sperm traits from Bonsmara and Nguni bulls following insemination. In this study, the average frozen-thawed sperm TM of 92.2 %, 81.0 %, 79.5 % and 82.8 % was recorded on ARC-Nguni, ARC- Bonsmara, Nguni Co-AI and Bonsmara Co-AI bulls, respectively. Individual cryotolerance on sperm motility variation was also evident in the present study. The successful of freezing can also be seen by assessed its recovery rate, by comparing the sperm TM of raw semen with post-thaw values. It was evident in the present study with highest recovery rate (98.9 %) in ARC- Nguni bulls. The post thaw sperm TM of 77.8 % was previously reported in Nguni bulls (Seshoka *et al*., 2016) and was numerically lower compared to the present findings. In addition, Awad (2011) also reported the total bull sperm motility rate of 72.4 % from frozen-thawed semen in an extender containing 3 % Glycerol used with tris-egg yolk. The present study used the 12 % of Glycerol with egg yolk citrate extender.

The success of sperm cryopreservation depends on many factors, including the type of cattle breed. In the present study, it was found that there were no individual variation especially within ARC-Nguni bulls. The bulls were kept under the same management practices throughout the study. In a study done by Farooq *et al*. (2013) on Cholistani bulls, the fresh

semen sperm motility recorded was 64.4 %, followed by a post-thaw value of 54.0 %, which was generally lower compared to the post-thaw results (92.2 %, 81.0 %, 79.5 % and 82.8 %) obtained in the present study. The type of semen extender, cryoprotectants concentration and freezing method (freezing curve) might be some of the cause of this variation with the other previous study results.

A significant difference was recorded between the sperm motility of Holstein semen frozen with Triladyl (31.8%) and a Biociphos (64.1 %) extender (Amirat *et al*., 2005), this might be the effect of breed and type of extender used on their study as compared to the current study results. The thawing of straws at 37 °C for 30 seconds resulted in a 65.2 % sperm motility in Friesian bulls (Al-Badry, 2012). A post-thaw sperm motility of 36.1 % and 42.6 % was also recorded in Holstein bulls (Stefanov *et al*., 2006; Masoumi *et al*., 2011). The post-thaw sperm motility has always been a commonly used trait for evaluation of frozen-thawed semen in bulls (Mathur *et al*., 2014). The evaluation of sperm motility rate is one of the most important traits for the assessment of an individual semen sample (Dorado *et al*., 2011). Sperm quality generally differs greatly from one bull to the other within the same breed and between breeds. Semen from certain bulls may be of acceptable quality following collection and processing, but might not survive well to the cryopreservation process (Person *et al*., 2007).

5.3 Synchronization and oestrous response in cows

In the present study, the oestrous response of 83 %, 90.8 % and 84.0 % was recorded for the Bonsmara, Nguni and Nguni type breeds, respectively, showing no breed differences. Mattoni and Ouedraogo (2000) recorded a lower percentage of oestrous response of 73 % on the Baoule and 64 % on the Zebu cows that exhibited signs of oestrous, following synchronization, compared to the present study. A comparable oestrous response rate of 89.6 % was recorded in

dairy cows by Xu *et al*. (1997), when PGF2alpha with or without supplementation of progesterone was used. The present study used a beef cattle breeds and the inclusion of CIDR® during oestrous synchronization protocol. Different synchronization protocols are associated with different estrous response in different cattle breeds. The higher oestrous responses of 100 % and 99 % have been previously reported in village cows by Maqhashu *et al*. (2016) when the same protocol was used as in the present study and 100 % in Nelore cows by Ali *et al*. (2014). Synchrony of estrous response is also dependent on synchrony of ovarian follicular waves among a group of cows/heifers undergoing estrous synchronization program. The current available protocols use varying strategies to induce follicular synchrony.

To date, the use of different methods of oestrous detection reported has resulted in a different detection efficiency (Roelofs *et al*., 2010). Many devices are used to help in oestrous detection, such as pedometers, mount devices, hormone measurements, etc. The current study used the heat mount detector and acceptable indication of oestrous response by cows were adequate in all the three cattle breeds. Beef herds that have more than 50 % anoestrous cows at the start of the breeding season may benefit from protocols that promote ovulation in response to GnRH, before initiating a FTAI program (Hill *et al*., 2014). The expression and detection of oestrous signs is crucial, so that TAI can be performed at the suitable time relative to ovulation. Unfortunately, the proportion of cows that stand to be mounted has been reported to reduce from 80 to 50 % and the period of oestrous to shorten from 15 to 5 hours over the past 50 years (Dobson *et al*., 2008). It was suggested that, stress must be kept minimum by appropriately feeding and housing high-production cows and prevent production diseases, etc (Dobson *et al*., 2008). The present study recorded the oestrous response of above 83 % in all the cattle breeds and showed the effectiveness of the synchronization protocol used.

5.4 Sperm fertility test *in vivo* by artificial insemination of cows following oestrous synchronization

An average pregnancy rate of 59.0 %, 37.1 % and 65.7 % for the Bonsmara, Nguni and Nguni type was recorded in the current study respectively, following semen evaluation prior to AI by CASA technology in Gametek Cryo-mobile laboratory. According to a newspaper titled "*Mobile lab does cows a service*" (Wild, 2014), before the establishment of the Gametek Cryo-mobile laboratory, the conception rate was substantially lower, averaging 40 %. With the Gametek Cryo-mobile laboratory, the conception rate has increased to 62 % because it was done on the field, which is a figure similar to the ones obtained by commercial farmers (Wild, 2014). A pregnancy rates of 53.3 % and 57.0 % following AI has been reported between the treatments groups in crossbred beef cattle (Howard *et al*., 2007) and was comparable with the results of Bonsmara and Nguni type cows used in the present study.

The overall conception rate in this study was 53.9 % which was higher than the results reported by Mattoni and Ouedraogo (2000) on Zebu and Baoule cattle breeds. Interestingly, Oliveira *et al*. (2012) reported also a lower conception rate of 49.1 %, 47.6 % and 52.7 % in Nelore cows following oestrous cycle synchronization and AI. This might be the effect of breed and the quality of semen used during AI. Our laboratory previously reported almost a similar pregnancy rate of 56.5 % in Nguni type cows following synchronization and timed AI (Maqhashu *et al*., 2016).

Reproductive failure in beef cows is considered to be one of the main economic loss causes for beef calves produced worldwide (Aono *et al*., 2013). The failure of cows to successfully establish and maintain pregnancy after insemination is one of the important limiting factors for the efficiency of beef cattle production systems in South Africa. The sperm motility is a vital

element of good viable sperm cell, since it has to travel through the female reproductive tract, to the fallopian tube where fertilization should take place (Govindaraju *et al*., 2012; Rodríguez *et al*., 2015). The frozen-thawed semen with acceptable post-thaw sperm motility (confirmed by CASA system) was deposited into the cows body of uterus in the present study and all the breeds followed the same protocol.

A higher ambient temperature was previously reported as one of the major factors associated with reduced fertility in farm animals (Hansen, 2009). It was documented that the quality of a cow oocyte and embryo are more compromised during hot season than cool season (Monty & Racowsky, 1987; AI-Katani *et al*., 2002). The current study was conducted during summer (natural breeding season) and the effect of ambient temperature might have played a negative role on oocyte and embryo further development. The elevated ambient temperature together with humidity causes changes in the animal physiology core temperature, being one of main factors responsible for low reproduction performance during hot seasons (Ju, 2005). A positive correlation was found between the cow rectal temperature (39 to 42 °C) and a high occurrence of abnormal embryos (Rivera *et al*., 2004).

5.5 Sperm fertility test during *in vitro* fertilization of slaughter house cow oocytes

The total IVF rate obtained ranged from 15 to 19 % in the present study. Thara and Nair (2007) reported an IVF rate of 2.8 to 28.2 % in beef cows, which was comparable with the current finding. A significant higher *in vitro* fertilization rate of 67.3 % was recorded when Holstein cows ovaries obtained from anonymous donors at a commercial abattoir were used (Wilson *et al*., 2006). Dairy cows ovaries were used on their study while present study used the beef cattle breed. Sex sorted sperm was used during IVF on their study while the present study used non-sexed sperm. The results are contradictory with the current findings. This may be due to the

effect of the breed and mediums that were used during IVF process. In the present study, age of the cows under current ordinary IVF procedures might be another element of lower fertilization rate of research importance, especially when cows of different ages and sources were used in the present study.

It has been reported that a sire can have a major effect on the outcome of IVF rate (Hillery *et al*., 1990) and also in terms of fertilization and blastocyst formation rate (Ward *et al*., 2003). The fertilization rate means ranged from 43.6 to 71.1 % (Costa *et al*., 2010) which was higher than that observed in the present study. The differences between studies in the effect of *in vitro* fertilization rate probably relate to differences in the handling of ovaries before to oocyte recovery (e.g. time from slaughter to oocyte recovery), method of oocyte recovery (aspiration or slicing) the size range of follicles from which oocytes were recovered.

The outcome of the IVF rate has been found to differ among sires (Shi *et al*., 1991; Zhang *et al*., 1999). Fertilization is a complex process requiring several steps that gametes must complete successfully (Ferraz *et al*., 2014). The IVEP technologies, especially in South African indigenous cattle, still have some challenges, which require further research (Nedambale & Mphaphathi, 2010). It was found that the presence of a high percentage of sperm that move rapidly and progressively in a bull ejaculate was positively and significantly correlated with the IVF (Ferraz *et al*., 2014). However, the finding on the present study was that, the sperm PM % was negatively correlated with the 8 cells cleavage stage, but the rapid sperm was positively correlated to IVF.

To date, we still do not have single *in vitro* sperm quality assessment method that can accurately predict sperm fertilizing potential. The CASA system was used to evaluate the post-thaw sperm

motility before IVF, in the present study. Although many efforts have been made, so far there are currently no available methods, or sperm assessment techniques that could accurately predict sperm fertilizing potential. If successful, such a method could contribute to improved herd fertility. There are still many unanswered questions regarding the likely, not yet established links between sperm quality and fertility i.e. the unknown traits of sperm quality that may be essential for fertilization. It is not yet clear if the CASA technology results (sperm motility traits) can be a successfully predicting tool to be used in synchronized cows during AI (just before the cows have to be inseminated) or IVF.

CHAPTER 6

GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 General conclusions

Different laboratories globally are moving away from subjective sperm evaluation to more objective analysis using CASA technology; therefore, CASA might bring standardized and uniform comparison for prediction of sperm fertility. Fertility is a multiparametric phenomenon that relies on the use of semen of sufficient quality and quantity for accurate timing of insemination, consequently appropriate herd management is essential. In this study, the average ejaculated semen volume of Bonsmara and Nguni bulls was 4.5 mL and 3.7 mL, semen pH was 7.4 and 7.1 and sperm concentration was 48.8 and 49.5 sperm/mL (x 10^9), respectively. The semen traits obtained in the present study was in agreement with the other previous authors (Chacur et al., 2012; Akhter *et al*., 2013).

The findings of this study showed that, more than 90 % of the Nguni bull sperm rate (fresh/raw semen) was recorded to be alive and normal, with an overall average of 92.1 %. The percentage of live sperm with mid-piece abnormalities differed significantly between the bulls ($P < 0.05$). There was no significant difference in live sperm with tail abnormalities on Nguni bulls. In contrast above 85 % of the Bonsmara bull sperm rate recorded were found to be alive and normal ($P<0.05$), with an overall average of 89.4 %. This suggest that the sperm damage might be more physiological than physical.

The individual Bonsmara bull’s total fresh (raw) sperm motility rate ranged from 86.5 to 93.9 % and with an overall average sperm motility rate of 91.3 %. There were no significant

differences in the sperm PM, NPM, RAP, MED and SLW rate. Similarly, individual Nguni bulls, total fresh (raw) sperm motility rate ranged from 89.5 to 95 % with an overall average of 93.0 %. There were no significant differences on fresh (raw) sperm PM, NPM, RAP, MED, SLW and STC rate. This result indicated homogeneity between the Bonsmara and Nguni cattle breeds.

The average sperm TM rate of frozen-thawed semen from ARC Nguni bulls was 92.0 % and higher compared to ARC Bonsmara 81.0 %, Nguni Co- AI 79.5 % and Bonsmara Co-AI 82.8 %. The sperm trajectories were regular and linear ($P < 0.05$), with the VCL (µm/sec), VSL (µm/sec) being similar to the VAP (µm/sec) and showed a moderate ALH (µm) on post-thawed semen for Bonsmara and Nguni bulls. In conclusion, the freezing-thawing process did not reduce the ARC- Nguni bull sperm TM post-thaw, compared with raw semen. However, a drastic decline was recorded on other breeds following semen thawing processes in the present study.

There was a statistical different on oestrous response of 83.0 %, 90.8 % and 84.0 % for the Bonsmara, Nguni and Nguni type cows, respectively ($P < 0.05$). However, Nguni type cows recorded a significant higher pregnant rate (65.7 %), compared to the Bonsmara (59.0 %) and Nguni (37.1 %) breeds ($P < 0.05$). The results showed that oestrous response and pregnant rate differs among Bonsmara, Nguni and Nguni type cows, even though they followed the same synchronization program. The sperm traits from Bonsmara and Nguni bulls were found to be related to *in vivo* conception rate when sperm traits were assessed by CASA technology. However, the pregnancy rate was lower in Nguni cows.

The total fertilization rate obtained ranged from 15 to 19 % in the present study, for Bonsmara and Nguni bulls. There was no difference in fertilization rate between the breeds (Bosmara and Nguni bulls) of used semen donors ($P > 0.05$). There were positive relationships ($r = 0.30$) recorded between sperm TM% and total fertilization rate. The sperm PM% and the 8 cell stage was negatively correlated ($r = -0.09$). The sperm traits from Bonsmara and Nguni bulls were found to be related to IVF rate when sperm traits were assessed by CASA technology. The low IVF rates obtained warrant more research in order to improve the IVM and IVF of cattle oocytes.

6.2 General recommendations

It is suggested that the following should be considered for future studies:

1. To conduct IVF with oocytes obtained through OPU from cows that are known for their fertility and are traceable. These techniques offer an alternative for producing offspring from genetic superior dams.
2. To use the GameteTek Cryo-Mobile laboratory to provide/serve (livestock reproduction) services to livestock farmers in the field since it is vital for rapid field work.
3. To use the CASA technology to quantify the sperm traits before artificial insemination (AI) of synchronized cows.
4. To identify which ovary has ovulated (with the use of an ultrasound scanner) and conduct FTAI on the site where ovulation occurred (intrauterine insemination).
5. To use the CASA technology to quantify the sperm traits prior to *in vitro* fertilization of cow's oocyte.

6.3 Implications

The CASA technology is a useful technique tool for identifying differences in sperm motility and velocity traits among bulls. Characterization of bull sperm cells based on those traits, could improve methods for assessing, selection of bull and improve the efficiency of AI and during IVF conditions.

QUANTIFICATION OF BULL SPERM TRAITS AS MEASURED BY CASA AND THE RELATIONSHIP TO PREGNANCY RATE FOLLOWING CONTROLLED BREEDING

by

MASINDI LOTTUS MPHAPHATHI

Study leader: Prof. T.L Nedambale

Co-study leader: Prof. J.P.C. Greyling

Department of Animal, Wildlife and Grassland Sciences

University of the Free State

Bloemfontein

Degree: M.Sc. (Agric)

Abstract

The biological importance of a bull's contribution through artificial insemination to reproductive efficiency is of great importance because the sperm represents half of the genetic composition of its progeny. Therefore, the main objectives of the current study were: (1) To characterise, compare and evaluate fresh (raw) and frozen-thawed semen of both Bonsmara and Nguni breeds using computer aided sperm analysis (CASA) technology, (2) to compare the oestrous synchronization response and conception rate of Bonsmara, Nguni and Nguni type cows following fixed timed artificial insemination (FTAI) with Bonsmara or Nguni semen, and (3) to find the relationship between cows conception rate (*in vivo* and *in vitro* fertilization) and bull sperm motility rate (sperm traits) assessed by CASA technology following insemination regardless of bull breed. Electro ejaculator method was used to collect semen from Bonsmara

(n = 4) and Nguni bulls (n = 4). Collected semen samples were evaluated for both macroscopic and microscopic traits.Following semen evaluation, the semen samples were either loaded into 0.25 or 0.5 mL straws and frozen using a programmable freezer. The semen straw was thawed at 37° C and analyzed by a CASA technology. In addition, purchased (from commercial AI center) frozen semen straws of Nguni and Bonsmara breed were also thawed. Sperm characteristics examined included total motility (rapid, medium and slow) progressive and non-progressive motility. Velocity characteristics included curvilinear and straight-line velocity, average path velocity, linearity, straightness, wobble, amplitude of lateral head displacement, beat cross frequency and hyperactive.

During *in vivo* sperm fertility test, 100 Bonsmara, 452 Nguni and 94 Nguni type cows were randomly selected and subjected to oestrous synchronization protocol and FTAI with frozen-thawed assessed semen by CASA before FTAI. Briefly at Day 0, cows were inserted with an intravaginal CIDR® and removed on Day 7. Prostaglandin was then administered on Day 08 and a heat mount detector was placed on the hind quarter of each cow. *In vitro* sperm fertility test, collected oocytes from slaughterhouse were *in vitro* matured (n = 360) and *in vitro* fertilized (1 x 10^6 sperm/mL) in 100 µL droplets (final volume) of BO-IVF medium per treatment bulls (Bonsmara or Nguni bull). The frozen/thawed semen straws of Bonsmara and Nguni bulls were randomly selected and were used under the same IVF conditions. The microscopic of thawed bulls sperm characteristics were examined by CASA prior to *in vitro* fertilization. Data was analyzed using ANOVA. Treatment means were compared using the Fisher's protected t-test least significant difference. The average ejaculated semen volume of Bonsmara and Nguni bulls was 4.5 mL and 3.7 mL, respectively. The Bonsmara semen pH was 7.2 to 7.8 and Nguni semen 6.9 to 7.2, with an average value of 7.4 and 7.1 semen pH recorded, respectively. Individual variation ejaculates (raw semen) of the Bonsmara and Nguni

bull's total sperm motility traits (ranged from 86.5 to 93.9 % and 89.5 to 95 %, respectively) were recorded. Sperm cell concentration differed significantly among individual Bonsmara bulls (48.8 × 10^9 sperm/mL). Most of the ejaculates with live and normal sperm were above 85.3 % and 90.1 % in the Bonsmara and Nguni bulls, respectively.

There was a significant difference on oestrous response for the Bonsmara (83.0 %), Nguni (90.8 %) and Nguni type cows (84.0 %), respectively. The Nguni type cows recorded a significant higher pregnancy rate (65.7 %), compared to the Bonsmara (59.0 %) and Nguni (37.1 %) breeds ($P < 0.05$). The sperm traits (TM, PM and RAP) were found to be positively correlated to conception rate ($r = 0.06$, 0.03 and 0.08, respectively). There was a significant difference on the average frozen-thawed sperm TM rate of ARC-Bonsmara (92.9 %), ARC-Nguni (92.2 %), CO-Nguni (85.2 %) and CO-Bonsmara (87.7 %). There was a positive correlation ($r = 0.52$) between oestrous response and pregnancy rate, for both Bonsmara, Nguni and Nguni type cows. There was a minimal fertilization rate following IVF with Bonsmara and Nguni breed bulls sperm.

There was significant relationship on sperm TM and fertilization rate for both Bonsmara and Nguni breed. In conclusion, Nguni cows had the highest oestrous response compared to Bonsmara cows; however, the pregnancy rate was lower in Nguni cows. The sperm traits from both Bonsmara and Nguni bulls were found to be related to *in vivo* conception and *in vitro* fertilization rate when sperm cells were assessed by CASA technology.

Key words: Cryopreservation, bull semen, extender, CASA, oestrous, synchronization, cows, FTAI, PD, IVF.

REFERENCES

Aboagla, E.M. & Terada, T., 2004. Effects of egg yolk during the freezing step preservative on the viability of goat spermatozoa. Theriogenology. 62, 1160-1172.

Acharya, T. & Devireddy, R.V., 2010. Cryomicroscopic investigations of freezing processes in cell suspensions. Open Biotechnol. J. 4, 26-35.

Acocks, J.P.H., 1988. Veld types of South Africa. National Botanical Institute, Pretoria, South Africa, 146.

AI-Katani, Y.M., Paula-Lopes, F.F. & Hansen, P.J., 2002. Effect of season and exposure to heat stress on oocytes competence in Holstein cows. J. Dairy. Sci. 85, 390-396.

Aisen, E., Medina, V. & Venturino, A., 2002. Cryopreservation and post thawed fertility of ram semen frozen in different trehalose concentrations. Theriogenology. 57, 1801-1808.

Aitken, J., 1994. On the future of the hamster oocyte penetration assay. Fertil. Steril. 62, 17-19.

Akhter, S., Azad, A.K., Rahman, Z. & Ashraf, A., 2013. Study on the Quality of Semen of Different Genetic Groups of Bull from Khulna Region of Bangladesh. Int. J. Pharm. Med. Res. 1 (1), 19-23.

Al-Badry, K.I., 2012. Effect of various thawing times and temperatures on frozen semen quality of Friesian bulls in Iraq. Inter. J. Anim. Vet. Adv. 4 (6), 384-388.

Alessandra, G., Michele, L.G., Angela, F., Flavia, P., Michele, N., Elena, D.M. & Fiorenza, M., 2010. Assessment of viability, chromatin structure stability, mitochondrial function and motility of stallion fresh sperm by using objective methodologies. J. Cell. Anim. Biol. 4 (2), 034-041.

Ali, M., Wahid, H., Rosnina, Y., Abas, M.O., Mohd, A.K. & Hishamfariz, M.A., 2014. Oestrus response and pregnancy rate after oestrus synchronization with exposure to light and heavy handling in Nelore cattle. Inter. J. Dev. Res. 4 (2), 369-376.

Amann, R.P. & Waberski, D., 2014. Computer-assisted sperm analysis (CASA): capabilities and potential developments. Theriogenology. 81 (1), 5-17.

Amann, R., 2004. Reflections on CASA after 25 years. J. Androl. 25, 317-325.

Ambrose, D.J., Colazo, M.G. & Kastelic, J.P., 2010. The applications of timed artificial insemination and timed embryo transfer in reproductive management of dairy cattle. R. Bras. Zootec. 39, 383-392.

Amirat, L., Anton, M., Tainturier, D., Chatagnon, G., Battut, I. & Courtens, J.J., 2005. Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. Reproduction. 129, 535-543.

Aono, F.H., Cooke, R.F., Alfieri, A.A. & Vasconcelos, J.L.M., 2013. Effects of vaccination against reproductive diseases on reproductive performance of beef cows submitted to fixed-timed AI in Brazilian cow-calf operations. Theriogenology. 79, 242-248.

Attia, S., Katila, T. & Andersson, M., 2016. The effect of sperm morphology and sire fertility on calving rate of Finnish Ayrshire AI bulls. Reprod. Domest. Anim. 51 (1), 54-58.

Awad, M.M., 2011. Effect of some permeating cryoprotectants on CASA motility results in cryopreserved bull spermatozoa. Anim. Reprod. Sci. 123 (3-4), 157-62.

Bage, R., Petyim, S., Larsson, B., Hallap, T., Bergqvist, A.S., Gustafsson, H. & Martinez, H.R., 2003. Oocytes competence in repeat-breeder heifers: effects of an optimized ovum pick-up schedule on expression of oestrus, follicular development and fertility. Reprod. Fert. Dev. 15, 115-123.

Bakhach, J., 2009. The cryopreservation of composite tissues: Principles and recent advancement on cryopreservation of different type of tissues. Organogenesis. 5 (3), 119-126.

Berlinguer, F., Madeddu, M., Pasciu, V., Succu S., Spezzigu, A., Satta, V., Mereu, P., Leoni, G.G. & Naitana, S., 2009. Semen molecular and cellular features: these parameters can reliably predict subsequent ART outcome in a goat model. Repro. Bio. Endocrin. 7, 125, 1-9.

Bissonnette N., Le´vesque-Sergerie, J.P., Thibault, C. & Boissonneault, G., 2009. Spermatozoal transcriptome profiling for bull sperm motility: a potential tool to evaluate semen quality. Soci. Reprod. Fert. 138, 65-80.

Blanco, J.M., Long, J.A., Gee, G., David E., Wildt, D.E. & Donoghue A.M., 2011. Comparative cryopreservation of avian spermatozoa: Benefits of non-permeating osmoprotectants and ATP on turkey and crane sperm cryosurvival. Anim. Reprod. Sci. 123, 242-248.

Blondin, P. & Sirard, M.A., 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocyte. Mol. Reprod. Dev. 41, 54-62.

Blondin, P., Bousquet, D., Twagiramungu, H., Barnes, F. & Sirard, M.A., 2002. Manipulation of follicular development to produce developmentally competent bovine oocytes. Biol. Reprod. 66, 38-43.

Bodmer, M., Janett, F., Hassig, M., den Daas, N., Reichert, P. & Thun, R., 2005. Fertility in heifers and cows after low dose insemination with sex-sorted and non-sorted sperm under field conditions. Theriogenology. 64, 1647-1655.

Bonsma, J., 2007. Livestock production – Man must measure. Agi Books, Cody, Wyoming, USA, pp 256.

Brito, L.F.C., Silva A.E.D.F., Rodrigues, L.H. & Kastelic, J.P., 2002. Effects of environmental factors, age and genotype on sperm production and semen quality in Bos indicus and Bos taurus AI bulls in Brazil. Anim. Reprod. Sci. 70 (3-4), 181-190.

Camargo, L.S.A., Viana, J.H.M., Sá, W.F., Ferreira, A.M., Ramos, A.A. & Vale Filho, V.R., 2006. Factors influencing in vitro embryo production. Anim. Reprod. 3 (1), 19-28.

Carvalho, N.A.T., Carvalho, E.L., Reis, M., Nichi, A.H., Souza, P.S. & Baruselli, P.S. 2008. Effect of early luteolysis in progesterone-based timed AI protocols in Bos indicus, Bos indicus x Bos taurus, and Bos taurus heifers. Theriogenology. 69 (2), 167-75.

Castellini, C., Bosco, A.D., Ruggeri S. & Collodel, G., 2011. What is the best frame rate for evaluation of sperm motility in different species by computer-assisted sperm analysis? Fertil. Steril. 96 (1), 24-27.

Chacur, M.G.M., Dias, H.S., Papa, P.M., Papa, F.O. & Melo-Onã, C.M., 2012. Effect of refrigeration systems upon frozen bull sperm viability assessed by computer-assisted sperm analysis and fluorescent probes. Ciências. Agrárias. Londrina. 33 (5), 1923-1930.

Cleland, D., Krader, P., McCree, C., Tang, J. & Emerson, D., 2004. Glycine betaine as a cryoprotectant for prokaryotes. J. Microbiol. Methods. 58, 31-38.

Collins-Lusweti, E., 2000. Performance of Nguni, Afrikander and Bonsmara cattle under drought conditions in the North West Province of Southern Africa. S. Afr. J. Sci. 30 (1), 33.

Costa, M.Z., Oliveira, L.Z., Resende, M.V., Lucio, A.C., Perini, A.P., Miguel, M.C.V. & Lima V.F.M.H., 2010. Induction of the acrosome reaction test to in vitro estimate embryo production in Nelore cattle. Arq. Bras. Med. Vet. Zootec. 62 (4), 771-777.

Coulter, G.H. & Foote, R.H., 1979. Bovine testicular measurements as indicators of reproductive performance and their relationship to productive traits in cattle: a review. Theriogenology. 11 (4), 297-311.

Dearing, C.G., Kilburn, S. & Lindsay. K.S., 2014. Validation of the sperm class analyser CASA system for sperm counting in a busy diagnostic semen analysis laboratory. Hum. Fertil. 17 (1), 37-44.

Dobson, H., Walker, S.L., Morris, M.J., Routly, J.E. & Smith, R.F., 2008. Why is it getting more difficult to successfully artificially inseminate dairy cows? Animal. 1104-1111.

Dorado, J., Rijsselaere, T., Muñoz-Serrano, A. & Hidalgo, M., 2011. Influence of sampling factors on canine sperm motility parameters measured by the Sperm Class Analyzer. Syst. Bio. Reprod. Medic. 57, 318-325.

El-Sheshtawy, R.I., Sisy, G.A. & El-Nattat, W.S., 2015. Effects of different concentrations of sucrose or trehalose on the postthawing quality of cattle bull semen. Asian. Pac. J. Reprod. 4 (1), 26-31.

Farooq, U., Ijaz, A., Ahmad, N., Rehman, H. & Zaneb, H., 2013. Investigations on semen quality and freezability of Cholistani breeding bulls - a preliminary study from Cholistan desert of Pakistan. The J. Anim. Plant. Sci. 23 (2), 359-363.

Farrell, P.B., Presicce, G.A., Brockett, C.C. & Foote, R.H., 1998. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenology. 49, 871-879.

Fenton, F.R. & Martinez, N.D., 1980. The optimum time for artificial insemination of cows in two climatic zones of Venezuela. Trop. Anim. Prod. 5 (3), 257-260.

Ferraz, M.A.M.M., Morató, R., Yeste, M., Arcarons, N., Pena, A.I., Tamargo, C., Hidalgo, C.O., Muiño, R. & Mogas, T., 2014. Evaluation of sperm subpopulation structure in relation to in vitro sperm-oocyte interaction of frozen-thawed semen from Holstein bulls. Theriogenology. 81, 1067-1072.

Ford, W.C., Williams, K.M., Harrison, S., Rees, J.M., Ray, B.D., McLaughlin, E.A. & Hull, M.G., 2001. Value of the hamster oocyte test and computerized measurements of sperm motility in predicting if four or more viable embryos will be obtained in an IVF cycle. Int. J. Androl. 24, 109-119.

Freneau, G.E., Chenoweth, P.J., Ellis, R. & Rupp, G., 2010. Sperm morphology of beef bulls evaluated by two different methods. Anim. Reprod. Sci. 118, 176-181.

Fricke, P.M., Caraviello D.Z., Weigel K.A. & Welle, M.L., 2003. Fertility of dairy cows after resynchronization of ovulation at three intervals following first timed insemination. J. Dairy. Sci. 86, 3941-3950.

Gączarzewicz, D., 2015. Influence of chamber type integrated with computer-assisted semen analysis (CASA) system on the results of boar semen evaluation. Pol. J. Vet. Sci. 18 (4), 817-824.

Gadea, J., 2003. Review: Semen extenders used in the artificial insemination of swine. Span. J. Agric. Res. 1, 17-27.

Galmessa, U., Mohanty, T.K., Raina, V.S., Gupta, A.K. & Prasad, S., 2014. Post-thawed and fresh spermatozoa motion characteristics of Sahiwal bulls under computer assisted semen analyser (CASA). Inter. J. Livest. Prod. 5, 65-70.

García-Vázquez, F.A., Gadea, J., Matás, C. & Holt, W.V., 2016. Importance of sperm morphology during sperm transport and fertilization in mammals. Asian J. Androl. 18, 844-850.

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

Geary, T.W., Whittier, J.C., Downing, E.R., LeFever, D.G., Silcox, R.W., Holland, M.D., Nett, T.M. & Niswender, G.D., 1998. Pregnancy rates of postpartum beef cows that were

synchronized using Syncro-Mate-B or the Ovsynch protocol. J. Anim. Sci. 76, 1523-1527.

Govindaraju, A., Dogan, S., Rodriguez-Osorio, N., Grant, K., Kaya, A. & Memili, E., 2012. Delivering value from sperm proteomics for fertility. Cells. Tissue. Res. 349, 783-793.

Hansar, E., Lemma, A. & Yilma, T., 2014. Pre-service ultrasonic and manual evaluation of the reproductive organs of dairy cows presumed to be in estrus. Springerplus. 3 (529), 1-5.

Hansen, P.J., 2009. Effects of heat stress on mammalian reproduction. Biol. Sci. 364 (1534), 3341-3350.

Hill, S.L., Perry, G.A., Mercadante, V.R.G., Lamb, G.C., Jaeger, J.R., Olson, K.C. & Stevenson, J.S., 2014. Altered progesterone concentrations by hormonal manipulations before a fixed-time artificial insemination CO-Synch + CIDR program in suckled beef cows. Theriogenology. 82 (1), 104-113.

Hillery, F.L., Parrish, J.J. & First, N.L., 1990. Bull specific effect on fertilization and embryonic development in vitro. Theriogenology. 33, p.249 (abstract).

Holt, C., Holt, W.V., Moore, H.D., Reed, H.C. & Curnock, R.M., 1997. Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. J. Androl. 18, 312-323.

Holt, W., Watson, P., Curry, M. & Holt, C., 1994. Reproducibility of computer aided semen analysis: comparison of five different systems used in a practical workshop. Fertil. Steril. 62, 1277-82.

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

Hossain, M.E., Khatun, M.M., Islam, M.M. & Miazi O.F., 2012. Semen characteristics of breeding bulls at the Central Cattle Breeding and Dairy Farm of Bangladesh. Bang. J. Anim. Sci. 41 (1), 1-5.

Howard, J.M., Manzo R., Frago, F., Falk, D.G., Glaze, J.B., Dalton, J.C. & Ahmadzadeh, A., 2007. Estradiol Cypionate did not affect pregnancy rates of beef cattle synchronized with a modified timed artificial insemination protocol. Prof. Anim. Sci. 23, 509-512.

Hung, P., Miller, M.G., Meyers, S.A. & Vande Voort, C.A., 2008. Sperm mitochondrial integrity is not required for hyperactivated motility, zona binding, or acrosome reaction in the Rhesus macaque. Biol. Reprod. 79, 367-375.

Isachenko, V., Maettner, R., Petrunkina, A.M., Sterzik, K., Mallmann, P., Rahimi, G., Sanchez, R., Risopatron, J., Damjanoski, I. & Isachenko, E., 2012. Vitrification of human ICSI/IVF spermatozoa without cryoprotectants: new capillary technology. J. Androl. 33, 462-468.

Ju, J.C., 2005. Cellular response of oocytes and embryos under thermal stress: hints to molecular signaling. Anim. Reprod. 2, 79-90.

Karunakaran, M. & Devanathan, T.G., 2017. Evaluation of bull semen for fertility-associated protein, *in vitro* characters and fertility. J. Appl. Anim. Res. 45 (1), 136-144.

Kathiravan, P., Kalatharan, J., Edwin, M.J. & Veerapandian, C., 2008. Computer automated motion analysis of crossbred bull spermatozoa and its relationship with in vitro fertility in zona-free hamster oocytes. Anim. Reprod. Sci. 104, 9-17.

Katila, T., 2001. In vitro evaluation of frozen-thawed stallion semen: a review. Acta Vet. Scand. 42, 199-217.

Kealey, C.G., MacNeil, M.D., Tess, M.W., Geary, T.W. & Bellows, R.A., 2006. Genetic parameter estimates for scrotal circumference and semen characteristics of Line 1 Hereford bulls. J. Anim. Sci. 84, 283-290.

Krause, W. & Viethen, G., 1999. Quality assessment of computer-assisted semen analysis (CASA) in the andrology laboratory. Andrologia. 31,125-9.

Kubovičová, E., Makarevich, A.V., Špaleková, E. & Hegedušová, Z., 2011. Motility and fertilizing ability of frozen-thawed ram sperm from two sheep breeds. Slovak. J. Anim. Sci. 44 (4), 134-139.

Kumar, K.V., Reddy, B.R. & Krishna, K.S., 2013. Comparison of different methods for assessing sperm concentration in infertility workup: A review. Intern. J. Med. Sci. 5 (9), 396-400.

Latif, M.A., Ahmed, J.U., Bhuiyan, M.M.U. & Shamsuddin, M., 2009. Relationship between scrotal circumference and semen parameters in crossbred bulls. Bang. Veter. 26 (2), 61-67.

Lenz, R.W., Kjelland, M.E., Vonder Haar, K., Swannack, T.M. & Moreno, J.F., 2011. A comparison of bovine seminal quality assessments using different viewing chambers with a computer-assisted semen analyzer. J. Anim. Sci. 89, 383-388.

Linde-Forsberg, C., Strom Holst, B. & Govette, G., 1999. Comparison of fertility data from vaginal vs. intrauterine insemination of frozen-thawed dog semen: a retrospective study. Theriogenology. 52, 11-23.

Liu, D.Y, Clarke, G.N., Baker, H.W., 1991. Relationship between sperm motility assessed with the Hamilton-Thorn motility analyzer and fertilization rates in vitro. J. Androl. 12, 231-239.

Lu, J.C., Huang, Y.F. & Lü N.Q., 2014. Computer-aided sperm analysis: past, present and future. Andrologia. 46 (4), 329-38.

Lusweti, E.C. 2000. Performance of Nguni, Afrikander and Bonsmara cattle under drought conditions in the North West Province of Southern Africa. S. Afr. J. Anim. Sci. 30 (1), 33.

Macaskill, C., 2016. Indigenous and locally developed breeds. Livestock. 1-4.

Machatkova, M., Hulinska, P., Reckova, Z., Hanzalova, K., Spanihelova, J. & Pospisil, R., 2008. In vitro production of embryos from high performance cows and the development of frozen-thawed embryos after transfer: a field study. Veter. Medic. 53 (7), 358-364.

Maiwashe, A., Nengovhela, N.B., Nephawe, K.A., Sebei, J., Netshilema, T., Mashaba, H.D., Nesengani, L. & Norris, D., 2013. Estimates of lactation curve parameters for Bonsmara and Nguni cattle using the weigh-suckle-weigh technique. S. Afr. J. Anim. Sci. 43 (1), 12-16.

Malik, A., Wahid, H., Rosnina, Y., Kasim. & Sabri, M., 2012. Effects of timed artificial insemination following estrus synchronization in postpartum beef cattle. Open. Vet. J. 2 (1), 1-5.

Mapiye, C., Chimonyo, M., Muchenje, V., Dzama, K., Marufu, M.C. & Raats J.G., 2007. Potential for value-addition of Nguni cattle products in the communal areas of South Africa: A review. Afr. J. Agric. Res. 2 (10), 488-495.

Maqhashu, A., Mphaphathi M.L., Seshoka, M.M., Ramukhithi, F.V., Seolwana, F.L., Masenya, M.B., Netshirovha, T.R., Mapeka, M.H., Nengovhela, N.B., Kanuya, N.L., Muchenje, V. & Nedambale, T.L., 2016. Comparison of oestrous synchronization response and pregnancy rate of village cows following timed artificial insemination in KwaZulu-Natal and Limpopo Provinces. Open. J. Anim. Sci. 6, 9-15.

Maroto Morales, A., García Álvarez, O., Ramón, M., Martínez Pastor, F., Fernández Santos M.R., Josefa Soler. A. & Julián Garde, J., 2016. Current status and potential of morphometric sperm analysis. Asian. J. Androl. 18, 863-870.

Martins, L.F., Pinho. R.O., Siqueira, J.B., Costa, D.S., Guimarães, S.E.F., Neto, T.M. & Guimarães, J.D., 2013. Hypoosmotic swelling test in young Nelore bulls classified as sound and unsound for breeding. Anim. Reprod. 10 (4), 684-688.

Masoumi, R., Towhidi, A., Javaremi A.N, Nabizadeh H. & Zhandi, M., 2011. Influence of $PGF_{2\alpha}$ on semen quality and libido in Holstein bulls. Turk. J. Vet. Anim. Sci. 35 (1), 1-6.

Mathur, A.K., Kumar, S., Prabhakar, J.H., Mandal, D.K., Ingale, H.R. & James, J., 2014. Motility of frozen thawed semen after thermal resistance test. Ind. J. Anim. Reprod. 35 (1), 18-20.

Mattoni, M. & Ouedraogo, A., 2000. A comparative study on the oestrus response to PGF_{2a} Analogue treatment, and conception rates according to time of artificial insemination, in Zebu (*Bos indicus*) and Baoule (*Bos taurus*) cattle. Trop. Anim. Health. Prod. 32, 127-134.

Menon, A.G., Barkema, H.W., Wilde, R., Kastelic, J.P. & Thundathil J.C., 2011. Associations between sperm abnormalities, breed, age, and scrotal circumference in beef bulls. Can. J. Vet. Res. 75, 241-247.

Mocé, E. & Graham, J.K., 2006. Cholesterol-loaded cyclodextrins added to fresh bull ejaculates improve sperm cryosurvival. J. Anim. Sci. 84, 826-833.

Monty, D.E. & Racowsky, C., 1987. In vitro evaluation of early embryo viability and development in summer heat-stressed, superovulated dairy cows. Theriogenology. 28, 451-465.

Morrell, J. & Humblot, P., 2016. Animal reproduction technologies-future perspectives. J. Agric. Sci. Tech. 6, 301-313.

Mortimer, S.T., van der Horst, G. & Mortimer, D., 2015. The future of computer-aided sperm analysis. Asian J. Androl. 17, 545-553.

Mphaphathi, M.L, Luseba, D., Sutherland, B. & Nedambale, T.L., 2012. Comparison of slow freezing and vitrification methods for Venda cockerel's spermatozoa. Open. J. Anim. Sci. 2 (3), 204-210.

Nasrin, S., Amin, M.R. & Alam, M.K., 2008. Evaluation of semen and non-return rate of bulls in artificial insemination (AI) center. Bang. J. Anim. Sci. 37 (2), 1-7.

Nedambale, T.L. & Mphaphathi, M.L., 2010. Beef Breeding in South Africa. 2nd edition. Chapter 8. Basic principles and advanced techniques to enhance reproductive rate. P 89-106.

Nedambale, T.L., 2014. Reproductive technology on the move. The Dairy Mail. P 117-119.

Oliveira, L.Z., Paes de Arruda, R., Cesar de Andrade, A.F., Celeghini, C. & Maria dos Santos, R., 2012. Assessment of field fertility and several in vitro sperm characteristics following the use of different Angus sires in a timed-AI program with suckled Nelore cows. Livest. Sci. 146, 38-46.

Orief, Y., Schultze-Mosgau, A.M.D., Dafopoulos, K.M.D. & Al-Hasani, S.M.D., 2005. Vitrification: will it replace the conventional gamete cryopreservation techniques? Middle. East. Fertil. Soc. J. 10 (3), 171-184.

Palmer, C.W. & Barth, A.D., 2003. Comparison of the BullMate™ sperm quality analyzer with conventional means of assessing the semen quality and breeding soundness of beef bulls. Anim. Reprod. Sci. 77, 173-185.

Paramio, M.T., 2010. *In vivo* and *in vitro* embryo production in goats. Small. Rumin. Res. 89, 144-148.

Patel, B.R. & Siddiquee, G.M., 2013. Physical and morphological characteristics of Kankrej bull semen. Vet. World. 405-408.

Person, C.N., Lester, T.D., Person, M.D. & Rorie, R.W., 2007. Effects of penicillamine, hypotaurine and epinephrine (PHE) on post-thaw bovine sperm parameters, as measured by computer-assisted sperm Analysis. Ark. Anim. Sci. Dep. Rep. 553, 47-50.

Phetudomsinsuk, K., Sirinarumitr, K., Laikul, A. & Pinyopummin, A., 2008. Morphology and head morphometric characters of sperm in Thai native crossbred stallions. Acta. Vet. Scand. 50 (41), 1-9.

Pieterse, M.C., Kappen, K.A., Kruip, T.A.A.M. & Taverne, M.A.M., 1988. Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries. Theriogenology. 30 (4), 751-762.

Polge, C., Smith, A. & Parkes, A.S., 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature.164, 666-666.

Pontes, J.H.F., Nonato-Junior, I., Sanches, B.V., Ereno-Junior, J.C., Uvo, S., Barreiros, T.R.R., Oliveira, J.A., Hasler, J.F. & Seneda, M.M., 2009. Comparison of embryo yield and pregnancy rate between in vivo and in vitro methods in the same Nelore (*Bos indicus*) donor cows. Theriogenology. 71, 690-697.

Quezada-Casasola, A., Martínez-Armendáriz, K.E., Carrera-Chávez J.M., Pérez-Eguía, E, Rodríguez-Alarcón, C.A. & Avendaño-Reyes, L., 2016. Effect of season on scrotal circumference, semen characteristics and testosterone serum concentration in Mexican Corriente and other beef breed bulls. Anim. Reprod. 13 (4), 787-794.

Rahman Md, A., Juyena, N.S., Ahmed, J.U., Ferdousy, R.N., Chakma, S., Rine Md, Z. & Tarif, A. Md. M., 2014. Evaluation of semen for breeding soundness of four different breeds of bull used for artificial insemination. J. Agric. Vet. Sci. 7 (9), 28-34.

Rivera, R.M., Dahlgren, G.M., de Castro e Paula, L.A., Kennedy, R.T. & Hansen, P.J., 2004. Actions of thermal stress in two-cell bovine embryos: oxygen metabolism, glutathione and ATP content, and the time-course of development. Reprod. 128, 33-42.

Rodríguez, C.M., Alvarez, M., Urueña, E.L., Alves, S.G., López, L.A., Tizado, J.E., Luis Anel, L. & de Paz, P., 2015. Head morphology of ram spermatozoa is associated with their ability to migrate in vitro and correlates with fertility. Reprod. Fertil. Dev. 28 (11), 1825-1837.

Roelofs, J., Lopez-Gatius, F., Hunter, R.H.F., Van Eerdenburg, F.J.C.M. & Hanzen, C., 2010. When is a cow in estrus? Clinical and practical aspects. Theriogenology. 74, 327-344.

Schafer, D.J., Bader, J.F., Meyer, J.P., Haden, J.K., Ellersieck, M.R., Lucy, M.C., Smith, M.F. & Patterson, D.J., 2007. Comparison of progestin-based protocols to synchronize oestrus and ovulation before fixed-time artificial insemination in postpartum beef cows. J. Anim. Sci. 85, 1940-1945.

Scheepers, S.M., Annandale, C.H. & Webb, E.C., 2010. Relationship between production characteristics and breeding potential of 25-month old extensively managed Bonsmara bulls. S. Afr. J. Anim. Sci. 40 (3), 163-172.

Schmidt, H. & Kamp, G., 2004. Induced hyperactivity in boar spermatozoa and its evaluation by computer-assisted sperm analysis. Soc. Reprod. Fertil. 128, 171-179.

Schoeman, S.J., 1989. Recent research into the production potential of indigenous cattle with special reference to the Sanga. S. Afr. J. Anim. Sci. 19 (2), 55-61.

Scholtz, M.M. & Theunissen, A., 2010. The use of indigenous cattle in terminal cross-breeding to improve beef cattle production in Sub-Saharan Africa. Anim. Genet. Res. 46, 33-39.

Seneda, M.M, Esper, C.R, Garcia, J.M., de Oliveira, J.A. & Vantini, R., 2001. Relationship between follicle size and ultrasound-guided transvaginal oocyte recovery. Anim. Reprod. Sci. 67, 37-43.

Seshoka, M.M., Mphaphathi, M.L. & Nedambale, T.L., 2016. Comparison of four different permitting and combination of two best cryoprotectants on freezing Nguni sperm evaluated with the aid of computer aided sperm analysis. Cryobiology. 72, 232-238.

Sharma, M., Singh, M., Kapoor, S. & Jasial, S., 2012. Inter relationship between some routine semen evaluation parameters in Jersey X local hill cattle crossbred bulls. Open. Vet. J. 2, 26-31.

Shi, D.S., Lu, K.H., Gordon, I. & Polge, C., 1991. Variation in cleavage and embryonic development of bovine oocytes in vitro fertilized with different bull ejaculates. Theriogenology. 35, 271.

Silva-Santosa, K.C., Santosa, G.M.G., Silotoa, L.S., Hertela, M.F., Andradea, E.R., Rubinb M.I.B., Sturionc, L., Melo-Sterzaa, F.A. & Senedaa, M.M., 2011. Estimate of the

population of preantral follicles in the ovaries of Bos taurus indicus and Bos taurus taurus cattle. Theriogenology. 76 (6), 1051-1057.

Siudzin´ska, A. & Łukaszewicz, E., 2008. Effect of semen extenders and storage time on sperm morphology of four chicken breeds. J. Appl. Poul. Res. 17, 101-108.

Somi, S.S., Kluger, E., Knapp, D., Klein, C. & Aurich, A., 2006. Effects of semen extender and semen processing on motility and viability of frozen-thawed dog spermatozoa. Theriogenology. 66, 173-182.

Song, K. & Lee, E., 2007. Modification of maturation condition improves oocyte maturation and *in vitro* development of somatic cell nuclear transfer pig embryos. J. Vet. Sci. 8 (1), 81-87.

Sosa, J.M., Senger, P.L. & Reeves, J.J., 2002. Evaluation of American Wagyu sires for scrotal circumference by age and body weight. J. Anim. Sci. 80, 19-22.

Spalekov, E., Makarevich, A.V. & Luka, N., 2011. Ram sperm motility parameters under the influence of epidermal growth factor. Vet. Med. Int. 2, 1-5.

Stefanov, R., Krumova, E., Angelova, M., Dolashka, P., Voelter, W. & Zachariev, Z., 2006. Artificial Insemination of sheep and cow with semen treated by Cu/ Zn-superoxide dismutase from the fungal strain *Humicola lutea* 103. World. J. Zool. 1 (1), 36-39.

Sundararaman, M.N. & Edwin, M.J., 2008. Changes in motility characteristics of goat spermatozoa during glycerol-equilibration and the relevance to cryopreservation. Asian. J. Cell. Biol. 3 (1), 22-33.

Sundararaman, M.N., Kalatharan, J. & Jawahar, K.T.P., 2012. Computer assisted semen analysis for quantification of motion characteristics of bull sperm during cryopreservation cycle. Vet. World. 5 (12), 723-726.

Taponen, J., 2009. Fixed-time artificial insemination in beef cattle. Acta. Vet. Scand. 51, (48), 1-6.

Tavares, L.M.T., Feitosa, W.B., De Oliveira, V.P., Nichi, M., Assumpção, M.E.O.D. & Visintin, J.A., 2011. In vitro development of bovine embryos cultured under different fetal calf serum concentrations and cell types. Braz. J. Vet. Res. Anim. Sci. 48 (1), 38-45.

Tejerina, F., Morrell, J., Petterson, J., Dalin, A.M. & Martinez, R., 2009. Routine assessment of motility of ejaculated stallion spermatozoa using a novel computer-assisted motility analyzer (Qualisperm™). Anim. Reprod. 6, 380-385.

Tekin, K. & Daşkin, A., 2016. Effect of different extenders on motility and some sperm kinematics parameters in Norduz goat semen. Turk. J. Vet. Anim. Sci. 40, 490-495.

Thara, K.M. & Nair, S.S.P., 2007. Sire effect on in vitro fertilizability of matured cattle oocytes. Ind. J. Biotechnol. 6, 421-422.

Van de Leemput, E.E., Vos Plam. P.L., Zeinstra, E.C., Bevers, M.M., van der Weijden, G.C. & Dieleman, S.J., 1999. Improved in vitro embryo development using in vivo matured oocytes from heifers superovulated with a controlled preovulatory L.H surge. Theriogenology. 52, 335-349.

Van der Westhuizen, R.R., Schoeman, S.J., Jordaan, G.F. & Van Wyk J.B., 2001. Heritability estimates derived from threshold analyses for reproduction and stability traits in a beef cattle herd. S. Afr. J. Anim. Sci. 31 (1), 25-32.

Veznik, O., Matouskova, D., Svecova, A. & Zajicova, A., 2001. The use of the computer technology for the evaluation of the strict morphological sperm analysis. Vet. Med. Czech. 46, 35-40.

Viana, J.H.M., Palhao, M.P., Siqueira, L.G.B., Fonseca, J.F. & Camargo, L.S.A., 2010. Ovarian follicular dynamics, follicle deviation, and oocyte yield in Gyr breed (*Bos indicus*) cows undergoing repeated ovum pick-up. Theriogenology. 73, 966-972.

Vilakazi, D.M. & Webb, E.C., 2004. Effect of age and season on sperm morphology of Friesland bulls at an artificial insemination centre in South Africa. S. Afr. J. Anim. Sci. 34 (1), 62-69.

Vincent, P., Underwood, S.L., Dolbec, C., Bouchard, N., Kroetsch, T. & Blondin, P., 2012. Bovine semen quality control in artificial insemination centers. Anim. Reprod. 9 (3), 153-165.

Vishwanath, R., Melis, J., Johnson, D.L. & Xu, Z.Z., 2004. Effect of timing of insemination of dairy cows with liquid semen relative to the observation of oestrus. New. Zeal. Soc. Anim. Product. 64, 140-142.

Vyt, P., Maes, D., Quinten, C., Rijsselaere, T., Deley, W., Aarts, M., de Kruif A. & Van Soom, A., 2008. Detailed motility evaluation of boar semen and its predictive value for reproductive performance in sows. Vlaams. Diergen. Tijds. 77, 291-298.

Walters, A.H., Eyestone, W.E., Saacke, R.G., Pearson R.E. & Gwazdauskas, F.C., 2004. Sperm morphology and preparation method affect bovine embryonic development. J. Androl. 25 (4), 554-563.

Ward, F., Rizos, D. & Boland, M.P., 2003. Effect of reducing sperm concentration during IVF on the ability to distinguish between bulls of high and low field fertility: work in progress. Theriogenology. 59, 1575-1584.

Watson, P.F., 1976. The protection of ram and bull spermatozoa by the low density lipoprotein fraction of egg yolk during storage at 5 ºC and deep freezing. J. Thermal. Biol. 1, 137-141.

Wild, S. Mobile lab does cows a service. Mail & Guardian. 21 November 2014.

Wilhelm, K.M., Graham, J.K. & Squires, E.L., 1996. Comparison of the fertility of cryopreserved stallion spermatozoa with sperm motion analyses, flow cytometric evaluation, and zona-free hamster oocyte penetration. Theriogenology. 46, 559-578.

Williams, S.W., Stanko, R.L., Amstalden, M. & Williams, G.L., 2002. Comparison of three approaches for synchronization of ovulation for timed artificial insemination in Bos indicus-influenced cattle managed on the Texas gulf coast. J. Anim. Sci. 80, 1173-1178.

Wilson, R.D., Fricke, P.M., Leibfried-Rutledge, M.L., Rutledge, J.J., Syverson Penfield C.M. & Weigel, K.A., 2006. In vitro production of bovine embryos using sex-sorted sperm. Theriogenology. 65, 1007-1015.

Woelders, H., Matthijs, A. & Engel, B., 1997. Effects of trehalose and sucrose, osmolality of the freezing medium and cooling rate on viability and intactness of bull sperm after freezing and thawing. Cryobiology. 35, 93-105.

Xu, Z.Z., Burton, L.J. & Macmillan, K.L., 1997. Reproductive performance of lactating dairy cows following estrus synchronization regimens with PGF_2 alpha and progesterone. Theriogenology. 47 (3), 687-701.

Zhang, B.R., Larsson, B., Lundeheim, N., Haard, M.G.H. & Rodriguez-Martinez, H., 1999. Prediction of frozen-thawed semen from young dairy bulls entering an AI-programme. Int. J. Androl. 22, 253-60.

Zhang, X., Catalano, P.N., Gurkan, U.A, Khimji, I. & Demirci, U., 2011. Emerging technologies in medical applications of minimum volume vitrification. Nanomedicine. 6 (6), 1115-1129.