Semen characteristics of free-ranging African elephants (*Loxodonta africana*) and Southern white rhinoceros (*Ceratotherium simum simum*) using Computer-aided sperm analysis, Electron microscopy and Genomics as diagnostic tools

by

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KEYWORDS

Sperm characteristics

Sperm motility

Sperm kinematics

Sperm morphology

Sperm morphometric

African elephant (*Loxodonta africana*)

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Computer-aided sperm/semen analysis (CASA)

Electron microscopy (EM)

Genomics

CatSper1 gene (*Loxodonta africana*)

ABSTRACT

Semen characteristics of free-ranging African elephants (*Loxodonta africana*) and Southern white rhinoceros (*Ceratotherium simum simum*) using Computer-aided sperm analysis, Electron microscopy and Genomics as diagnostic tools

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The survival of free-ranging (*in situ)* African elephant and Southern white rhinoceros populations are currently being challenged on a daily basis in Africa. Reproductive health is considered a vital component of species conservation. Conservation of the last mega land mammals may ultimately require intervention by breeding management or combined with assisted reproductive technologies (ART). There is a strong case for gathering baseline information, both physiological and biological, of any species, as opportunities arise.

During this study a total number of 21 ejaculates collected over two seasons from 12 free-ranging African elephant bulls were characterised, as well as 10 ejaculates collected from 10 free-ranging Southern white rhinoceros bulls from two populations. Ejaculates were collected from adult bulls by means of electroejaculation under anaesthesia. Routine semen analysis was combined with Computer-aided sperm analysis (CASA), Computer-aided sperm morphology analysis (CASMA), Transmission electron microscopy (TEM) and Genomics as diagnostic tools. Additionally, sperm functionality within different media was investigated and sperm subpopulation classification according to the motion pattern displayed. The results presented is based on the evaluation and classification of ≈ 45 000 individual African elephant spermatozoa and ≈ 18 000 individual Southern white rhinoceros spermatozoa.

The average elephant ejaculate contained a total number of 47 x 10^9 spermatozoa (volume of 56 ± 38mL x concentration of 818 ± 750 x 10^6/mL) that recorded a total motility of 81 ± 29% of which 62 ± 26% were progressively motile. CASA recorded velocities for curvilinear velocity (VCL 241 ± 58µm/s), straight-line velocity (VSL 173 ± 181µm/s) and average path velocity (VAP 201 ± 54µm/s), and kinematics at straightness of track (STR 86 ± 85%), linearity of track (LIN 67 ± 16%), amplitude of lateral head displacement (ALH 4 ± 0.75µm) and beat cross frequency (BCF 21 ± 3Hz). Structural analysis revealed 68 ± 11% of the spermatozoa were viable (intact plasma membrane) and 77 ± 11% maintained acrosome integrity. Ejaculates contained 55 ± 14% morphologically normal spermatozoa, CASMA measured sperm head lengths at 6.83 ± 0.26µm and width 3.32 ± 0.18µm (total head area of 20.17 ± 1.96µm^2) of which 38.95 ± 0.92% is covered by an acrosomal cap.

The average rhinoceros ejaculate contained a total number of 1.1 x 10^9 spermatozoa (volume of 24 ± 24mL x concentration of 83 ± 96 x 10^6/mL) that recorded a total motility at 82 ± 8% of which 28 ± 23% were progressively motile. CASA recorded velocities for VCL (85 ± 29µm/s), VSL (44 ± 25µm/s) and VAP (69 ± 30µm/s, and kinematics at STR (63 ± 14%), LIN (51 ± 16%), ALH (2 ± 0.16µm) and BCF (16 ± 6Hz). Structural analysis revealed 73 ± 10% of the spermatozoa were viable (intact plasma membrane) and 76 ± 4% maintained acrosome integrity. Ejaculates contained 62 ± 14% morphologically normal spermatozoa, CASMA measured sperm head lengths at 5.5 ± 0.17µm and width 2.9 ± 0.19µm (total head area of 14.8 ± 1.43µm^2) of which 36.3 ± 0.59% is covered by an acrosomal cap.

Based on a Boolean argument and CASA data exploration it was possible to derive elephant and rhinoceros CASA cut-off criteria to sort between activated and hyperactivated motile spermatozoa. For the genomic component of this study, the CatSper1 (*Loxodonta africana)* gene was identified, sequenced and verified in a free-ranging (natural) African elephant population. Multivariate analysis (MVA) was applied to examine the associations between the semen and sperm parameters and the traits they accounted for in this study. Our understanding of wildlife reproductive sciences can substantially progress as the analytical techniques applied and the combination thereof is expanded. This investigation presents a new set of comprehensive semen and sperm threshold values for future investigations.

July 2016

DECLARATION

Hereby I, the undersigned, declare that the thesis *"Semen characteristics of free-ranging African elephants (Loxodonta africana) and Southern white rhinoceros (Ceratotherium simum simum) using Computer-aided sperm analysis, Electron microscopy and Genomics as diagnostic tools."* is my own work, that it has not been submitted previously for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Ilse Luther

31 July 2016

Signed: ……... Date: …….

“If I have seen further than others, it was by standing on the shoulders of Giants”

Isaac Newton

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To the foundation on which all past, present and future giants stand;

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µL	Microliter
µm	Micrometre
AC	Acrosome
ALH	Amplitude of lateral head displacement
AN	Annulus
APV	Apoptotic vesicle
ART	Assisted Reproductive Techniques
AV	Artificial Vagina
AX	Axoneme
BC	Berlin Cryoextender
BCF	Beat Cross Frequency
BSA	Bovine Serum Albumin
CAP	Capitulum
CASA	Computer-Aided Sperm Analysis
CASMA	Computer-Aided Sperm Morphology Analysis
CD	Cytoplasmic Droplet
CPP	Coiled Principal Piece
DCT	Distal Centriole
DD	Dag Defect
EE	Electroejaculation
EY	Egg Yolk
EY_H	Egg yolk and Ham's F10
EY_I	Egg yolk and INRA96®
FB	Fibrous Sheath
H	Ham's F10
HA	Hyperactive Motility
HA C	Hyperactive Circular Motility
HA S	Hyperactive Starspin Motility
I	INRA96®
IF	Implantation Fossa

IU	Unit International
IUCN	International Union for Conservation of Nature
IZW	Institute for Zoo and Wildlife Research, Berlin, Germany
kV	Kilovolt
LIN	Linearity
LIN	Linearity of Track
M	Midpiece
mA	Milli Ampere
mg	Milligram
mL	Millilitre
MS	Manual Stimulation
N	Nucleus
Non-HA	Non-Hyperactive Motility
NRF	National Research Foundation of South Africa
NT	Neat
NT_BO	Neat semen and BO media
NT_H	Neat and Ham's F10
NT_I	Neat and INRA96®
NZG	National Zoological Gardens of South Africa
ODF	Outer Dense Fibres
PAS	Post Acrosomal Sheath
PCT	Proximal Centriole
pH-	Negative phase contrast objective
PM	Progressive Motility
PM	Sperm Plasma Membrane
PP	Principal Piece
RNE	Redundant Nuclear Envelope
S.A.	South Africa
SCA®	Sperm Class Analyser®
STR	Straightness of Track
TEM	Transmission Electron Microscopy
TM	Total Motility
US	University of Stellenbosch
UWC	University of the Western Cape

V	Volt
V	Nuclear Vacuoles
v/v	Volume per Volume
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VSL	Straight-Line Velocity
W/V	Weight per Volume
WOB	Wobble
α	Alpha

CONTENTS

CHAPTER 1

INTRODUCTION TO STUDY

1.1 GENERAL INTRODUCTION

The future well-being of earth's environment and the survival of species is an ever-increasing concern (Andrabi & Maxwell, 2007; Milligan, *et al*., 2009). The nature and pace of environmental changes brought about by humankind have been reported to have an intensified effect on climate, nutrition, predators, pathogens, pollutants, and or combination thereof. These effects cascades further down to present additional factors to the survival and selection pressures on various wildlife species (Holt & Pickard, 1999; Milligan, *et al.*, 2009). Many species are currently under threat of accelerated extinction due to anthropogenic causes of poaching and habitat fragmentation rather than through slower and natural evolutionary processes (Leibo & Songsasen, 2002; Milligan, *et al.*, 2009; Hermes, *et al*., 2013).

According to the IUCN Red List classification, the African elephant (*Loxodonta africana*) is listed as "Vulnerable*"* and the Southern white rhinoceros (*Ceratotherium simum simum*) as "Near Threatened" (Emslie, 2011; IUCN, 2013). Population surveys performed by the ICUN in 2013 estimated African elephant populations to consist of a maximum of 637 600 individuals, and Southern white rhinoceros populations at a maximum of 20 405 individuals living in the wild (IUCN, 2013). One of the greatest wildlife threats in Africa is the rapid advancement of illegal markets trading in wildlife products (Wemmer, *et al.*, 1996; Cousins, *et al.*, 2008; IUCN, 2013; Wittemeyer, *et al.*, 2015).

Considering the current rate of poaching in South Africa, annual mortalities exceed the annual birth rate numbers recorded for Southern white rhinoceros (Poaching, 2015). While elephant populations are currently growing within remaining natural African habitats, an estimated 35,000 elephants were poached for their ivory during the 2014 in Africa (Wittemeyer, *et al.*, 2015). If this current trend of overharvesting is maintained these free-ranging mega-species will be extinct within 20 years.

Fundamentally, the best way to conserve biodiversity is through habitat preservation and natural breeding. Despite current advances in national efforts, biological diversity is still experiencing an accelerated loss in species numbers (Cousins, *et al.*, 2008; IUCN, 2013). These facts have highlighted the importance of effective conservation strategies in South Africa and has shed light on the need to expand and improve efforts to maintain healthy, viable populations of endangered mammals in the country. Animal breeding is considered an important component of conservation management, as stipulated in the terms of the Convention of Biodiversity (Holt & Picard, 1999; Andrabi & Maxwell, 2007). The IUCN recommends that captive breeding programmes should be founded before the *in situ* population becomes so unstable that any interference will further aggravate its decline (Swaisgood, *et al*., 2009; IUCN, 2013). The ideology of captive breeding includes the maintenance of self-sustaining populations as genetic reservoirs, should the need arise for the re-introduction or population supplementation of threatened species (Swaisgood, *et al.*, 2009; IUCN, 2013). Under the umbrella concept of animal breeding or animal reproductive techniques is included the application of Assisted Reproductive Technologies (ART). These technologies can assist to overcome limited natural breeding opportunities exaggerated by demographic bottlenecks. Furthermore, these attempts can only strengthen efforts of conservation and biodiversity by ensuring that viable populations of genetic reservoirs remain intact in both *in situ* (in natural, or wild locations) and *ex-situ* (in captivity) populations.

The main objective of ART, comprising a wide variety of expertise and technologies, is to produce healthy offspring (Holt & Picard, 1999; Fickel, *et al*., 2007; Andrabi & Maxwell, 2007; Flint & Woolliams, 2008; O' Brien, *et al.*, 2009; Hermes, *et al.,* 2009; Hildebrandt, *et al*., 2012; Hermes, *et al.*, 2013; Monfort, 2014). In mammals, sperm preservation is combined with ART such as embryo and oocyte transfer, sperm sexing and cryopreservation of embryos. Once successful gamete preservation has been achieved, artificial insemination (AI) using fresh or frozen sperm has become the method of choice to ensure fertilisation (Holt & Picard, 1999; Fickel, *et al.*, 2007; Andrabi & Maxwell, 2007; Flint & Woolliams, 2008; O' Brien, *et al.*, 2009; Hermes, *et al.*, 2009; Hildebrandt, *et al.*, 2012; Hermes, *et*

al., 2013). Successful captive breeding and ART are also dependent on the availability of high quality, fresh, chilled or frozen semen (Holt & Picard, 1999; Graham, 2005; Fickel, *et al.*, 2007). The accurate assessment of semen quality of such a sample is vital in order to estimate the probability of fertilisation, as much time, resources and effort can be wasted by using poor quality semen samples (Hermes, *et al.*, 2005; Holt, *et al.*, 2007; O' Brien, *et al*., 2009). The spermatozoon is a complex cell that possesses distinctive traits fundamental for fertilisation. It is required to locate an oocyte, to transfer its genetic material and to initiate the development of an embryo (Graham, 2005; Babcock, 2007). Male mammals store sperm in an inactive state to conserve irreplaceable resources (Fawcett, 1970; Weiss & Greep, 1977; Hodgson, *et al.*, 1990). The relative abundance or absence of organelles within a spermatozoon is usually a good indication of sperm function (Fawcett, 1970; Weiss & Greep, 1977; Hodgson, *et al.*, 1990). Sperm motility, viability and morphology are also important sperm characteristics, since abnormalities in either may result in the complete or partial elimination of sperm at several natural barriers in the female reproductive tract (Nöthling & Irons, 2008; Saacke, 2008).

Once ejaculated sperm are released into the female reproductive tract, they undergo capacitation and become hyperactive (Yanagimachi, 1969; Saurez, 1988). Subsequent and irregular hyperactive motility provides the spermatozoa with the force required to free itself from the oviductal reservoir and penetrate the cumulus oophorous and zona pellucida that surround the oocyte (Yanagimachi, 1969; Yanagimachi, 1994; Saurez, *et al.*, 1993; Ho & Saurez, 2001; Ren, *et al.*, 2001; McPartlin, *et al.,* 2009; Ren & Xia, 2010). The physiological changes that occur during hyperactivation and acrosome reaction of spermatozoa are vital for a spermatozoon to acquire fertilisation potential and are collectively known as capacitation (Mortimer, *et al.*, 1998). The assessment of the reproductive performance of males is critical for optimising the propagation of selected species of importance or of endangered wildlife species. This has not been done for many wildlife species in South Africa, limiting the benefits of these technologies to the conservation of threatened species. The usefulness and importance of ART in this respect has not been properly utilised. All mammals, even those taxonomically closely related, exhibit species-specific and even population-specific reproductive and endocrine norms and

traits. Therefore male reproductive evaluations should be focussed to successfully collect and preserve spermatozoa of individual species (Wildt, *et al.*, 1995; Wildt, 1996; Estep & Dewsbury, 1996; Andrabi & Maxwell, 2007).

1.2 AIMS AND OBJECTIVES

The aim of this project was to optimise and apply relevant male reproductive assessment technologies and methodologies as diagnostic tools in order to evaluate and characterise the populations of spermatozoa within the ejaculates collected from two free-ranging large land mammals, the African elephant and Southern white rhinoceros.

The main objectives of this study were:

1. To study the motility characteristics of spermatozoa within ejaculates collected from free-ranging adult African elephants and Southern white rhinoceros populations. Additionally, the effect of various semen extenders and/or dilution media during CASA analyses were compared.
2. To study and describe the morphological and structural characteristics of individual spermatozoa within these ejaculates of African elephants and Southern white rhinoceros.
3. To differentiate sub-populations of motile spermatozoa according to the multidimensional motion patterns displayed by spermatozoa within these African elephant and Southern white rhinoceros ejaculates.
4. To investigate the possibility of identifying, isolating and sequencing the CatSper1 gene (first of the CatSper family involved in hyperactivation) for the African elephant (*Loxodonta africana*).

1.3 OVERVIEW OF THESIS CHAPTERS TO FOLLOW

This thesis is divided into eight chapters, namely a general introduction, literature overview and four research chapters. A discussion and general conclusion together with a complete reference list are included at the end of the thesis.

CHAPTER 2: LITERATURE OVERVIEW

This chapter entails an overview of the literature on conservation and assisted reproductive technologies and a general background on the African elephant and the Southern white rhinoceros. Emphasis was placed on the sperm structure, sperm motility and the importance of hyperactivation (HA) during fertilisation, ultrastructural evaluation with the use of transmission electron microscopy (TEM) and the development of CASA technologies.

CHAPTER 3: MATERIALS AND METHODS

The collection, general handling and evaluation of semen samples collected from free-ranging African elephants and Southern white rhinoceroses is described. Semen samples were evaluated for macroscopic (colour, viscosity and volume) and microscopic (sperm concentration, motility and kinematic parameters, sperm morphology and vitality) characteristics. Motion pattern analysis was used to determine the CASA cut-off values to identify and classify the percentage of the hyperactivated (HA) sperm populations within a semen sample. Sperm organelles were evaluated at an ultrastructural level by means of transmission electron microscopy (TEM).

CHAPTER 4: RESULTS FOR AFRICAN ELEPHANT (*Loxodonta africana*)

The data represents 21 ejaculates collected from 12 free-ranging African elephant bulls during two seasons, September 2009 and April 2010. The results include data collected from 21 macroscopic (colour, viscosity, volume) and 155 microscopic (concentration, motility, kinematic parameters, functionality, viability, acrosome integrity, morphology and sperm head morphometric) analyses. Also included in the results are TEM micrographs of seven elephant ejaculates chosen randomly.

CHAPTER 5: RESULTS FOR SOUTHERN WHITE RHINOCEROS (*Ceratotherium simum simum*)

The data represents 10 ejaculates collected from 10 free-ranging Southern white rhinoceros bulls collected at two different locations (two populations) during April 2010. The results include data collected from 10 macroscopic (colour, viscosity, volume) and 71 microscopic (concentration, motility,

kinematic parameters, functionality, viability, acrosome integrity, morphology and sperm head morphometric) analyses. Also included in the results are TEM micrographs of three ejaculates chosen randomly.

CHAPTER 6: SEQUENCING CATSPER1 GENE FOR THE AFRICAN ELEPHANT (*Loxodonta africana)*

The collection of samples for gene sequencing of CatSper1 (first of the CatSper family involved in hyperactivation) including primer design, DNA extraction and PCR is described in this chapter. The results for the isolation and sequencing of the CatSper1 gene for the African elephant (*Loxodonta africana*) are also presented.

CHAPTER 7: MULTI-VARIATE ANALYSIS AS A TOOL TO INVESTIGATE POTENTIAL RELATIONSHIPS AMONG RECORDED SEMEN AND SPERM CHARACTERISTICS OF THE AFRICAN ELEPHANT (*Loxodonta africana)*

Exploratory factor analysis (EFA) was used to investigate underlying relationships among measured variables. The main aim of this chapter was, therefore, to determine the relationship between volume, concentration, motility, kinematic parameters, sperm head morphometric, sperm morphology and acrosome integrity by using various statistical exploratory graphs and statistical modelling. Due to analysis restrictions only the elephant dataset was sufficient for MVA analysis during this study.

CHAPTER 8: DISCUSSION

The combined findings of this study presented in chapters 4-7 and the methodology used in chapter 3 are discussed, taking the available literature into account. A hypothesis is proposed regarding the current semen and sperm characteristics of free-ranging African elephant and Southern white rhinoceros populations based on the semen and sperm parameters analysed during this study.

1.4. RESEARCH OUTPUTS

1.4.1 PUBLICATIONS

1. Standing sedation with medetomidine and butorphanol for manual semen collection in captive male African elephants (*Loxodonta Africana*).

   I. Lueders, B. Tindall, D. Young, G. Van Der Horst, S. Botha, I. Luther, L. Maree, H.J. Bertschinger. *The Veterinary Journal* (2015) Volume 206, page 190-192.

   ISSN 1090-0233. http://www.sciencedirect.com/science/article/pii/S1090023315003056

2. CatSper1 gene sequence for the African elephant (Loxodonta africana) KR856273 – KR856279. *Genebank* (2015)

   I. Luther, C. Labuschange, L. Maree, A. Kotze, G. van der Horst

1.4.2 CONFERENCE PRESENTATIONS

1. Semen evaluation in the African elephant, *Loxodonta africana,* by making use of computer-aided semen analysis and electron microscopy. (Oral presentation)

   Gerhard Van Der Horst, Ilse Luther, Frank Goeritz, Robert Hermes, Jitte Dietrich, Imke Lueders, Adrian Tordiffe, Paul Bartels, Romain Potier, Barbara Baker, Willie Theison, JJ Van Altena, Douw Grobler and Thomas Hildebrandt.

   International Elephant Foundation Symposium, Pretoria, South Africa. January 2010.

2. Sperm motility patterns quantified in the African elephant, *Loxodonta africana*: What is the significance? (Oral presentation)

   Gerhard Van Der Horst, Ilse Luther, Frank Goeritz, Robert Hermes, Jitte Dietrich, Imke Lueders, Adrian Tordiffe, Paul Bartels, Romain Potier, Barbara Baker, Willie Theison, JJ Van Altena, Douw Grobler and Thomas Hildebrandt.

   International Elephant Foundation Symposium, Pretoria, South Africa. January 2010.

3. Sperm Morphometric in the African elephant, *Loxodonta africana. (*Poster Presentation)

   Gerhard Van Der Horst, Ilse Luther, Frank Goeritz, Robert Hermes, Jitte Dietrich, Imke Lueders, Adrian Tordiffe, Paul Bartels, Romain Potier, Barbara Baker, Willie Theison, JJ Van Altena, Douw Grobler and Thomas Hildebrandt.

International Elephant Foundation Symposium, Pretoria, South Africa. January 2010.

4. Semen evaluation in the African Elephant, *Loxodonta africana.* (Oral presentation)

   Gerhard Van Der Horst, Ilse Luther, Frank Goeritz, Robert Hermes, Jitte Dietrich, Imke Lueders, Adrian Tordiffe, Paul Bartels, Romain Potier, Barbara Baker, Willie Theison, JJ Van Altena, Douw Grobler and Thomas Hildebrandt.

   First Annual NZG Research Symposium, Pretoria, South Africa. November 2010.

5. Semen evaluation in the African elephant, *Loxodonta africana* by making use of computer-aided semen analysis and electron microscopy. (Oral presentation)

   Ilse Luther, Liana Maree, Antoinette Kotze, Gerhard van der Horst.

   Third Annual NZG Research Symposium, Pretoria, South Africa. November 2012.

6. Computer-aided semen analysis (CASA): Application and usefulness in semen quality assessment in free-ranging African elephants, *Loxodonta africana*. (Oral presentation)

   Ilse Luther, Liana Maree, Antoinette Kotze, Gerhard van der Horst.

   Fourth Annual NZG Research Symposium, Pretoria, South Africa. November 2013.

7. The role of Assisted Reproductive Technologies in research and conservation. (Oral presentation)

   Ilse Luther, Imke Lueders, Adrian Tordiffe, Gerhardus Scheepers, Gerhard Van Der Horst.

   Wildlife Group Congress, Pretoria, South Africa. February 2014.

CHAPTER 2

LITERATURE OVERVIEW

2.1 INTRODUCTION

South Africa covers approximately 2% of global land area and with its abundance of species (10% global total of plant species, 6% mammal species, 7% bird species, 5% reptile species and 6% insect species), is internationally recognised as a country prolific in biological diversity (Cousins, *et al*., 2008; Abensperg-Traun, 2009; Mora, *et al*., 2011; IUCN, 2013). The aim of conservation is defined as "…ensuring the survival of species of animals, plants and micro-organisms in their inherent environments" (Swaisgood, *et al.,* 2009; Andrabi & Maxwell, 2007; Flint & Woolliams, 2008; IUCN, 2013). One of the greatest wildlife threats in Africa is the rapid advancement of illegal markets in wildlife products (Wemmer, *et al.*, 1996; Cousins, *et al.*, 2008; IUCN, 2013).

Habitat and population fragmentation further influence the survival of species by interfering with their natural ability to reproduce or limiting their ability to find suitable mates (Hermes, *et al.*, 2013). As the natural habitat or inherent environment of a species is fragmented or destroyed, the remaining individuals become more isolated and subsequently genetic diversity diminishes (Holt & Picard, 1999; Andrabi & Maxwell, 2007; Milligan, *et al.*, 2009). Inbreeding further reduces individual survival due to impaired physiological functions affecting normal spermatogenesis and the ability to sustain normal embryo development (Holt & Picard, 1999; Andrabi & Maxwell, 2007; Milligan, *et al.*, 2009). Successful natural breeding of captive populations is dependent on the basic knowledge of a species' biology, ecology, social structure, reproductive cycle, seasonality, implantation, placentation, gestation, parturition, maternal behaviour, neonatal care, nutrition, disease susceptibilities, and causes of endangerment (Holt & Pickard, 1999; Leibo & Songsasen, 2002; Flint & Woolliams, 2008; Monfort, 2014).

In combination with natural breeding, artificial insemination (AI) is warranted when: natural breeding is ineffective; mating pairs are incompatible; physical limitations not associated with genetic defects are present; or for transferring genetic information without translocating animals (Swaisgood, *et al.*, 2009; Hildebrandt, *et al*., 2012). Unfortunately, many captive African elephants and Southern white rhinoceros populations are not self-sustaining (Schwarzenberger, *et al.*, 2001; Hermes, *et al*., 2004; Swaisgood, *et al.*, 2009; Hildebrandt, *et al*., 2012; O'Brien, *et al*., 2013). In fact, many mammals fail to reproduce successfully in captivity due to a variety of reasons: 1) limited number of breeding bulls available since bull husbandry is restricted; 2) decrease in the genetic diversity of captive populations due to the limited number of available reproductively active males; 3) acyclic females or variable estrus cycle lengths; and 4) mating failure due to sibling relationship or mate choice problems (Schwarzenberger, *et al.*, 2001; Hildebrandt, *et al.*, 2012). For the captive Southern white rhinoceros, male infertility and ovarian pathologies are severe problems and the main causes of their low reproductive rate (Schwarzenberger, *et al.*, 2001; Hermes, *et al.*, 2004).

To date, our inability to utilise ART to salvage our dwindling wildlife populations, particularly in our endangered species, is complicated due to insufficient knowledge of the reproductive biology of the majority of wildlife species (Holt & Pickard, 1999; Holt, 2000; Comizzoli, *et al.*, 2000; Flint & Woolliams, 2008; O' Brien, *et al.*, 2009; Monfort, 2014). Our very basic, or in most cases non –existent, knowledge of the reproductive biology of a majority of wildlife species remains our greatest limitation (Holt, 2000; Holt & Pickard, 1999; Flint & Woolliams, 2008; O' Brien, *et al.*, 2009; Hermes, *et al.*, 2013; Monfort, 2014). Currently most of the available reproductive biology information is derived from human, domestic and laboratory animals (Asa, 1996; Hermes, *et al.*, 2013; Leibo & Songsasen, 2002). In general, the male reproductive system has been studied far less than the female reproductive system (Holt, 2000; Holt & Pickard, 1999; Flint & Woolliams, 2008; O' Brien, *et al.,* 2009; Hermes, *et al.*, 2013; Monfort, 2014).

2.2 THE DEVELOPMENT OF ASSISTED REPRODUCTIVE TEGNOLOGIES

The aim of this section is to provide a brief overview of the development of assisted reproductive technologies (ART) aimed at enhancing small fragmented and captive populations of endangered species with specific reference to key events for elephant and rhinoceros. Holt and Pickard (1999), Comizzoli *et al*. (2000), Leibo and Songsasen (2002), Durant (2009), Holt *et al.* (2014), provide excellent overviews on this topic. The study of mammalian gametes began around 1675 when Antonie van Leeuwenhoek described the spermatozoon of a dog, and in 1827 when von Baer discovered the oocyte, also of a dog (Nöthling, 2006). Lazzaro Spallanzani performed the first verifiable artificial insemination (AI) around 1780 in a bitch (Nöthling, 2006; Durrant, 2009). However, in 1866 the thoughts of Mantegazza regarding the value of assisted reproduction were visionary when he wrote: "If the human sperm can be preserved for more than four days at the temperature of melting ice without undergoing any change, then it is certain that scientists of the future will be able to improve breeds of horses and cattle without having to spend enormous sums of money in transporting thoroughbred stallions and bulls. It will be possible to carry out artificial insemination with frozen sperm sent rapidly from one locality to another. It should also be feasible for a husband who dies on the battlefield to fertilise his wife and thus to have legitimate sons even after his own death" (cited by Nöthling, 2006).

In 1799, the first successful artificial insemination was reported in humans (Nöthling, 2006; Durrant, 2009). By 1912, AI in domestic animals began and by 1930s bovine semen cooperatives were organised (Durrant, 2009). The development of the artificial vagina (AV) in 1914 and the electro-ejaculator (EE) in 1936 enabled routine semen collection and evaluation (Durrant, 2009). When Polge, *et al.,* (1949) discovered the cryoprotectant properties of glycerol, the breeding industry was revolutionised. Reports facilitating the long-term storage of fowl spermatozoa at – 79 °C (Polge, *et al.*, 1949; Nöthling, 2006; Holt, 2013), as well as spermatozoa collected from stallions (Krause & Grove, 1967) and primates (Roussel & Austin, 1967) soon followed.

Early efforts employed to collect semen samples from elephant and rhinoceros included manual penile and/or rectal massage techniques that resulted in successful collections from only a few animals (Jainudeen, *et al.*, 1971; Schaffer, *et al*., 1998; Roth, 2001). In general, the collection of elephant and rhinoceros semen has proven to be difficult and the collection of good quality sperm samples has been somewhat unreliable (Roth, 2001; Roth, *et al*., 2005; Hermes, *et al.*, 2005; Roth, 2006; Behr, *et al.,* 2009; Thongtip, *et al.*, 2006; O'Brien, *et al.*, 2013). Attempts at electroejaculation have proven to be more successful in elephant (Howard, et al., 1984) and rhinoceros since the design of the rectal probes used was modified to accommodate the male rhinoceros urogenital tract (Behr, *et al*., 2009; Hildebrandt, *et al.*, 2012). Ultrasonography has been used to evaluate reproductive tracts and, during electroejaculation, to monitor changes associated with artificially stimulated ejaculation in male rhinoceros (Schaffer, *et al.*, 1998; Hermes, *et al.,* 2005) and African elephant (Hildebrandt, *et al*., 2006; Lueders, *et al.*, 2015).

The process of AI is complex and requires thorough, species-specific knowledge of: female reproductive cycles, relationship of pituitary/gonadal events to ovulation, effective ovulation induction, semen collection, processing, capacitation and short/long-term storage, effective insemination dose, female reproductive anatomy and the effects of anaesthesia on sperm transport and ovulation (Goodrow, 2001). Significant advances in endocrine analyses, ultrasonography evaluations and behavioural profiling have resulted in a number of remarkable discoveries regarding the reproductive biology in these mega-vertebrates that have been hailed as major breakthroughs in assisted reproduction (Hildebrandt, *et al.*, 2006). The first elephant calf from fresh sperm AI was born in 1999 (Asian), and then in 2000 two more fresh sperm AI calves (African) were born (Schmitt, *et al*., 2001). AI in elephants is performed endoscopically in trained elephants and has already produced more than 40 calves (Hildebrandt, *et al.*, 2012). Furthermore, frozen semen AI has been successful in the birth of Asian (Thongtip, *et al.*, 2009) and African elephants calves (Hildebrandt, *et al.*, 2012; Hermes, *et al.,* 2013).

Fresh and frozen semen AI in captive rhinoceros populations (zoos) has proven to be successful (Hermes, *et al.*, 2009). In 2007, the first rhinoceros calf was born from AI using fresh semen and in 2009, the first post-thaw semen AI rhinoceros calf was born. Similarly, AI with fresh semen has become an important captive breeding tool in elephants (Hildebrandt, *et al.,* 2012). The application of ART to wildlife breeding management will play a decisive role in the future of species survival, presenting the possibility of cryopreserved gametes and embryos being exchanged between captive collections and small populations in various countries to diversify the gene pool. Successful results have also been recorded in the genetic and reproductive management of captive Bottlenose dolphins with the birth of six calves, all of a predetermined sex (O' Brien, *et al.,* 2009).

2.3 LARGE MAMMALS OF SOUTHERN AFRICA:

2.3.1 THE AFRICAN ELEPHANT *(Loxodonta africana*)

The largest land mammals, Elephantidae represents the only living family of the order Proboscidea. This order consist of the genera *Loxodonta* with two species; the African elephant (*Loxodonta africana*) and African Forest Elephant (*Loxodonta cyclotides*), and *Elephas*, with one species, the Asian Elephant *(Elephas maximus*) (Skinner & Smithers, 1990). The African elephant is very adaptable and can easily move between various habitats from savannahs to forests (Skinner & Smithers, 1990; Spinage, 1994; Blanc, *et al.*, 2008). Habitat destruction and illegal poaching are seriously encroaching on the existence of all three elephant species within their natural habitat and roaming areas. The African elephant was listed as *"Vulnerable"* in the 2004 IUCN Red List. Prior to the 2004 assessment, the species was listed as *"Endangered"* (Blanc, *et al.,* 2008). Although the number of populations may be declining in some parts of their natural habitat ranges, an average current growth of 4% per annum was recorded for Eastern and Southern elephant populations that account for over two-thirds of all known elephants in Africa (Blanc, *et al.*, 2008).

In animals over 20 years, it is estimated that the average shoulder heights of a male elephant are 2.35m and a female elephant 2.1m. Exceptional individual males reach a value of 3m and females 2.5m (Skinner & Smithers, 1990). The trunk of the elephant is a remarkable organ with many uses and is set between two ivory tusks that are elongated upper incisor teeth (Skinner & Smithers, 1990; Spinage, 1994). Their ears are well-developed triangular lobes and constitute 20% of their body surface area (Skinner & Smithers, 1990). Adult elephant bulls have very large ear flaps, with recorded measurements of a nearly 2m vertical height and a breadth of 1.2m that assist with thermoregulation by dispersing the heat generated within the ears (Skinner & Smithers, 1990). Elephants have a temporal gland situated on each side of the head which produce copious amounts of secretions, particularly when the animals are stressed. The secretions from these glands can often be seen as dark marks on the skin (Skinner & Smithers, 1990).

Elephants are sociable animals that live in family groups, usually consisting of an adult female with her offspring and a number of closely related females with their offspring (Skinner & Smithers, 1990; Spinage, 1994). Occasionally, family groups bond together to form a herd. Bulls tend to herd together temporarily and very old bulls live solitary lives. Sexual activity in the male elephant is predominantly associated with musth, a state or condition in mature African elephant bull, which refers to a set of physical, physiological and behavioural characteristics, including elevated androgen levels (Poole, 1987; Skinner & Smithers, 1990; Ganswindt, *et al.*, 2010). Only when a female is in estrus will a bull join the female herd, especially bulls in musth (Skinner & Smithers, 1990; Spinage, 1994). Elephant bulls reach puberty at the age of about ten and sexual maturity at the age of about 15 years of age. Bulls are considered “socially mature” At the age of approximately 35 years and only then will their attempts to mate with cows in estrus be successful against other bulls (Skinner & Smithers, 1990). Elephant bulls in musth can be identified by a greenish discoloration of the penis and sheath, and by continuous urine dribbling that is associated with a strong odour (Poole, 1987; Ganswindt, *et al.,* 2010). Enlargement of the temporal glands with copious secretion is an additional sign of musth (Poole, 1987; Skinner & Smithers, 1990; Ganswindt, *et al.*, 2010).

Female elephant reaches puberty between the ages of 9-18 years. During favourable conditions, females may conceive at the age of about nine years (Skinner & Smithers, 1990). It is estimated that an African elephant cow has a “reproductively active life” for up to approximately the age of 52 years, if conditions are favourable. This amounts to a 40-year period of continuous cycles of estrus, mating, conception, 22 month pregnancies, parturition, lactation and rearing of calves (Skinner & Smithers, 1990; Spinage, 1994).

Offspring success is primarily determined by male–male competition and the outcome based on their physical strength and weaponry or sperm competition. Male African elephants have life-history traits of longevity, intermediate growth, and large sexual dimorphism, which suggest a high degree of size-dependant competition for mates, favouring older and larger males (Ramussen, *et al.*, 2008; Ganswindt, *et al.,* 2010). Dominance rank and fighting success are determined by the size and the age of the bulls, suggesting an increased paternity success with age (Ramussen, *et al.*, 2008). However, a male in musth is dominant to all other bulls, except a larger bull in musth (Poole, 1987; Skinner & Smithers, 1990; Ramussen, *et al.,* 2008; Ganswindt, *et al.*, 2010). Paternity analyses have revealed that approximately 75 – 80% of offspring can be attributed to male elephants in musth (Ramussen, *et al.*, 2008; Ganswindt, *et al.*, 2010). Elephants exhibit polygamous mating that involves female choice of mates and male-male competition for access to females (Ramussen, *et al.,* 2008; Ganswindt, *et al.*, 2010). The female African elephant has a long, 16-week estrus cycle, with only a brief receptive period of one week and a 4 – 5-year interval between births, making sexually active females a limited resource (Ramussen, *et al.*, 2008).

2.3.2 THE SOUTHERN WHITE RHINOCEROS (*Ceratotherium simum simum*)

The white rhinoceros is the second largest land animal on earth today, evolving from the black rhinoceros in relatively recent historical times, eventually spreading large numbers throughout Africa, Eurasia and North America (Emslie, 2011). The family Rhinocerotidae is comprised of five species; *Dicerorhinus sumatrensis* (Sumatran rhinoceros), *Rhinoceros unicornis* (Indian rhinoceros) and *Rhinoceros sondaicus* (Javan rhinoceros) that are found in Asia, and *Ceratotherium simum* (white rhinoceros) and *Diceros bicornis* (black rhinoceros) which are found in Africa. Populations in both Africa and Asia have declined rapidly in recent decades due to loss of habitat and poaching (Houck, 2001). The Rhinocerotidae is one taxon that could significantly benefit from a reliable method of semen collection since four of the five rhinoceros species (black rhinoceros, greater one-horned rhinoceros, Sumatran rhinoceros, and Javan rhinoceros) are endangered (Emslie, 2011; IUCN, 2013). The IUCN conservation status of the Southern white rhinoceros is currently indicated as “near threatened”. Their natural population structure is 19% adult bulls, 26% adult cows, 32% sub-adults and 23% calves. Due to their long calving interval, a maximum of 40% of the adult cows, or 10.4% of the total female population calves per annum (Emslie, 2011). Therefore, preserving spermatozoa from representatives of the current population is an important component of a broader species conservation strategy.

Adult male rhinoceros reach a shoulder height of up to 1.8m with a mass of 2000 – 2300kg. Rhinoceroses have a barrel-shaped body with short, thickset limbs. Bulls are about 30% larger than cows. Their characteristic features include a long head with two continuously growing horns on the maxilla, a longer one in front and a shorter one behind (Skinner & Smithers, 1990; Emslie, 2011). The horns do not have a bone core and consist of a compact mass of tubular keratin fibres growing directly from the skin. The horns of cows are thinner but generally longer than those of a bull (Emslie, 2011). Rubbing of the horn against tree trunks and rocks is frequently observed in rhinos, especially bulls that cause the tip of the horn to wear away. This reduces the growth of the horn to a maximum of 120 cm at the prime age of 28 – 30 years (Emslie, 2011).

The main difference between the white and black rhinoceros lies in the shape of their lips. The white rhino has a broad, flat lip approximately 20 cm wide used for grazing, compared to the prehensile upper lip of the black rhino used to grasp twigs of the woody plants they eat whilst browsing (Skinner & Smithers, 1990). The outline of the back also differs between the two species, and while the white rhinoceros has a well-developed, obvious nuchal hump, this is completely absent in the black rhino (Skinner & Smithers, 1990). White rhinoceros are grazers that prefer feeding on short, sweet palatable grass and use their broad and square lips to pluck the grass. Rhinos do not ruminate as they are monogastric (Skinner & Smithers, 1990; Emslie, 2011).

White rhinoceros are semi-social and occur in small family groups that usually consist of a single dominant or territorial bull, subordinate bulls, cows and their offspring. The dominant bulls occupy clearly defined territories that they defend with great ferocity against trespassing bulls from neighbouring territories (Skinner & Smithers, 1990; Emslie, 2011). Communication within the species relies heavily on olfactory signals (urine and dung components). Rhinoceros' sensitive sense of smell allows individual animals to detect other members of their community when they come across dung (Skinner & Smithers, 1990). Vocalisation and subtle displays are used for direct communication. Even though white rhinoceroses have preputial glands in the region of the penis and vulva, olfactory communication appears to be limited to the odours of the urine and dung (Skinner & Smithers, 1990). Rhinoceros bulls mark their territories by urine spraying or defecating on latrines located along their boundaries. Territorial bulls trespassing into the territory of an adjacent bull normally take avoidance action and serious fights are averted. However, if a female in estrus accompanies a territorial bull, serious fighting may follow (Skinner & Smithers, 1990). Subordinate bulls are tolerated, providing they remain submissive, and thus several subordinate bulls may live in a territory of a single dominant bull. On the other hand, cows may have home ranges that overlap with those of other cows and several territorial bulls (Skinner & Smithers, 1990). White rhinoceros breed at any time of the year. Bulls can become territorial at around 12-13 years of age. Bulls tend to form a close attachment with a cow in pro-estrus for a considerable time before mating (Skinner & Smithers, 1990). It has also been reported

that bulls will actively prevent females from leaving their territories during this time. They will chase, squeal or even clash horns with a pro-estrus cow until she remains in his territory. The dominant bull will actively drive off subordinate bulls showing interest in pro-estrus females during this time (Skinner & Smithers, 1990). Females breed from the age of four years and based on the hormone profiles of captive white rhinoceros has an oestrous cycle length of approximately 28 days. They have a gestation period of about 16 months and the birth weight of calves is approximately 40kg. Calves are weaned at approximately one year-old and separate from their mothers at about two or three years of age (Skinner & Smithers, 1990).

2.4 MALE REPRODUCTION

The reproductive life of an animal starts with puberty, a complex process that culminates in fertility potential. During this time, important changes within the hormone profiles trigger the growth and development of the gonads and germ cells; this includes the development of species-specific secondary sex characteristics (Setchell & Breed, 2006). Contributing factors that can affect puberty include the nutritional plane level and the maintenance of an individual's critical body weight. Increased levels of nutrition will lead to exponential growth, in turn leading to the rapid onset of puberty (Asa, 1996; Estep & Dewsbury, 1996; Barth, 2007; Chenoweth & Kastelic, 2007). The reproductive tract of a male mammal is composed of two testicles, an excurrent duct system (efferent ducts, epididymis, and ductus deferens), accessory sex glands and a penis. Testicular function includes the synthesis and secretion of androgens, especially testosterone and the production of spermatozoa. The Leydig cells responsible for androgen production lie between the interstitial spaces in the seminiferous tubules (Asa, 1996; Setchell & Breed, 2006; Cooper & Hausman, 2009). Sequentially increased androgen levels stimulate testicular Sertoli cells responsible for initiation and maintenance of normal spermatogenesis (Asa, 1996; Setchell & Breed, 2006). In males, the increased levels of luteinizing hormone (LH), follicle stimulating hormone (FSH) and prolactin (PRL) secreted from the anterior pituitary, in combination with the androgen, testosterone, produced by Leydig cells, is directly

associated with an increase in the mass of the testes and accessory sex organs. Furthermore, in the male mammal the onset of puberty is associated with the first production of spermatozoa (Asa, 1996; Chenoweth & Kastelic, 2007; Goyal & Memon, 2007).

During ejaculation, spermatozoa pass from the epididymis through the *Vas deferens*/*Ductus deferens*, whereafter accessory fluids are added and then the semen expelled via the penile urethra (Asa, 1996; Setchell & Breed, 2006). Major accessory sex glands include the seminal vesicles, the prostate and the bulbourethral (Cowper's) glands, with only the prostate glands found to be universal amongst mammals (Asa, 1996; Chenoweth & Kastelic, 2007; Goyal & Memon, 2007; Cheng, *et al.*, 2010). Semen consists of spermatozoa that are suspended in a seminal plasma containing sperm metabolites derived from the testes, epididymis and accessory sex glands (Hafez, 1987; Barth, 2007). Spermatozoa that are not ejaculated are phagocytosed or leaked into the bladder. The longevity of epididymal sperm varies greatly amongst species (Asa, 1996; Setchell & Breed, 2006; Barth, 2007). In the majority of mammals, the testes descend from the abdominal cavity into a scrotum (Setchell & Breed, 2006). However, the degree of testicular descent varies amongst orders and families. For example, in elephants there is virtually no testicular migration observed. This is the same for hyraxes and elephant shrews (Skinner & Smithers, 1990; Setchell & Breed, 2006). In whales and dolphins the testes migrate to the caudal abdominal cavity. Testes of hedgehogs and moles migrate just through the wall of the abdominal cavity and in pigs the testes form a sub-anal swelling (Skinner & Smithers, 1990; Setchell & Breed, 2006). The testes of rhinoceros are located in the dorsal aspect of the preputial fold and are, similar to the stallion, positioned horizontally. A pronounced external (scrotal) swelling is seen in primates and ruminants (Setchell & Breed, 2006). These differences in testicular location have been widely debated, but remain unexplained (Setchell & Breed, 2006).

2.4.1 THE AFRICAN ELEPHANT *(Loxodonta africana*)

The testes of this mammal are located next to the kidneys and are accordingly referred to as intra-abdominal testes (Figure 2.1). The testes of an elephant make up about 0.1% of a bull's body weight and grow continuously throughout life (Perry, 1953; Skinner & Smithers, 1990). In an adult African elephant bull, the epididymal portion of the tubule is extremely convoluted within a thick tissue cord located around the medial surface of the testis (Perry, 1953; Short, *et al.*, 1967; Holt, *et al.*, 1980; Jones & Holt, 1981). A short distance caudal to the testis, the epididymis (Figure 2.2) becomes reduced to the thickness of a finger and for most of its remaining length remains fairly uniform in size and follows a direct course to the junction with the duct of the seminal vesicle on either side near the opening of the urethra (Perry, 1953; Short, *et al.*, 1967; Holt, *et al.*, 1980; Jones & Holt, 1981). The prostate glands are lobulated and situated near the base of the seminal vesicles at about the same level, and open into the urethra via several ducts. Cowper's glands (bulbourethral glands) are situated further down the urethra and their ducts open some distance below the actual level of the glands (Perry, 1953; Short, *et al.*, 1967). The penis is large and very extensible and, while there is no true prepuce, the glans penis is concealed by a fold of skin (Perry, 1953; Short, *et al.*, 1967).

2.4.2 THE SOUTHERN WHITE RHINOCEROS (*Ceratotherium simum simum*)

The basic reproductive anatomy of rhinoceros (Figure 2.3) is unique as their characteristics represent that of several domestic species combined. Schaffer and co-workers (2001) reported that to assess rhinoceros fertility, current domestic reproductive techniques will have to be modified to accommodate this unique morphology (Schaffer, *et al.*, 2001). Lateral penile flaps are distinctive in the rhinoceros and tapir. These flaps are usually located inside the vagina during intromission and significantly increase the diameter of the penis when erect (Schaffer, *et al.*, 2001). The testes are located on the dorsal aspect of the preputial fold, and as in the stallion, are positioned horizontally. The testes of these species are not visually apparent on live animals but can be located *in situ* by palpating in a horizontal line along the dorsal preputial fold from the inguinal area to the caudal border of the prepuce (Schaffer, *et al.*, 2001).

The testes are enclosed in a thick connective tissue capsule (Figure 2.4). The epididymis (head, body and tail) is loosely attached to the testes and has a relatively large calibre of epididymal ducts (Schaffer, *et al.*, 2001). Rhinoceros accessory glands are accessible per rectum and have characteristics similar to a variety of domestic species. The vesicular glands are paired and multi-lobed and the seminal vesicles are similar to those in the bull. The triangular, bi-lobed prostate is similar to that of the stallion and the gland lacks an ampulla as seen in the boar (Schaffer, *et al.,* 2001). The prominent bulbourethral gland is elongated and irregular in the white rhinoceros and covered by a thick muscular layer. The dilation of the posterior urethra of the rhinoceros is similar to the ampulla in other species, where semen accumulates before ejaculation. Monitoring of this region by means of ultrasound during electroejaculation has resulted in the successful collection of semen samples from the rhinoceros (Schaffer, *et al.,* 2001).

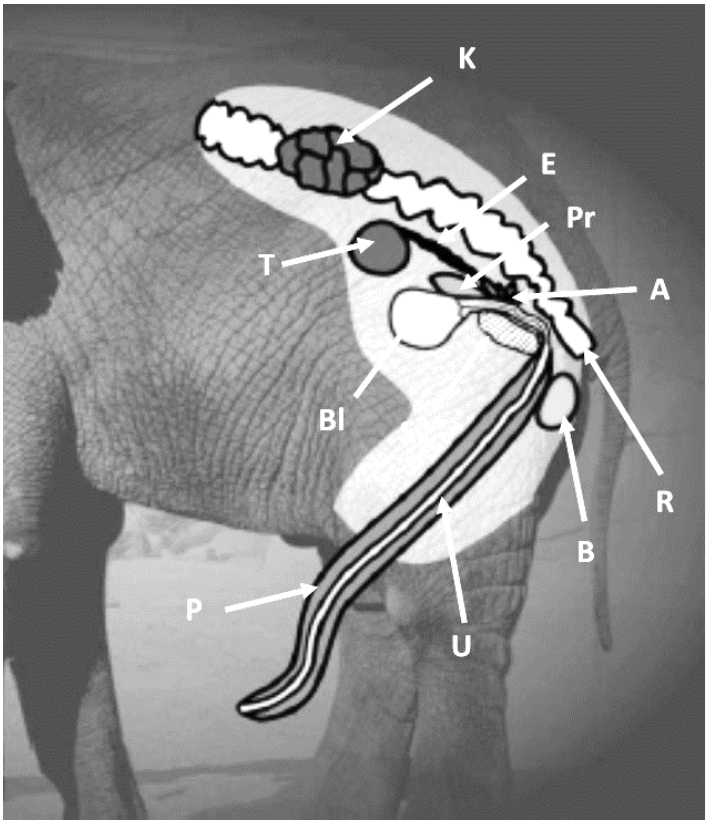


Figure 2.1: Topography of the male African elephant reproductive tract: P = penis; U = urethra; T = testis; E = epididymis; B = bulbourethral gland; Pr = prostate; A = ampulla; Sv = seminal vesicles, Bl = bladder; K = kidney; R = rectum (Photo credit: T. Hildebrandt)

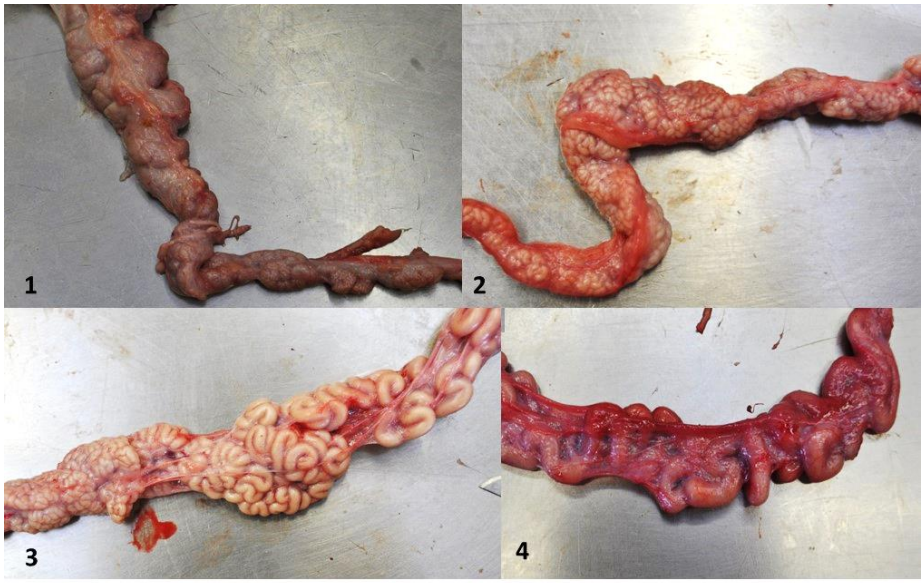


Figure 2.2: The epididymis of an adult African elephant bull. Leaving the medial aspect of the testis, the proximal cauda (images 1 & 2), leading to the distal cauda (image 3) then into the vas deference (image 4) close to the opening of the ampulla) clearly illustrate the complexity of the coiling and how the epididymis increase in diameter from cranial to caudal.

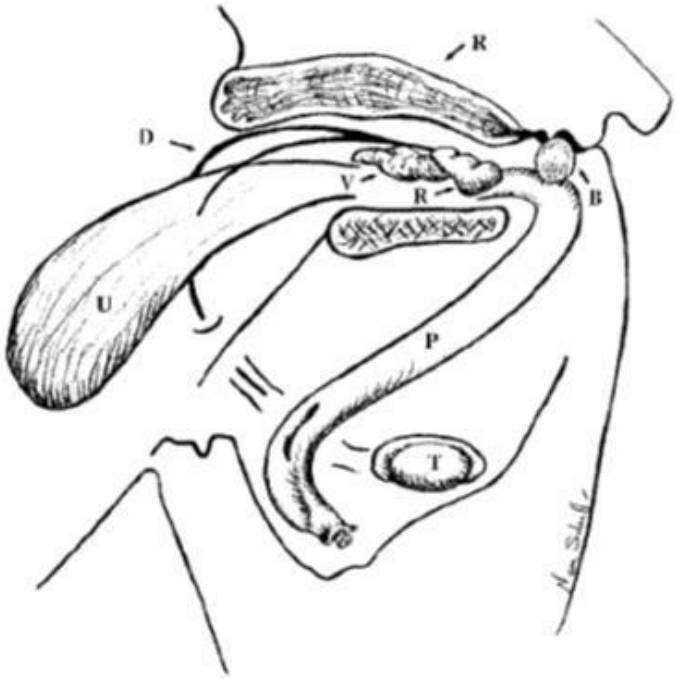


Figure 2.3: Topography of the male Southern white rhinoceros reproductive tract. P = penis; U = urinary bladder; T = testis; D = *Ductus deferens*; B = bulbourethral gland; R = prostate; V = vesicular gland (Schaffer, et al., 2001).

Figure 2.4: The testes of a Southern white rhinoceros are located in the dorsal aspect of the preputial fold and are enclosed in a thick, white connective tissue capsule. Once this capsule has been removed the testis and loosely attached epididymis are clearly visible (Photo credit: L. Maree).

2.5 THE SPERMATOZOON

Spermatozoa are specialised cells that function briefly as unicellular organisms although they are produced by multicellular organisms (Hodgson, *et al.*, 1990). A mammalian spermatozoon must conserve a copy of the genetic material from the individual it originated from, be efficiently motile and must be able to fuse with an oocyte from the same species (Fawcett, 1970; Barth & Oko, 1989; Cooper & Hausman, 2009).

2.5.1 SPERMATOGENESIS AND SPERMIOGENESIS

The male gamete is moulded during the process of spermatogenesis. Spermatogenesis refers to the process whereby spermatogonial stem cells at the base of the seminiferous tubules divide and differentiate to give rise to spermatozoa at the luminal free surface (Weiss & Greep, 1977). Spermatogenesis involves three processes, namely' spermatocytogenesis, meiosis and spermiogenesis (Polanski & Kubiak, 1999; Chenoweth & Kastelic, 2007). Spermiogenesis refers specifically to the differentiation of newly formed spermatids into mature spermatids just prior to their release into the tubular lumen (Fawcett, 1970; Weiss & Greep, 1977; Barth & Oko, 1989; Cooper & Hausman, 2009). This process generally involves a series of four structural progressions that develop simultaneously: 1) Golgi bodies of the spermatid produce small vesicles which fuse to form the acrosome; 2) centrioles are positioned posterior to the nucleus to form tail microtubules; 3) mitochondria are positioned at the anterior end of the developing tail and are eventually arranged into a helix; 4) the acrosome and nucleus take on their final form (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009).

Excess cytoplasm and organelles no longer required by the spermatozoon are shed as part of the distally migrating cytoplasmic droplet to enable spermatozoa to adopt their characteristic shape (Weiss & Greep, 1977; Hodgson, *et al.*, 1990). Spermiation is the process by which mature spermatids are released from the supporting somatic Sertoli cells into the lumen of the seminiferous tubule (O'Donnel, 2011). During spermiation, cohorts of spermatids extend into the tubule lumen while the

Sertoli cell cytoplasm surrounding them gradually recedes (O'Donnel, 2011). Spermiation ends with the disengagement of the spermatid (now known as the spermatozoon) into the lumen and phagocytosis of the remainder of the residual body by the Sertoli cell (O'Donnel, 2011). This seems to be a critical determinant of the number of spermatozoa entering the epididymis, and consequently the sperm content of the ejaculate (O'Donnel, 2011). When spermatozoa are released from Sertoli cells, they are transported to the *Rete testis* and subsequently into the efferent ducts that join the epididymal duct. Within the efferent duct and head (caput) of the epididymis, fluid is reabsorbed, whereas the body (corpus) of the epididymis has a secretory function and is involved in sperm maturation. In the tail (cauda) of the epididymis, spermatozoa are stored in a relatively inactive form (quiescent state) until ejaculation occurs (Polanski & Kubiak, 1999; Chenoweth & Kastelic, 2007; Cooper & Hausman, 2009).

2.5.2 STRUCTURE OF THE MAMMALIAN SPERMATOZOON

The structure of spermatozoa has been adapted to meet a number of requirements which are common to most species. Consequently, all spermatozoa are fundamentally similar in structure and function (Hodgson, *et al.*, 1990). The relative abundance or absence of organelles within a spermatozoon is usually a good indication of sperm function; for instance, metabolically active cells will usually possess numerous mitochondria (Fawcett, 1970; Weiss & Greep, 1977; Hodgson, *et al.,* 1990; Maree, 2011). The mature mammalian spermatozoon is composed of a head, neck and tail (consisting of a principle piece, midpiece and end piece) (Figure 2.5).

2.5.2.1. Sperm head:

The head of the mammalian spermatozoon primarily comprises the nucleus that is usually flattened dorso-ventrally and pyriform in shape. Internally, it is a well-organised structure that coordinates the arrangement of genetic material and localises nuclear functions (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009). Laminas provide the loci for chromatin attachment and organises

the protamines into functional nuclear bodies. In the nucleus, chromatin is arranged into large loops of DNA which are bound to the lamina matrix at specific regions by lamina-binding proteins in the chromatin (Weiss & Greep, 1977; Cooper & Hausman, 2009). Small cavities or irregular shaped deformities called, “nuclear vacuoles” are randomly observed in the sperm nucleus on electron micrographs. These vacuoles are classified as localised defects formed during condensation of the chromatin (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009). A cap-like structure, the acrosome, covers nearly two-thirds of the anterior region of the nucleus. The acrosome contains glycoproteins and numerous lysosomal enzymes (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009).

The caudal region of the nucleus displays a cavity, the implantation fossa that provides attachment to the conforming capitulum of the connecting piece. The shape of the implantation fossa is characteristic for each species and lined by two closely opposed layers of the nuclear envelope (Fawcett, 1970). The redundant nuclear envelope (RNE) is closely applied to the nucleus except near the junction of the caudal aspect of the nucleus. Here it is often deflected away from the condensed chromatin and extends back into the neck region to form a long membranous fold or tightly wound scroll (Fawcett, 1970). The RNE is closely associated with the proximal mitochondria. It remains unknown whether the calcium (Ca^{2+}) required for hyperactivation of spermatozoa normally comes from both the RNE and the plasma membrane channels or whether the RNE serves instead to modulate hyperactivated motility (Suarez, 2008).

2.5.2.2 Sperm flagellum - midpiece:

The neck, which connects the head to the midpiece consists of a pair of centrioles and a connecting piece of nine segmented columns that seems to merge with the nine outer dense fibres of the rest of the sperm tail. Anteriorly to the basal plate of the nucleus is the “articular” component of the connecting piece called the capitulum. The capitulum is usually elongated in the transverse axis and continuous on either side with two major columns (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman,

2009). In the interior of the connecting piece, immediately beneath the capitulum, is the proximal centriole. The distal centriole serves as the basal body of the flagellum during early spermiogenesis (Fawcett, 1970). It is commonly accepted that the distal centriole is a key component for all motile flagella, as it serves as the kinetic centre and is the site of origin of the flagellar wave (Fawcett, 1970). The midpiece extends from the neck to the annulus, which originates as a ring-like structure that migrates from the base of the distal centriole down the flagellum during spermiogenesis (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009).

In the midpiece, the mitochondria are elongated and arranged end-to-end in a helix around the core of the contractile elements. The number of gyres in a favourable longitudinal sections of the mitochondrial sheath can be determined with considerable accuracy from electron micrographs (Fawcett, 1970). These cytoplasmic organelles are specifically devoted to energy metabolism and the production of adenosine triphosphate (ATP). The energy derived from the breakdown of carbohydrates and fatty acids is converted to ATP through the process of oxidative phosphorylation (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009). Mitochondria are selectively positioned in locations of high-energy use and consist of an inner and outer mitochondrial membrane separated by an inter-membrane space (Cooper & Hausman, 2009). The inner membrane forms numerous folds (cristae) that extend into the interior (or matrix) of the organelle. Each of these components plays distinct functional roles, with the matrix and inner membrane representing the major working compartments of mitochondria. The matrix contains the mitochondrial genetic system as well as the enzymes responsible for the central reactions of oxidative metabolism (Weiss & Greep, 1977; Cooper & Hausman, 2009).

2.5.2.2 Sperm flagellum - principal piece

The main portion of the flagellum is the principal piece that is enclosed by the fibrous sheath and extends from the annulus to nearly the end of the sperm tail. It is the longest section of the flagellum and the most effective component in locomotion (Fawcett, 1970; Weiss & Greep, 1977; Cooper &

Hausman, 2009). Flagella are microtubule-based projections that are responsible for movement. Their movement results from the sliding of microtubules driven by the action of dynein motors. Sperm motility is dependent on a fundamental structure called the axonemal complex or axoneme is composed of microtubules and their associated proteins (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009). The microtubules in mammalian spermatozoa are arranged in a characteristic "9 + 2" pattern (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009). A central pair of microtubules and nine double microtubules (doublets) are surrounded by an additional row or cylindrical bundle of uniformly spaced outer dense fibres (9 + 9+ 2), believed to be the combined accessory motor elements while also providing structural support and strength (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009). The outer microtubule doublets are connected to the central pair by radial spokes and to each other by links of the protein nexin. In addition, two dynein arms are attached to each doublet. The dynein arms have a rounded outer contour and taper towards the corresponding doublet of the axoneme (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009).

In the proximal part of the sperm tail, peripheral to the axoneme, the outer dense fibres have a petal-like shape and radial symmetry when seen in cross section. The form of the nine outer fibres differs amongst species when examined in cross-section. However, in many species, three fibres with designated numbers 1, 5, and 6 are distinctly larger than the other fibres (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009). These fibres are always thicker in the proximal part of the midpiece and progressively reduce in diameter with the gradual tapering of the principal piece of the sperm flagellum (tail). In most species, fibres 3 and 8 end in the first part of the principal piece, and their place is taken by the inward extensions of the longitudinal columns (thickenings) of the fibrous sheath (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009). The remaining seven fibres, those with the smallest initial diameter terminate first, while the thicker fibres 1, 5 and 6 extend further along the tail and are the last to terminate. The end piece is the terminal section of the tail that consist of randomly distributed axonemal microtubules (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009).

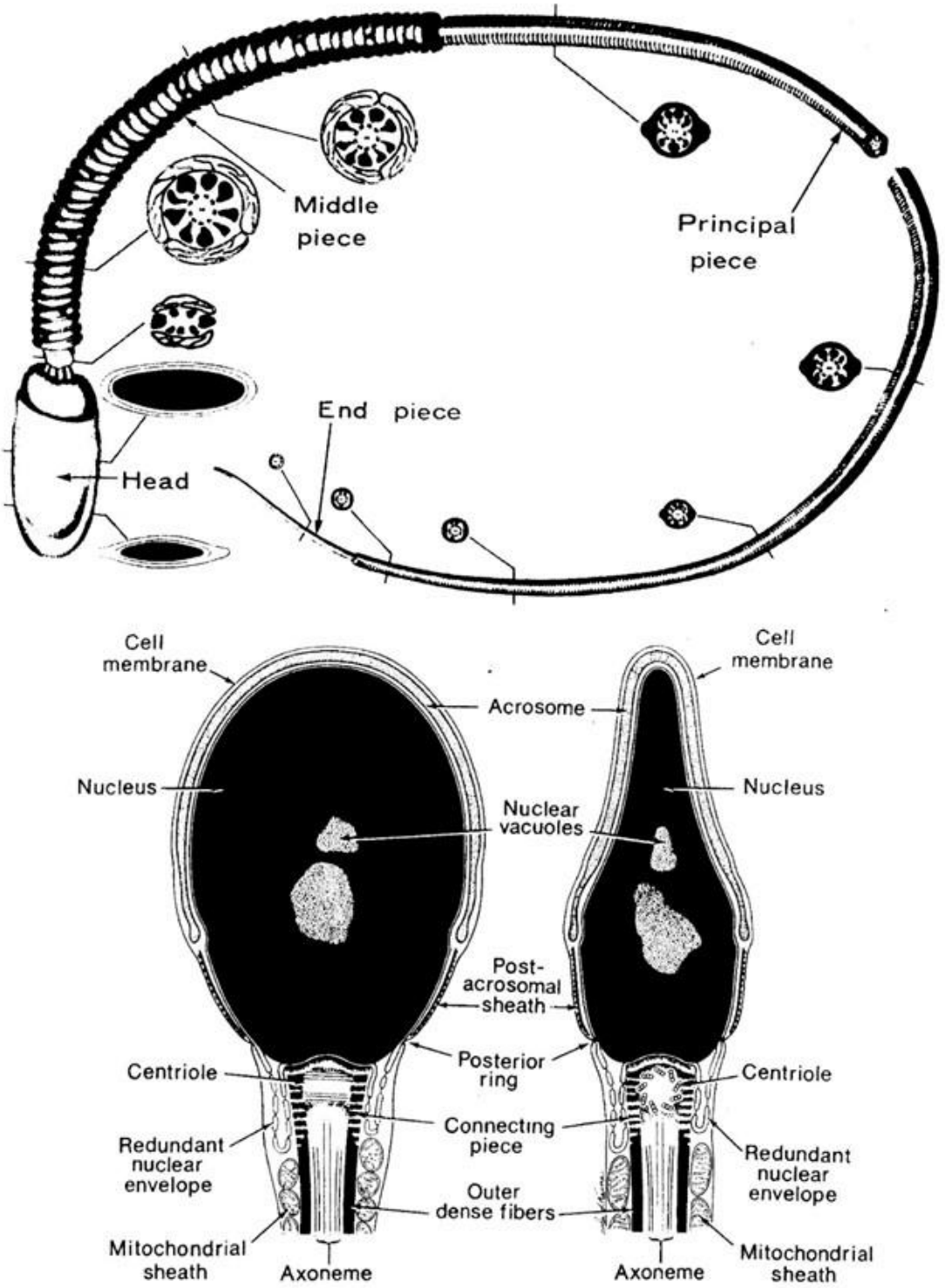


Figure 2.5: A schematic drawing of the mammalian spermatozoon as revealed by electron microscopy, showing transverse sections at different locations along its length (top image). The bottom images are illustrations of the head and neck region of the mammalian spermatozoon (Weiss & Greep, 1977).

A plasma membrane surrounds all cells, both prokaryotic and eukaryotic. It defines the boundary of the cell and separates its internal contents from the environment by serving as a selective barrier to the passage of molecules (Fawcett, 1970; Weiss & Greep, 1977). Other proteins of the plasma membrane control the interactions between cells of multicellular organisms and serve as sensors through which the cell receives signals from its environment. The plasma membrane thus plays a dual role: to isolates the cytoplasm and mediate interactions between the cell and its environment (Saacke, 2008).

2.5.3 THE PROCESSES OF CAPACITATION

Capacitation is the term used to cover a complex series of biochemical and physiological events the spermatozoon has to undergo before it can fertilise an oocyte. This process includes the display of hyperactivated motility, acquisition of a capacity to respond to unique signals emanating from the oocyte, and the acrosome reaction (AR). The AR is an exocytotic event during which cholesterol is released from the inner and outer acrosomal membranes allow these membranes to fuse (Mortimer, *et al.*, 1998; Gordon, 2003). Hyperactivation is characterised by an increase in the amplitude of the flagellar bend, usually one-sided, producing a beat pattern that is highly asymmetrical. Both head and tail regions of the spermatozoon thus perform specific functions before fertilisation takes place (Mortimer, *et al.,* 1998; Ren, *et al.,* 2001).

Yanagimachi (1969) reported the vigorous whiplash beating of the flagella of spermatozoa close to the site of fertilisation. He proposed that this hyperactive motility plays a significant role in the ability of a spermatozoon to fertilise an oocyte (Yanagimachi, 1969; Yanagimachi, 1994; Ho & Saurez, 2001; Ho & Suarez, 2003). Hyperactivated motility is the swimming pattern shown by most spermatozoa retrieved from the oviductal ampulla at fertilisation. Hyperactivated spermatozoa throw their flagella into deeper bends and their beating is usually less symmetrical than that found in the flagella of activated spermatozoa as shown in Figure 2.6 (Yanagimachi, 1994; Ho & Saurez, 2001; Ren & Xia, 2010).

Hyperactivated sperm have been observed to swim in circles on microscope slides, including extremely asymmetrical bends that compose figure-of-eight movement patterns. These erratic paths are due to the intermittent production of deep flagellar bends (Saurez, *et al.*, 1993; Ho & Saurez, 2001). It is suggested that this could represent the switching back and forth between an activated and hyperactivated state, thus defining hyperactivation as being biphasic (Johnson, *et al.*, 1981). Other factors that affect flagellar beating pattern is the rate of rotation of a spermatozoon's flagellum along the longitudinal axis. This can account for the variation in the patterns of activation and hyperactivation observed amongst species (Ho & Saurez, 2001; Ho & Suarez, 2003; Suarez, 2008). Rolling is part of sperm kinetics, whereby under capacitating conditions, rolling increases and spermatozoa swim in helical patterns. During hyperactivation, rolling fades and spermatozoa trace out small circles (Saurez, 1988). Rolling that occurs once per beat cycle produces a helical path while intermittent rolling causes a change in the swim direction (Ho & Saurez, 2001; Ho & Suarez, 2003). Another morphological factor affecting swimming patterns is the thickness of the outer dense fibres in the flagellum. Species with smaller outer dense fibres (humans, mice) show a smaller radius of curvature in flagellar bend compared to species with larger outer dense fibres (rat, Chinese hamster) where a large radius of curvature was recorded in the flagellar bends (Ho & Saurez, 2001).

Initiation and maintenance of hyperactivation is associated with a change in the calcium (Ca^{2+}) concentration in the sperm flagellum, which is acquired from extracellular Ca^{2+} accessible in the female reproductive tract (Ho & Saurez, 2001; Quill, 2003; Babcock, 2007; Jin, *et al.*, 2007; Suarez, 2008; Singh & Rajender, 2014). As sperm travel from the vagina (pH ≈ 5) to the cervical mucus (pH ≈ 8) they undergo intracellular alkalinization (Jin, *et al.*, 2007). Alkalinization dramatically increases the conductivity of the cation channels and the Ca^{2+} influx is thus enhanced which, in turn, increases flagellar bending (Jin, *et al.*, 2007; Qi, *et al.*, 2007; Suarez, 2008; Singh & Rajender, 2014).

The molecular mechanisms of chemotaxis are still largely unknown and no specific has factor has been identified. However, there is evidence that the source of the signal is follicular fluid. Progesterone is also involved and Ca^{2+} plays a limited role (Ho & Saurez, 2001; Ren, *et al.*, 2001; Suarez, 2008; McPartlin, *et al.*, 2009; Ren & Xia, 2010). It is hypothesised that the follicular fluid released during ovulation also contains chemo-attractants, released from the cumulus-oocyte complex, that draw the spermatozoa towards the oocyte (Ho & Saurez, 2001; Ren, *et al.*, 2001; Suarez, 2008; McPartlin, *et al.*, 2009; Ren & Xia, 2010). The final barrier between the spermatozoa and the egg, and only a minority of spermatozoa reach this site, is the thick layer of glycoprotein surrounding the oocyte termed the zona pellucida (ZP). These ZP glycoproteins stimulate an increase in the Ca^{2+} concentration (Ho & Saurez, 2001; Suarez, 2008; Ren & Xia, 2010). Progesterone secreted from the cumulus cells triggers a Ca^{2+} influx into sperm and also induces the acrosome reaction (AR) (Ho & Saurez, 2001; Ren, *et al.*, 2001; Suarez, 2008; McPartlin, *et al.*, 2009; Ren & Xia, 2010). Every strategic response from the spermatozoon to penetrate the egg coating and reach the egg membrane by combining mechanical forces and chemical digestions involved Ca^{2+} entry. Upon entry through the egg membrane the spermatozoon releases sperm "factors" that prompts a wave of Ca^{2+} signalling the initiation of egg activation and embryonic development (Ren & Xia, 2010; Singh & Rajender, 2014).

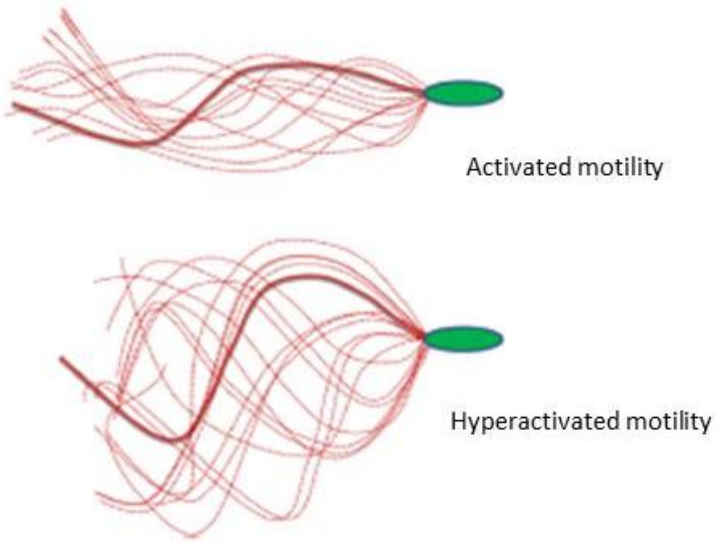


Figure 2.6: Hyperactivated sperm display vigorous and asymmetrical flagellar bending essential to propel the cell through the viscous oviductal fluid and to penetrate the protective layer of the oocyte. Activated sperm show only basal motility and are unable to reach the fertilisation destination (Singh & Rajender, 2014).

2.6 SEMEN AND SPERM QUALITY RELATED TO SPERM FUNCTION AND FERTILITY

Laboratory methods must include techniques to evaluate and test sperm characteristics relevant for both fertilisation and embryo development (Salisbury, *et al*., 1978; Rodriques-Martinez & Barth, 2007). Parameter evaluation methods used can often correctly estimate the fertilising potential and capacity of a sperm sample by scoring, measurement, and classification of sperm quality (Gordon, 2003; Holt & Van Look, 2004; Rodriques-Martinez & Barth, 2007). Sperm samples are therefore subjected to *in vitro* tests used to discriminate the ability of sperm populations to undergo specific steps crucial for normal fertilisation and embryonic development (Holt & Van Look, 2004; Rodriques-Martinez & Barth, 2007). Sperm evaluation is performed in an attempt to reduce the relationship between sperm parameters measured and fertilisation potential (Holt & Van Look, 2004; Rodriques-Martinez & Barth, 2007). Currently an array of tests are available, ranging from simple visual assessment to detailed molecular analysis, but no single test is able to determine the overall fertilisation potential of spermatozoa (Gordon, 2003; Holt & Van Look, 2004; Rodriques-Martinez & Barth, 2007). Over a period of many years semen quality has been based on a subjective evaluation of various parameters outlined below.

2.6.1 EJACULATE COLOUR AND VISCOSITY

Normal semen samples range from white, ivory to yellow in colour (Hafez, 1987; Barth, 2007). This range of normal colours seen in semen is mainly a result of sperm concentration but other factors such as diet also contribute. More diluted samples will appear greyish-white compared to the rich cream or ivory colour of highly concentrated semen samples (Howard, *et al.,* 1984; Hafez, 1987; Barth, 2007). Abnormal colour of ejaculates includes the following: a) watery-yellow, which usually indicates urine contamination, particularly problematic during electro-stimulation; b) Red, which indicates fresh blood, usually associated with penile trauma or acute inflammation in the genital tract; c) brown to black, revealing the presence of old blood from proximal parts of the genital tract and d) green to brown, resulting from faeces or green pus from a possible infection (Hafez, 1987; Barth,

2007). Semen viscosity and consistency are determined by sperm concentration in domestic bulls but in species other than ruminants, only the consistency is described. The consistency of a semen sample can range from thin watery or milky to thick creamy (Howard, *et al.*, 1984; Hafez, 1987; Barth & Oko, 1989; Barth, 2007; Goyal & Memon, 2007).

2.6.2 EJACULATE VOLUME

Ejaculate volume provides meaningful information of a male's capacity to produce sperm (Barth, 2007). It is important to understand that the volume of an ejaculate may be influenced by a number of factors, including 1) age of the animal. Younger males usually produce smaller volumes of ejaculate when compared to mature males under the same conditions. 2) Collection method will also affect the volume obtained. An ejaculate is only considered to be representative of a male's full production potential when semen is collected by means of an artificial vagina (AV), whereas volumes attained from electroejaculation (EE) depend on the duration of stimulation and selection of the various fractions (ejaculates collected through EE are usually larger). 3) The frequency of collection, where frequent collection over short periods of time will also reduce the ejaculate volume. 4) Lastly, completeness of an ejaculation is also directly proportional to the volume produced (Hafez, 1987; Barth, 2007).

2.6.3 SPERM CONCENTRATION

Sperm concentration refers to the number of spermatozoa per millilitre (mL) of semen. The sperm count is the total number of spermatozoa within an ejaculate. Both parameters are important and should be calculated (Hafez, 1987). The number of spermatozoa per mL of semen is a highly variable semen characteristic, not only between animals but also between ejaculates collected from the same animal (Hafez, 1987; Barth & Oko, 1989; Barth, 2007). The fertility potential of a male is also dependent on the number of spermatozoa released per insemination. With semen evaluation of stallions, boars and rams, the total number of spermatozoa per ejaculate is used to estimate the

potential sperm output, while in domestic bulls the capacity to produce sperm is determined by measuring the scrotal circumference (Barth, 2007). To determine the total number of spermatozoa in an ejaculate, the concentration must first be determined by taking a fixed volume of raw semen and mixing it with a fixed volume of diluent. This can be accomplished with the use of a haemocytometer, spectrophotometer, densitometer or CASA (Hafez, 1987; Barth & Oko, 1989; Barth, 2007). The haemocytometer has the advantage of being the cheapest way to manually count sperm, although it is not the most accurate. Spectrophotometers and densitometers evaluate fluid opaqueness and have the potential to give false readings due to the presence of debris or other non-sperm matter (Love, 2008).

2.6.4 SPERM MOTILITY

Sperm motility is the most widely used indicator of mammalian sperm function since it is the main feature of potentially fertile spermatozoa. Sperm motility is readily quantified as well as qualified and reflects several structural and functional competencies, including essential aspects of sperm metabolism (Hafez, 1987; Barth, 2007; Patryka, *et al.,* 2012; Alvarez, *et al.*, 2014). During internal fertilisation in mammals spermatozoa are guided through the confines of the oviduct and interact with the tubal epithelium. Consequently, sperm interaction within these boundaries plays a role during the process of fertilisation (Alvarez, *et al*., 2014). Three different regulatory mechanisms, namely chemotaxis, thermotaxis and rheotaxis, have been proposed during the process of guiding spermatozoa through the narrow oviductal isthmus of mammals (Alvarez, *et al.*, 2014). Spermatozoa can be described as ‘pushers’ when evaluated from a hydrodynamic point of view as they are micro-swimmers with a motor in the back (the flagellum) and a passive load at the front (the head). The typical field of flow around a pusher is characterised by the backward flow in the rear as fluid is being pushed backwards by the flagellum and the forward flow in the front due to the head being pushed along (Figure 2.7 A) (Lauga & Powers, 2009; Alvarez, *et al.*, 2014). Their surrounding medium and enclosing environment (Figure 2.7 B) rules the kinetics of micro-swimmers’ (sperm, bacteria and algae) motility. Micro-swimmers are small organisms and cells, micrometres in scale, that swim at a

low Reynolds number (*Re*). *Re* highlights the importance of fluid inertia; large *Re* values (*Re*> 10^4) indicate fast turbulent flow; whereas small *Re* values *(Re* < 1) indicate slow creeping flow*. Re* for micro-swimmers like sperm is approximately 0.01 (Lauga & Powers, 2009; Alvarez, *et al.*, 2014). In short, the navigation of micro-swimmers occurs at low *Re* numbers where viscous forces dominate over inertial forces (Alvarez, *et al.*, 2014). As a result, swimming occurs when two symmetries are broken: temporal (irreversible motion of the flagella beat) and spatial (friction anisotropy of the flagellum) as depicted in Figure 2.8. (Alvarez, *et al.*, 2014).

Motility provides important information on the fertilisation potential of a spermatozoon once it has reached the mucus-rich utero tubal junction which may possibly act as a barrier to sperm with poor motility. Simultaneously, this internal process of sperm selection drives sperm competition (Contri, *et al.*, 2010). The activation of motility is a prerequisite for hyperactivation, that is, if a spermatozoon cannot swim it cannot become hyperactivated (Suarez, 2008). Sperm motility has long been considered a major criterion in the assessment of male fertility (Holt & Van Look, 2004; Maree & Van der Horst, 2013). The motility of freshly ejaculated spermatozoa is characterised by the flagellum that generates a symmetrical and low amplitude waveform that pushes the spermatozoon in a relatively straight-line (Turner, 2003; Maree & Van der Horst, 2013). Sperm motility is expressed as the percentage of total motile or progressively motile spermatozoa (Patryka, *et al.*, 2012).

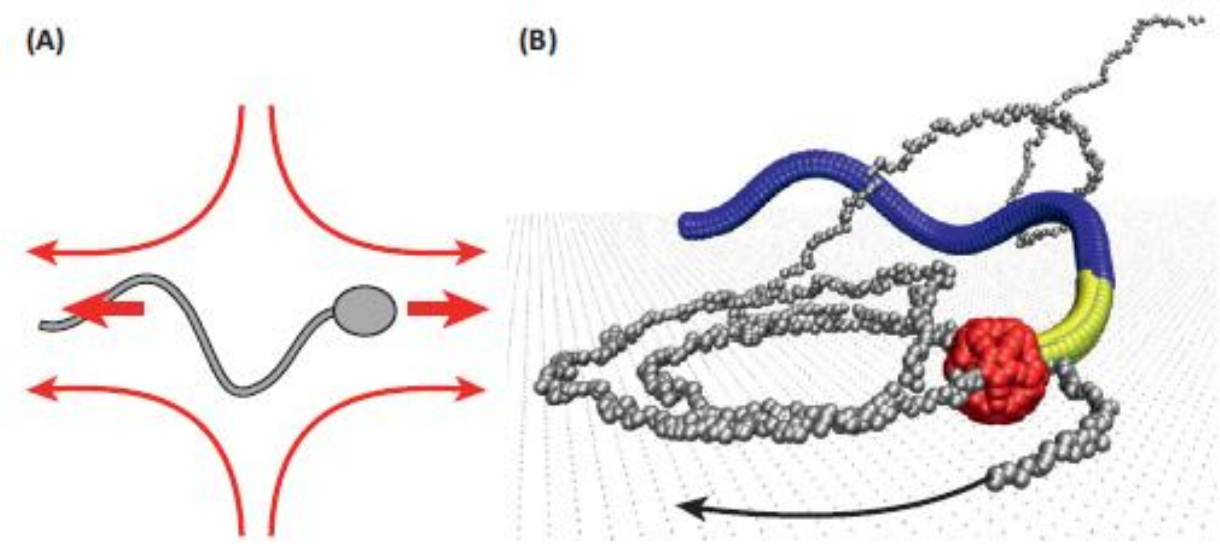


Figure 2.7: Hydrodynamics of micro-swimmers. (A) Schematic flow (indicated by red arrows) around a sperm swimming in a bulk fluid. Sperm fall in the category of micro-swimmers called "pushers". (B) Sperm with a curved flagellum or located close to a wall swim in helical trajectory in the bulk (indicated by small grey beads) on particle-based mesoscale hydrodynamic simulations (From Alvarez *et a*l., 2014).

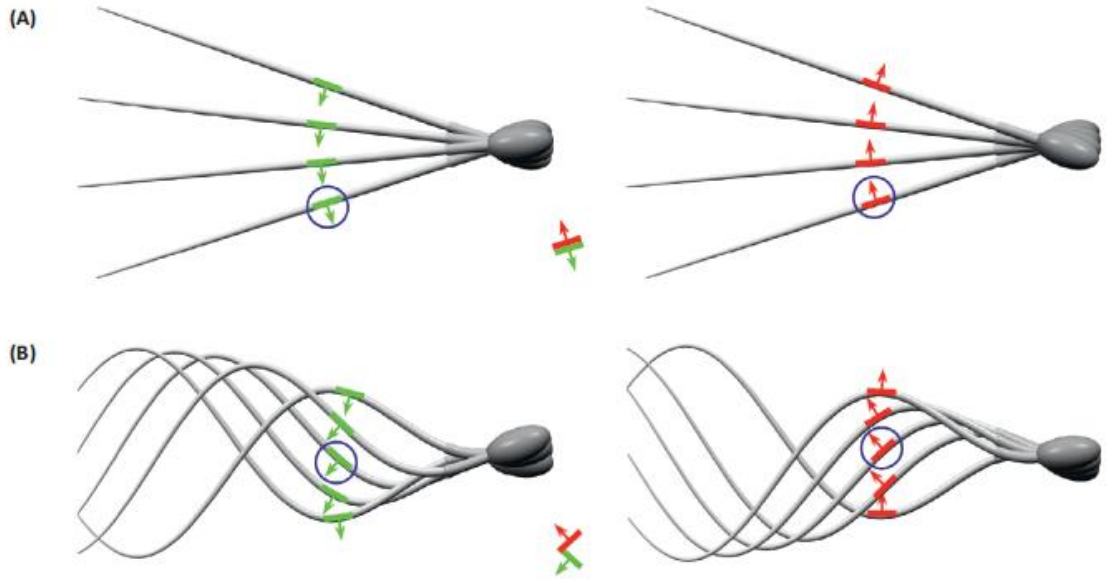


Figure 2.8: Breaking the symmetry at low Reynolds numbers: **(A)** Forces imposed by a small portion of the flagellum on the fluid by the red and green arrows. Since the two halves of a beat cycle are identical, forces applied during one-half of the cycle (green) are compensated for during the other half (red) and the micro-swimmer will not move forward. **(B)** Sperm break the temporal symmetry and the cycles differ, because the travelling wave propagates in only one direction and, as a result, the micro-swimmer will move forward. (From Alvarez *et a*l., 2014).

2.6.5 STRUCTURAL INTEGRITY OF SPERMATOZOA

The accurate determination of sperm morphology, vitality, acrosome integrity and morphometric assessments depends on careful preparation, fixation and staining procedures when handling spermatozoa (Garcia-Herreros, *et al.*, 2006; Henkel, *et al.*, 2007; Van der Horst, *et al.*, 2009; Van der Horst & Maree, 2009). Due to the fact that staining procedures can affect sperm dimensions significantly, one of the most important criteria for staining solutions and methods is that the processes involved should cause as little change as possible to the true form and structural integrity of the spermatozoa (Van der Horst & Maree, 2009).

2.6.5.1 Plasma membrane integrity (Viability)

A functional, intact sperm plasma membrane is a pre-requisite for sperm viability and function (Salisbury, *et al*., 1978). The plasma membrane forms a boundary around the sperm cytoplasm while maintaining the chemical gradient relevant for sperm metabolism and the ability to interact with its surroundings. A viable sperm plasma membrane is vital for normal cellular homeostasis as a lack of interaction with the surrounding environment means no fertilisation (Salisbury, *et al.*, 1978; Rodriques-Martinez & Barth, 2007). Eosin-nigrosin is a vital sperm stain that has been used for decades to evaluate the integrity of the cell plasma membranes of spermatozoa (Campbell, *et al.*, 1956; Jainudeen, *et al.,* 1971; Nöthling & Irons, 2008). Eosin does not penetrate functionally intact membranes. Spermatozoa that remain white after exposure to eosin are considered alive whereas red stained spermatozoa that have absorbed the stain are considered dead (Campbell, *et al.,* 1956; Jainudeen, *et al.,* 1971; Nöthling & Irons, 2008).

2.6.5.2 Acrosome integrity

Evaluation of sperm morphology also requires that sperm be differentially stained to allow a clear distinction between the boundaries of the acrosome, the head, the midpiece and the tail (Nöthling & Irons, 2008; Van der Horst & Maree, 2009; Maree, *et al.*, 2010). The presence of an intact acrosome is essential for the acrosome reaction to occur during capacitation and ultimately for fertilisation to

take place (Fawcett, 1970; Weiss & Greep, 1977; Barth, 2007; Cooper & Hausman, 2009). Spermac® and eosin-nigrosin stained smears can be used to assess acrosome integrity by means of phase-contrast microscopy or differential interference contrast microscopy (Barth & Oko, 1989; Nöthling, 2006).

2.6.5.3 Sperm morphology

Morphological and ultrastructural analysis of spermatozoa are useful tools in reproductive biology and phylogenetic studies. The percentage of morphologically normal sperm in a semen sample is a highly predictive of the potential fertility of the male (Barth & Oko, 1989; Brito, *et al*., 2003; Nöthling & Irons, 2008). Previous studies have shown that morphological defects may originate during spermatogenesis, spermiogenesis, epididymal transfer, epididymal storage and retention in the ampulla, as well as during ejaculation or after ejaculation (Barth & Oko, 1989; Nöthling & Irons, 2008). Structural defects recorded may indicate severe widespread malfunction of the spermatogenic epithelium, including, for example, irregular nuclear division during meiosis, abnormal transformation of the nucleus, Golgi vesicles, centriole or mitochondria of the spermatid during spermiogenesis, failure of the cytoplasmic droplet to migrate towards the annulus during epididymal maturation, inability to maintain flagellar coherence during epididymal migration, deterioration of membrane stability during epididymal storage, disintegration of senescent spermatozoa in the epididymis or ampulla, and failure of the seminal plasma to induce detachment of distal cytoplasmic droplets (Barth & Oko, 1989; Nöthling & Irons, 2008). After ejaculation, exposure of spermatozoa to an environment that is hypotonic or too cold induces bent flagella, whereas manipulations and robust mechanical handling may cause detached heads or broken tails. *In vitro* incubation or the freeze-thaw process may also cause bent tails and acrosomal changes (Barth & Oko, 1989; Nöthling & Irons, 2008).

Various schemes for the morphological classification of spermatozoa have been proposed, with one of the earliest schemes classifying sperm according to primary or secondary abnormalities. Primary abnormalities are identified when the “occurrence is assumed to be due to disorders in the

spermiogenic epithelium" and secondary abnormalities as "appearing as a result of some unphysiological conditions affecting sperm after they have left the spermiogenic epithelium" (Nöthling & Irons, 2008). A later system that was later recognised classified spermatozoa according to the occurrence of major or minor defects. When between 10 – 15% primary (major) defects are recorded, sperm function is correlated with impaired fertility (Barth, 2007; Nöthling & Irons, 2008). A scheme for the assessment of bovine sperm morphology proposed by Saacke in 1994 suggested that the morphological defects of a spermatozoon should be classified as compensable or uncompensable (Saacke, 2008; Nöthling & Irons, 2008). Spermatozoa with compensable defects either do not reach the site of fertilisation or if they do, cannot initiate the activation of a zona vittaline block to prevent polyspermy (Saacke, 2008; Nöthling & Irons, 2008). Spermatozoa with uncompensable defects are able to reach the oocyte, penetrate and activate the block to polyspermy, but are unable to complete the fertilisation process or maintain normal embryo development (Saacke, 2008; Nöthling & Irons, 2008).

Various stains such as Diff-Quick®, Papanicolaou®, Schorr® and other groups of closely related rapid stains, including Spermac® and eosin-nigrosin are used to stain sperm smears prior to evaluation under bright-field microscopy (Barth, 2007; Nöthling & Irons, 2008; Maree, *et al.*, 2010). Unstained sperm smears can be evaluated by using phase-contrast microscopy after fixation with glutaraldehyde or formal-saline (Barth & Oko, 1989; Nöthling & Irons, 2008).

2.6.5.4 Sperm head morphometric analysis

Computer automated sperm morphology analysis (CASMA) has been developed for both morphologic and morphometric assessment of spermatozoa. CASMA morphometric analysis can be used to measure the size of different components of a sperm cell (Amann & Katz, 2004; Van der Horst & Maree, 2009). SpermBlue® stain has been shown to be easy to use that allows for accurate morphology and morphometric analysis in various species (Van der Horst & Maree, 2009).

2.6.5.5 Transmission electron microscopy (TEM)

TEM provides a structural basis of accounting for abnormalities or general normality of spermatozoa within a semen sample as well as the evaluation of acrosomal morphology (Barth & Oko, 1989). Studies on sperm morphology and the underlying ultrastructural defects are important for understanding the reproductive biology of species and the information collected during these studies can be used to investigate reproductive problems (Brito, et al., 2010). Light microscopy only allows for limited resolution, thus the analysis of detailed cell structure requires the use of more powerful microscopic techniques, namely electron microscopy (Hodgson, *et al.,* 1990; Cooper & Hausman, 2009).Two types of electron microscopy methods, transmission and scanning, are widely used to study cells. In principle, transmission electron microscopy is similar to the observation of stained cells with the bright-field light microscope. Specimens are fixed and stained with salts of heavy metals, which provide contrast by scattering electrons (Fawcett, 1970; Weiss & Greep, 1977; Hodgson, *et al.,* 1990). Electron microscopy was developed in the 1930s and first applied to the evaluation of biological specimens by Albert Claude, Keith Porter and then by George Palade, in the 1940s and 1950s (Cooper & Hausman, 2009).

Due to the fact that the wavelength of electrons is shorter than that of light, the electron microscope can achieve a much greater resolution than that obtained with the light microscope (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009). The wavelength of electrons in an electron microscope can be as short as 0.004nm, about 100,000 times shorter than the wavelength of visible light. Theoretically, this wavelength could yield a resolution of 0.002nm, but such a resolution cannot be obtained in practice because the resolution is determined not only by wavelength, but also by the numerical aperture of the microscope lens (Hodgson, *et al.*, 1990; Cooper & Hausman, 2009). Consequently, for biological samples the practical limit of resolution of the electron microscope is 1 to 2nm. Although this resolution is much less than that predicted simply from the wavelength of electrons, it represents more than a hundred-fold improvement on the resolving power of the light microscope (Hodgson, *et al.,* 1990; Cooper & Hausman, 2009).

2.7 COMPUTER-AIDED SPERM ANALYSIS (CASA) TECHNOLOGIES

This section provides a brief overview of the development of CASA technology and the application thereof as a diagnostic tool in spermatology studies. Excellent overviews regarding CASA are provided in cited literature (Davis & Katz, 1993; Verstegen & Onclin, 2002; Amann & Katz, 2004; Holt, *et al.*, 2007; Amann & Waberski, 2014; Mortimer, *et al.,* 2015). Since the 1950s, when the phase contrast microscope was first used, it has become the primary instrument for observing and evaluating sperm (Amann & Katz, 2004). Phase contrast microscopy is an integral part of the development of computer-aided sperm analysis (CASA) since the optics allow for high-contrast visualisation and edge detection of each translucent cell (Amann & Katz, 2004).

CASA is an automated system where phase contrast microscope optics allows a software programme to digitise and record the motion track of every spermatozoon detected within a microscopic field. CASA motility evaluation consists of a series of successive images of motile spermatozoa with the use of image standard acquisition rates (frames s^{-1}; Hz). Computer software scans these images in sequence using algorithms to identify individual spermatozoa (Davis, *et al.,* 1992; Davis & Katz, 1993; Van der Horst, 1995; Amann & Katz, 2004; Holt, *et al.*, 2007; McPartlin, *et al.*, 2009; Broekhuijse, *et al.,* 2011; Maree & Van der Horst, 2013). CASA has also made an automated analysis of sperm morphology, morphometric, vitality and DNA fragmentation possible (Davis & Katz, 1993; Amann & Katz, 2004) CASA parameters are modelled and refined mathematically to best describe the motion characteristics of each sperm as it travels through a microscopic field. These sperm kinematic parameters include curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL), linearity (LIN) and straightness (STR) of motion tracks, flagellar beat frequency (BCF) and the amplitude of the lateral head displacement (ALH) expected to reflect the physiological status of individual spermatozoa (Abaigar, *et al.*, 1999; Suarez, 2008; Abaigar, *et al.*, 2011). CASA has greatly improved semen analysis and our understanding regarding the dynamics of sperm kinematics (Van der Horst, 1995; Amann & Katz, 2004; Holt, *et al.*, 2007; Abaigar, *et al.*, 2011; Broekhuijse, *et al.,* 2011; Maree & Van der Horst, 2013).

CASA furthermore increases the objectivity, precision and reliability of sperm motility assessment (Davis & Katz, 1993; Mortimer, et al., 1998; Amann & Katz, 2004; Holt, et al., 2007; McPartlin, et al., 2009). CASA systems are, however, not ready-to-use devices and the results depend largely on the experience of the user, the technical settings used, together with the preparation and handling of the semen sample. The availability of data recorded by CASA also enables the comparison of results, for example sperm motility, and makes it possible to find subtle differences between individuals, treatments or ejaculates (Davis & Katz, 1993; Cancel, *et al.*, 2000; Amann & Katz, 2004; Holt, *et al.*, 2007; McPartlin, *et al.*, 2009; Broekhuijse, *et al.*, 2011; Maree & Van der Horst, 2013).

The use of CASA systems has allowed for the evaluation of motile and hyperactivated sperm subpopulations within ejaculates collected from different species in the quest to understand semen quality and the possible potential of a spermatozoon to reach the site of fertilisation (Abaigar, *et al.*, 1999; Holt, *et al.*, 2007). With well-designed and controlled CASA studies, together with their applications, the potential power of CASA is revealed with the use of appropriate methodologies (Davis, *et al.*, 1992; Davis & Katz, 1993; Amann & Katz, 2004). In previous studies, CASA has been successfully applied in the analysis of hyperactive motility in hamster, rats and stallions (Ho & Saurez, 2001; Cancel, *et al.*, 2000; McPartlin, *et al.*, 2009) , as well as the classification of sperm subpopulations in the domestic boar (Abaigar, *et al.,* 1999; Broekhuijse, *et al.,* 2011) and the gazelle antelope (Abaigar, *et al.,* 1999; Abaigar, *et al.,* 2011). Furthermore, CASA studies allowed for the quantification of epididymal flushed semen obtained from the domestic bull (Goovaerts, *et al.,* 2006; Contri, *et al.,* 2010) and the domestic cats (Fillers, *et al.*, 2008). CASA results have been reported to be repeatable in humans and rabbits studies (Farrel, *et al.*, 1995). CASMA has also been successfully applied in domestic boar (Garcia-Herreros, *et al.*, 2006) and humans (Van der Horst & Maree, 2009; Maree, *et al.*, 2010) sperm head morphometric studies.

Since species differ in movement patterns, specific thresholds are required for a CASA system to identify e.g. hyperactivated spermatozoa within an ejaculate of each species evaluated. The incidence of hyperactivation in spermatozoa can be measured by setting cut-off values for the kinematic

parameters that distinguish between the subgroups of motile spermatozoa within an ejaculate (Mortimer, *et al.*, 1998; Ho & Saurez, 2001; McPartlin, *et al.*, 2009). The general procedure is to incubate spermatozoa under *in vitro* fertilisation conditions that support capacitation and compare swimming patterns with spermatozoa not exposed to capacitating conditions (Abaigar, *et al.,* 1999; Ho & Suarez, 2003; McPartlin, *et al.*, 2009; Abaigar, *et al.,* 2011). The examiner then identifies measurements affected by the capacitating conditions and uses the information to set threshold or cut-off values to identify hyperactivated spermatozoa (Mortimer, *et al.,* 1998; Suarez, 2008; McPartlin, *et al.*, 2009).Cut-off values are usually set for a minimum curvilinear velocity, a maximum path linearity (minimum curvature of path trajectory) and a minimum for the amplitude of lateral head displacement (ALH). It is important to note that CASA systems operate by measuring movements of the head, which measures flagellar beat cross frequency (BCF) indirectly (Mortimer, *et al.,* 1998; Ho & Saurez, 2001; McPartlin, *et al.,* 2009). The motility playback feature present in some CASA systems must be utilised to check the accuracy of the evaluation and re-adjust thresholds if necessary (Suarez, 2008). The interpretation of the results obtained can validate the usefulness of this technology in practical research. In practice (and industry) it might assist in determining the potential of a spermatozoon to reach the site of fertilisation (Davis, *et al.*, 1992; Van der Horst, 1995; Verstegen & Onclin, 2002; Holt, *et al.*, 2007; O' Brien, *et al.*, 2009; Maree & Van der Horst, 2013). In summary, CASA allows for objective evaluation of semen quality, using standardised methodologies. Accurate semen evaluation can be performed and computer aided systems can greatly assist in standardising data capturing methodologies (Hafez, 1987; Barth & Oko, 1989; Barth, 2007; Holt, 2000).

2.8 CONCLUSION

The successful application of Assisted Reproductive Technology (ART) holds the potential to propagate small fragmented populations of wild endangered species. This includes the possibilities of obtaining more offspring from selected individuals to ensure genetic diversity, and the reduction of intervals between generations (Comizzoli, *et al.*, 2000; Holt & Pickard, 1999; Andrabi & Maxwell, 2007; Milligan, *et al.*, 2009; Monfort, 2014; Holt & Brown, 2014). The combined use of semen

cryopreservation and artificial insemination (AI) has the potential to lengthen the reproductive capability of an individual beyond their natural lives (Holt & Pickard, 1999; Hildebrandt, *et al.*, 2012; Comizzoli, *et al.*, 2000). The application of ART in the conservation of endangered wildlife species has grown in popularity over the last 25 years (Pickard & Holt, 2007; Monfort, 2014). To increase the genetic representation under the guidance of Species Survival Plans (O'Brien, *et al.*, 2013), the successful application of semen cryopreservation and AI justify the collection of semen from *in situ* populations to enhance breeding programs of *ex situ* populations (Hermes, *et al.*, 2004; Hildebrandt, *et al.*, 2007; Holt, *et al.*, 2007; O' Brien, *et al.*, 2009). The assessment of the reproductive performance of males is critical for optimising the propagation of selected species of importance or of endangered wildlife species (Wildt, et al., 1995; Wildt, 1996; Estep & Dewsbury, 1996; Andrabi & Maxwell, 2007).

Spermatozoa within a single ejaculate have different structural and functional characteristics that may be associated with differences in quality and fertility potential (Holt & Van Look, 2004; Abaigar, *et al.*, 2011). In this study, we used routine semen analysis and CASA to determine the motility and kinematic parameters, and CASMA and TEM to investigate the structural integrity of spermatozoa within semen samples collected from free-ranging African elephant and Southern white rhinoceros within South Africa. Generated datasets were further explored by means of pattern analysis (classification of spermatozoa according to the motion patterns) to measure the descriptors without losing their information content.

CHAPTER 3

MATERIALS AND METHODS

3.1 ETHICAL STATEMENT

All procedures were performed in agreement with and approval by the Ethics Committees of the University of the Western Cape (Project Number: 94/1/00), the National Zoological Gardens of South Africa (Project Number: P10/06) and the Internal Committee of Ethics and Animal Welfare of the Leibniz Institute for Zoo and Wildlife Research (Approval Number: 2009-08-01).

3.2 SOURCE OF BIOMATERIAL SAMPLES

The opportunity for the collection of biomaterial and data for this study arose during a joint research project within South Africa. The project involved international collaboration among several participants from the Department of Medical Biosciences, University of the Western Cape (UWC), Bellville, The National Zoological Gardens of South Africa (NZG), Pretoria, S.A., The Leibniz Institute for Zoo and Wildlife Research (IZW), Berlin, Germany, ZooParc de Beauval, Saint Aignan sur Cher, France, Pittsburgh Zoo and PPG Aquarium, Pittsburgh, United States of America.

A total number of 21 free-ranging adult African elephant bulls and 10 free-ranging Southern white rhinoceros was evaluated during this study. Biomaterial was collected in the spring month of September 2009 at the end of the dry season, from seven African elephants and in the following year during the autumn month of April 2010, another fourteen elephants were sampled. In April 2010 and at the end of the rainy season, semen from 10 free-ranging Southern white rhinoceros were also sampled and evaluated. The free-ranging elephant bulls were located at Phinda Private Game Reserve, KwaZulu-Natal, S.A. (S27°46.657” E32°20.942”). Six of the white rhinoceros bulls located at Pragtig Private Game Farm, Musina, South Africa (S22°20’30.007” E30°2’31.2072”) and the other

four bulls were located at Mziki Private Game Farm, Beestekraal, South Africa (S25°25.208" E27°31.551").

3.3 ANAESTHESIA OF ANIMALS

All sampling procedures of African elephants and Southern white rhinoceros commenced after the individuals were under general anaesthesia. Individual bulls were identified by using records of ear notch images. The immobilising combinations used in this study had been successfully applied in previous studies (Hermes, *et al*., 2005; Hermes, *et al*., 2009; Hildebrandt, *et al.*, 2012; Hermes, *et al.*, 2013). Anaesthesia dosages were determined after body weight estimations. The immobilising combination was delivered in a 3 mL plastic dart (Dan-inject, Fritz Rohr, Skukuza, S.A.) by means of a dart gun (Dan-inject, Fritz Rohr, Skukuza, S.A.) from a helicopter (Figure 3.1). Qualified veterinary staff performed all the darting and subsequent drug administration. An experienced veterinarian frequently monitored the general well-being, heart rate and breathing pattern of individual animals under anaesthesia during the sampling process.

3.3.1 AFRICAN ELEPHANTS (*Loxodonta africana*)

The dosages ranged from 12 to 20mg of etorphine hydrochloride (9.8mg, M99, Novartis, Johannesburg, S.A.) combined with 30 to 40mg of azaperone (40mg/mL, Stresnil®, Jassen Pharmaceutica, Johannesburg, S.A.) delivered intramuscularly (IM). Reversal of the anaesthesia was done by administering 26 to 42mg of diprenorphine (12mg, Revivon M5050, Novartis, Johannesburg, S.A.) intravenously (IV) in combination with 100 to 300mg of naltrexone hydrochloride (50mg/mL, Trexonil®, Wildlife Pharmaceuticals, Pretoria, S.A.), with half of the dose administered IV and half intramuscularly (IM).

3.3.2 SOUTHERN WHITE RHINOCEROS (*Ceratotherium simum simum*)

Anaesthesia wasachieved using a combination of 30.0mg Midazolam (5mg/mL, Dormicum, Roche, Isando, S.A.) and 4 to 5 mg etorphine hydrochloride (9.8mg, M99, Novartis, Johannesburg, S.A.) applied IM via a dart gun. This was followed by 10 to 20.0mg butorphanol (50mg/mL, Kyron Laboratories, Johannesburg, S.A.) which was administered into the ear vein, once the animal was recumbent, to partially antagonise the etorphine and facilitate breathing during the sampling process. The reversal of anaesthesia was accomplished by administering 50.0mg naltrexone hydrochloride (50mg/mL, Trexonil®, Wildlife Pharmaceuticals, Pretoria, S.A.), with 25mg administered IV and 25mg IM.

Figure 3.1: African elephant bulls were located, identified and anaesthesia dosages determined after body weight estimations. The immobilising combination was delivered in a plastic dart by means of a dart gun from a helicopter (Photo credit: P. Salvaggio).

3.4 BIOMATERIAL COLLECTION TECHNIQUES

3.4.1 SEMEN COLLECTION

During anaesthesia assessment of the male reproductive system was done by means of ultrasonography (Voluson *i,* GE Medical Systems, Zipf, Austria, with 2-5 MHz and 4-8.5 transducers) as described in previous studies (Hermes, *et al.*, 2005; Hermes, *et al.*, 2009; Hildebrandt, *et al*., 2012; Hermes, *et al.*, 2013). The electroejaculation procedure was performed immediately thereafter (Electro-ejaculator: Seager model 14, Dalzell USA systems, The Plains, VA, USA). A handheld probe (125mm in length and 105mm in diameter) (Dalzell USA Medical systems, The Plains, VA, USA) especially designed for elephants and rhinoceroses (Figure 3.2 A) (Hermes, *et al.*, 2005; Hermes, *et al.,* 2009; Hildebrandt, *et al.,* 2012; Hermes, *et al.,* 2013) was used. Mounted within the probe are three longitudinal electrodes that have been slightly raised (Hermes, *et al.*, 2005; Hermes, *et al.,* 2013). The increased diameter of the probe allows for optimal contact between the electrodes of the probe and the rectal mucosa lining, following administration of a rectal enema (Hermes, et al., 2005). The probe was inserted into the rectum with the electrodes positioned ventrally.

During the ultrasound evaluation, the correct positioning of the probe was determined (Figure 3.2 B). This was done to ensure optimal stimulation of the prostate and other accessory sex glands, as well as to avoid stimulating the urinary bladder (Hermes, *et al.*, 2005; Hermes, *et al.*, 2009; Hildebrandt, *et al.*, 2012; Hermes, *et al.,* 2013). The electroejaculation procedure consisted of a sequence of electric stimuli based on the principle of rest and stimulation, of more or less equal duration. The pattern of stimulation was adapted to the response of the bull, as it could vary between individuals for both elephant and rhinoceros. The erect penis (extruded from the sheath) was manipulated manually and a condom placed over the penis to collect different fractions of the ejaculate during electro-stimulation (Figure 3.2 C). A condom consisting of a modified rectal glove containing a 50mL conical tube (Greiner®, Sigma-Aldrich, Johannesburg, South-Africa) protected by insulation foam to insulate the collected semen and accordingly protect it from temperature shock was used. These condoms

were replaced after each set of stimuli to allow the collection of different fractions of the ejaculate and to avoid possible urine contamination of the collected fractions (Figure 3.3). After each set of stimulation, the pelvic and penile aspects of the urethra were massaged manually as shown in Figure 3.2 D.

3.4.1.1 African elephants (*Loxodonta africana*)

African elephants respond well to 12 - 16 sets of electric stimulations where the voltage (15 - 30V) and amperage (450 – 1000mA) were increased in increments (Hildebrandt, *et al.*, 2012; Hermes, *et al.*, 2013).

3.4.1.2 Southern white rhinoceros (*Ceratotherium simum simum*)

For the Southern white rhinoceros, three to four sets of electric stimuli with increasing voltage (5 - 20V) and amperage (200 – 900mA) were sufficient to collect semen (Hermes, *et al.*, 2005; Hermes, *et al.*, 2009).

Figure 3.2: (A) The electroejaculation procedure performed to collect semen samples with a specially designed hand-held probe for elephants and rhinoceroses. **(B)** The reproductive tracts were evaluated by means of ultrasound. **(C)** A condom consisting of a modified rectal glove containing a 50 mL conical tube protected by insulation foam was used to collect the ejaculates. **(D)** After each set of stimulation, the pelvic and penile aspects of the urethra were massaged manually (Photo credit: P. Salvaggio & L. Olivier).

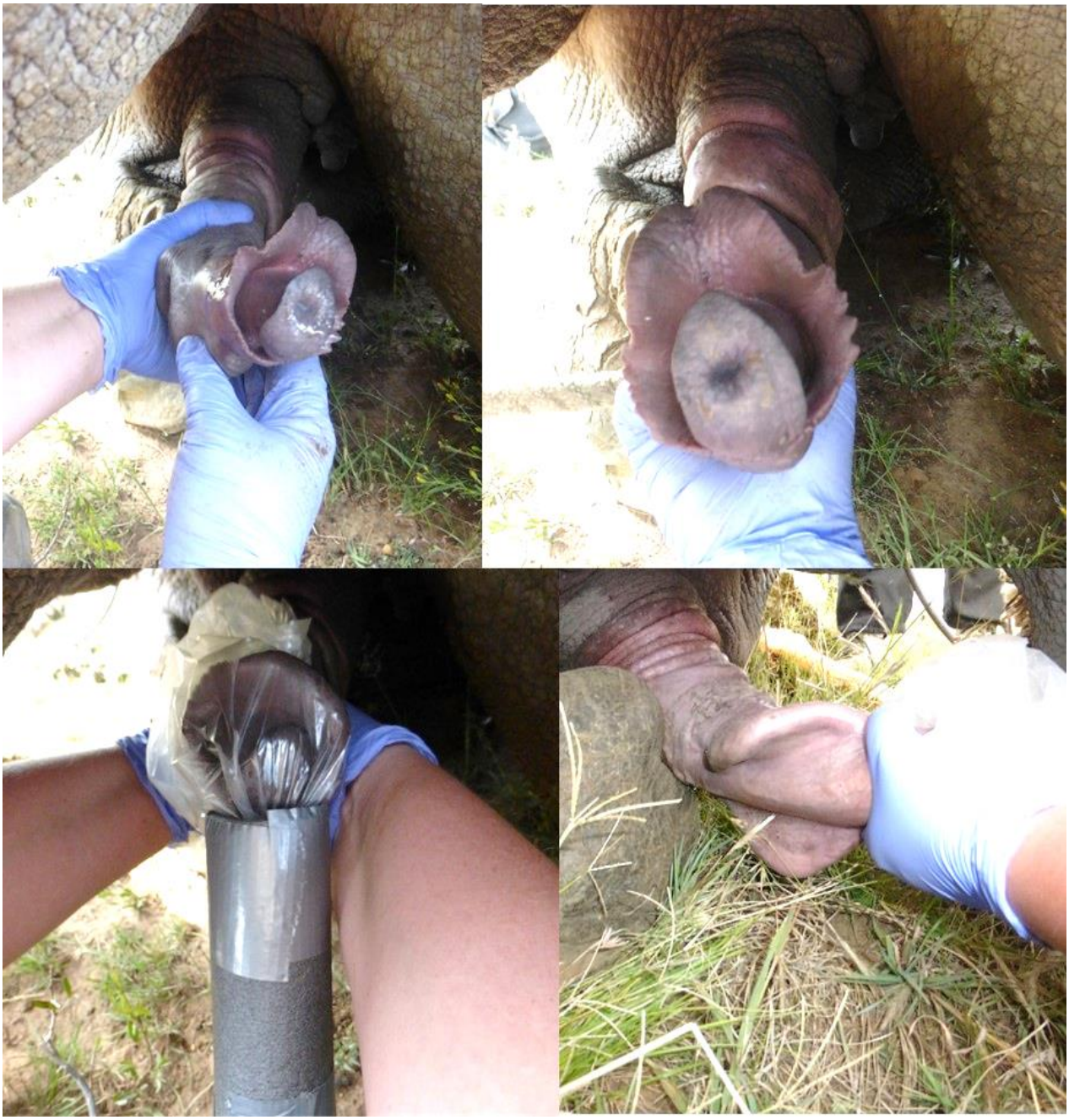


Figure 3.3. The erect penis of a Southern white rhinoceros, manually manipulated (extruded from sheath) to place a condom over the penis tip. These condoms were replaced after each set of stimuli to allow for the collection of different fractions of the ejaculate and to avoid possible urine contamination of the collected fractions.

3.4.2 BLOOD COLLECTION

While under sedation, a minimum of 3mL blood was collected from an ear vein, in an EDTA vaccutainer® blood tube, from each individual at the time of semen collection. These samples were used for genomics (discussed in Chapter 6) (Figure 3.4 D). Samples collected were kept at 5°C, and transported to the laboratory, decanted into Nunc® cryotubes (Sigma-Aldrich, Johannesburg, S.A.), and stored at - 80°C until analysis.

3.4.3 SAMPLE HANDLING, TRANSPORT AND PROCESSING

A 2mL aliquot of neat semen was immediately removed after collection to determine sperm concentration and to evaluate neat (NT) sperm motility (Figure 3.4. C). The colour and viscosity of the collected semen sample was evaluated and described (Figure 3.4 A). The remainder of the ejaculate was diluted (1:1) with pre-heated (± 37°C) egg yolk (EY) based Berliner Cryomedium (BC). This Tris-egg yolk based extender is composed of 2.41% (w/v) TES, 0.58% (w/v) Tris, 0.1% (w/v) fructose, 5.5% (w/v) lactose, 15.6% (v/v) egg yolk and 20IU α-tocopherol/mL (Hermes, *et al.*, 2013). After dilution, the samples were placed in a shipping box containing a water bag, heated to 35°C in order to minimise temperature shock to the spermatozoa (Figure 3.4 E). All the collected sperm samples were transported by means of the helicopter to the field laboratory. All sperm samples were evaluated within 30 minutes of collection.

3.4.4 GENERAL HANDLING AND PROCESSING PROCEDURE OF SPERM SAMPLES

Upon arrival in the field laboratory, within 30 minutes after collection, sperm samples were immediately processed for evaluation as presented in the schematic diagram (Figure 3.5). All disposables and slides were clearly labelled with the identity of the animal, date and the media abbreviation, if relevant. All sperm samples, either neat (NT) or extended with egg yolk (EY) were initially screened for motility and the sperm concentration determined after 10x dilution with Ham's F10 media (100µl of sperm sample in 900µl of Ham's F10 media).

Ham's F10 media was chosen as control medium due to its proven capability to maintain sperm functions of various species for long periods of time (Brinders, 1994; Mahadevan, *et al.*, 1997; Maree & Van der Horst, 2013). Ham's F10 medium (Sigma-Aldrich, Johannesburg, S.A.) was supplemented with 3 % bovine serum albumin (BSA) (Sigma-Aldrich, Johannesburg, S.A.). INRA96® (IMV Technologies, Taurus, Pretoria, S.A.) is a commercial milk-based extender routinely used for both fresh and frozen storage of equine sperm (Imrat, *et al*., 2013). Similar to the original recipe published by Kenny *et al.* (1975), milk based extenders are easy to prepare, inexpensive and can be stored in frozen form and result in acceptable fertility rates in mares (Aurich, 2008).

Hyperactivated motility (HA) was induced with the use of BO sperm wash media, as described by Bracket and Olifant (1975). Available sperm sub-samples (either EY or NT) collected from the elephant population during season 2 and of both rhinoceros populations, was incubated with Bracket and Olifant media (BO) containing 10mM caffeine. The BO media used in this study contained 6mg/mL fatty acid-free bovine serum albumin, 5mg/mL BSA fraction V and 10mM caffeine (Brackett & Oliphant, 1975).

In this study the following sperm sample dilutions and evaluation media were used: Egg Yolk (EY), Egg yolk and BO (EY_BO), Egg yolk and Ham's F10 (EY_Ham's), Egg Yolk and INRA96® (EY_INRA), Neat and BO (NT_BO), Neat and Ham's F10 (NT_H Ham's) and Neat and INRA96® (NT_INRA). All samples were initially analysed for motility with SCA® CASA (Sperm Class Analyser, version 4.1. or 5.4, Microptic S.L., Barcelona, Spain) after a five-minute incubation period. The motility and kinematic parameter values recorded in the control group with Ham's F10 media (either EY or NT) were used to compare against the parameter values recorded for INRA96® and BO (10mM caffeine) media (either EY or NT).

Figure 3.4: (A) The colour and viscosity of the collected semen sample were evaluated and recorded **(B)** All sperm motility and kinematic parameter assessments were performed by means of computer-aided sperm analysis systems (CASA). **(C)** A 2mL aliquot of neat semen was immediately removed after collection to determine sperm concentration and to evaluate neat (NT) sperm motility. **(D)** Blood was collected from an ear vein. **(E)** All collected samples were placed in a shipping box and transported to the laboratory. **(F)** Semen smears were prepared for morphological and morphometric evaluation (Photo credit: P. Salvaggio).

3.5 MACROSCOPIC SEMEN EVALUATION

3.5.1 EJACULATE COLOUR AND VISCOSITY

Each ejaculate collected was evaluated for appearance before further dilution. Semen samples with a normal colour were classified as follows: milky, yellow, clear or grey. Any viscosity and/or abnormal colours were recorded and potential contamination, for example mucous, pus, debris, urine or faecal matter classified (Howard, et al., 1984; Barth, 2007). Viscosity was subjectively assessed using a 1 to 5 score system where 1 was watery and 5 highly viscous.

3.5.2 EJACULATE VOLUME

The volume of all the fractions collected was measured in graduated conical tubes (Greiner®, Sigma-Aldrich, Johannesburg, S.A.) and the total volume recorded for each ejaculate. As a correction factor, the recorded volume was divided by 2 (to compensate for the 1:1 dilution with BC media in the field) and 2mL added (to compensate for the 2mL neat aliquot removed for evaluation). The final estimated volume was recorded for each ejaculate collected.

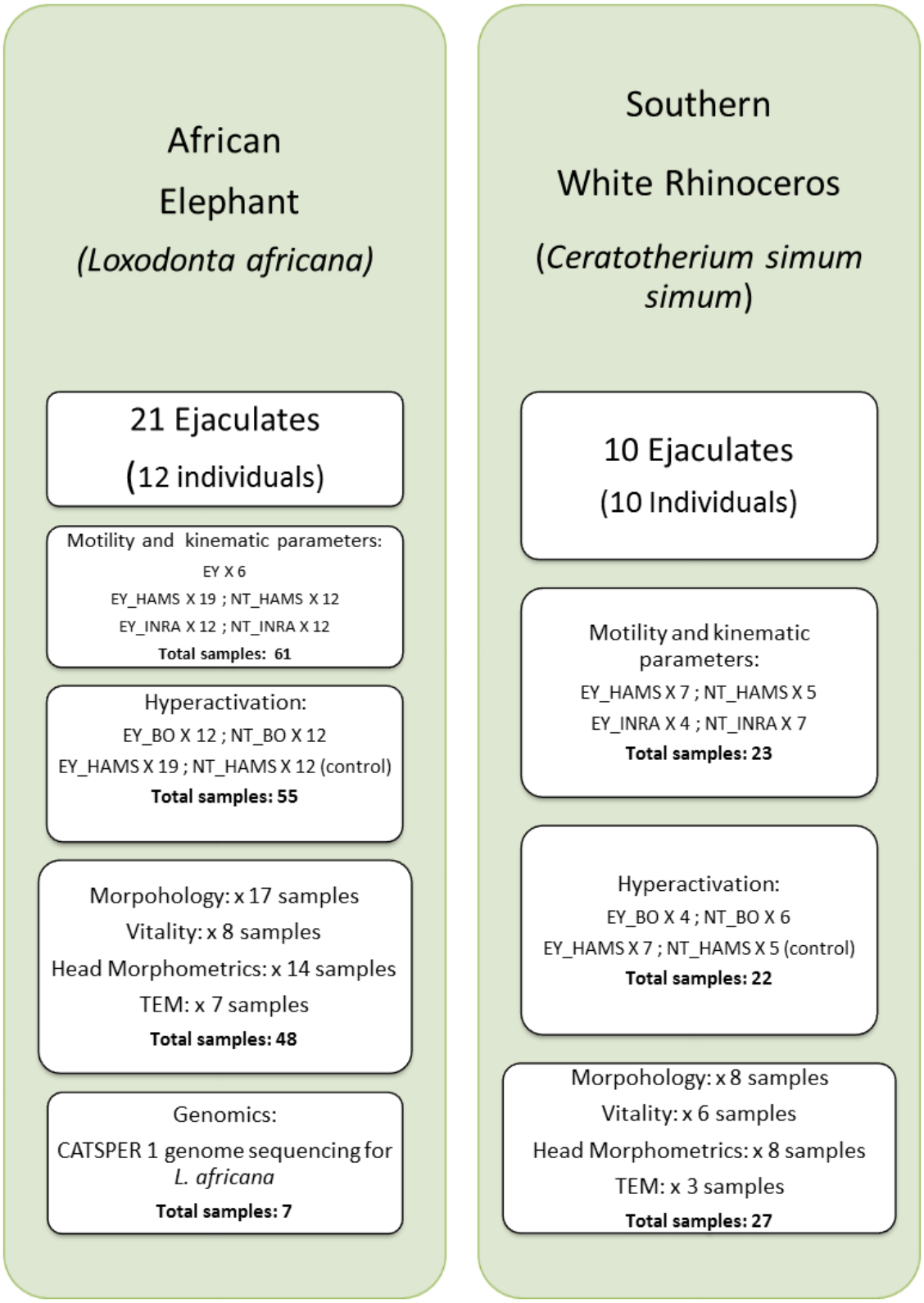


Figure 3.5: Schematic diagram of samples evaluated during this study. Egg yolk (EY); Egg-yolk and Ham's F10 (EY_ Ham's); Egg-yolk and INRA96® (EY_INRA); Egg-yolk and BO (EY_BO); Neat and Ham's F10 (NT_ Ham's); Neat and INRA96® (NT_INRA) and Neat and BO (NT_BO).

3.6 MICROSCOPIC SEMEN EVALUATION

3.6.1 SPERM CONCENTRATION

The sperm concentration of neat (undiluted) semen is generally too high in most mammals for CASA analysis (Van der Horst, 1995). The optimum concentration to evaluate a semen sample by means of CASA (SCA) is at a range of 10-30 x 10^6 spermatozoa per mL. Dilutions varied from 10x to 1000x (depending on sperm concentration) using Ham's F10 medium (Sigma-Aldrich, Johannesburg, S.A.).

3.6.2 CASA EQUIPMENT AND PARAMETER SETTINGS (ANALYSIS PROPERTIES)

All sperm motility and kinematic parameter assessments were done by means of automated image analysis techniques using computer-aided sperm analysis. The Sperm Class Analyser (SCA®) (Microptic SL, Barcelona, Spain) version 4.1.0.0 or 5.4.0.0 was connected to a Basler 312fc digital camera (Microptic SL, Barcelona, Spain) mounted (C-mount) onto a Nikon Eclipse 50i microscope (IMP, Johannesburg, S.A.). A 10x negative phase lens was used and the microscope stage was electronically temperature controlled (HS-50, IMP, Johannesburg, S.A.) and pre-set to 37°C (Figure 3.4 B).

All disposables such as slides and pipette tips used were pre-heated to 37°C. Sperm motility was captured at 50 frames per second. Negative phase optics was used and a 10x negative phase contrast objective (Ph-) was employed. Leja® 20 (Leja® slide, Leja® Products B.V., Netherland) is a disposable glass chamber with a defined volume (2µl) and depth of 20µm that is used during automated sperm analysis. The shape of the chamber is rectangular and loaded by capillary action. The depth of the chamber provides a monolayer of cells, which is important for the focal depth and image acquisition without interfering with sperm motion characteristics. Analysis properties used to measure the motility parameters were set according to the particular species (Van der Horst, 1995; Van der Horst, *et al.*, 1999) (Table 3.1).

Table 3.1: The SCA® property settings selected for each species analysed during this study.

	African elephant	Southern white rhinoceros
Particle area	15 < PA < 80	4 < PA < 75
Velocity intervals	10 < S < 65 < M< 120 < R	10 < S < 65 < M< 120 < R
Progressivity	70 % of straightness (STR)	75 % of straightness (STR)
Circular	< 50 % LIN	< 50 % LIN
Connectivity	14	12
Average path velocity (VAP)	7	5
S = slow, M = medium, R = rapid		

3.6.2.1 Southern white rhinoceros (*Ceratotherium simum simum*)

The two populations of rhinoceros were inadvertently analysed at two different frame rates and despite small sample sizes, no statistical difference was found for the kinetic parameters between the two frame rates. In addition, ROC curve analysis did not reveal clear cut-off points between the two frame rates for the different kinematic parameters of the two rhinoceros populations. Accordingly, the data was combined. Subsequently, no comparisons were made between the two rhino populations for motility and kinematic parameters, and only the effect of different media was compared using the combined data.

3.6.3 ANALYSES OF MOTILITY AND KINEMATIC PARAMETERS

A total of 10 CASA motility and kinematic parameters were assessed including total motility (TM), progressive motility (PM), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), the amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) (see Table 3.2). During sperm analysis under field conditions, swim-up samples were used to evaluate the functionality of spermatozoa.

However, this technique was time-consuming and impractical (especially when the next elephant bull was located and prepared for sampling within the same hour). The sperm selection methodology for functional motility evaluation was adapted accordingly from a sperm swim-up technique to a sperm flushing technique (Figure 3.6 A): The sperm flushing technique (van der Horst, unpublished) is based on first loading a small volume of sperm in the opening of the chamber and then flushing the semen/sperm deeper in the chamber. Virtually all sperm are accordingly displaced to the back of the chamber and sperm then gradually swim into the interface of semen/medium. After several minutes, many sperm are seen swimming in the medium component only. This could be readily visualised as no seminal plasma components (numerous granules) other than sperm were present. This technique is not only very easy and rapid but also similar in principal to a swim-out in a medium when compared to a swim-up.

For every evaluation, a 20µm deep chamber Leja® slide (Leja® Products B.V., Nederland) was filled with four µl of a sperm sample and/or media. Data was recorded after a five-minute incubation period while keeping the slides at a temperature of 37.5°C. During analyses, a minimum of 5 fields (or 500 spermatozoa across the filled chamber was captured. Fields were randomly captured and the only selection was to ensure that there were no semen/yolk particles in the background and that sperm were fully in contact with the medium. On the assumption that there were no particles in the background the type of motility recorded was mainly as a result of interaction with the media. The methodology involves loading a Leja 20 chamber of 3 to 5µL with one µl of a neat (NT) or egg yolk (EY) sperm sample at the opening of the chamber. Immediately thereafter two µl of Ham's F10, INRA96® or BO (10mM caffeine) media is pipetted into the opening of the chamber, causing the sperm droplet to disperse and displace (flush) towards the opposite opening of the chamber (Figure 3.6 B). A representation of the displaced and dispersed motile sperm populations within the chamber is depicted in Figure 3.6 C. Every neat (NT) and/or egg yolk (EY) semen sample extended with either Ham's F10, INRA96® or BO (10mM caffeine) media was analysed.

Table 3.2 Descriptions, abbreviations, units and definitions for CASA (SCA®) motility and kinematic parameters measured.

Parameter		Definition	Unit
Total Motility	**TM**	Total number of motile spermatozoa	%
Progressive Motility	**PM**	Total number of progressively motile spermatozoa	%
Curvilinear velocity	**VCL**	Time-average velocity of sperm head along the entire trajectory	µms-1
Average path velocity	**VAP**	Time-average velocity of sperm head along the smoothed trajectory	µms-1
Straight-line velocity	**VSL**	Time-average velocity of sperm head projected along straight-line from first to last coordinates	µms-1
Linearity of track	**LIN**	Ratio of projected length to total length of curvilinear trajectory, LIN = VSL/VCL x 100	%
Straightness of tract	**STR**	Expression of the straightness of average path, STR = VSL/VAP x 100	%
Wobble	**WOB**	Expression of the degree of oscillation of the curvilinear path about its smooth trajectory, WOB = LIN/STR x 100	%
Amplitude of lateral head displacement	**ALH**	Maximum amplitude of lateral distances of the sperm head trajectory about its smooth trajectory	µm
Beat cross frequency	**BCF**	Frequency which moves sperm head back and forth	Hz

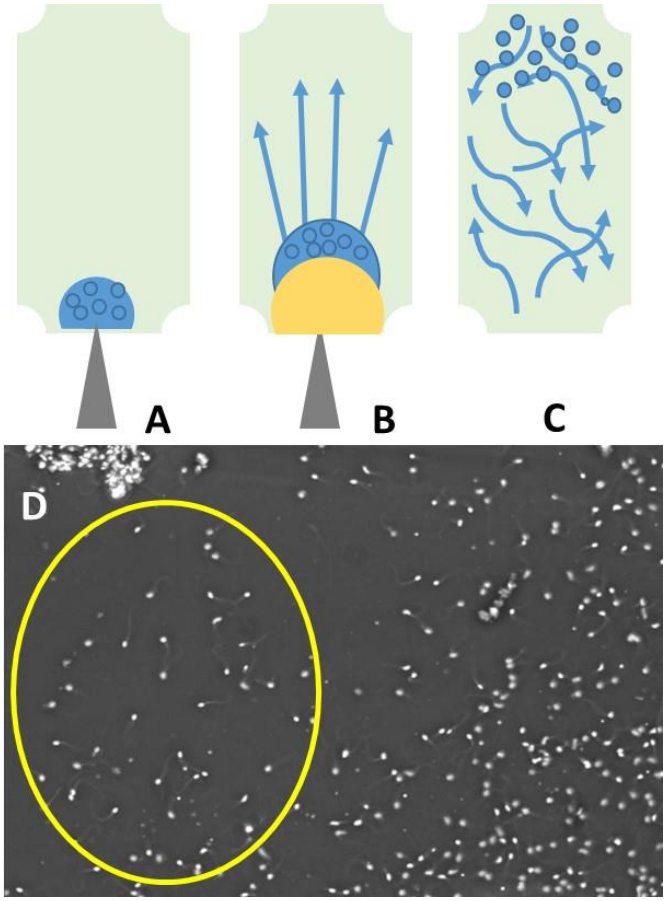


Figure 3.6: Annotation of semen flushing technique used during the functional evaluation of spermatozoa in this study**. (A)** Load one µl of the EY or NT semen sample at the opening of the Leja® chamber. **(B)** Thereafter, load with either four µl of Ham's F10 or BO (10mM caffeine) or INRA96® to disperse and displace droplet into the chamber. **(C)** Accordingly, most spermatozoa swim away from the sperm droplet and disperse in media **(D)** Screenshot of CASA analysis to illustrate the interface between semen and diluted sperm (yellow circle).

The neat (NT) and egg yolk (EY) sperm samples collected from both elephant and rhinoceros contained small particles, presumed to originate from the seminal plasma component of the ejaculate. The SCA® system automatically detects the sperm head (visualised as a white dot with phase contrast microscopy) and makes rapid and accurate measurements of various sperm motility and kinematic parameters (Maree & Van der Horst, 2013). Accordingly, motility should only be measured in an open field, when possible. Fields with any debris or clumps of sperm were avoided to ensure accurate analysis, and an example is shown in Figure 3.6 D. After the motility assessment, all the captured tracks were visually verified for accuracy and any incorrect tracks were deleted (e.g., due to spermatozoa colliding or debris incorrectly recorded as immotile spermatozoa) and final motility percentages and ranges recorded.

3.6.4 HYPERACTIVATED MOTILITY ANALYSIS IN SPERM POPULATIONS USING CASA

3.6.4.1 Pattern analysis and the classification of motile spermatozoa

The 'reanalyse field' feature of the SCA CASA system was applied to evaluate and select individual African elephant and white rhinoceros spermatozoa exhibiting any deviations from the straight-line motion pattern. Special interest was given to individual spermatozoa that portrayed any circular or starspin motion patterns within the NT_Ham's, NT_BO, EY_Ham's and EY_BO sperm samples during CASA analysis. Individual spermatozoa were grouped according to Group 1: Straight-line (non-HA) pattern, Group 2: Linear (Intermediate) pattern, Group 3: HA Circular pattern (HA C) and Group 4: HA Starspin pattern (HA S). This arbitrary method was used to classify the kinematic parameters of individual spermatozoa according to their motion pattern classification. The kinematic parameters, VCL (curvilinear velocity), VSL (straight-line velocity), STR (straightness of track), LIN (linearity of track), ALH (amplitude of lateral head displacement) and BCF (beat cross frequency), that are suspected to be indicators of hyperactivation were selected (Abaigar, *et al*., 1999; Abaigar, *et al*., 2011). The pattern recognition method was used to determine cut-off values to estimate the percentage of spermatozoa displaying hyperactivated motility compared to patterns displaying a

straight line or linear type movement pattern. The diameter of the circular and starspin patterns was also determined by means of measuring the diameters of the circular/start spin type patterns.

3.6.4.2 CASA cut-off values for hyperactive motility analyses

The motion pattern displayed by individual elephant and rhinoceros spermatozoa was used as a basis (Boolean argument) to derive species-specific CASA cut-off values. Used to sort and determine the percentage of spermatozoa displaying hyperactivated motility within the collected elephant and rhinoceros ejaculates collected. Receiver operating characteristics (ROC) analyses discriminate quantitatively between the distribution per motion pattern group on the basis of the highest sensitivity and specificity. ROC Cut-off values or criterion values allow the possibility of establishing motile and hyperactivated populations using specific cut-off points (Zweig & Campbell, 1993).

The extracted data from each motion pattern group was analysed by ROC curve analysis. For overall hyperactivation (HA) analyses, ROC cut-off values were obtained from analysing straight-line (non-HA) motion pattern data and comparing it to the combined circular (HA C) and starspin (HA S) pattern data. Kinematic parameters that revealed a high sensitivity and specificity (> 90%) cut-off during ROC curve analysis were considered for the use in a Boolean argument. The Boolean argument in this study was applied to distinguish between Non-HA and HA motile spermatozoa and is indicated in Chapter 4, Figure 4.14 for the African elephant and in Chapter 5, Figure 5.12 for the Southern white rhinoceros.

In ROC curve analysis, the true positive rate (sensitivity) was plotted as the function of the false positive rate (100-specificity) for different cut-off points. Each plot represents a sensitivity/specificity pair corresponding to a particular threshold (Zweig & Campbell, 1993). A test with perfect discrimination (where there is no overlapping in data distribution) has a ROC plot that passes through

the upper left corner (100% sensitivity, 100% specificity). Therefore, the closer the ROC is to the upper left corner the higher the overall accuracy of the test (Zweig & Campbell, 1993). The value for the area under the ROC curve (AUC) can be interpreted as follows: an area of 0.84 means that a randomly selected individual from the positive group has a test value larger than that for a randomly chosen individual from the negative group in 84% of the instances (Zweig & Campbell, 1993). When a variable cannot distinguish between two groups and if there is no difference in the distributions of data, the area will be equal to 0.5 (the ROC curve will coincide with the diagonal). When there is a perfect separation of data for the two groups and there is no overlapping of the distributions of data, the area under the ROC curve equals one, (the ROC curve will reach the upper left corner of the plot).

3.6.4.3 Hyperactivated motility analysis of sperm samples in different media

Hyperactivation (HA) analyses combined with the use of HA inducing media (BO containing 10Mm caffeine) could possibly assess sperm functionality. Available sub-samples of EY or NT sperm samples collected were incubated for five minutes with BO (containing 10mM caffeine) to attempt to induce HA motility. All Elephant (collected season 2) and rhinoceros (both populations) sperm samples (NT_Ham's, NT_BO, EY_Ham's or EY_BO) were initially analysed with SCA® CASA after a five-minute incubation period. The derived ROC cut-off values obtained after HA C and HA S motion pattern data was combined to measure the total percentage of HA motile spermatozoa within the sperm samples analysed. This allowed for the development of novel CASA sort criteria for African elephant and Southern white rhinoceros during this study (Boolean argument applied) to measure the percentage HA motility. A restriction of < 30µm for VCL was set to ensure that the percentage of hyperactivated motility expressed and recorded was only of the motile sperm population within the sperm samples analysed. During total HA analyses every NT_Ham's, NT_BO, EY_Ham's and EY_BO sperm sample captured was reanalysed (SCA® version 5.4, Microptic, Spain) and the final HA percentage recorded.

3.6.5 STRUCTURAL INTEGRITY ANALYSIS OF SPERMATOZOA

All semen samples were processed for staining within 30 minutes after collection. The evaluation and classification of sperm morphology and plasma membrane integrity were based on criteria originally described for the domestic bull (Campbell, *et al.*, 1956; Barth & Oko, 1989; Nöthling & Irons, 2008). An eosin-nigrosin smear was made by mixing pre-heated (37°C) eosin–nigrosin (Section Reproduction, Faculty of Veterinary Science, University of Pretoria, Pretoria, S.A.) and semen on a pre-heated microscope slide (Figure 3.4 F).

A ratio of 2:1 (stain to semen) usually resulted in good smears but depended on the concentration of spermatozoa. Nigrosin stain was used as the background stain, and produced sufficient contrast to visualise the eosinophilic sperm (Campbell, et al., 1956; Barth & Oko, 1989; Nöthling, 2006). Smears were made on a warm slide (37°C) and dried to minimise the exposure time of live cells to the stain as cold stress and hypo-osmolarity increases the proportion of spermatozoa with flagellar defects. Ha

3.6.5.1 Plasma membrane integrity (Viability) and acrosome integrity

Stained spermatozoa were evaluated (for morphology and acrosome integrity) using bright field microscopy (for viability evaluations). All examinations and differential counts were done under oil immersion at x1000 magnification counting 100 cells per slide.

A live spermatozoon, at the time of staining, does not allow penetration of the eosin stain through the plasma membrane and appears white against the dark nigrosin background. If the plasma membrane of a spermatozoon is damaged at the time of staining, the eosin stain will penetrate and stain the spermatozoon red against the dark nigrosin background. The numbers of red (damaged) and white (intact) spermatozoa were recorded (Live/Dead Ratio). An example of bovine spermatozoa illustrates the difference between intact and damaged membrane integrity (Figure 3.6). Additionally, if the post-acrosomal region of spermatozoon stain red but the acrosomal cap remained white the spermatozoon was also classified as dead.

Classification of the acrosome integrity was done as follow: If the acrosome had a smooth and intact appearance the spermatozoa were classified as; intact acrosome. If an acrosome is lost, and the shoulders are visible in the equatorial region or if the acrosome is “ruffled” in appearance the spermatozoa were classified as; reacted acrosome as illustrated in Figure 3.7.

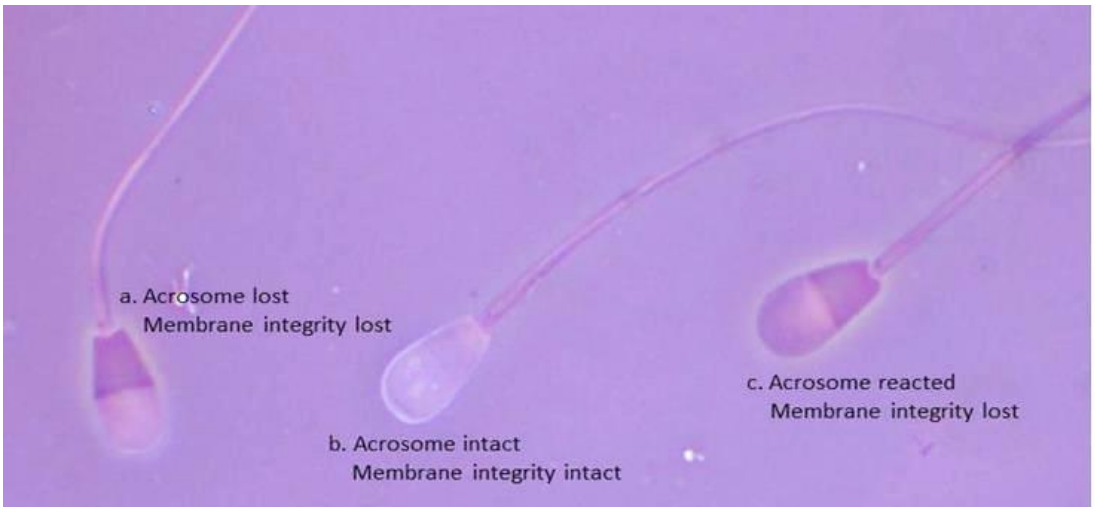


Figure 3.7: An example of bovine spermatozoa illustrating the difference between intact and damaged membrane integrity and intact and reacted acrosomes.

3.6.5.2 Sperm morphology

For the sperm morphology analyses the stained and mounted spermatozoa were classified as either being normal or abnormal in appearance (structural morphology) using bright field microscopy. One hundered cells were counted and classified per slide. A standardised datasheet routinely used for the classification of sperm morphological defects in the domestic bull was adapted for use during this study (Barth & Oko, 1989; Nöthling & Irons, 2008). This data capture sheet (Figure 3.8) allowed for the detailed evaluation and classification of 28 different types of head, midpiece or tail defects recorded in spermatozoa during morphologicaL evaluation. Illustration of these defects and their classification is shown in Figure 3.9 (Nöthling & Irons, 2008).

3.6.5.3 Sperm head morphometric

SpermBlue® fixative and stain (Microptic SL, Barcelona, Spain) stain sperm differentially with a clear background that allows for accurate CASA analysis (van der Horst & Maree, 2009). Two semen smears were prepared per sample and allowed to air dry before staining (Figure 3.4 F). The dried smears were immersed in SpermBlue® fixative (Microptic SL, Barcelona, Spain) for 10 minutes. After completion of the fixation step, the slides were placed at an angle of about 60 - 80° to drain off any excess fixative. Thereafter the smears were immersed in the SpermBlue® (Microptic SL, Barcelona, Spain) stain for 15 minutes. After staining, the excess is stain drained off once again, as for the fixative at an angle. The slides are then dipped into distilled water for about three seconds; this was done very gently to prevent sperm being washed off. This washing step allowed for the removal of any excess blue background colour.

The slides were then placed at an angle of about 60 - 80° to drain any excess fluid and allowed to air dry. The quality of the stain was evaluated immediately under bright field optics at 400 x magnification. If the nuclear staining was insufficient, the smear was re-stained for another 3 - 5 minutes (van der Horst & Maree, 2009). If the staining quality was satisfactory, the smears were mounted with a coverslip using Entallan® (Merck, Johannesburg, S.A.) mounting medium.

Mounted slides were evaluated with the SCA® morphology module using bright field optics under a 100x oil immersion objective. 100 cells were counted per slide. During the automated sperm morphology analysis, eight sperm morphometric parameters were rapidly and accurately measured using the x60 objective: sperm head length, head width, head perimeter, head surface area, head ellipticity, head elongation, head regularity and the percentage acrosome coverage over the head (van der Horst & Maree, 2009).

3.6.5.4 Ultrastructure analysis of spermatozoa morphology by means of transmission electron microscopy (TEM)

All available sperm sub-samples collected by electroejaculation from free-ranging elephants (n = 7) and rhinoceroses (n = 3) were processed for TEM. Unfortunately, not all ejaculates or individuals were represented during TEM analysis due to the initial low sperm concentration of some ejaculates as well as the restricted amount allocated to this study. From the semen collected by EE for transmission electron microscopy standard preparation techniques was used for the fixation of material and preparation of grids (Lebelo & van der Horst, 2010). Sample preparation involved the pipetting 200-400 µl of the initial semen fraction into a microcentrifuge tube for centrifugation at 300 g for 10 minutes and room temperature. Thereafter the supernatant was discarded and the sample re-suspended and fixed in 500 µl of 2.5% phosphate buffered glutaraldehyde. The fixed samples were subsequently processed for TEM that included contrasting with lead citrate and uranyl acetate. Specimen preparation involved three main steps: Fixation in 2.5% phosphate buffered glutaraldehyde and 1% osmium tetroxide to preserve the material; dehydration to remove any free water and thus allow the infiltration of a non-aqueous embedding medium that hardens the sample into a dry block by polymerization. A Reichert ultramicrotome (SMM Instruments, Johannesburg, South Africa) with a diamond knife (Agar Scientific, Randburg, South Africa) was used to prepare silver to gold sections. The resin block was cut into thin (50nm) sections to allow imaging in the electron microscope (Hodgson, *et al.*, 1990).

The production of the electron micrographs required the examination of the thin sections on copper grids using a Jeol JEM 1011 transmission electron microscope at 80 kV (Advanced Laboratory Solutions, Johannesburg, South Africa). A digital camera was fitted onto the microscope and by means of the ITEM software package (Megaview III, Advanced Laboratory Solutions, Johannesburg, South Africa) electron micrographs were produced. Various magnifications (ranging from 5 000 x to 80 000 x) were employed to produce the electron micrographs of the various components of spermatozoa and seminal content.

3.7 STATISTICAL ANALYSIS

For basic statistical analysis, Medcalc® version 10.4.0.0 (Mariakerke, Gent, Belgium) was used. Every ejaculate collected was considered an individual sample during statistical analyses. The analyses performed tested the data sets collected for normality in the parametric (normal) and non-parametric distributions. Levene's test for equality of variances was applied and when $p > 0.05$ one-way analysis of variance analysis (ANOVA) were performed for parametric data distributions. Any significant differences ($p \leq 0.05$) indicated in the ANOVA table between groups were analysed further using the Student-Newman-Keuls test for pairwise comparisons (van der Horst & Maree, 2009; Maree & Van der Horst, 2013).

If Levene's test gave $p \leq 0.05$, independent T-tests with equal or unequal variances (depending on the F-test result) were employed for individual differences. In subsets of data that appeared to have non-parametric data distributions, the Kruskal-Wallis test was used and when required the Mann-Whitney test for independent samples was used. Data was represented as mean, ± standard deviation, median, minimum and maximum values in the tables while $p \leq 0.05$ was considered significant (van der Horst & Maree, 2009; Maree & Van der Horst, 2013).

SPERM MORPHOLOGY	
Smear ID	
1	Teratoid/Therathogenic head
2	Double head
3	Macrocepahlic head
4	Microcephalic head
5	Rolled/Crested head
6	Pyriform head
7	Tapered (narrow) head
8	Diadem (nuclear crater)
9	Narrow base head
10	Abnormal base head
11	Other abnormal head shapes
12	Abnormal loose heads
13	Acrosome
14	Stump Tail
15	Segmental aplasia or malpositioned mitochondria
16	Mitochondrial defects (cork screw)
17	Other midpiece
18	Tightly coiled Dag
19	Dag-like defect
20	Midpiece reflex
21	Coiled principal piece
22	Proximal droplet
23	Distal droplet
24	Fractured tails
25	Normal loose heads
26	Altered Acrosome
27	Bent midpiece
28	Bent principal piece
Total Morphologically Normal	
Total Morphologically Abnormal	

Figure 3.8: Datasheet used to classify and describe sperm morphological abnormalities during eosin-nigrosin and SpermBlue® semen smear evaluations (modified from Nöthling and Irons, 2008).

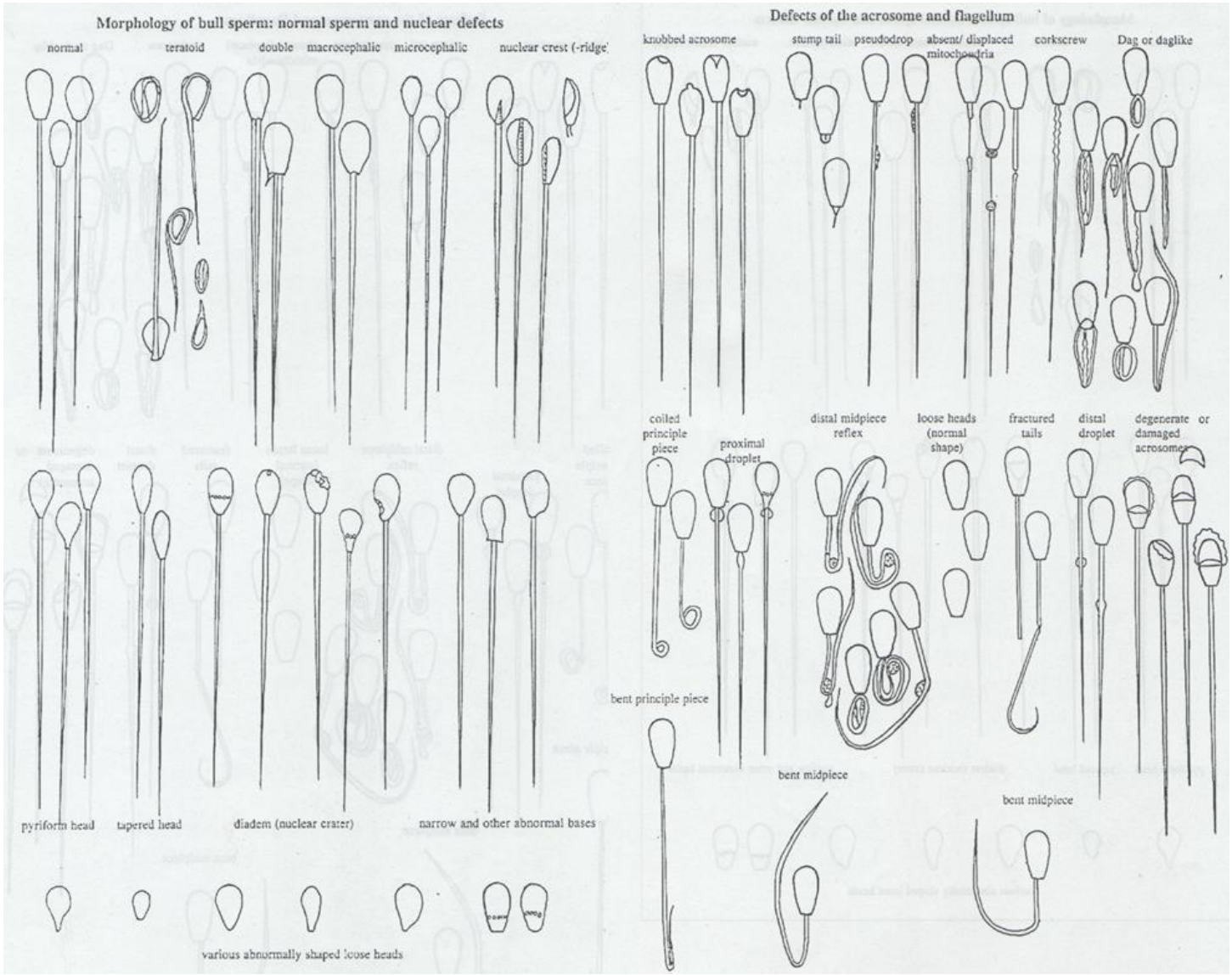


Figure 3.9: Schematic drawings used as a reference to classify sperm morphological abnormalities during eosin-nigrosin and SpermBlue® semen smear evaluations (Nöthling & Irons, 2008).

CHAPTER 4

AFRICAN ELEPHANT (*Loxodonta africana*) SEMEN AND SPERM CHARACTERISTICS

A series of comprehensive semen evaluation techniques was performed on 21 ejaculates collected over two seasons from 12 mature free-ranging elephant bulls aged 17 to 35 years. Seven of these ejaculates were collected and evaluated during season 1 (September 2009), and 14 ejaculates during season 2 (April 2010). Listed in Table 4.1 is the general ejaculate data per individual during both seasons.

Table 4.1 Overview of 21 African elephant ejaculates collected during season 1 (September 2009) (n=7) and season 2 (April 2010) (n=14). The data represents each individual's identification, date and location of collection, the season of collection, age*, shoulder height*, half girth*, ejaculate volume, sperm concentration and total number of spermatozoa per ejaculate collected (*Additional information).

Date	Location of Collection	ID	Name	Season	Age (years)	Shoulder Height (meter)	Half Girth (meter)	Total Volume (mL)	Sperm Concentration ($x10^6$/mL)	Total # sperm per ejaculate ($x10^9$)
11/9/2009	Phinda	LA 1a	Steve	1	35	3.1	2.7	13	563	73
12/9/2009	Phinda	LA 2a	Madube	1	25	2.96	2.3	17	45	8
12/9/2009	Phinda	LA 3a	No 3	1	17	2.53	1.83	47	1583	744
12/9/2009	Phinda	LA 4a	Rasputin	1	20	2.5	1.8	22	10	22
13/9/2009	Phinda	LA 7a	Seven	1	25-27	3.09	2.8	83	1134	941
14/9/2009	Phinda	LA 8a	Righthook	1	33	2.96	2.65	133	2000	2660
14/9/2009	Phinda	LA 9a	Looney	1	25	3.2	2.48	6	1500	90
12/4/2010	Phinda	LA 1b	Righthook	2	34	2.98	2.34	227	80	182
12/4/2010	Phinda	LA 2b	Bump	2	32	3.2	2.46	130	150	195
12/4/2010	Phinda	LA 3b	Looney	2	26	3.21	2.4	2.7	0	0
12/4/2010	Phinda	LA 5b	No name	2	-	2.99	2.17	57	2025	1160
13/4/2010	Phinda	LA 6b	No 3	2	18	2.53	1.83	68	472	321
13/4/2010	Phinda	LA 7b	Smiley	2	24	2.59	1.65	31	0	0
14/4/2010	Phinda	LA 8b	Righthook	2	34	2.98	2.34	67	712	477
14/4/2010	Phinda	LA 9b	Young 1	2	-	2.49	1.83	31	1095	340
14/4/2010	Phinda	LA 10b	Bump	2	32	3.2	2.46	38	750	285
15/4/2010	Phinda	LA 11b	Young 1	2	-	2.49	1.83	23	787	181
15/4/2010	Phinda	LA 12b	No name	2	-	2.99	2.17	55	1815	998
16/4/2010	Phinda	LA 14b	Rex	2	25	3.03	2.05	79	1387	1096
16/4/2010	Phinda	LA 15b	No name	2	-	2.99	2.17	28	225	63
16/4/2010	Phinda	LA 16b	Steve	2	36	3. 1	2.2	11	847	93

4.1 MACROSCOPIC SEMEN EVALUATION

4.1.1 EJACULATE COLOUR AND VISCOSITY

In general, it was difficult to quantify the colour and viscosity of the semen samples collected. The appearance of the fractions of the ejaculates ranged from being clear, watery-grey to milky in colour (Figure 4.1). The ejaculates were fairly viscous in consistency with marble formation recorded in the majority of the ejaculates collected. Good quality semen was indicated by a white colour.

However, in some bulls, there were fractions that were too viscous to process, had a distinct odour and yellow colour indicating urine contamination, or contained no spermatozoa. Very viscous fractions were recorded in sperm samples collected from LA 9b (Young 1) and LA 12b (No name). These fractions were discarded and not used in this study. The seminal traits of the African elephant ejaculates were defined on a similar basis as for seminal traits of domestic animals (for example bulls, rams and stallions) (Howard, et al., 1984; Barth, 2007).

Figure 4.1: Example of an ejaculate collected from an African elephant bull depicting differences in colour, ranging from a milky (left) to a clear (right) appearance (Photo credit: I. Lueders).

4.1.2 EJACULATE VOLUME

An average ejaculate volume of 45.9 ± 47mL (range: 6-133mL) was obtained from 7 bulls during season 1 (September 2009). The lowest ejaculate volume was recorded for LA 3b (Looney) and the highest for LA 8a (Righthook). During season 2 (April 2010), 14 semen samples recorded a mean volume of 60.6 ± 58mL (range: 2.7-227mL). The lowest and highest ejaculate volumes were again recorded for LA 4b (Looney) and for LA 1b (Righthook), respectively. There was no significant difference (p = 0.4555) recorded between ejaculates volumes of the two seasons (Figure 4.2 A). A summary of the semen volume data is portrayed in Table 4.2.

Table 4.2 Ejaculate volumes and sperm concentrations of 21 African elephant ejaculates collected during two different seasons. Season 1: September 2009 (n=7) and season 2: April 2010 (n=14). The data presented as mean ± standard deviation (SD) and median.

	Season 1						Season 2						Both Seasons					
Parameters	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.
Volume (mL)	7	**46**	±47	22	6	133	14	**61**	±58	47	2.7	227	21	**56**	±38	54	2.7	227
Concentration (million\mL)	7	**976**	±784	1134	10	2000	14	**739**	±655	731	0	2025	21	**818**	±750	690	0	2025

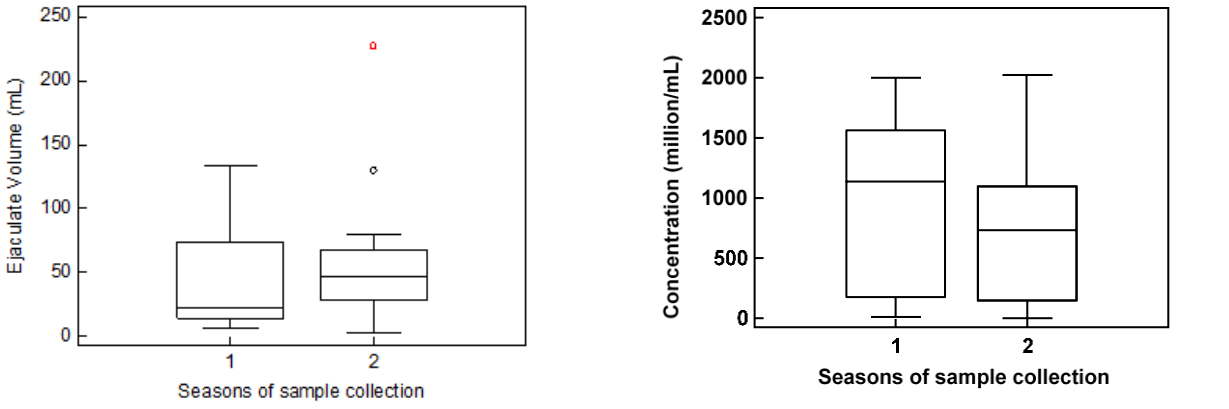


Figure 4.2: Box-and-whisker plots illustrating **(A)** ejaculate volumes (mL) **(B)** and sperm concentrations (10^6/mL) recorded for free-ranging African elephant ejaculates collected during season 1 (September 2009) and season 2 (April 2010).

4.2. MICROSCOPIC SPERM EVALUATION

4.2.1. SPERM CONCENTRATION

The average concentration recorded for the elephant ejaculates collected by means of electroejaculation (EE) was 818 ± 750 x 10^6/mL during this study. The sperm concentrations recorded during the two different seasons revealed no significant difference (p = 0.379; F-ratio = 0.539) when compared, as shown in Table 4.2 and Figure 4.2 B. Ejaculate fractions collected from LA 4b (Looney) and LA 7b (Smiley) contained no spermatozoa in season 2. In the same period, ejaculate fractions collected from LA 5b (Young 1) recorded the highest sperm concentration at 2025 x 10^6 spermatozoa /mL. The ejaculate with the highest total number of spermatozoa (sperm concentration X ejaculate volume) was collected during season 1 from LA 8a (Righthook) yielding 266 x 10^9 sperm (Table 4.1 and Figure 4.3).

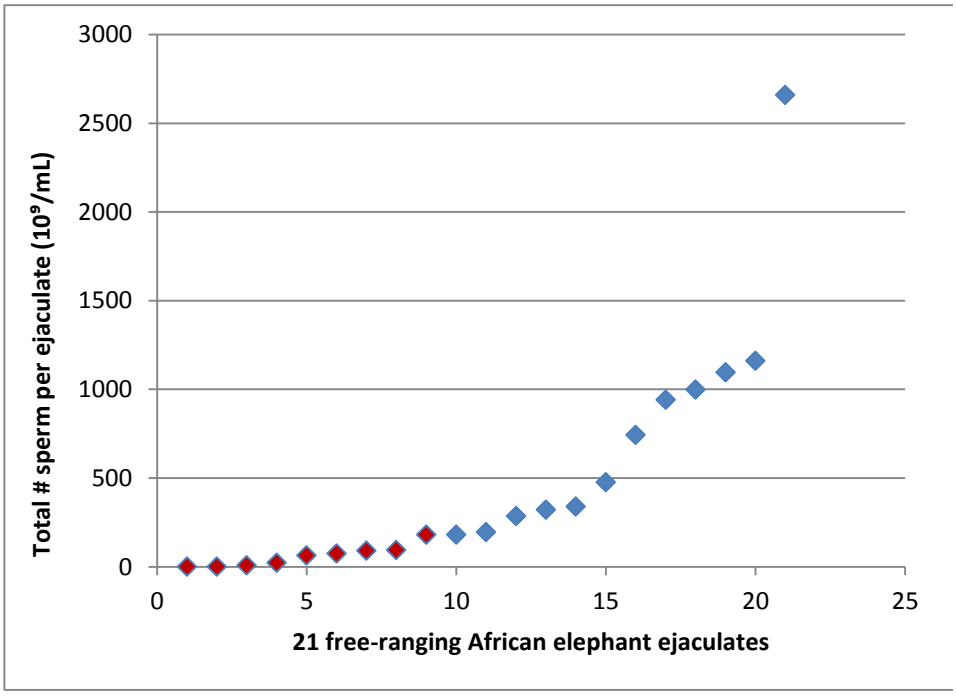


Figure 4.3: Scatter plots representing the total number of spermatozoa (x10^9/mL) per ejaculate recorded (ejaculate volume X ejaculate concentration) for all 21 free-ranging African elephant ejaculates collected by means of electroejaculation during season 1 (red = September 2009, n = 7) and season 2 (blue = April 2010, n=14) arranged from the lowest to the highest concentration.

4.2.2. MOTILITY AND KINEMATIC PARAMETER VALUES TO ESTABLISH THE QUALITY OF SPERM SAMPLES

To assess the quality of ejaculates, aliquots were extended with egg yolk (EY) and diluted with Ham's F10 medium (EY_Ham's). These diluted aliquots were used as control or reference samples in order to obtain threshold values. Motility and kinematic parameter values obtained from 19 EY_Ham's semen samples for season 1 (n=7) and for season 2 (n=12) are presented in Table 4.3. Data recorded per individual ejaculate is presented in Table 4.4.

Analyses of EY_Ham's samples revealed an average for total motility of 80.53 ± 29.65% of which 61.92 ± 26.98% of the spermatozoa were progressively motile. Total motility ($p = 0.006$; $F = 9.832$) and progressive motility ($p = 0.0021$; $F = 6.478$) differed significantly between the two season (Figures 4.4 A, B and C). Sperm samples collected during season 2 scored on average a higher percentage of progressively motile spermatozoa per sample at 77.64 ± 7.51%, compared to the average score of 57.98 ± 25.36% recorded during season 1. Looking at individual bulls, the lowest percentage recorded for progressive motility was 17.6% in LA 3a (No 3) in season 1, compared to the lowest percentage of 67% recorded by LA 10b (Bump) in season 2 (Table 4.4.).

Average speeds of 241 ± 58.47µm/s for curvilinear velocity (VCL), 172 ± 49.04µm/s for straight-line velocity (VSL) and 200.98 ± 54.52µm/s average path velocity (VAP) were recorded for the motile sperm populations. These parameters were significantly different between the two seasons when comparing VCL ($p = 0.002$; $F = 13.682$), VSL ($p = 0.003$; $F = 11.621$) and VAP ($p = 0.004$; $F = 10.975$) with velocities recorded during season 2 being much higher compared those recorded during season 1 (Figure 4.4 D, E and F). The highest individual VCL of 309µm/s recorded for LA 10b was during season 2 and the lowest VCL of 70µm/s from LA 2a was recorded during season 1 (Table 4.4). Overall, data collected from LA 2a during season 1 recorded the lowest values for all the velocity parameters VCL, VSL and VAP. For the progression kinematic parameters, only LIN ($p = 0.04$; $F =$

4.96) was significantly different when the data of the two seasons were compared. Linearity (LIN) of the tracks recorded by the motile sperm population averaged 66.98 ± 16.38%. Season 1 recorded a range for LIN at 8.5% - 73.6% compared to the range of 62.2% - 87.6% for season 2. No significant difference was found in any of the data for STR ($p = 0.252$; $F = 1.409$) and WOB ($p = 0.378$; $F = 0.818$) recorded during the two different seasons. Analysis of the data for the amplitude of lateral head displacement (ALH) revealed that there was a significant difference ($p = 0.04$; $F = 4.924$) between the two seasons. ALH measurements of season 1 recorded a population average of 3.22 ± 0.99µm compared the 3.95 ± 0.42µm measurements recorded during season 2. Beat cross frequency (BCF) recorded a population average of 20.66 ± 3.05Hz with no significant difference ($p = 0.1763$) between the two seasons compared.

In summary, the average EY_H sperm sample contained 80% motile spermatozoa of which 62% were progressively motile and reached average curvilinear velocities of 241µm/s while maintaining 67% linearity of the progression and 86% straightness of track during which the flagella displayed an average beat cross frequency of 20Hz causing a 3.68µm lateral displacement of the spermatozoon's head (Table 4.3).

Table 4.3 Population averages of motility and kinematic parameter measurements recorded during season 1 (September 2009) and season 2 (April 2010) in semen samples extended with egg yolk and further diluted with Ham's F10 medium (EY_H) for CASA evaluation. The data is presented as sample number (N), mean ± standard deviation (SD), median, minimum and maximum values.

	Season 1						Season 2						Both Seasons					
Parameters	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max
Total Motility (%)	7	**51.5**[a]	±32.63	32.2	18.2	99,0	12	**97.45**[b]	±3.01	98.5	91.3	100	19	**80.53**	±29.65	97.2	18.2	100
Progressive motility (%)	7	**34.97**[a]	±27.19	26.7	3.8	67.4	12	**77.64**[b]	±7.51	77.2	67	93.8	19	**61.92**	±26.98	72.4	3.8	93.8
Non-progressive motility (%)	7	**16.52**	±11.12	12.5	3.5	31.6	12	**19.81**	±8.12	21.95	5.4	33	19	**18.61**	±9.18	20.2	3.5	33
VCL (µm/s)	7	**191.24**[a]	±70.13	206,0	69.8	288.7	12	**270.02**[b]	±20.41	266.2	245.8	308.6	19	**241**	±58.47	254	69.8	308.6
VSL (µm/s)	7	**132.97**[a]	±55.29	139.4	44.5	196.9	12	**196.02**[b]	±25.87	200.8	156	254.7	19	**172.8**	±49.04	181.6	44.5	254.7
VAP (µm/s)	7	**157.45**[a]	±63.91	156.2	56.7	244.7	12	**226.36**[b]	±26.97	222.75	190	273.8	19	**200.98**	±54.52	213.3	56.7	273.8
LIN (%)	7	**57.05**[a]	±22.57	63.7	8.5	73.6	12	**72.76**[b]	±7.88	71.35	62.2	87.6	19	**66.98**	±16.38	69.2	8.5	87.6
WOB (%)	7	**80.7**	±8.71	81.6	66.7	92.5	12	**83.53**	±5.06	83.2	75.3	92.4	19	**82.49**	±6.55	82.4	66.7	92.5
STR (%)	7	**83.67**	±5.71	85.5	74.9	89.2	12	**86.83**	±5.53	86.5	78	94	19	**85.67**	±5.66	85.5	74.9	94
ALH (µm)	7	**3.22**[a]	±0.99	3.4	1.6	4.4	12	**3.95**[b]	±0.42	3.95	3.1	4.6	19	**3.68**	±0.75	3.8	1.6	4.6
BCF (Hz)	7	**19.38**	±3.16	19.2	13.4	23.8	12	**21.4**	±2.85	20.55	17.4	27.1	19	**20.66**	±3.05	20.2	13.4	27.1

[a, b] values in rows labelled with different superscript letters were significantly different ($p < 0.05$)

VCL = Curvilinear velocity, VSL = Straight line velocity, VAP = Average path velocity, LIN = Linearity of tract, WOB = Wobble, BCF = Beat cross frequency, ALH = Amplitude of lateral head displacement

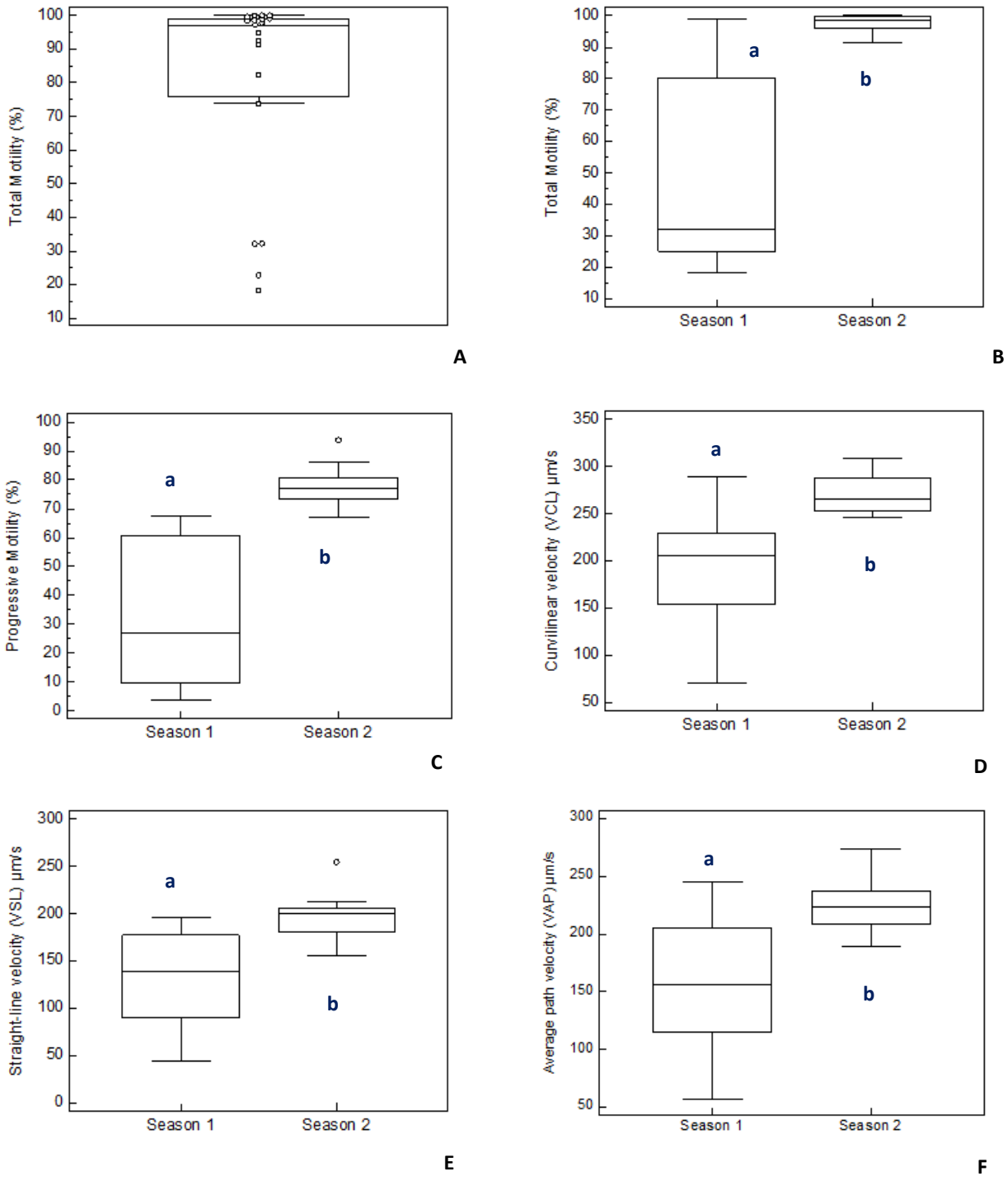


Figure 4.4: Box-and-whisker plots of the motility and kinematic parameters recorded for African elephant sperm samples extended with egg yolk and diluted with Ham's F10 medium (EY_H). (**A**) Total motility (%) recorded for population; (**B**) Total motility (%) recorded during each season; **(C)** Progressive motility (%); (**D**) Curvilinear velocity (VCL µm\s); (**E**) Straight-line velocity (VSL µm\s); (**F**) Average path velocity (VAP µm\s) recorded during season 1 (September 2009, n=7) and season 2 (April 2010, n=12). Data plots labelled with different letters in the same graph were significantly different ($p < 0.05$).

Table 4.4 CASA motility and kinematic parameters values recorded (at 50 frames per second) from individual sperm samples extended in egg yolk and diluted in Ham's F10 medium (EY_H) and collected during season 1 (September 2009) and season 2 (April 2010).

Elephant ID	ID	Total Motile %	Proggressive Motily %	Non-Progressive Motily %	VCL µm/s	VSL µm/s	VAP µm/s	LIN %	STR %	WOB %	ALH µm	BCF Hz
LA 1a	Steve	93	81	12	149	80	107	54	75	72	3	19.1
LA 2a	Madube	96	68	28	70	45	57	64	79	81	2	13.4
LA 3a	No 3	41	18	23	225	180	211	74	86	86	4	19.7
LA 4a	Rasputin	81	77	4	169	139	156	9	89	93	2	19.2
LA 7a	No 7	39	26	13	232	168	189	73	89	82	3	23.8
LA 8a	Righthook	73	68	5	206	121	137	59	88	67	4	21.5
LA 9a	Looney	99	67	32	289	197	245	68	81	85	4	19
LA 1b	Righthook	98	74	24	246	156	190	63	82	77	4	24.4
LA 2b	Bump	99	94	5	265	203	215	76	94	81	4	27.1
LA 5b	No name	95	68	27	257	160	194	62	83	75	5	19.6
LA 6b	No 3	100	80	20	275	202	234	76	89	85	4	20.3
LA 8b	Righthook	99	72	27	267	181	220	68	82	82	4	19.7
LA 9b	Young 1	98	82	16	253	182	204	72	89	81	4	25
LA 10b	Bump	100	67	33	309	214	274	69	78	89	4	18.4
LA 11b	Young 1	98	86	12	291	255	274	88	93	92	3	20.8
LA 12b	No Name	91	78	13	254	207	225	82	92	89	4	17.4
LA 14b	Rex	100	76	24	291	191	232	66	82	80	5	22.4
LA 15b	No name	92	80	12	246	200	213	81	94	87	4	20.2
LA 16b	Steve	98	74	24	287	203	241	71	84	84	4	21.5

VCL = Curvilinear velocity, VSL = Straight line velocity, VAP = Average path velocity, LIN = Linearity of tract, WOB = Wobble, BCF = Beat cross frequency, ALH = Amplitude of lateral head displacement

4.2.3. COMPARISON OF CASA RECORDED MOTILITY AND KINEMATIC PARAMETERS OF ALL SPERM SAMPLES EVALUATED IN DIFFERENT MEDIA

CASA derived motility and kinematic parameters were analysed for each sperm sample in seven different media. A summary of the motility and kinematic parameter data is indicated in Table 4.5 for Egg Yolk (EY), Egg Yolk and BO (EY_BO), Egg Yolk and Ham's F10 (EY_Ham's), Egg Yolk and INRA96® (EY_ INRA), Neat and BO (NT_BO), Neat and Ham's F10 (NT_Ham's) and Neat_INRA96® (NT_ INRA). Due to constraints indicated by the conditions in the field and the availability of sufficient samples, not all treatments were conducted simultaneously on all samples in true split-sample fashion. The motility and kinematic values recorded in Ham's F10 medium were used as a reference to compare values recorded in BO (10mM caffeine) and INRA96® media of the same ejaculate, respectively.

The highest average total percentage motility was recorded at 97.3 ± 4.68% in NT_ INRA amongst all media groups compared. Similarities were recorded between the EY_ Ham's and EY_ INRA as well as between NT_ Ham's and NT_ INRA groups ($p = 0.09$; $F = 2.283$). Overall, total motility percentages were high (above 80%) amongst all groups compared, except for the low total motility recorded in EY only at 34.50 ± 25.03%. The total motile percentages recorded were similar between EY_BO and NT_BO groups (Figure 4.5 A). Progressive motile sperm percentages were significant ($p = 0.176$; $F = 1.713$) between EY_ Ham's and NT_Ham's (61.92 ± 26.98% and 77.03 ± 6.71%) as well as between EY_ INRA and NT_INRA (66.73 ± 19.81% and 71.97 ± 7.98%) The highest average of progressively motile spermatozoa per sample was recorded in NT_ Ham's at 77.03 ± 6.71%, compared to lowest percentage of 15.55 ± 17.65% recorded in EY only (Figure 4.5 B). EY, EY_BO and NT_BO sperm samples recorded lower percentages for progressive motility (< 42%) compared to EY_Ham's and NT_Ham's (> 61%) sperm samples. The non-progressive motility percentages recorded in EY_BO and NT_BO (> 51%) sperm samples were higher when compared to the EY_ Ham's and NT_ Ham's averages recorded (> 20%) (Table 4.5).

VCL ‘s recorded for EY_ Ham’s and NT_ Ham’s (241 ± 58.47µm/s and 278 ± 48.41µm/s) and between EY_ INRA and NT_ INRA (206.84 ± 46.32µm/s and 203.87 ± 39.07µm/s) were significantly different ($p = 0.002$; $F = 5.856$). Overall, higher VCL’s were recorded in Ham’s F10 samples compared to INRA96® samples groups. The lowest VCL average was recorded in EY at 95.8 ± 38.96µm/s. Similarities in VCL’s were recorded amongst EY_BO and NT_BO and EY_INRA and NT_INRA media groups (Figure 4.5 D). Significant differences were recorded for VSL ($p = 0.013$; $F = 3.941$) between EY_Ham’s and NT_Ham’s (172.7 ± 49.04µm/s and 198.5 ± 42.58µm/s) and between EY_INRA and NT_INRA (154.2 ± 44.82µm/s and 140.6 ± 34.83µm/s). On average, EY_Ham’s and NT_Ham’s recorded the highest VSL’s while the lowest VSL’s were recorded in EY only (95.8 ± 38.96µm/s) and EY_BO and NT_BO (87.48 ± 26.78µm/s and 88.23 ± 34.52µm/s) media groups (Figure 4.5 E). VAP averages were significantly different ($p = 0.040$; $F = 2.980$) between INRA96® and Ham’s F10 media groups. Recorded VAP averages were higher in EY Ham’s and NT_ Ham’s (200.9 ± 54.42µm/s and 224.7 ± 48.7µm/s) compared to EY_INRA and NT_INRA (168.8 ± 38.84µm/s and 179.8 ± 53.03µm/s). The lowest VAP was recorded for EY only at 73.57 ± 39.94µm/s. Similarities were recorded for VAP between NT_BO and NT_ INRA (142.9 ± 38.66µm/s and 168.8 ± 38.84µm/s) (Figure 4.6 F).

No significant differences were recorded betwenn EY_Ham’s and NT_Ham’s and EY_INRA and NT_INRA groups for parameters LIN ($p = 0.753$; $F = 0.401$), STR ($p = 0.553$; $F = 0.706$) and WOB ($p = 0.549$; $F = 0.712$) (Figure 4.6 A and B). The average LIN in EY_BO (57.67 ± 13.15%) and NT_BO (46.39 ± 15.62%) was lower compared to the averages recorded in EY_ Ham’s (85.67 ± 5.66%) and NT_ Ham’s (71.55 ± 10.19%). A similar trend was observed amongst the groups for parameter STR. Comparison of ALH measurements recorded for EY Ham’s and NT_Ham’s and EY_INRA and NT_INRA were significantly different ($p = 0.003$). EY Ham’s and NT_Ham’s (3.68 ± 0.75µm and 4.10 ± 0.78µm) recorded significant higher ALH values compared to EY_INRA and NT_INRA (3 ± 0.51µm and 3.11 ± 0.95µm) media groups. The highest population average for ALH was recorded in EY_BO at 5.09 ± 3.38µm and the lowest in EY only at 2.17 ± 3.38µm (Figure 4.6 C).

BCF data compared between all groups exhibited the lowest averages in EY_BO (11.75 ± 2.61 Hz) and the highest in EY_ Ham's (20.66 ± 3.05Hz) (Figure 4.6 D). In summary, media group comparison revealed the highest number of motile spermatozoa in NT_INRA, however, the highest number of progressively motile spermatozoa was recorded in NT_Ham's. Similarities were recorded for total motility, non-progressive motility, LIN, STR and ALH parameters between Ham's F10 and INRA96® (EY and NT) media groups. Comparison between Ham's F10 and BO (EY or NT) indicated distinct differences. Non-progressive motility, VSL, LIN and STR parameters were lower in BO (10mM caffeine) media compared to Ham's F10. However, the lowest total motility, progressive motility, VCL, VSL and ALH was recorded in EY only media.

Table 4.5 Summary of motility and kinematic parameters recorded during evaluation of semen ejaculates collected from all free-ranging African elephants during both seasons. The data in the table is presented as sample number (N), mean ± standard deviation (SD), median, minimum and maximum values.

	Egg Yolk (EY)						Egg yolk_ Bo (EY_Bo)						Egg yolk_Hams (EY_Hams)						Egg yolk_Inra (EY_Inra)				
Semen Parameters	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.
Total Motility (%)	6	**34.50**^{a}	25.03	44.6	1	56.6	12	**93.60**^{b}	5.35	95	80.6	99.7	19	**80.53**^{c}	±29.65	97.2	18.2	100	12	**88.78**^{c}	±21.25	98.15	26.5	100.1
Progressive motility (%)	6	**15.55**^{a}	17.65	10.8	0	45.7	12	**37.71**^{a}	15.23	41.7	10.6	61.8	19	**61.92**^{b}	±26.98	72.4	3.8	93.8	12	**66.73**^{b}	±19.81	70.1	8.7	81.9
Non-progressive motility (%)	6	**18.95**^{a}	18.13	15.45	1	50	12	**55.89**^{b}	17.13	54.75	30.4	85.1	19	**18.61**^{a}	±9.18	20.2	3.5	33	12	**22.05**^{a}	±7.21	20.75	9	33.3
VCL (µm/s)	6	**95.80**^{a}	38.96	92.9	47.8	158	12	**190.59**^{b}	34.09	194.25	123.7	237.3	19	**241.00**^{c}	±58.47	254	69.8	308.6	12	**206.84**^{b}	±46.32	189.7	142.4	284
VSL (µm/s)	6	**56.30**^{a}	41.11	51.15	16.2	131.1	12	**87.48**^{a}	26.78	94.5	30.9	115.6	19	**172.80**^{b}	±49.04	181.6	44.5	254.7	12	**154.21**^{c}	±44.82	137.25	93.1	229.2
VAP (µm/s)	6	**73.57**^{a}	39.94	68.8	32.5	143.9	12	**153.29**^{b}	34.8	157.7	66.7	199.6	19	**200.98**^{c}	±54.52	213.3	56.7	273.8	12	**179.81**^{d}	±53.03	168.3	101.2	268.3
LIN (%)	6	**53.48**^{a}	18.97	56	31.9	83	12	**47.49**^{a}	10.85	47.25	32.4	67.2	19	**66.98**^{b}	±16.38	69.2	8.5	87.6	12	**67.28**^{b}	±21.39	73	4.4	84.1
WOB (%)	6	**74.12**^{a}	11.54	74.8	56.7	91	12	**79.85**^{abcd}	10.23	80.8	53.9	94.1	19	**82.49**^{abcd}	±6.55	82.4	66.7	92.5	12	**85.93**^{b}	±9.22	89.25	66.9	94.5
STR (%)	6	**70.40**^{a}	14.98	74.5	49.8	91.1	12	**57.67**^{a}	13.15	58	38.8	81.6	19	**85.67**^{b}	±5.66	85.5	74.9	94	12	**86.03**^{b}	±4.68	85.8	79.7	91.9
ALH (µm)	6	**2.17**^{a}	1.18	2.45	0	3.2	12	**5.09**^{b}	3.38	4.25	2.2	15.3	19	**3.68**^{b}	±0.75	3.8	1.6	4.6	12	**3.00**^{a}	±0.51	3	2.2	4.2
BCF (Hz)	6	**11.85**^{a}	6.36	13.05	0	17.6	12	**11.75**^{b}	2.61	12.1	8.3	15.5	19	**20.66**^{c}	±3.05	20.2	13.4	27.1	12	**16.14**^{d}	±2.54	16.4	10.7	20

a,b,c,d,e values labelled with different superscript letters were significantly different (p>0.05)

Table 4.5 (continued)

	Neat_Bo (NT_Bo)						Neat_Hams (NT_Hams)						Neat_Inra (NT_Inra)					
Semen Parameters	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.
Total Motility (%)	12	**93.60**^{c}	±7.72	96.05	77.3	99.7	12	**96.66**^{d}	±6.27	99.05	77.9	100	12	**97.31**^{d}	±4.68	98.55	83	100
Progressive motility (%)	12	**41.98**^{b}	±15.57	44	13.9	73.1	12	**77.03**^{d}	±6.71	77.8	64.5	87.9	12	**71.97**^{c}	±7.98	70.55	56.8	81.4
Non-progressive motility (%)	12	**51.63**^{b}	±16.96	50.25	23.1	85.5	12	**19.63**^{a}	±9.97	21.1	0.6	34.5	12	**25.34**^{a}	±7.94	23.7	15.9	39.9
VCL (µm/s)	12	**194.85**^{b}	±36.71	208.55	141	257.5	12	**278.09**^{d}	±48.41	281.9	173.7	342.1	12	**203.87**^{e}	±39.07	205.75	145.4	265.3
VSL (µm/s)	12	**88.23**^{a}	±34.52	84.15	39.4	165.6	12	**198.55**^{d}	±42.58	208.15	126.6	251.9	12	**140.64**^{e}	±34.83	129	92.9	212.3
VAP (µm/s)	12	**142.90**^{b}	±38.66	147.7	83.6	191	12	**224.74**^{e}	±48.70	233.3	151	299.9	12	**168.80**^{b}	±38.84	156.45	119.9	240.7
LIN (%)	12	**46.39**^{a}	±15.62	45.55	20.8	74.2	12	**71.55**^{b}	±10.19	70.55	57.5	90.7	12	**71.83**^{b}	±10.22	73.15	55.3	83.9
WOB (%)	12	**73.18**^{c}	±13.40	77.2	49.5	88	12	**83.39**^{b}	±8.28	85.15	68.6	95.7	12	**86.18**^{d}	±9.61	90	69.6	95.2
STR (%)	12	**62.52**^{a}	±14.29	61.7	41.8	87.3	12	**85.60**^{b}	±5.88	83.9	77.2	96	12	**83.19**^{b}	±5.30	82.35	77	94.7
ALH (µm)	12	**4.01**^{b}	±1.41	3.65	2.2	7.7	12	**4.10**^{b}	±0.78	4.25	2.5	5.3	12	**3.11**^{a}	±0.95	2.7	1.9	4.9
BCF (Hz)	12	**15.64**^{a}	±2.68	14.95	10.6	20	12	**19.54**^{c}	±1.97	19.85	16.6	22	12	**15.63**^{a}	±2.43	15.05	11.6	20.8

a,b,c,d,e values labelled with different superscript letters were significantly different (p>0.05)

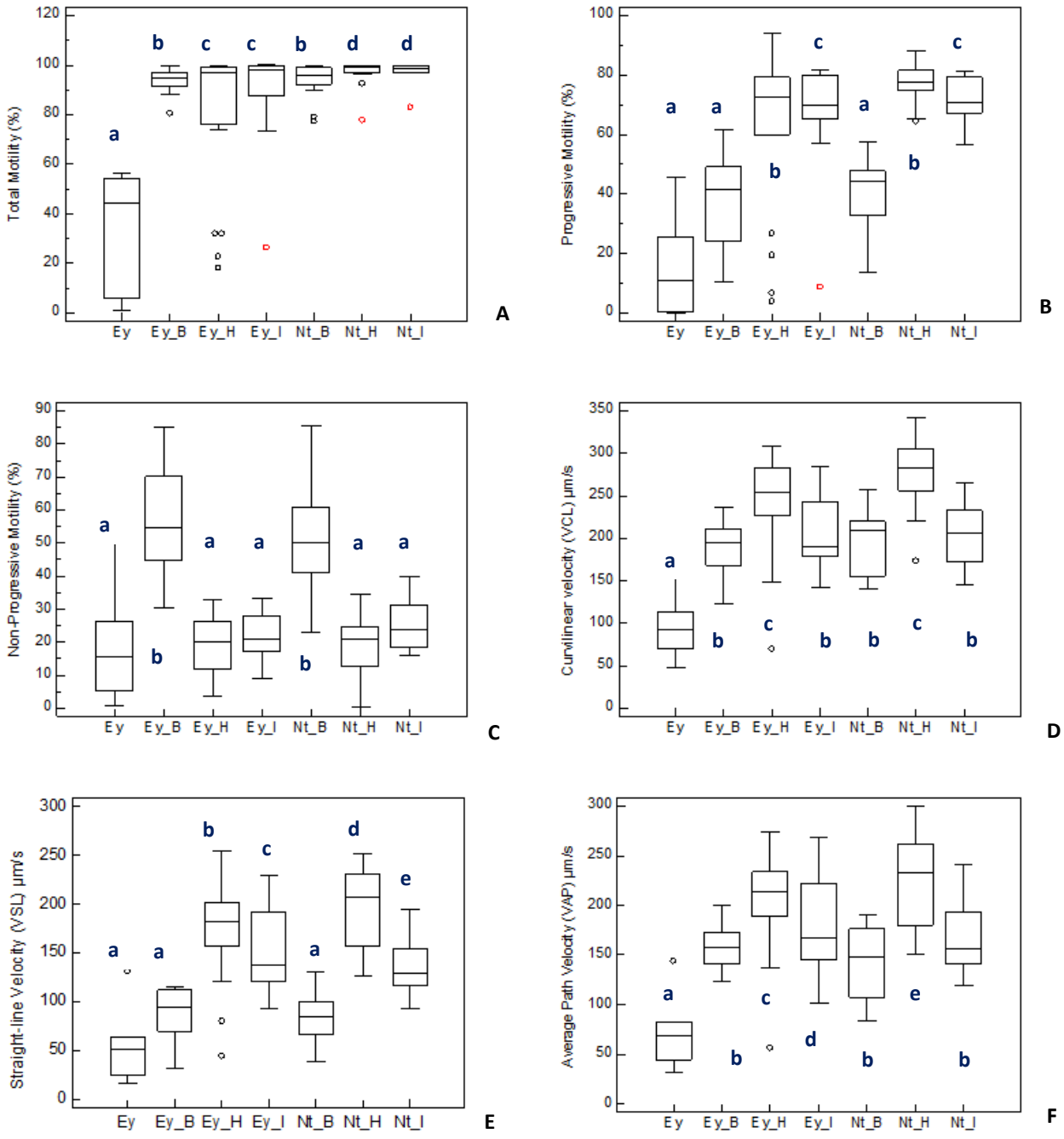


Figure 4.5: Box-and-whisker plots illustrating **(A)** Total Motility (%) **(B)** Progressive Motility (%) **(C)** Non-Progressive Motility (%), **(D)** Curvilinear Velocity (VCL µm/s), **(E)** straight-line velocity (VSL µm/s) and **(F)** average path velocity (VAP µm/s) values of semen in different media. EY (Egg Yolk), EY_B (Egg Yolk and BO), EY_H (Egg Yolk and Ham's F10), EY_I (Egg Yolk and INRA96®), (NT_B) Neat and BO, NT_H (Neat and Ham's F10) and NT_I (Neat and INRA96®). Data plots labelled with different letters in the same graph were significantly different ($p < 0.05$).

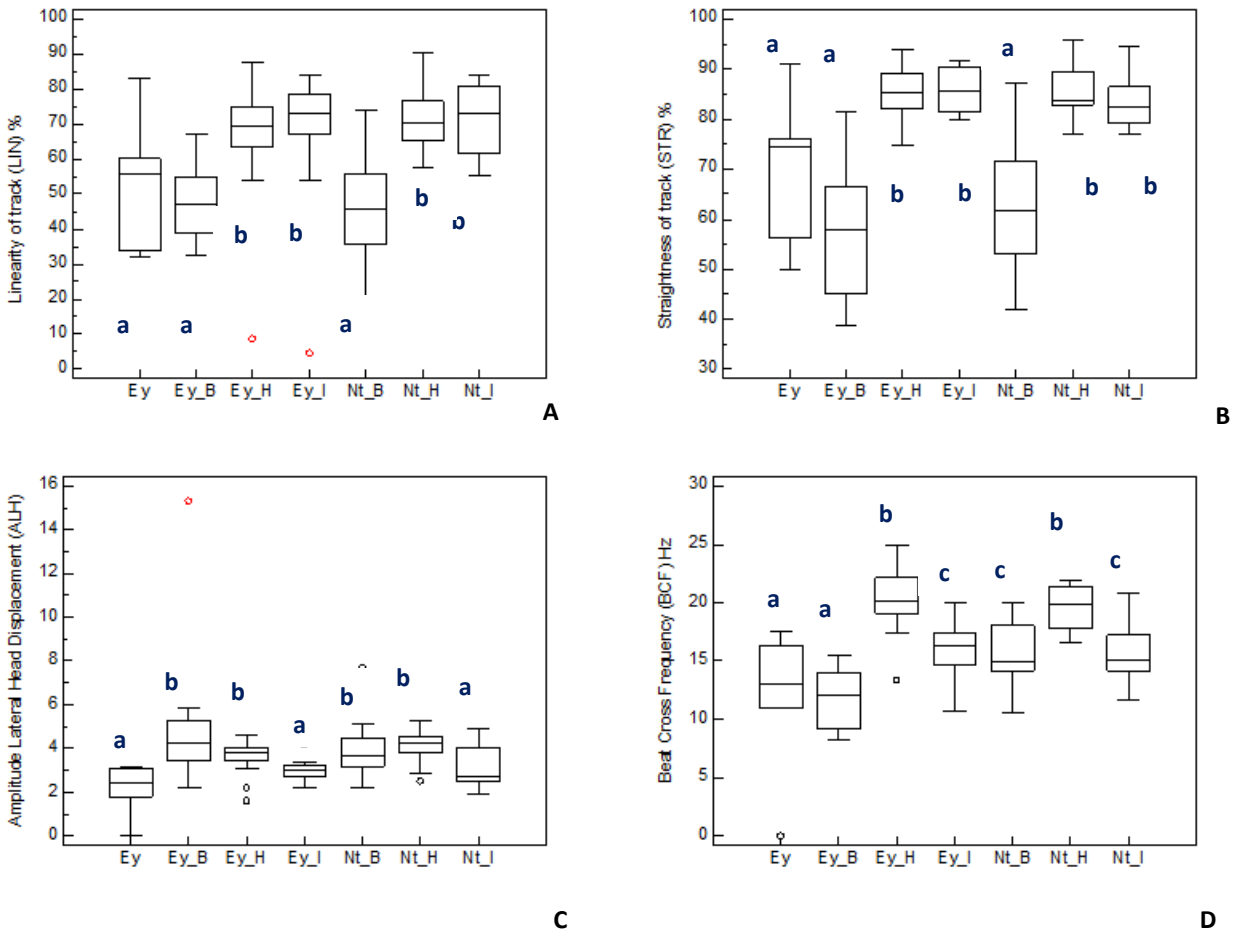


Figure 4.6: Sperm analyses by means of CASA showing **(A)** The linearity of track (LIN %) recorded **(B)** The straightness of the track (STR %) **(C)** The amplitude of head displacement (ALH µm) together with **(D)** The beat cross frequency (BCF Hz) recorded of sperm samples in different media. EY (Egg Yolk), EY_B (Egg Yolk and BO), EY_H (Egg Yolk and Ham's F10), EY_I (Egg Yolk and INRA96®), (NT_B) Neat and BO, NT_H (Neat and Ham's F10) and NT_I (Neat and INRA96®). Data plots labelled with different letters in the same graph were significantly different ($p < 0.05$).

4.2.4. HYPERACTIVATED MOTILITY ANALYSIS IN SPERM POPULATIONS USING CASA

4.2.4.1 Pattern analysis and the classification of motile spermatozoa

Individual spermatozoa in either EY or NT sperm samples incubated in either BO (10mM caffeine) or Ham's F10 media were analysed for the classification of individual spermatozoa according to the motion pattern displayed. For every individual spermatozoon classified, the VCL, VSL, LIN, STR, ALH and BCF parameter values were recorded and grouped accordingly. As illustrated in screen images A - D (Figure 4.7) fields were analysed and the motion patterns of individual spermatozoa designated as Group 1: Straight-line (non-HA) pattern (Figure 4.8). Group 2: Linear (Intermediate) pattern, Group 3: HA Circular pattern (HA C) and Group 4: HA Starspin pattern (HA S) (Figure 4.9).

Extracted parameter values were plotted to analyse the frequency distribution of the data recorded per motion pattern depicted in Figures 4.10 and 4.11. A data summary of the parameters recorded per motion pattern group is presented in Table 4.6. Statistical analysis of the above-described motion patterns recorded several similarities and differences in progression parameters amongst the four pattern groups compared (Table 4.6 and Figure 4.12).

Distribution based analysis allowed for the graphic illustration of parameter data recorded from individual spermatozoa. The distribution of the parameter data for every individual spermatozoon per motion pattern group classified (Non-HA, Intermediate, HA C and HA S) is presented as data dots in the scatter plots of Figures 4.10 and 4.11.

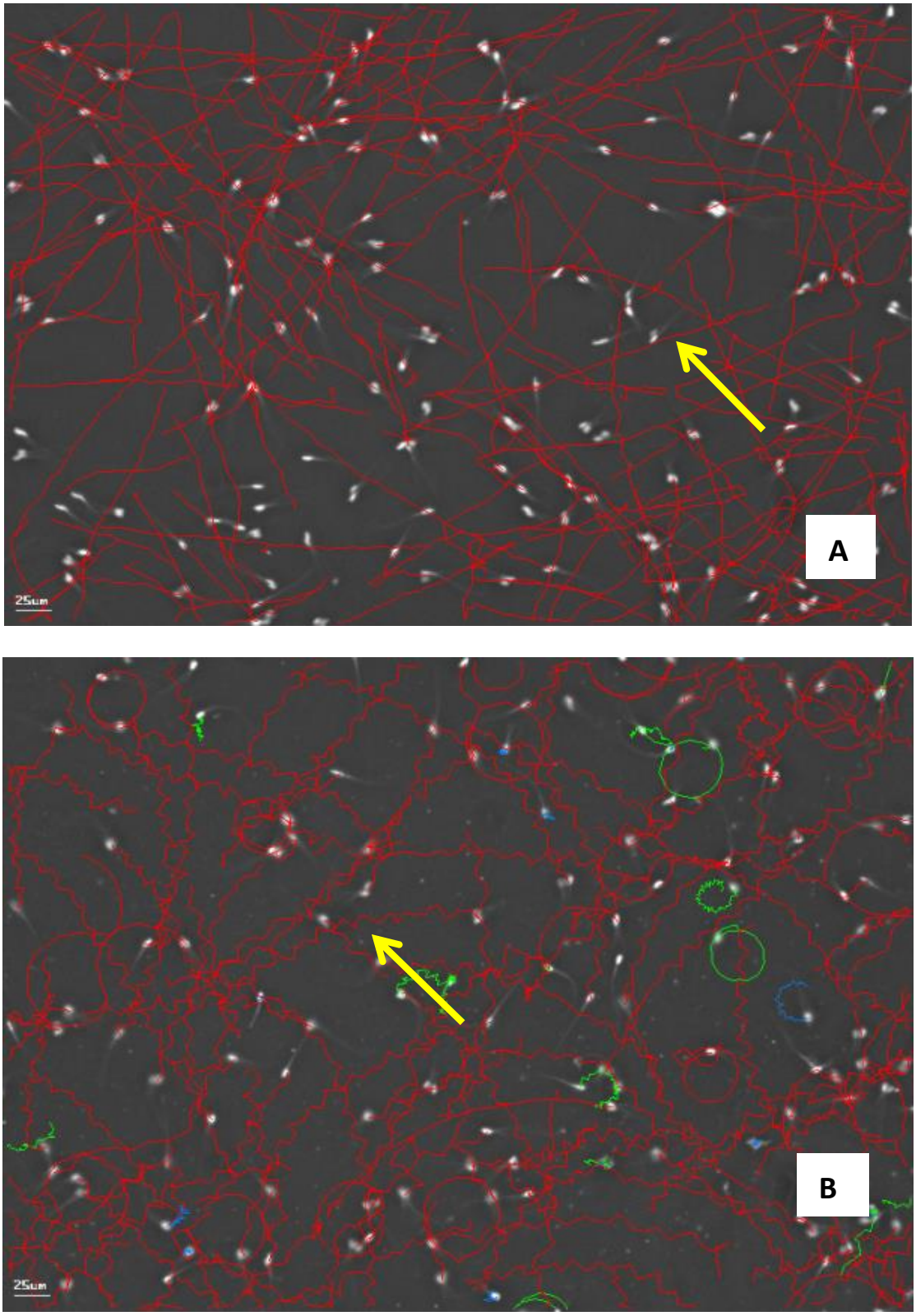


Figure 4.7: A selection of various SCA® screen images recorded during analysis showing sperm motion tracks or patterns recorded from various individual African elephant spermatozoa. Yellow arrows indicate various motion patterns displayed by individual spermatozoa. **(A)** An example of a straight-line (non-HA) pattern. **(B)** Motility becomes less progressive, this type of motion pattern displayed by spermatozoa is classified as linear (intermediate).

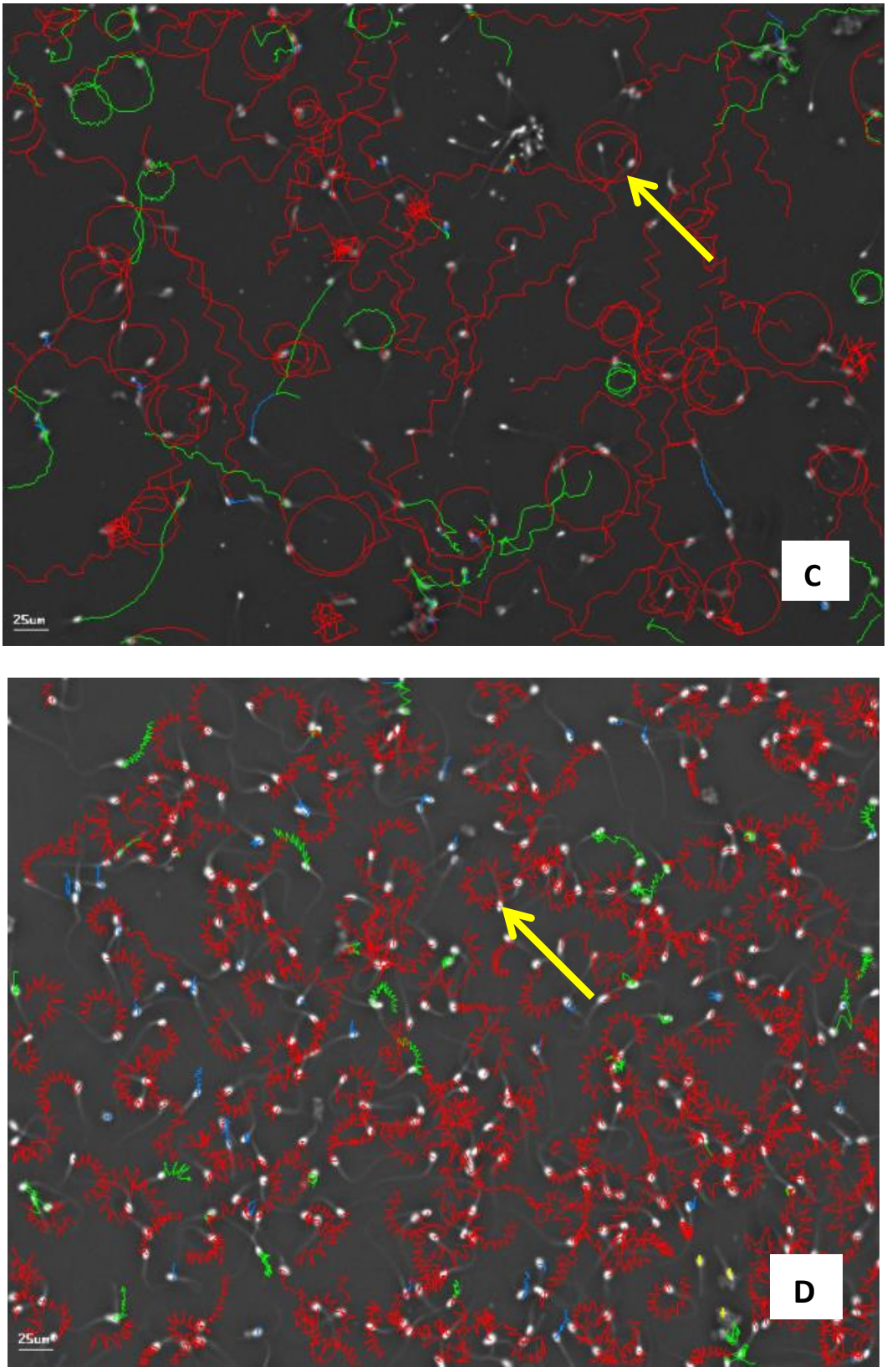


Figure 4.7: (continued) (C) An example of a circular motion pattern (HA C). **(D)** Starspin motion patterns (HA S) recorded of spermatozoa displaying hyperactivated motility.

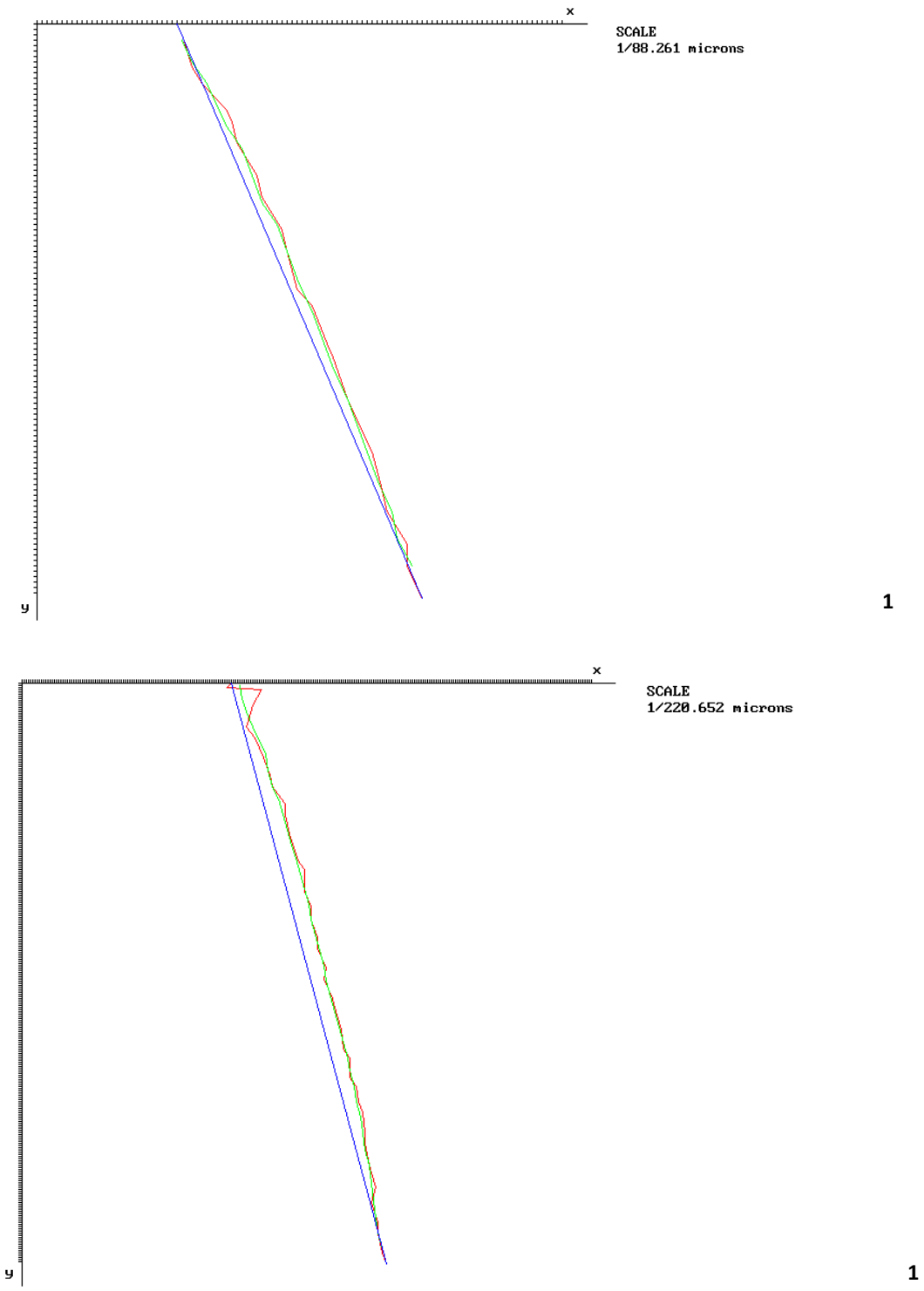


Figure 4.8: Group 1: Spermatozoa exhibiting a straight-line motion pattern or trajectory. The red line represents the actual sperm path also referred to as the curvilinear velocity (VCL µm/s), the green line represents the straight-line velocity (VSL µm/s) and the blue line the average path velocity (VAP µm/s). This visual analysis allowed for the classification of individual motion patterns.

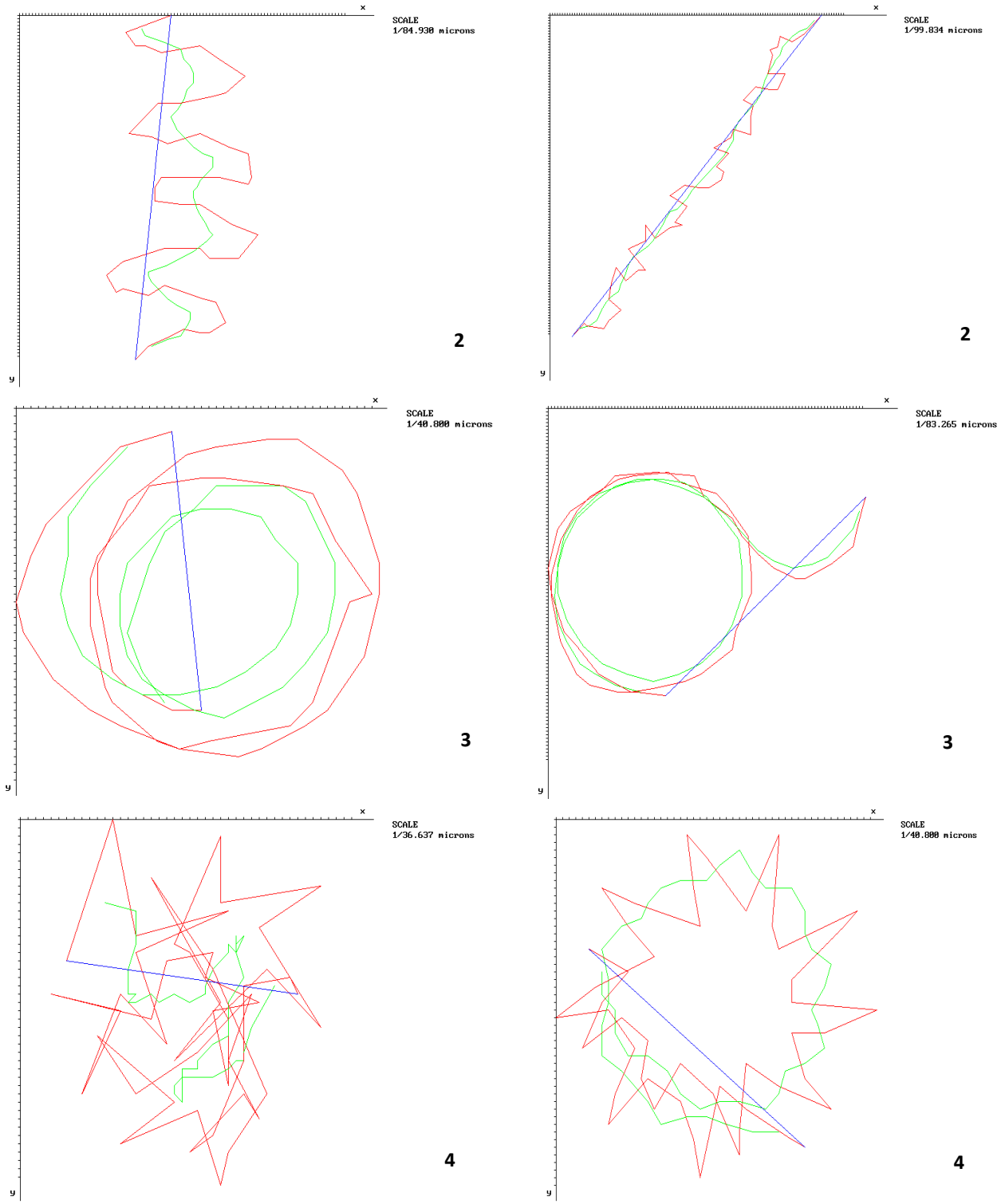


Figure 4.9: Visual analysis allowed for the classification of motion patterns displayed by individual spermatozoa, under the conditions imposed. **(2)** Group 2: Linear motion pattern. **(3)** Group 3: Circular motion patter. **(4)** Group 4: Starspin motion pattern. Red line = curvilinear velocity (VCL µm/s), green line = straight-line velocity (VSL µm/s) and blue line = average path velocity (VAP µm/s).

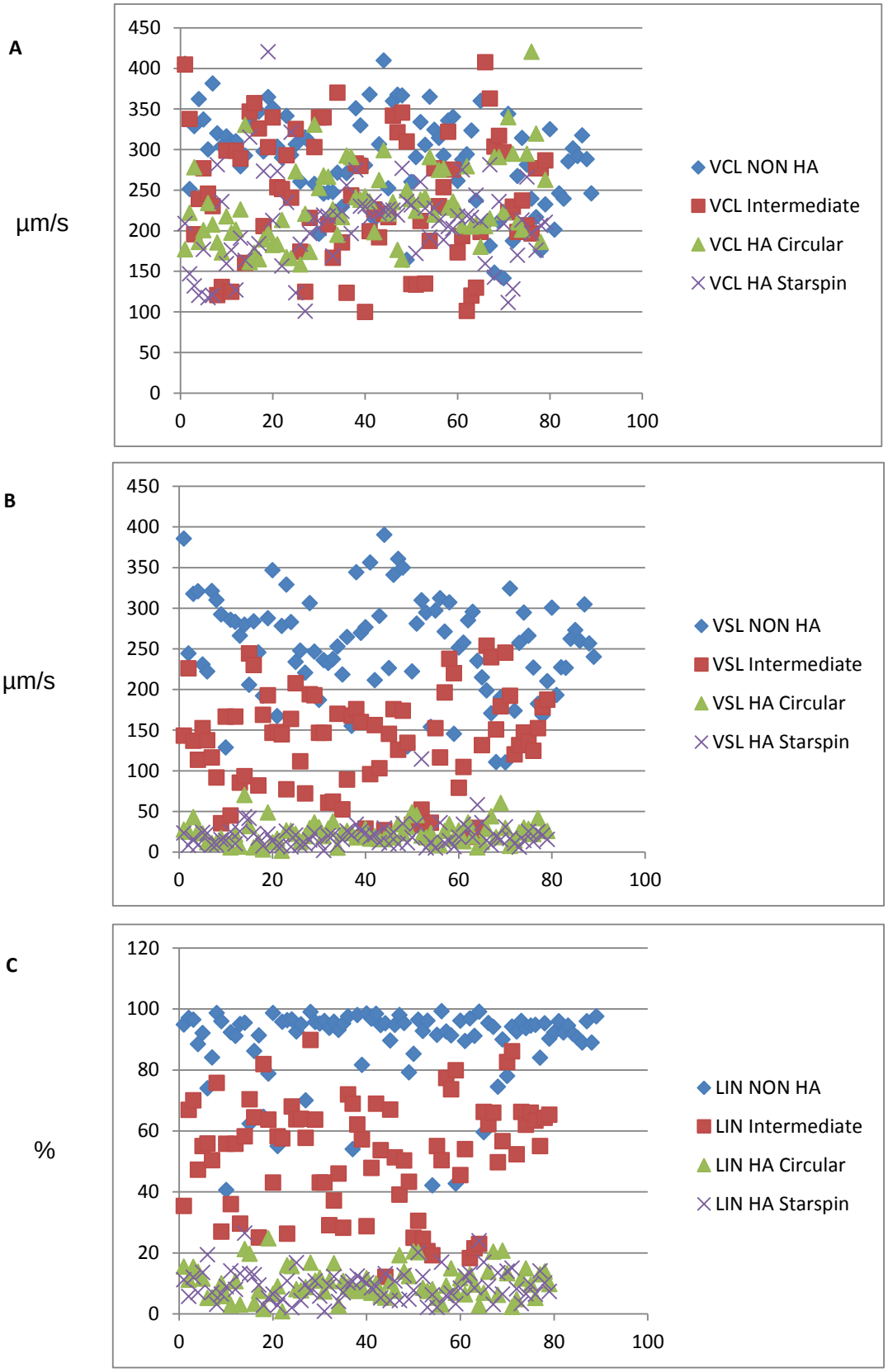


Figure 4.10: Distribution based analysis of progression parameters recorded from individual spermatozoa of African elephant displaying hyperactive (HA) characteristics. Data dots in scatter graphs represents **(A)** The VCL (curvilinear velocity µm/s) **(B)** The VSL (straight-line velocity µm/s) and **(C)** LIN (Linearity of the track %) recorded for every: 1. Straight-line (NON HA) (n = 79), 2. Linear (Intermediate) (n = 89), 3. Circular (HA C) (n = 79), and 4. Starspin (HA S) (n = 79) motion pattern.

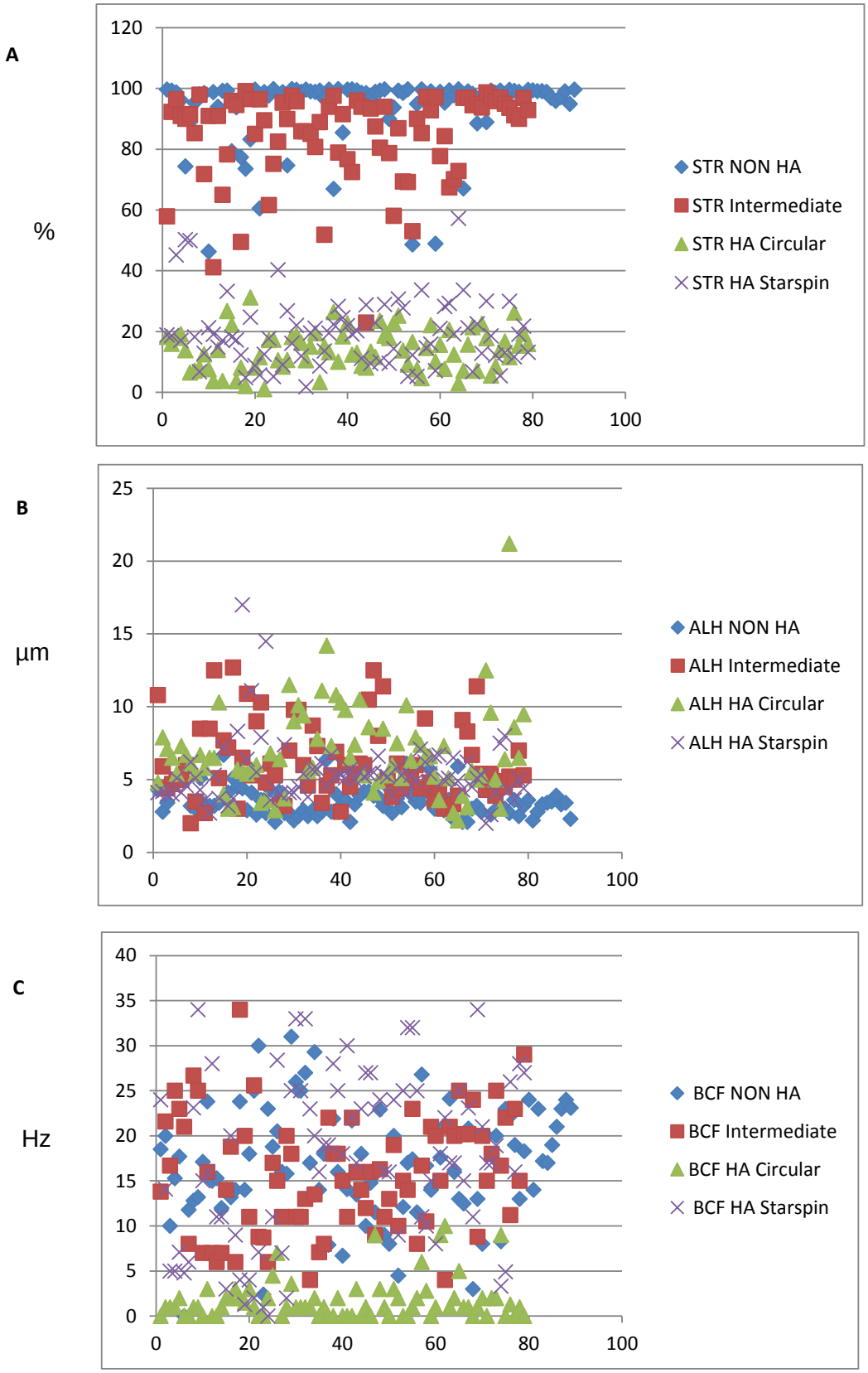


Figure 4.11: Distribution based analysis of STR, ALH and BCF recorded from individual spermatozoa of African elephant displaying hyperactive (HA) characteristics. Data dots in scatter graphs represents **(A)** STR (straightness of track %) **(B)** ALH (amplitude of the lateral head displacement µm) and **(C)** BCF (beat cross frequency Hz) recorded for every: 1. Straight-line (NON HA) (n = 79), 2. Linear (Intermediate) (n = 89), 3. Circular (HA C) (n = 79), and 4. Starspin (HA S) (n = 79) motion pattern.

Table 4.6 Summary table of African elephant sperm populations parameters classified according to individual motion patterns displayed. The data in the table is presented as sample number (N), mean ± standard deviation (SD), and median, minimum and maximum values for Curvilinear velocity (VCL µm/s), Straight line velocity (VSL µm/s), Linearity of track (LIN %), Straightness of tract (STR %), Amplitude of lateral head displacement (ALH µm) and Beat cross frequency (BCF Hz) and pattern diameters.

	1. STRAIGHT-LINE (NON-HA)						2. LINEAR PATTERN (INTERMEDIATE*)						3. HA CIRCULAR PATTERN (HA C)						4. HA STARSPIN PATTERN (HA S)					
Parameters	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.
VCL (µm/s)	89	**289.8**[a]	±57.23	294.4	142	410	79	**244.71**[b]	±76.95	240.8	100	408	79	**231.74**[b]	±49.79	222.8	159	421	79	**208.75**[c]	±52.98	212.9	101	421
VSL (µm/s)	89	**254.6**[a]	±61.07	257.6	110	390	79	**131.91**[b]	±61.66	143.2	19	254	79	**23.31**[c]	±12.62	22.9	1.7	70.2	79	**19.84**[d]	±14.88	17.3	1.9	114
LIN (%)	89	**88.94**[a]	±13.12	94.1	41	99.3	79	**52.51**[b]	±18.17	55.8	12	89.8	79	**10.19**[c]	±5.25	9.6	0.8	24.8	79	**9.3**[c]	±4.98	8.9	0.8	26.5
STR (%)	89	**93.32**[a]	±11.92	98.7	46	99.8	79	**84.52**[b]	±15.06	90	23	99.1	79	**13.8**[c]	±6.61	13.8	1	31.2	79	**19.34**[d]	±10.94	18.5	1.8	57.2
ALH (µm)	89	**3.54**[a]	±1.01	3.3	2.1	6.7	79	**6.18**[b]	±2.587	5.4	2	12.7	79	**6.79**[c]	±2.95	6.4	2.2	21.2	79	**5.45**[d]	±2.19	5.1	2	17
BCF (Hz)	89	**16.97**[a]	±6.18	17.1	0	31	79	**15.8**[a]	±6.41	16	4	34	79	**1.72**[b]	±2.26	1	0	10	79	**17.28**[a]	±9.34	17	0	34
Diameter (µm)	-	-	-	-	-	-	-	-	-	-	-	-	79	**33.83**	±10.81	32	1	64	73	**31.83**	±12.93	29	15	124

[a,b,c] values labelled with different superscript letters in the same row were significantly different ($p<0.05$)

* Spermatzoa with straight-line patterns but with increased ALH classified under 2. Linear pattern (intermediate*) group

HA = Hyperactive

In general, spermatozoa within the straight-line (non-HA) population recorded on average the highest values for VCL, VSL, LIN and STR parameters amongst all four groups (Table 4.6). The VCL's recorded ranged from 208.75 ± 52.98µm/s to 289.8 ± 57.23µm/s among the four motion patterns compared. On average, the highest VCL's were recorded in the straight-line (non-HA) pattern group, but the highest individual VCL was measured at 421 µm/s in group 4: HA starspin pattern (Table 4.6). Statistical analysis indicated similarities for VCL's recorded between group 2: linear (intermediate) and group 3: HA circular pattern when comparing the various groups (Figure 4.12 A). On average, the highest VSL was recorded in group 1: straight-line (non-HA) at 245.6 ± 61.07µm/s followed by group 2: linear (intermediate) at 244.71 ± 76.95µm/s. VSL's dropped to 23.31 ± 12.31µm/s in group 3: HA circular and the lowest VSL was recorded in group 4: HA starspin pattern group at 19.84 ± 14.88µm/s (Table 4.6). The VSL's were significantly different ($p < 0.0001$) between the four pattern group compared (Figure 4.12 B).

Linearity (LIN) percentages of group 3: HA circular (10.91 ± 5.25%) and group 4: HA starspin (9.3 ± 4.93%) exhibited similarities when compared (Figure 4.12 C). As expected, the highest population average for LIN was recorded in Group 1: straight-line (non-HA) recorded at 88.94 ± 13.12%. The STR percentages were significantly different ($p < 0.0001$) among the four pattern groups (Figure 4.12 D). Group 1: straight-line (non-HA) showed the highest population average for STR at 93.32 ± 11.92%, followed by group 2: linear (intermediate) at 84.52 ± 15.06% (Table 4.6). Analysis of data recorded for ALH revealed a significant difference ($p < 0.0001$) among the four pattern groups compared (Figure 4.12 E). The highest ALH's were recorded in group 3: HA circular at 6.79 ± 2.95µm (Table 4.6). The BCF's were similar amongst group 1: straight-line, group 2: linear (intermediate) and group 4: HA starspin while the BCF's recorded in group 3: HA circular was significantly lower (Figure 4.12 F).

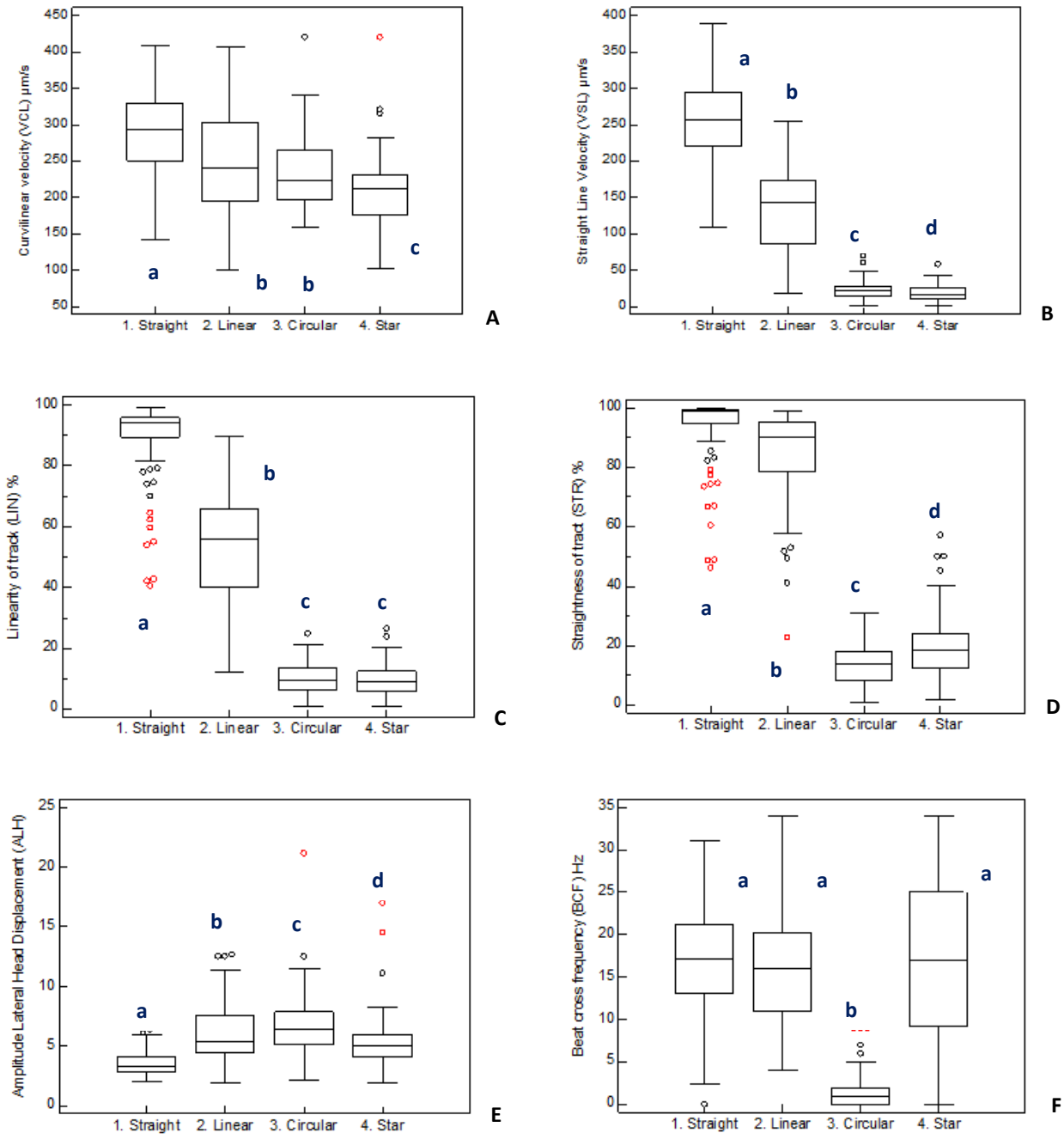


Figure 4.12: Box and whisker plots of the kinematic parameters recorded for hyperactivated spermatozoa of the African elephant showing: **(A)** curvilinear velocity (VCL µm/s) **(B)** straight-line velocity (VSL µm/s) **(C)** linearity of tracks (LIN %) **(D)** Straightness of track (STR %) **(E)** Amplitude of lateral head displacement (ALH µm) and **(F)** beat cross frequency (BCF Hz) of the four motion patterns. Data plots labelled with different letters in the same graph were significantly different ($p < 0.05$). Data from individual spermatozoa recorded during CASA assessment were evaluated and grouped according to motion pattern: 1. Non-hyperactivated straight-line motion track (NON-HA), 2. Linear (Intermediate), 3. Circular (HA C), and 4. Starspin (HA S) motion pattern.

In summary, applied pattern analyses allowed for the grouping of progression parameter data per individual elephant spermatozoon classified. Velocities recorded for VCL ranged from 289.8 – 208.75µm/s a decrease in every consecutive pattern group was detected from non-HA, to intermediate, and from HA C to HA S. Scatter plots showed a general grouping in the distribution of the data dots for VCL's recorded per pattern group (Figure 4.10 A). However, for VSL the distribution of data dots for non-HA and Intermediate (254.6µm/s and 131.91µm/s) grouped together, while data dots for HA C and HA S (23.31µm/s and 19.84µm/s) grouped together on the scatter plot (Figure 4.10 B).

For both LIN and STR parameters the distribution clearly grouped data dots for Non-HA and Intermediate pattern groups together and those for HA C and HA S grouped together (Figure 4.10 C and 4.11 A). LIN decreased from 88.94% and 52.51% to 10.19% and 9.3% in every consecutive pattern group from non-HA and intermediate, to HA C and HA S. STR also decreased from 93.32% and 84.52% to 13.8% - 19.34% from non-HA and intermediate, to the HA C and HA S pattern groups. ALH increased from 3.54µm (in non-HA) to 6.18µm (in intermediate) and 6.79µm (in HA C) then decreased to 5.45µm (in HA S). Scatter plot analyses indicated a general grouping in the distribution of the ALH data dots per pattern group (Figure 4.11 B). The distribution of the BCF data dots grouped non-HA, intermediate and HA S (16.87Hz, 15.8Hz and 17.28Hz) during scatter plot analyses (Figure 4.11 C). A significant decrease was recorded for BCF in the HA C (1.72Hz) pattern group compared to the non-HA, intermediate and HA S pattern groups.

Taking the results of the scatter plots and pattern group analyses into consideration, the parameters VCL, LIN and STR proved to be sensitive parameters for distinguishing between motile sperm populations based on the motion pattern displayed (Figures 4.10 B, 4.10 C and 4.11 A).

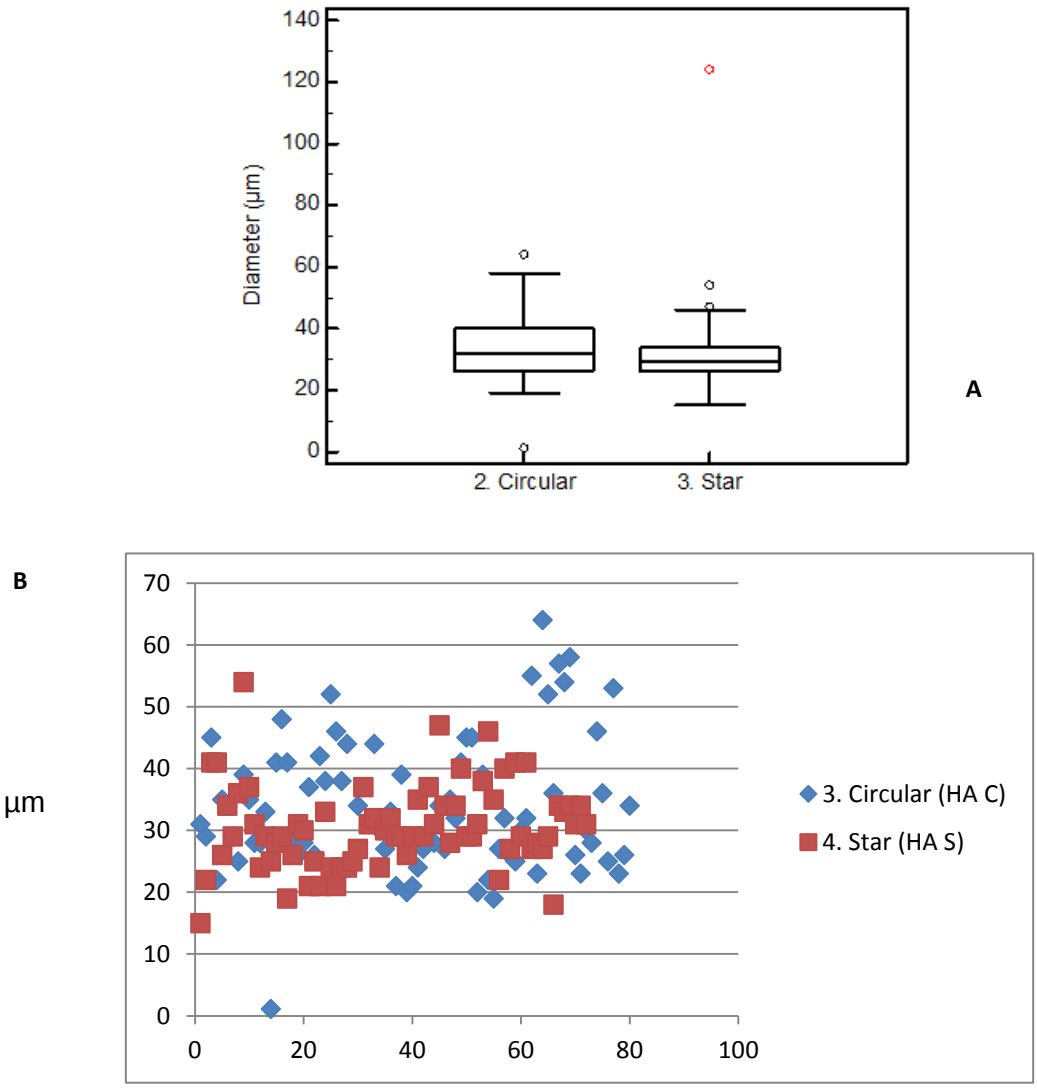


Figure 4.13: Distribution based analysis of the diameter (µm) measurements recorded from hyperactivated African elephant spermatozoa with circular (HA C) (n = 79) or starspin (HA S) (n = 79) motion patterns. **(A)** Box and whisker plots and **(B)** Data dots in a scatter graph represent the diameters of the motion patterns recorded by spermatozoa displaying hyperactive (HA) characteristics.

Data collected from the individual diameters measured of both circular and starspin motion pattern groups (Table 4.6) revealed no difference between diameters for the two patterns (Figure 4.13).

4.2.4.2 CASA Cut-off values for hyperactive motility analysis

The extracted data from each pattern group was arranged for ROC curve analysis. Parameter criteria for CASA were derived from a comparison of straight-line pattern (non-HA) data to HA circular (HA C) and HA starspin (HA S) pattern data, combined during ROC analysis (Table 4.7). The cut-off criterion for VCL (Figure 4.14 A) and BCF (Figure 4.14 E) revealed low sensitivity and specificity (> 75%). However, VSL (Figure 4.14 B), STR (Figure 4.14 C), and LIN (Figure 4.14 D) displayed a high sensitivity and specificity (>90%) for cut-off criterion with no data overlapping (AUC = 1) (Table 4.7).

4.2.4.3 Hyperactivated motility analysis of sperm samples in different media

CASA derived cut-off values (Figure 4.14 F) were used to distinguish between the motile and hyperactivated elephant sperm populations within every captured NT_Ham's, NT_BO, EY_Ham's and EY_BO sperm sample. The sort criteria was set and all CASA analysed field were re-analysed and accordingly sorted all spermatozoa as either motile or HA motile. The data collected per sperm sample analysed is presented in Table 4.8. The type of motility recorded as a result of interaction with the media is depicted as bar graphs in Figures 4.15 and 4.17. Captured SCA® screen images illustrates the difference in sperm motion tracks or patterns recorded under the different conditions imposed. Analysed egg yolk (EY) extended sperm samples in either BO (10mM caffeine) (EY_BO) or Ham's F10 (EY_Ham's) media are depicted in Figure 4.16 A and B. Neat sperm samples (NT) extended in either BO (10mM caffeine) (NT_B) or Ham's F10 (NT_Ham's) media are presented in Figure 4.16 C and D.

HA motility percentages recorded in egg yolk (EY) samples ranged from 4% in EY_Ham's compared to 29% recorded for EY_BO (used to stimulate HA). HA percentage recorded ranged from 0% - 22.8% (in EY_Ham's) to 8% - 53.6% (in EY_BO). In neat sperm (NT) samples HA motility percentages ranged from 4% in NT_Ham's compared to 20% in NT_BO. HA percentage recorded ranged from

1.2% - 9.8% (in NT_Ham's) to 4.2% - 35.6% (in NT_BO). The highest individual HA percentage was recorded for LA 2 (Bump) at 53.6% in EY_BO compared to 0% for the same animal in EY_Ham's. A consecutive ejaculate collected from Bump (LA 10) recorded HA percentages at 22.8% (in EY_Ham's) and 38.5% (in EY_BO).

During this study, three successive ejaculates collected from Young 1 were analysed for HA motility during season 2. HA percentages were recorded for ejaculates LA 5 at 4.7% and 13.5% (in EY_Ham's and EY_BO, in ejaculate LA 9 at 4% and 21.5% (in EY_Ham's and EY_BO), and in ejaculate LA 11 at 0.5% and 20.6% (in EY_Ham's and EY_BO). When using neat semen, ejaculates collected from Young 1 recorded HA percentages in LA 5 at 9.8% and 20.3% (in NT_Ham's and NT_BO), and in ejaculate LA 9 at 2.8% and 16.4% (in NT_Ham's and NT_BO). These results suggest inter and intra variation in functional sperm populations between ejaculates collected from the same individual. With the exception of LA 1 (Righthook), LA 2 (Bump), and LA 11 (Young 1) all other elephant sperm samples analysed for HA motility in either NT_Ham's, NT_BO, EY_Hams or EY_BO media groups defined a percentage of the motile sperm population as HA. The difference recorded for HA motility percentages between NT_Ham's and NT_BO and EY_Hams and EY_BO media groups ranged from 16% - 24%.

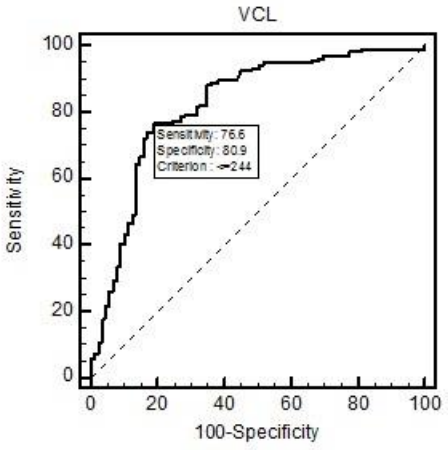
Table 4.7 ROC analyses of African elephant sperm parameter data classified according to individual motion patterns displayed. 1) Straight-line non-hyperactive (non-HA), 3) Hyperactive circular (HA C), 4) Hyperactive starspin (HA S) patterns and 5) Total HA (HA C and HA S combined). The data collected is presented in the table as number of samples (N), area under the curve (AUC), minimum (Min.) and maximum (Max.) values for Curvilinear velocity (VCL µm/s), Straight line velocity (VSL µm/s), Linearity of track (LIN %), Straightness of tract (STR %) and Beat cross frequency (BCF Hz) parameters.

	1. STRAIGHT-LINE (NON-HA)						3. HA CIRCULAR PATTERN (HA C)						4. HA STARSPIN PATTERN (HA S)						5. TOTAL HA (HA C + HA S)					
Parameters	N	ROC Criterion	% Sensitivity / % Specificity	AUC	Min.	Max.	N	ROC Criterion	% Sensitivity / % Specificity	AUC	Min.	Max.	N	ROC Criterion	% Sensitivity / % Specificity	AUC	Min.	Max.	N	ROC Criterion	% Sensitivity / % Specificity	AUC	Min.	Max.
VCL (µm/s)	89	**≤ 245.8**	68.8 / 80.9	0.774	142	410	79	**≤ 242.8**	69.6 / 80.9	0.787	159	421	79	**≤ 236.4**	82.3 / 84.3	0.857	101	421	158	**≤ 244**	76.6 / 80.9	0.822	101	421
VSL (µm/s)	89	**≤ 152.4**	88.2 / 94.4	0.972	110	390	79	**≤ 70.2**	100 / 100	1	1.7	70.2	79	**≤ 58.2**	98.7 / 100	1	1.9	114	158	**≤ 70.2**	99.4 / 100	1	1.7	114
LIN (%)	89	**≤ 73.7**	97 / 89.9	0.981	41	99.3	79	**≤ 24.8**	100 / 100	1	0.8	24.8	79	**≤ 26.5**	100 / 100	1	0.8	26.5	158	**≤ 26.5**	100 / 100	1	0.8	27
STR (%)	89	**≤ 93.9**	88.2 / 79.8	0.934	46	99.8	79	**≤ 31.2**	100 / 100	1	1	31.2	79	**≤ 57.2**	100 / 96.6	0.999	1.8	57.2	158	**≤ 45.2**	98.1 / 100	0.999	1	57
BCF (Hz)	89	**≤ 11.2**	54.9 / 86.5	0.673	0	31	79	**≤ 7**	94.9 / 94.4	0.982	0	10	79	**≤ 11**	67.1 / 13.5	0.524	0	34	158	**≤ 11**	66.2 / 86.5	0.727	0	34

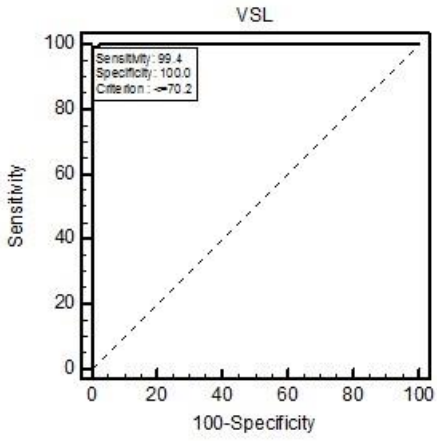
Total % HA : HA C + HA S combined

HA = Hyperactived motility

AUC = Area under ROC curve



A



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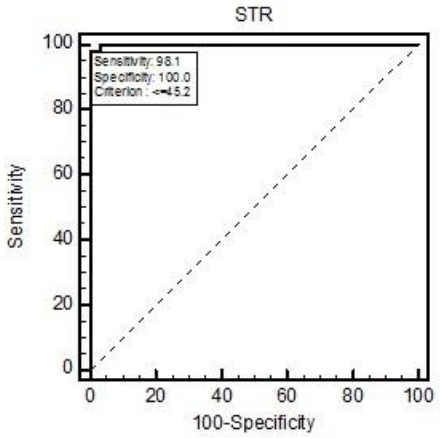

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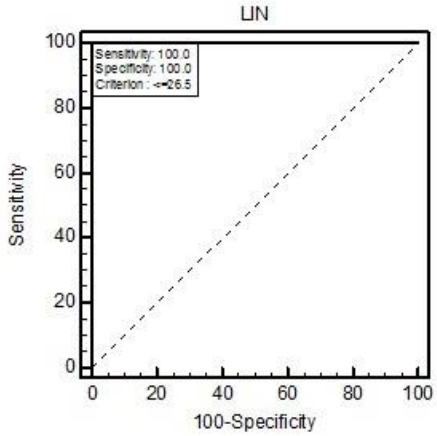

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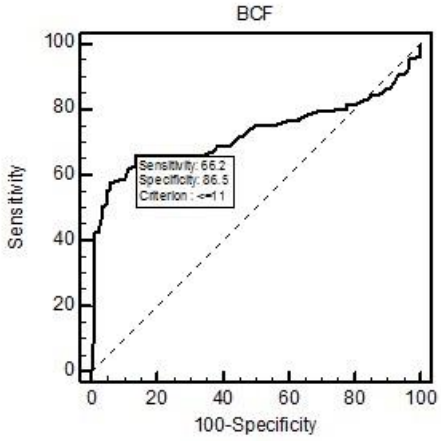

E

African Elephant	
SCA sort	**% HA**
VCL	> 100 and < 500
VSL	> 1 and < 70.2
STR	> 1 and < 26.5
LIN	> 1 and < 45.2
Restriction	
VSL	< 30

F

Figure 4.14: ROC criterion derived to assess hyperactivated motility in African elephant sperm samples by comparing the straight-line pattern to the HA circular (HA C) and HA starspin patterns combined. Cut-off criteria for CASA **(A)** VCL (curvilinear velocity µm/s) **(B)** VSL (straight-line velocity µm/s) **(C)** LIN (linearity of track) **(D)** STR (straightness of track %) and **(E)** BCF (beat cross frequency Hz) **(F)** ROC derived cut-off criterion derived by applying The Boolean argument to determine the total percentage of spermatozoa displaying hyperactivated motility in each sample evaluated.

Table 4.8 The total percentage hyperactivated motility (HA) measured per sperm sample analysed using CASA cut-off criterion derived from motion pattern analyses. HA results recorded in either egg yolk (EY) samples with Ham's F10 or BO (10mM caffeine) media (EY_Ham's and EY_BO) or in neat (NT) with Ham's F10 or BO (10mM caffeine) media (NT_Ham's and NT_BO). Results presented as percentage (%) and population average.

ID	Name	Total % HA (EY_Ham's)	Total % HA (EY_BO)	Total % HA (NT_Ham's)	Total % HA (NT_BO)
LA 1	Righthook	0.5	8	-	-
LA 2	Bump	0	53.6	-	-
LA 5	Young 1	4.7	13.5	9.8	20.3
LA 6	No 3	2.9	43.6	4.8	35.6
LA 8	Righthook	4.5	24.4	3.8	30.3
LA 9	Young 1	4	21.5	2.8	16.4
LA 10	Bump	22.8	38.5	-	-
LA 11	Young 1	0.5	20.6	-	-
LA 12	No name	3.8	32.9	1.2	4.2
LA 14	Rex	-	-	3.9	12.9
Population average:		**5 %**	**29 %**	**4 %**	**20 %**
HA = Hyperactivated motility					
NT_Ham's (neat semen and Ham's F10), NT_BO (neat semen and BO), EY_Ham's (egg yolk semen and Ham's F10), EY_BO (egg yolk semen and BO)					

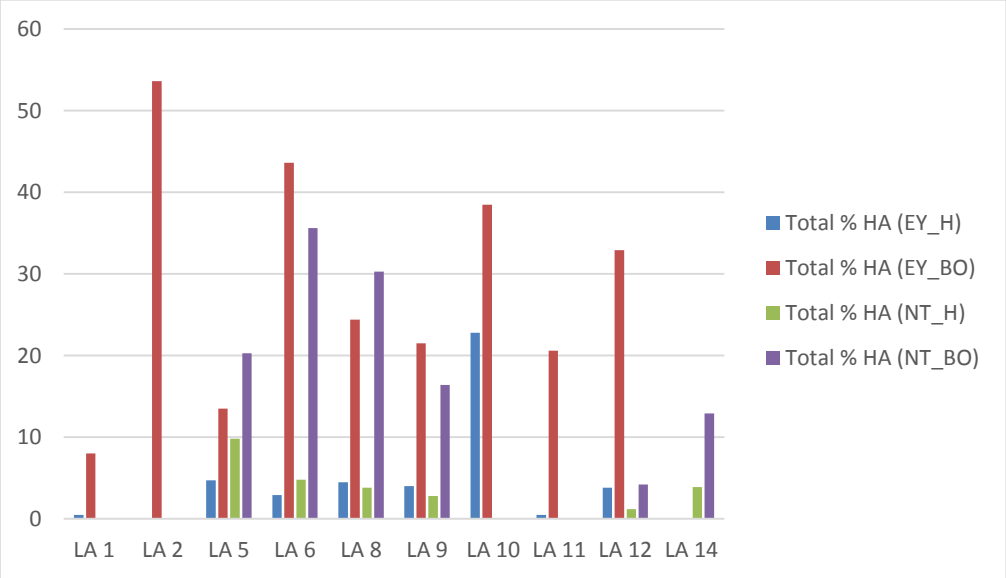

Figure 4.15: A comparison of total percentage hyperactivated motility (HA) measured per sperm sample analysed. Data recorded in either egg yolk (EY) extended samples with Ham's F10 media or BO (10mM caffeine) (EY_H and EY_BO) or from neat (NT) extended samples with Ham's F10 media or BO (10mM caffeine) (NT_H and NT_BO). Samples measured on CASA with cut-off criterion derived after applied motion path pattern and ROC analyses.

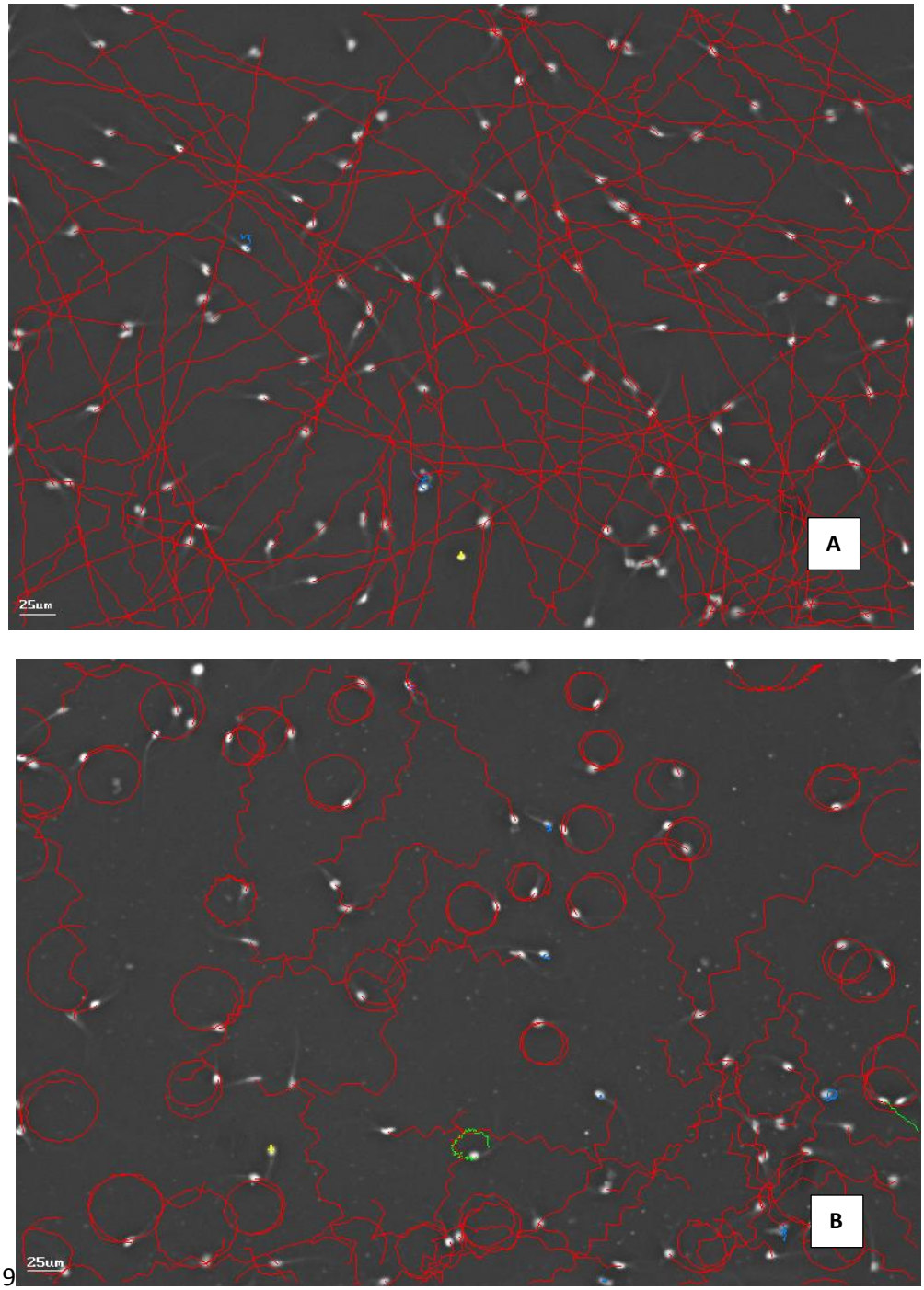

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Figure 4.16: SCA® screen images) showing the difference in sperm motion tracks or patterns recorded in **(A)** egg yolk (EY) extended sperm samples with Ham's F10 (EY_Ham's), and in **(B)** egg yolk (EY) extended sperm samples with BO (10mM caffeine) (EY_BO).

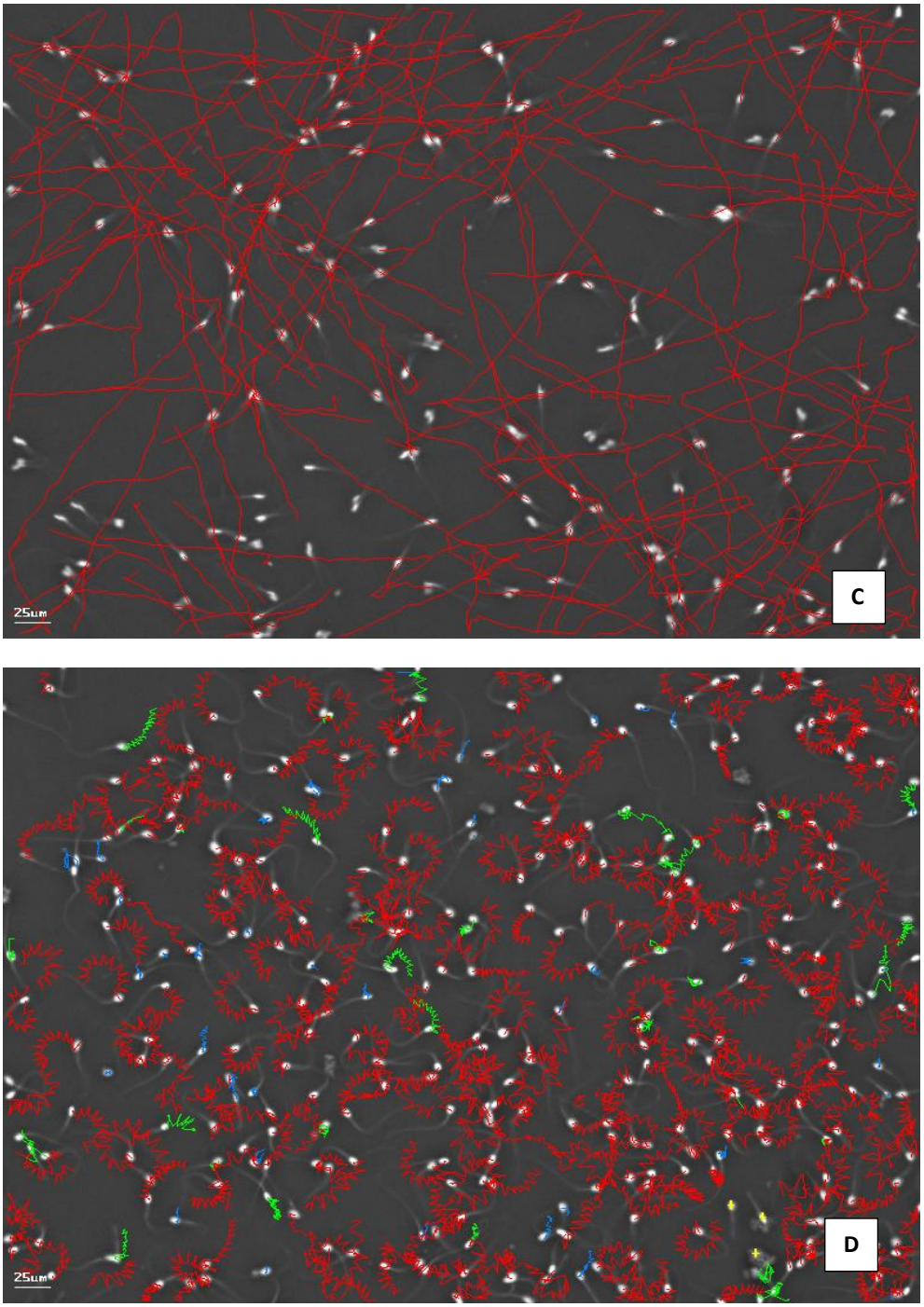


Figure 4.16: (continued) SCA® screen images showing the difference in sperm motion tracks or patterns recorded. **(C)** In neat sperm samples (NT) extended with Ham's F10 (NT_Ham's) and in **(D)** neat sperm samples (NT) extended with BO (10mM caffeine) (NT_BO).

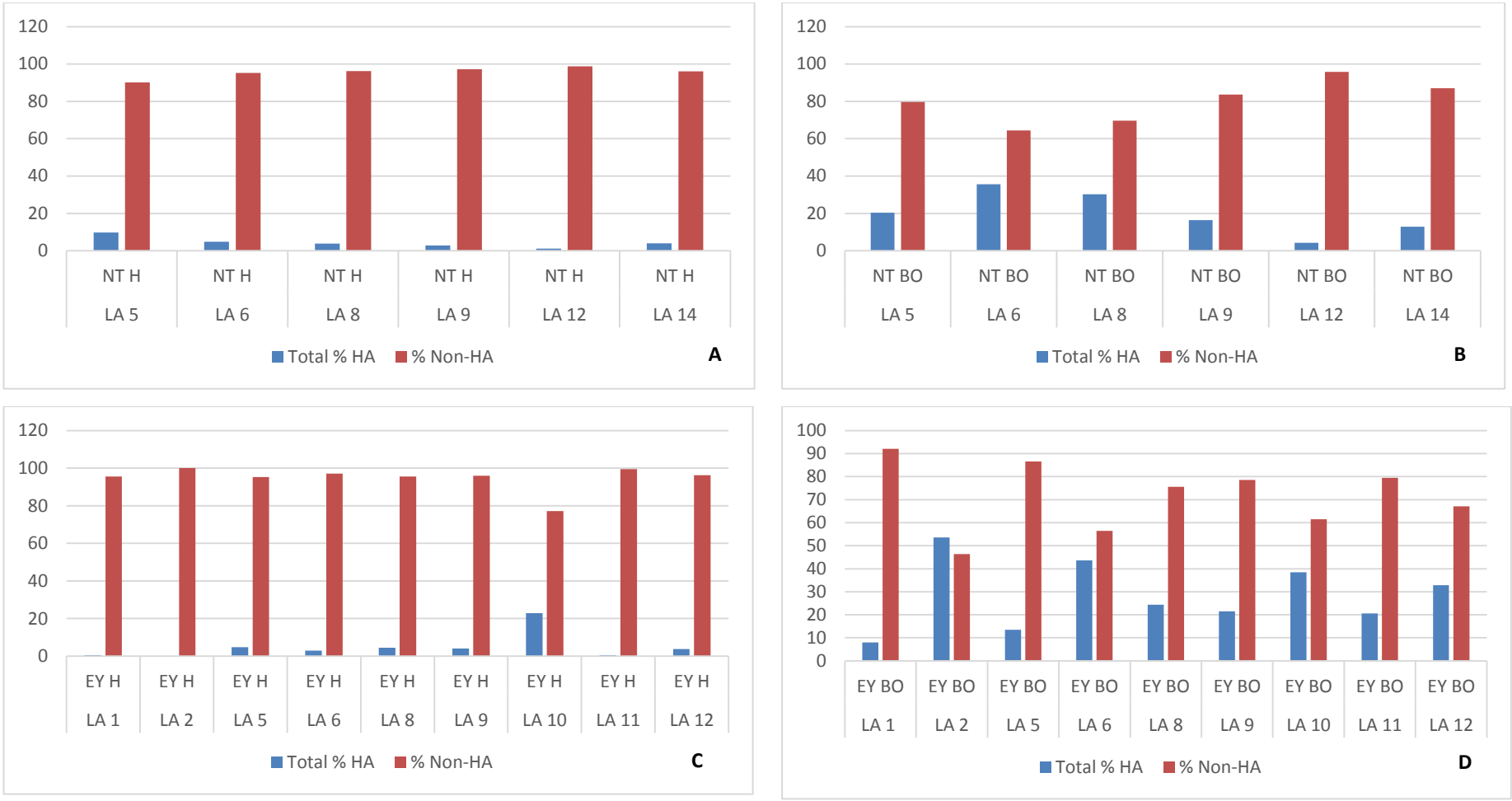


Figure 4.17: Bar graphs representing percentage hyperactivated (% HA) motility vs. percentage non-hyperactivated (% Non-HA) spermatozoa for each sperm sample analysed. Data recorded from neat (NT) samples extended with Ham's F10 media or BO (10mM caffeine) **(A)** NT_H and **(B)** NT_BO or in the egg yolk (EY) extended samples with Ham's F10 media or BO (10mM caffeine) **(C)** EY_H **(D)** EY_BO. Measured by CASA after cut-off criterion was derived following applied motion path pattern and ROC analysis.

4.2.5. STRUCTURAL INTEGRITY ANALYSIS OF SPERMATOZOA

4.2.5.1 Plasma membrane integrity (Viability) and acrosome integrity

In general, the sperm samples evaluated recorded high percentages of spermatozoa with the plasma membrane (68 ± 11.88%) and acrosomes (77 ± 11.31%) intact. Analysis of the data collected is presented in Table 4.9 and data recorded per ejaculate presented in Table 4.10. The highest individual percentage for intact plasma membranes (86%) and intact acrosomes (88%) was recorded for LA11b (Young 1). Examples of the classification of spermatozoa according to viability and acrosome integrity are illustrated in Figure 4.18.

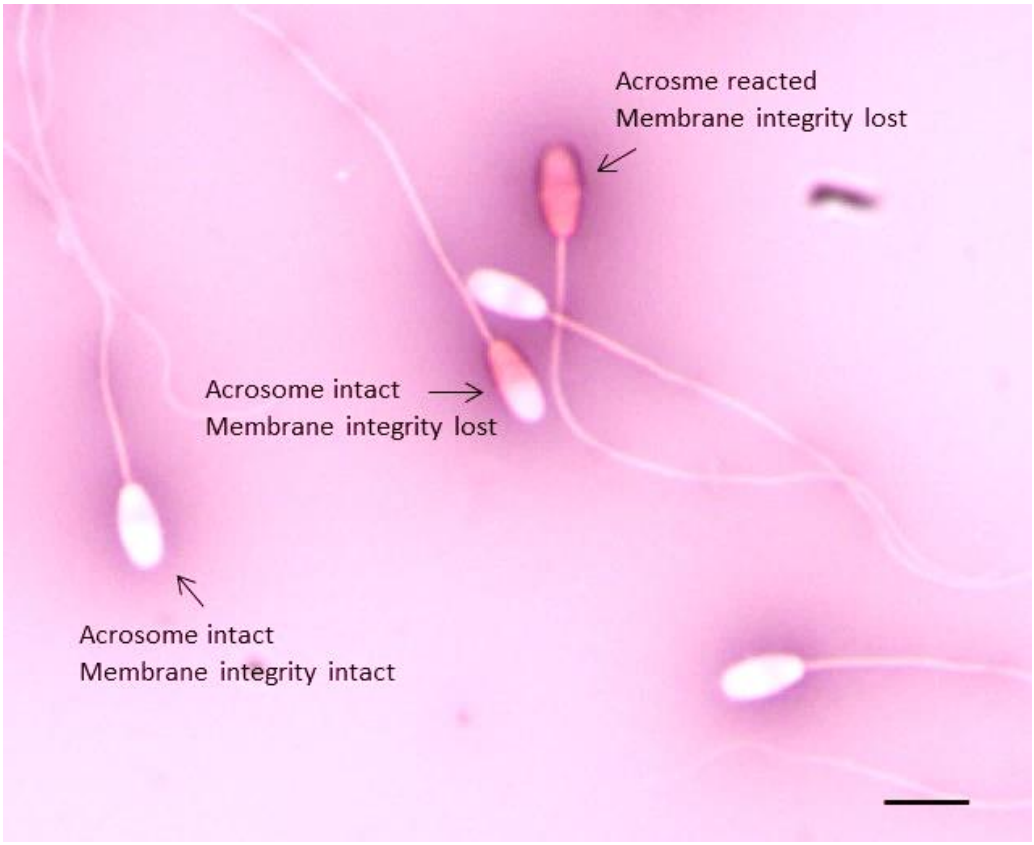


Figure 4.18: Eosin-nigrosin smear of African elephant sperm to illustrate the classification of membrane integrity and acrosome integrity of spermatozoa during microscopic evaluation (Scale bar = 7 µm).

Table 4.9 Summary of membrane integrity (vitality) and acrosome integrity parameter values of African elephant spermatozoa. Data is presented as sample number (N), mean ± standard deviation (SD) and median, minimum (min.) and maximum (max.).

Season 2						
Parameters	N	Mean	±SD	Median	Min.	Max.
Membrane integrity intact (%)	8	**68**	±11.88	70	54	86
Membrane integrity lost (%)	8	**32**	±11.88	30	14	46
Acrosome intact (%)	8	**77**	±11.31	81	50	88
Acrosome reacted (%)	8	**23**	±11.31	19	12	50

Table 4.10 Overview of individual viability and acrosome integrity results recorded per African elephant sperm sample analysed.

Elephant ID	Name	% Viability Live	% Viability Dead	% Acrosome Intact	% Acrosome Reacted
LA 6b	No 3	72	28	72	28
LA 8b	Righthook	56	44	78	22
LA 9b	Young 1	78	22	82	18
LA 10b	Bump	54	46	66	34
LA 11b	Young 1	86	14	88	12
LA 12b	No Name	70	30	84	16
LA 14b	Rex	82	18	84	16
LA 16b	Steve	70	30	82	18

4.2.5.2 Sperm morphology

The mature spermatozoon of the African elephant is divided into a head and tail (flagellum) region. The fundamental parts of the head are the nucleus and the covering acrosome. The tail (flagellum) region contains the neck situated just below the head at the implantation socket. The midpiece (or mitochondrial sheath) extends from the neck to the annulus. From here, the principal piece extends from the annulus to nearly the end of the tail. The end piece completes the flagellum structure (Figure 4.19). Spermatozoa with imperfections in any of these regions were classified under abnormal morphology and the nature of the imperfection was classified accordingly. Examples of abnormalities recorded are illustrated in Figure 4.21.

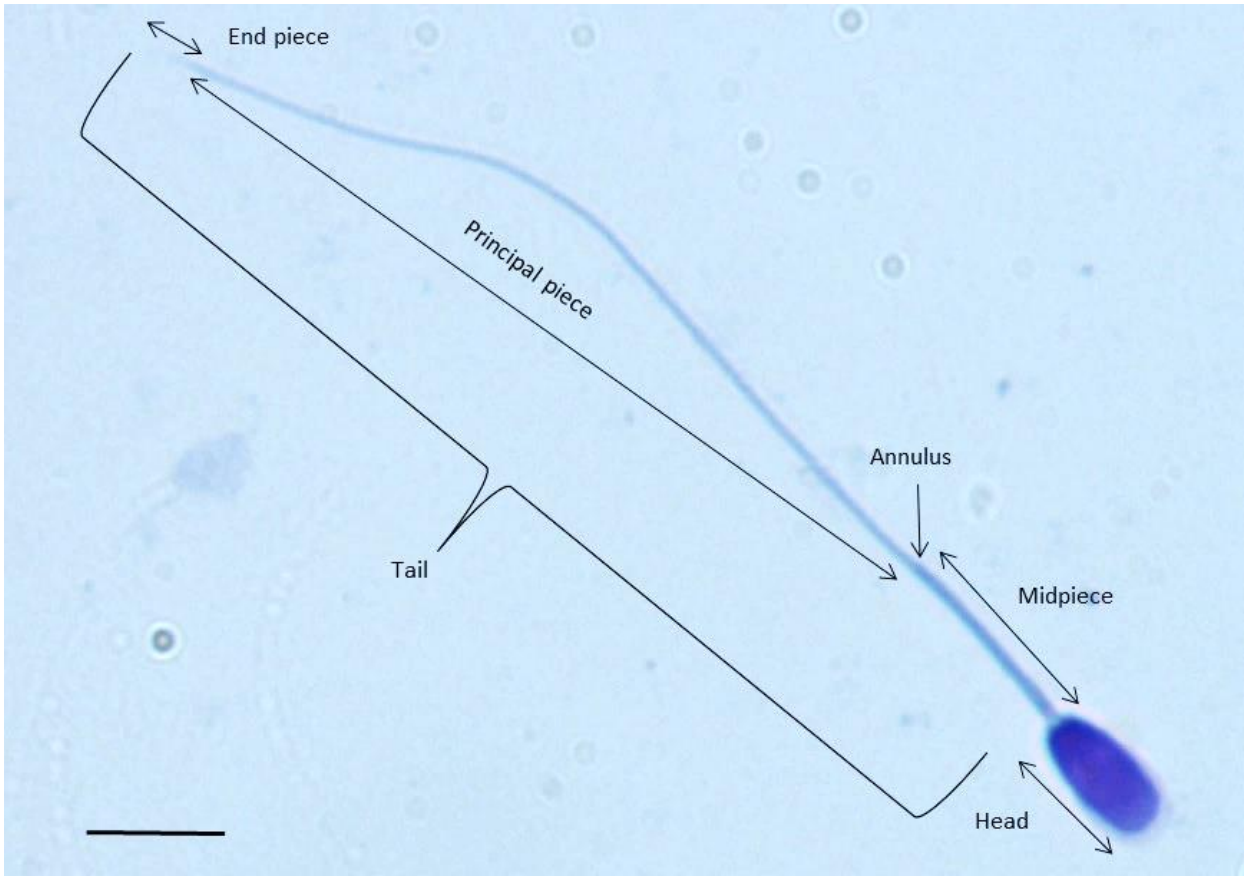


Figure 4.19: An example of an African elephant spermatozoon depicting normal morphology. Spermatozoa were stained with SpermBlue® stain in order to identify and evaluate each sperm component individually (Scale bar = 7µm).

No significant difference in percentage normal morphology ($p = 0.760$; $F = 0.097$) or abnormal morphology ($p = 0.001$) was found between the two seasons. Data recorded for detailed sperm morphology analysis is presented in Table 4.11. There were, however, significant differences in the types of abnormalities recorded when the two seasons were compared. A significant difference was recorded between the two seasons in the number of head defects ($p = 0.015$; $F = 7.343$, Figure 4.20 A). The average number of head defects recorded during season 1 was 24 ± 24.13% compared to only 9 ± 6.78% in season 2 (Table 4.10). No significant difference ($p = 0.579$) was recorded for midpiece abnormalities between season 1 (12 ± 7.80%) and season 2 (16 ± 10.69%). Tail defects compared between seasons were significantly different ($p = 0.03$; $F = 5.773$). Higher percentages of tail defects were recorded during season 2 (21 ± 11.33%) compared to season 1 (9 ± 4.91%, Figure 4.20 B).

The most prevalent sperm defects recorded during season 1 were loosehead, dag defects (bent/coiled tails within common surrounding plasma membrane), midpiece reflex, bent midpiece, coiled principal piece and bent principal piece. Similar defects were prevalent during season 2 with the addition of teratoid, abnormal loose heads and proximal and distal cytoplasmic droplets. The proportion of all other sperm defects recorded was low (double head, diadem, pyriform, stump tail, mitochondrial defect). Individual morphology data and the classification of the defects recorded are listed in Table 4.12.

Table 4.11 Population averages recorded of African elephant sperm morphology evaluations over two seasons. Data is presented as sample number (N), mean ± standard deviation (SD), median, minimum (min.) and maximum (max.).

	Season 1						Season 2						Both Seasons			
Parameters	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median
Normal Morphology (%)	7	**58**	±21.79	56	34	85	10	**54**	±11.29	56	30	68	17	**55**	±14.21	56
Abnormal Morphology (%)	7	**42**	±21.79	44	15	66	10	**46**	±9.38	44	32	63	17	**45**	±13.37	44
Head (%)	7	**24**[a]	±24.13	13	4	66	10	**9**[b]	±6.78	10	1	24	17	**15**	±17.08	6
Midpiece (%)	7	**12**	±7.80	9	5	23	10	**16**	±10.69	15	4	44	17	**14**	±9.66	10
Tail (%)	7	**9**[a]	±4.91	8	2	15	10	**21**[b]	±11.33	20	6	40	17	**16**	±11.0	14

[a, b] values labeled in rows with different superscript letters were significantly different ($p < 0.05$)

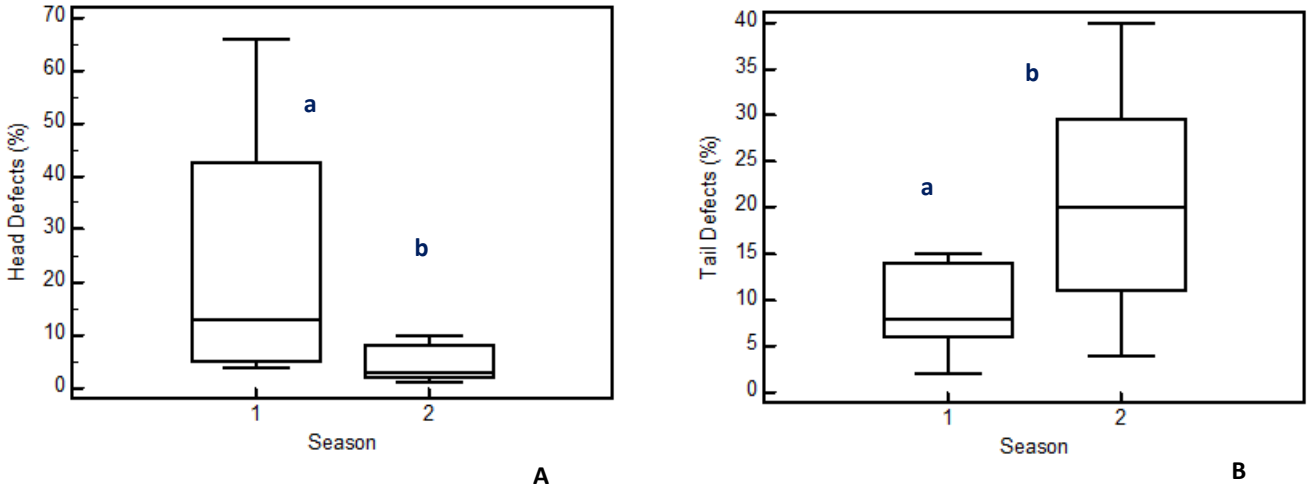


Figure 4.20: (A) The percentage of head defects (%) and **(B)** the percentage of tail defects (%) recorded for all African elephant semen samples evaluated during season 1: September 2009 and during season 2: April 2010, represented as box-and-whisker plots. Data plots labelled with different letters in the same graph were significantly different ($p < 0.05$).

Table 4.12 Individual morphology percentages and classification of defects recorded for African elephant sperm samples.

Elephant ID	Name	Shoulder Height (meter)	Concentration ($x10^6$/ml)	% Normal Morphology	% Head Defects	% Midpiece Defects	% Tail Defects	Comments
LA 1a	Steve	3.1	562,5	35	47	10	8	Diadem (nuclear crater), loose heads, coiled principal piece, midpiece reflex and bent principl piece
LA 2a	Madube	2.96	45	75	4	7	14	Majority loose heads, coiled principal piece and midpiece reflex
LA 3a	No 3	2.53	1582,5	40	29	23	8	Diadem (nuclear crater), loose heads, Dag defect, bent midpiece & pricipal piece
LA 4a	Rasputin	2.5	10	56	8	21	15	Majority loose heads, Dag defect, midpiece reflex & bent midpiece
LA 7a	No 7	3.09	1134	78	13	7	2	Diadem (nuclear crater), loose heads, bent midpiece & pricipal piece
LA 8a	Righthook	2.96	2000	34	66	0	0	Loose heads
LA 9a	Looney	3.2	1500	85	4	5	6	Majority loose heads, Dag defect, coiled principal piece & bent midpiece
LA 1b	Righthook	2.98	80	65	1	15	19	Midpiece reflex, bent midpiece and bent principal piece
LA 5b	Young 1	2.99	2025	60	10	10	20	Teratoid, abnormal heads, loose heads, Dag defect, proximal droplets, bent midpiece & principal piece
LA 6b	No 3	2.53	472	60	12	22	6	Teratoid, abnormal heads, loose heads, Dag defect, proximal & distal droplets, bent midpiece & principal piece
LA 8b	Righthook	2.98	712	37	24	9	30	Majority loose heads, Dag defect, midpiece reflex, bent midpiece & principal piece
LA 9b	Young 1	2.49	1095	56	8	6	30	Teratoid, loose heads, Dag defect, midpiece reflex, bent midpiece & principal piece
LA 10b	Bump	3.2	750	68	4	18	10	Teratoid, loose heads, Dag defect, midpiece reflex, bent midpiece
LA 11b	Young 1	2.49	787	30	16	44	10	Abnormal heads, loose heads, Dag defect, midpiece reflex, proximal & distal droplets
LA 12b	No Name	2.99	1815	56	10	8	26	Abnormal heads, loose heads, midpiece reflex, bent midpiece & principal piece
LA 14b	Rex	3.03	1387	58	10	22	10	Abnormal heads, loose heads, midpiece reflex, distal droplets, bent midpiece & principal piece
LA 16b	Steve	3.1	847	54	3	15	28	Abnormal heads, Dag defect, midpiece reflex, coiled principal piece, bent midpiece & principal piece

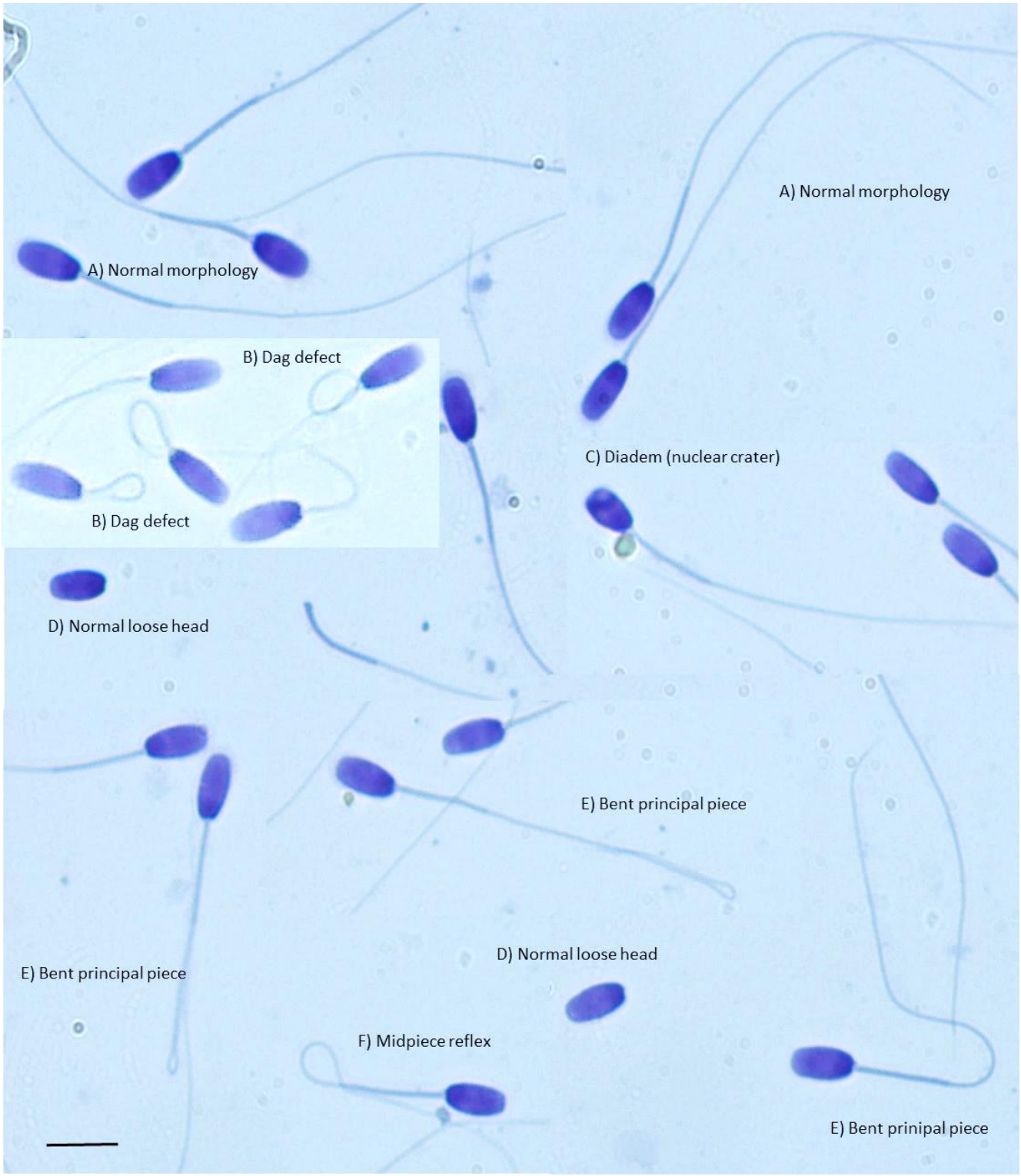


Figure 4.21: A collection of images showing examples of normal and abnormal morphology recorded during evaluation of semen samples collected from free-ranging African elephants. SpermBlue® stained smears evaluated at 1000x magnification (Scale bar = 7µm).

4.2.5.3 Sperm head morphometric analyses

During morphometric analyses, measurements of head length, width, area, ellipticity, elongation, perimeter, regularity, roughness and acrosome coverage of individual spermatozoa per ejaculate evaluated was recorded by means of computer-aided morphometric analysis as illustrated in Figure 4.23. Population averages recorded of the data collected per parameter are presented in Table 4.13 and individual data per ejaculates in Table 4.14. African elephant spermatozoa had an average head length of 6.83 ± 0.26µm and width of 3.32 ± 0.18µm, making up a total head area of 20.17 ± 1.96µm^2. The acrosome covered approximately 38.97 ± 0.92% of the total head area. Acrosome coverage between the two season were also significantly different (p = 0.009; F = 9.061) (Figure 4.22 C). Acrosome measurements were larger during season 2 (39.32 ± 0.77%) compared to season 1 (38.11 ± 0.66%).

Data analysis of parameters indicated no significant difference in the measurements for head length (p = 0.470; F = 0.553), width (p = 0.221; F = 1.643), total area (p = 0.068; F = 3.9), ellipticity (p = 0.591; F = 0.303), elongation (p = 0.591; F = 0.303) or perimeter (p = 0.221; F = 1.642) when compared over the two seasons. However, head regularity (p = 0.012; F = 8.279) and roughness (p = 0.029; F = 5.947) recorded a significant difference when data over the two seasons were compared (Figure 4.22 A and B).

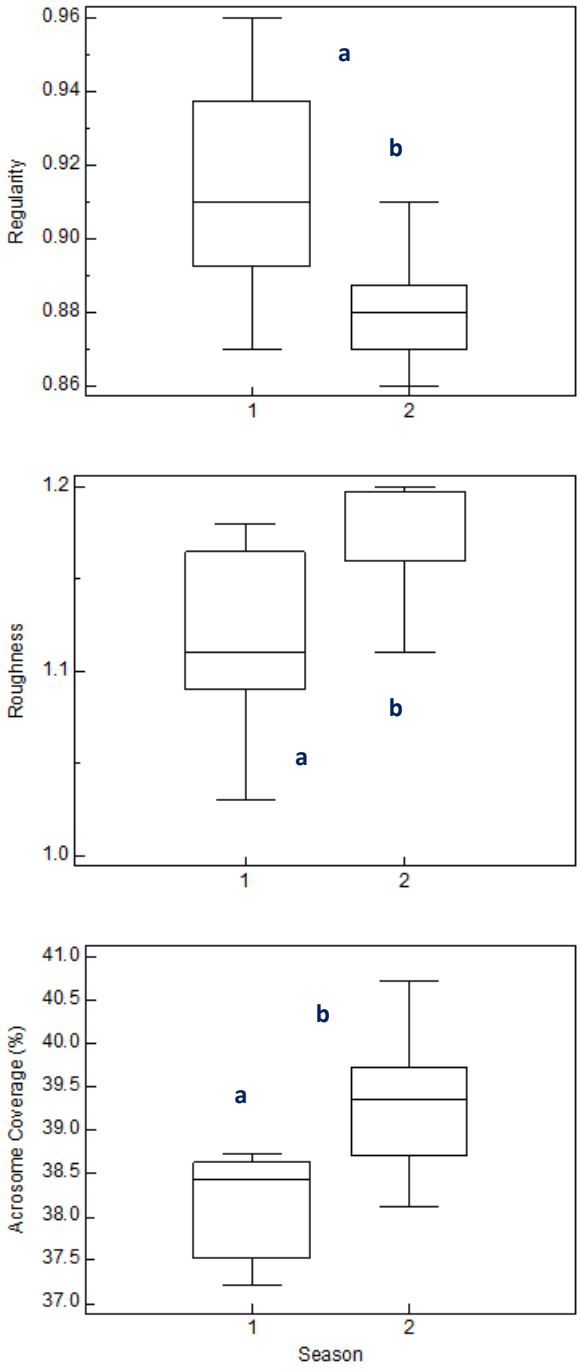


Figure 4.22: Box-and-whisker plots representing sperm head morphometric data for African elephant and illustrating **(A)** head regularity **(B)** head roughness and **(C)** the percentage of acrosome coverage (%) recorded from all samples evaluated during season 1 (September 2009) and during season 2 (April 2010). Data plots labelled with different letters in the same graph were significantly different ($p < 0.05$).

Table 4.13 Average sperm head morphometric data for the African elephant. Data is presented as sample number (N), mean ± standard deviation (SD), median, minimum (min.) and maximum (max.).

	Season 1						Season 2						Both Seasons		
Parameters	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median
Length (µm)	5	**6.73**	±0.45	6.88	6.07	7.12	9	**6.85**	±0.18	6.82	6.6	7.22	14	**6.83**	±0.26	6.87
Width (µm)	5	**3.23**	±0.23	3.22	2.87	3.47	9	**3.35**	±0.15	3.31	3.12	3.67	14	**3.32**	±0.18	3.3
Area (µm²)	5	**18.84**	±2.59	19.44	14.43	21.29	9	**20.76**	±1.36	20.33	18.35	23.39	14	**20.17**	±1.96	20.26
Ellipticity	5	**2.09**	±0.13	2.14	1.89	2.24	9	**2.06**	±0.09	2.09	1.93	2.21	14	**2.07**	±0.10	2.1
Elongtion	5	**0.35**	±0.026	0.36	0.31	0.38	9	**0.34**	±0.016	0.35	0.32	0.36	14	**0.35**	±0.019	0.35
Perimeter (µm)	5	**14.5**	±0.78	14.83	13.17	15.19	9	**14.9**	±0.46	14.8	14.09	15.7	14	**14.78**	±0.58	14.82
Regularity	5	**0.91**[a]	±0.03	0.91	0.87	0.96	9	**0.88**[b]	±0.014	0.88	0.86	0.91	14	**0.89**	±0.02	0.88
Roughness	5	**1.11**[a]	±0.05	1.11	1.03	1.18	9	**1.17**[b]	±0.028	1.16	1.11	1.2	14	**1.15**	±0.04	1.16
Acrosome coverage (%)	5	**38.11**[a]	±0.66	38.42	37.21	38.73	9	**39.32**[b]	±0.77	39.36	38.12	40.71	14	**38.95**	±0.92	38.76
[a, b] values labelled in rows with different superscript letters were significantly different ($p < 0.05$)																

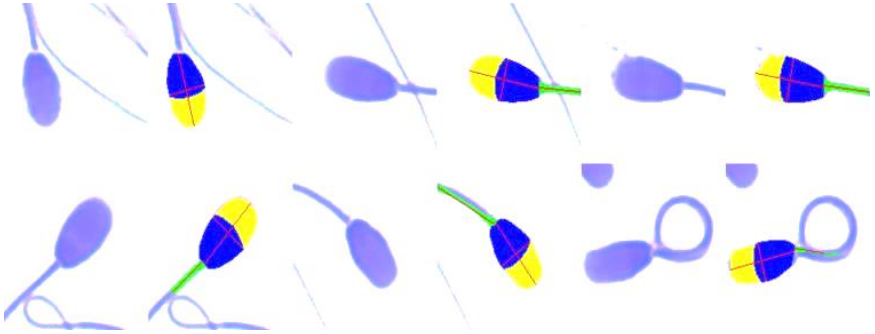


Figure 4.23: Computer-aided morphometric sperm head analysis of African elephants. An image of each sperm assessed is shown on the left and its morphometric assessment on the right. Red lines represent head length and width measurements. The yellow shaded area represents the acrosome in a cap-like shape. Green structures indicate part of the midpiece and measures essentially midpiece width and the angle that the tail attached to the head (not considered in this investigation).

Table 4.14 Individual morphometric analysis data for African elephant sperm samples.

Elephant ID	Name	Head Length (µm)	Head Width (µm)	Head Area (µm²)	Head Perimeter	Head Ellipticity	Head Elongation	Head Roughness	Head Regularity	% Acrosome Coverage
LA 2a	Madube	7.12	3.2	19.34	14.83	2.24	0.38	1.11	0.93	38.6
LA 3a	No 3	6.89	3.41	21.29	15.19	2.03	0.34	1.16	0.87	38.73
LA 4a	Rasputin	6.56	3.47	19.74	14.51	1.89	0.31	1.18	0.91	38.42
LA 7a	No 7	6.87	3.22	19.44	14.84	2.14	0.36	1.11	0.9	37.62
LA 8a	Righthook	6.07	2.87	14.43	13.17	2.18	0.36	1.03	0.96	37.21
LA 1b	Righthook	7.22	3.51	22.64	15.69	2.06	0.35	1.16	0.88	40.26
LA 6b	No 3	6.98	3.29	20.33	15	2.13	0.36	1.14	0.89	40.71
LA 8b	Righthook	6.75	3.21	19.84	14.57	2.15	0.36	1.16	0.87	38.65
LA 9b	Young 1	6.63	3.45	20.82	14.77	1.93	0.32	1.2	0.87	39.36
LA 10b	Bump	6.81	3.35	20.19	14.78	2.04	0.34	1.16	0.9	38.78
LA 11b	Young 1	6.73	3.47	21.15	14.91	1.94	0.32	1.2	0.87	38.68
LA 12b	No Name	6.6	3.12	18.35	14.09	2.21	0.36	1.11	0.91	38.12
LA 14b	Rex	6.87	3.29	20.33	14.64	2.11	0.35	1.19	0.88	39.74
LA 15b	No name	6.97	3.31	21.26	15.01	2.11	0.36	1.19	0.86	39.64

4.2.5.4 Ultrastructural analysis of African elephant spermatozoa by means of transmission electron microscopy (TEM)

The inclusion of TEM was not to re-describe the spermatozoon at the ultrastructural level as previously done by Jones (1973) but to confirm the major ultrastructural features of the spermatozoa of the elephant ejaculates collected. Furthermore, we aimed to confirm and/or describe any other abnormal sperm or characteristics that were not detected during bright field microscopy. The electron micrographs were processed from ejaculates collected from seven African elephant bulls over two seasons. Most of the ultrastructural features observed were similar among different elephants. Table 4.15 includes some of the major semen and sperm characteristics recorded of the ejaculates processed for TEM analyses.

As indicated in section 4.2.5.2, the spermatozoon of the African elephant includes a head and tail (flagellum). The sperm head is composed of the nucleus, acrosome and post-acrosomal region. The nucleus was mostly uniformly electron dense and is enclosed by the nuclear envelope (Figure 4.24). A small number of sparsely distributed nuclear craters were observed in some samples (Figures 4.25 & 4.32). The acrosome covers the anterior part of the head (Figures 4.24 & 4.25). Longitudinal sections revealed the acrosome consisted of two distinct parts, a thicker anterior segment covering the anterior portion of the nucleus and a narrower caudal portion around the equatorial segment (Figure 4.24). The outer acrosomal membrane is situated beneath the plasma membrane and is continuous at the posterior margin with the inner acrosomal membrane, which is closely applied to the nuclear membrane (Figures 4.24 & 4.25). The posterior end of the nucleus formed a concave implantation fossa covered by a thick layer of very dense material, the basal plate. Immediately beneath was the connecting piece. The connecting piece is formed by the capitulum (Figure 4.26). The capitulum has a convex shape that conformed to the concavity of the basal plate lining the implantation fossa of the nucleus. The space between the capitulum and basal plate was sparsely filled with moderate electron dense material. The segmented longitudinal columns with their cross striated fibrous protein is located just beneath the capitulum (Figure 4.26).The proximal centriole is

positioned at about a 45-degree angle to the tail (flagellum) axis (Figure 4.24). The distal centriole (Figure 4.28) serves as the kinetic centre that extends posteriorly from the capitulum and the segmented columns run caudally and eventually contribute to the formation of the outer dense fibres of the flagellum. The tail is the longest part of the spermatozoon and includes the midpiece, principal piece and end piece. Some micrographs showed the end-to-end arrangement of the mitochondrial gyres of the midpiece forming a helix around the dense fibres (Figure 4.27). The annulus marks the border between the most caudal gyre of mitochondria and the most anterior part of the fibrous sheath of the principal piece (Figures 4.29).

For the most part along the length of the flagellum, the axoneme is surrounded by nine outer dense fibres, each associated with a pair of microtubule doublets. The core of the flagellum consists of the axoneme and the outer dense fibres. The shape of the outer dense fibres varied from petal-like to round structures (Figure 4.30). The difference in size of the outer dense fibres was apparent as the outer dense fibres 1, 5 and 6 were larger than the others in the midpiece region (Figure 4.30). The outer dense fibres are surrounded by the mitochondrial gyres in the midpiece (Figure 4.30) and by a fibrous sheath in the principal piece (Figure 4.31) of the flagellum. In longitudinal sections, the fibrous sheath is characterised by a series of electron dense ribs. The outer dense fibres 3 and 8 fused with the fibrous sheath but can still be recognized as thickenings in the fibrous sheath in line with the inner doublets of the axoneme in the first part of the principal piece (Figure 4.31). Radial spokes extend from the central doublets to the peripheral doublets of the axoneme (Figure 4.31). Dynein arms were visible between the pairs of peripheral doublets. The plasma membrane surrounds the entire spermatozoon and is usually seen loosely attached around the acrosomal region of the head and around sections of the flagellum and this is generally considered an artefact of TEM processing (Figures 4.24, 4.30 & 4.31).

Structural abnormalities observed by TEM were the occurrence nuclear craters in the chromatin (Figure 4.32) and of flagella either at the end of the midpiece (midpiece reflex or bent midpiece) or at the principal piece (a coiled principal piece or bent midpiece) with residual cytoplasmic droplets present (Figure 4.33). Coiling of the flagellum usually involved a rolled up tail in the midpiece area and transverse sections through this area clearly indicated several transverse sections of the axoneme and associated fibres all contained within a common plasma membrane (Dag defect) (Figure 4.34). Apoptotic vesicles showing blebbing and breakdown of cellular organelles were also observed (Figure 4.35).

Table 4.15 Characteristics of African elephant semen and sperm samples processed for transmission electron microscopic (TEM). Samples processed were collected during season 1 (September 2009 – end of dry season) and season 2 (April 2010 – end of wet season).

Semen characteristic of samples processed for electron microscopy										
TEM images Figure #	Elephant ID	Name	Volume (ml)	Conc. million/ml	% Normal Morphology	% Head defect	% Midpiece defect	% Tail defect	% Acrosome intact	% Acrosomal coverage
4.24	LA 1a*	Steve	13	563	35	47	10	8	-	-
4.25	LA 16b**	Steve	11	847	54	3	15	28	82	-
4.26	LA 3a*	No 3	47	1582	40	29	23	8	-	38.73
4.27	LA 6b**	No 3	68	472	60	12	22	6	72	40.71
4.28	LA 8a*	Righthook	133	2000	34	66	-	-	-	37.21
4.29	LA 9a*	Looney	6	1500	85	4	5	6	-	-
4.30	LA 5b*	Young 1	57	2025	60	10	10	20	80	38.93
* a value refers to samples collected during season 1 in September 2009										
** b value refers to samples collected during season 2 in April 2010										

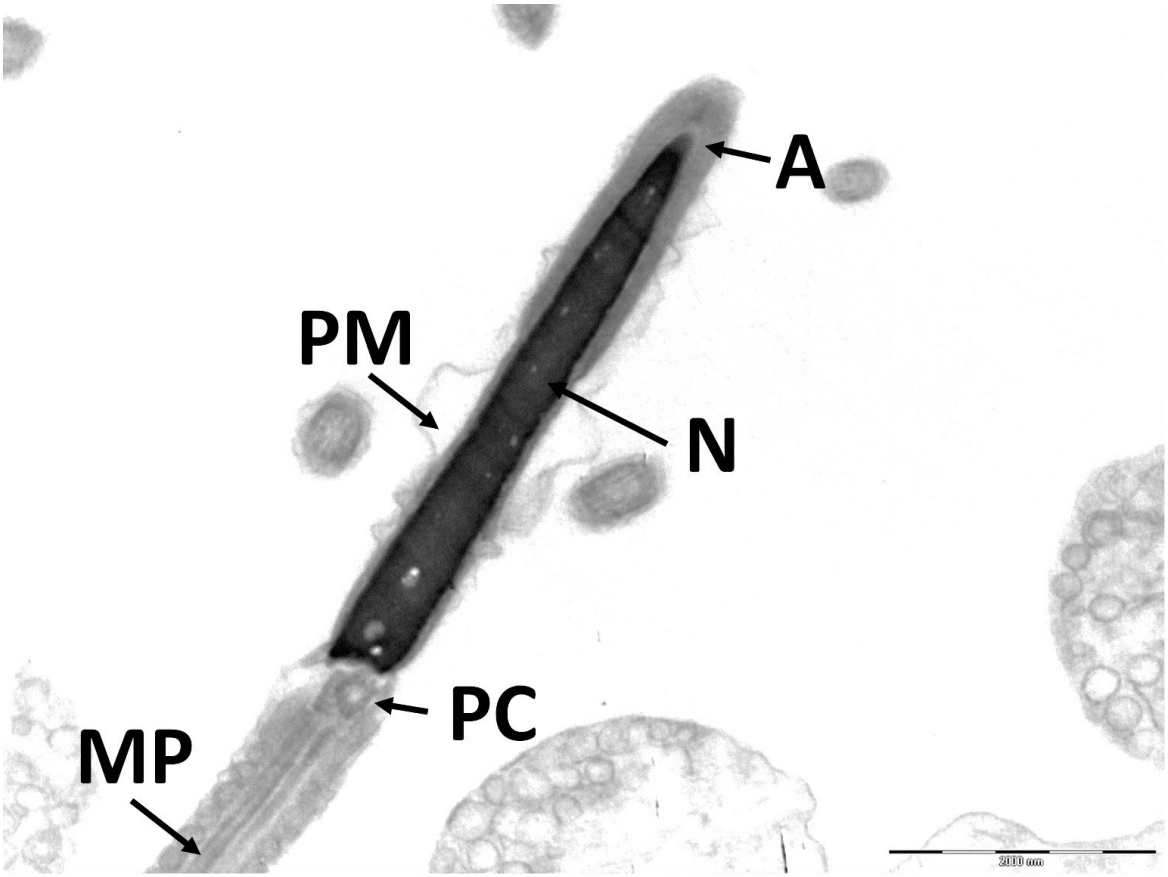


Figure 4.24 Longitudinal section showing the acrosome (A), nucleus (N) and plasma membrane (PM) of the sperm head and below the proximal centriole (PC) and midpiece (MP) (Scale bar = 2µm).

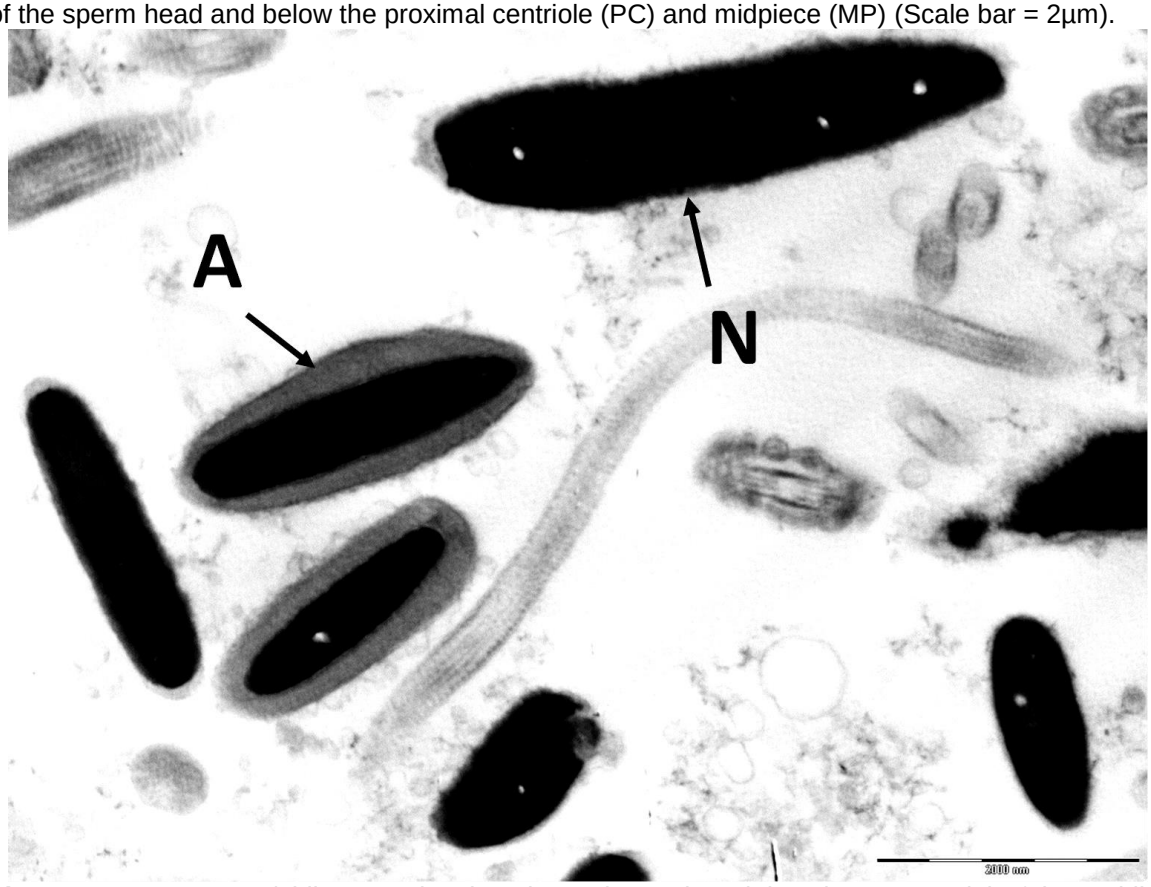


Figure 4.25 Transverse/oblique section that shows the nucleus (N) and acrosome (A) of the paddle-shape elephant sperm head (Scale bar = 2µm).

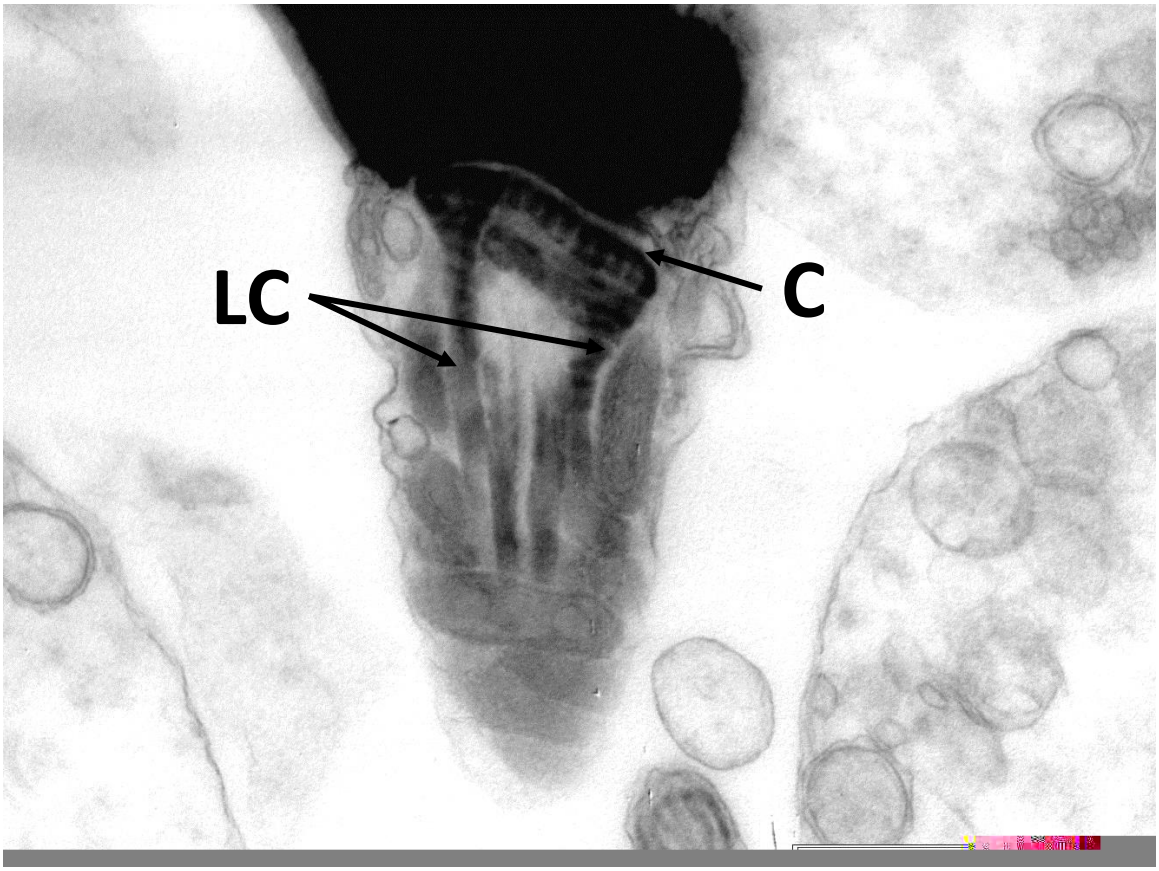


Figure 4.26 Diagonal section showing the capitulum (C) and longitudinal columns (LC) with cross-striated fibrous proteins (Scale bar = 1µm).

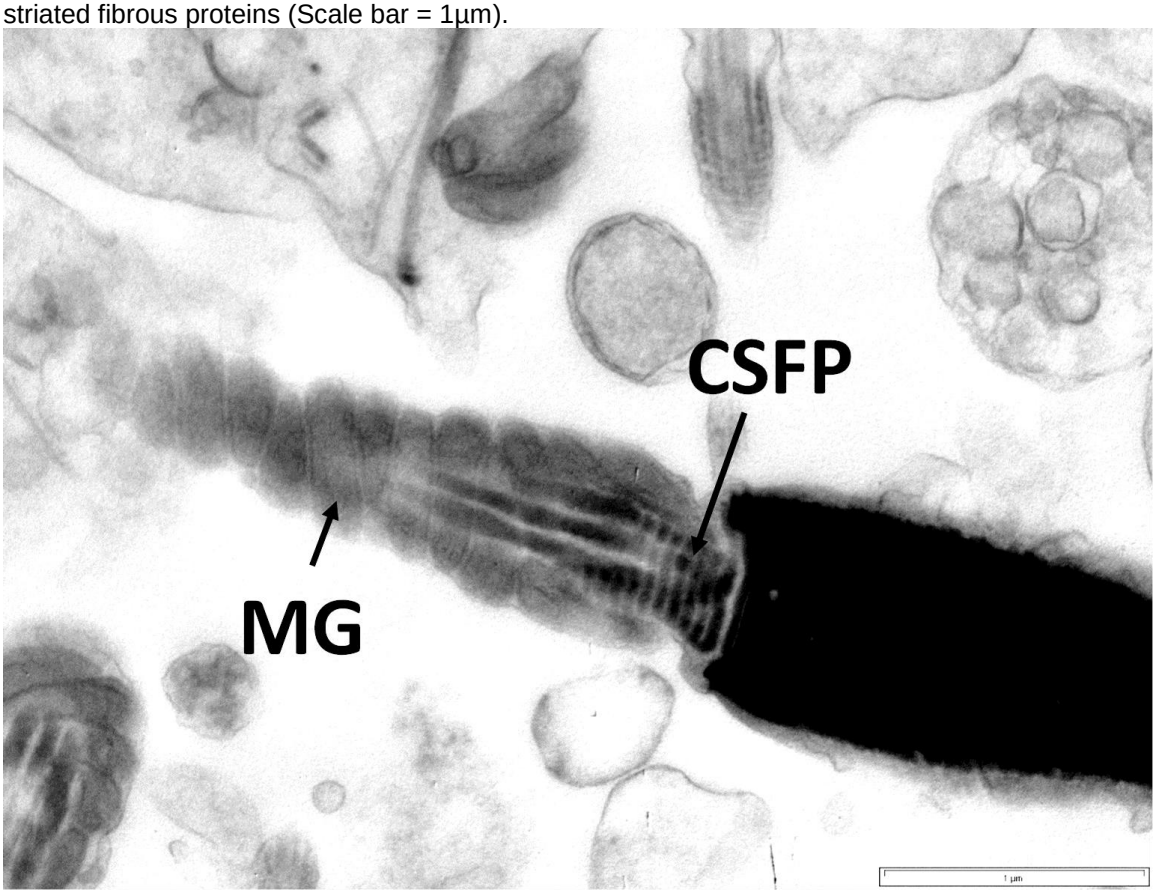


Figure 4.27 Diagonal section showing the cross-striated fibrous proteins (CSFP) and mitochondrial gyres of the midpiece of an elephant spermatozoon (Scale bar = 1µm).

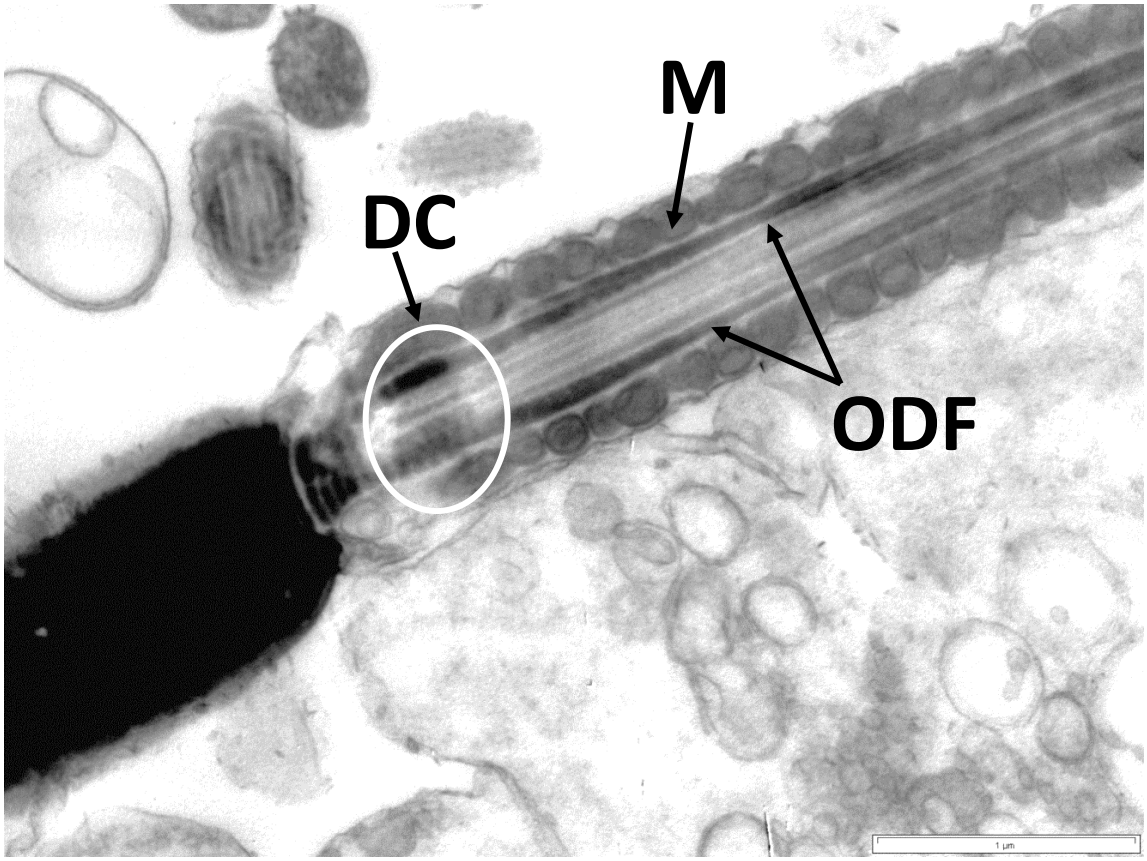


Figure 4.28 Longitudinal section of the midpiece showing the distal centriole (DC), the outer dense fibres (ODF) and mitochondria (M) (Scale bar = 1µm).

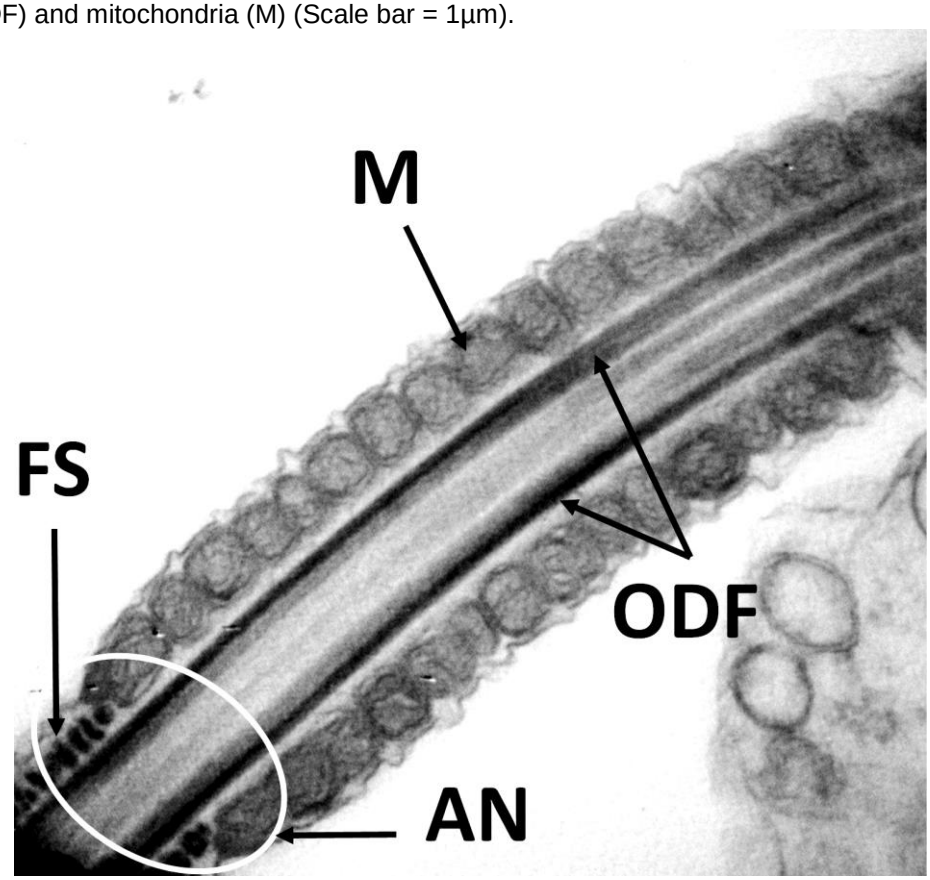


Figure 4.29 Longitudinal section of the midpiece and principal piece showing the outer dense fibres (ODF) surrounded by mitochondria (M) in the midpiece. The annulus (AN - encircled white) marks the end of the midpiece and shows the start of the fibrous sheath (FS) of the principal piece.

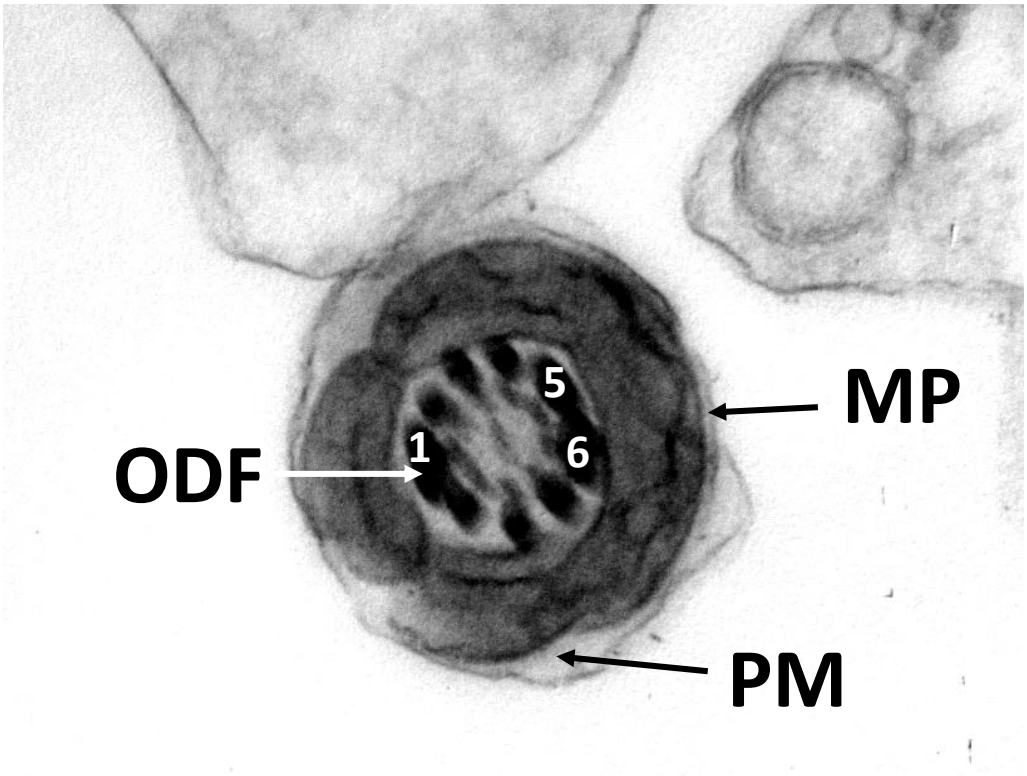


Figure 4.30 Cross section showing the outer dense fibres (ODF) of the midpiece (MP) surrounded by plasma membrane (PM). The difference in size was apparent in the outer dense fibres (ODF) 1, 5 and 6.

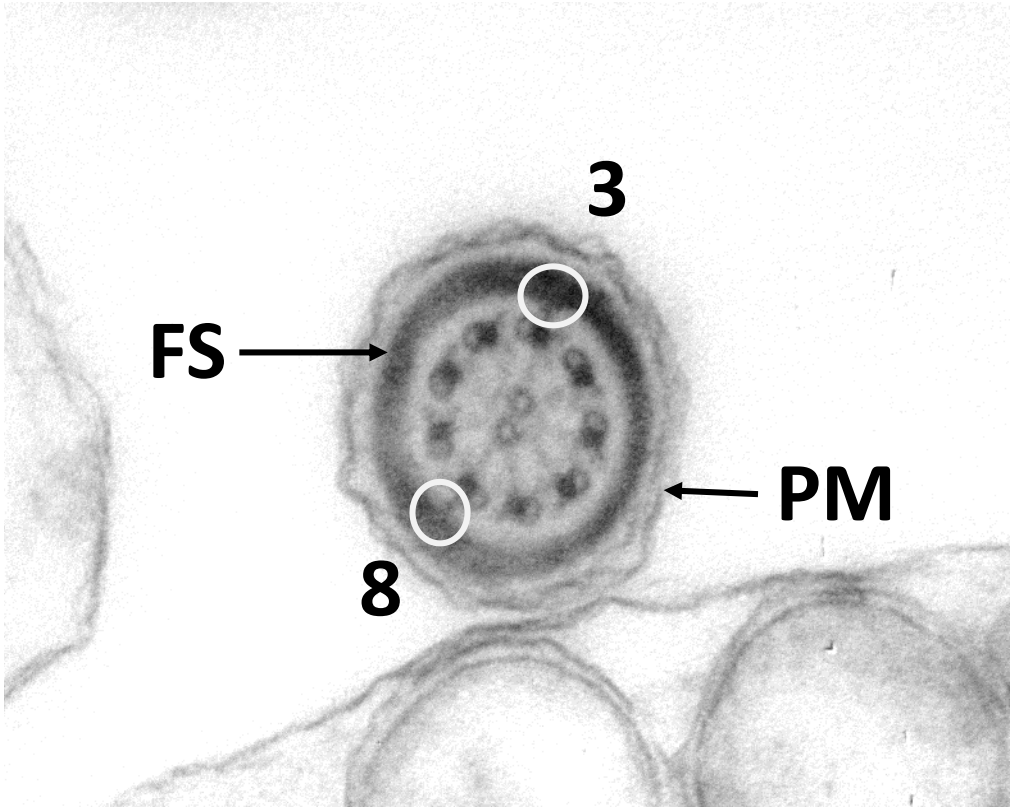


Figure 4.31 Cross section of the principal piece of flagellum showing the outer dense fibres 3 and 8 as inward extensions of the fibrous sheath (FS) surrounded by plasma membrane (PM).

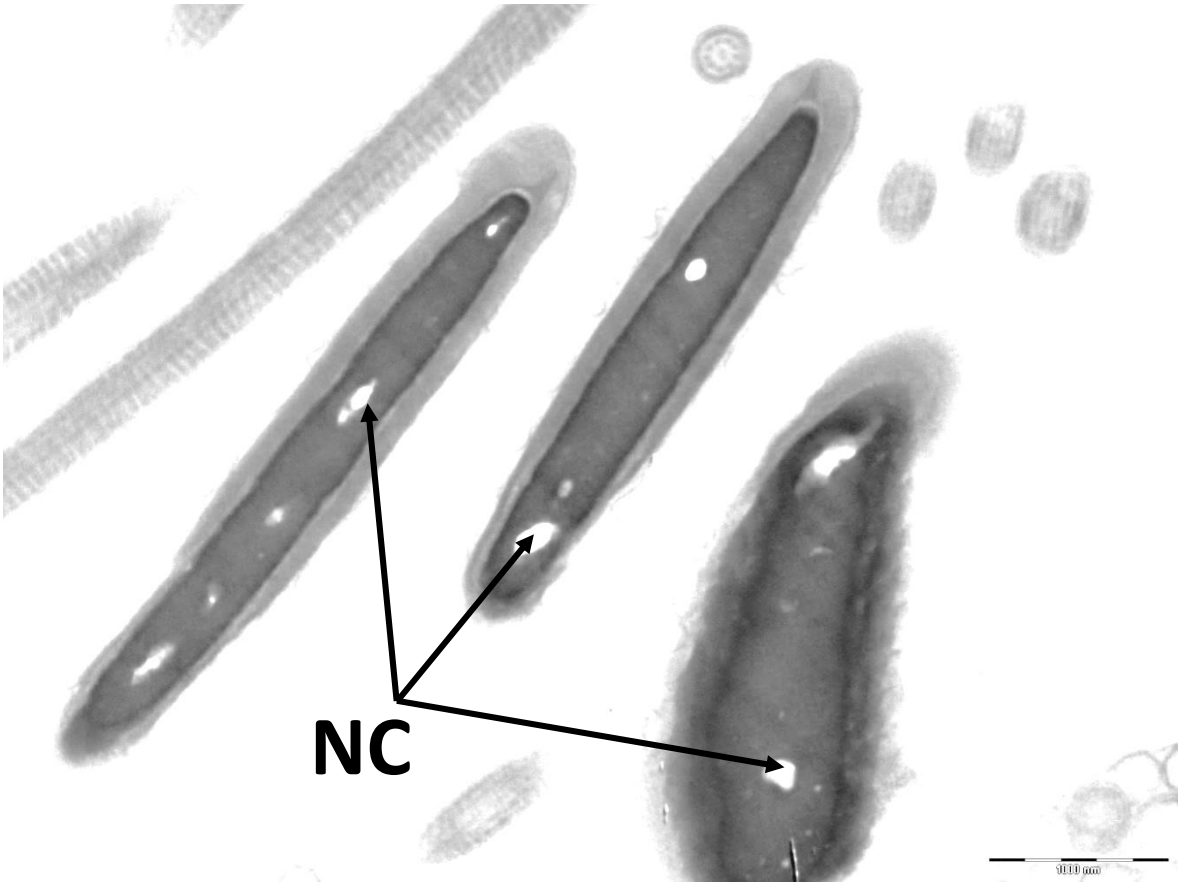


Figure 4.32 Transverse/ oblique section showing the occurrence of nuclear craters (NC) (Scale bar = 1µm).

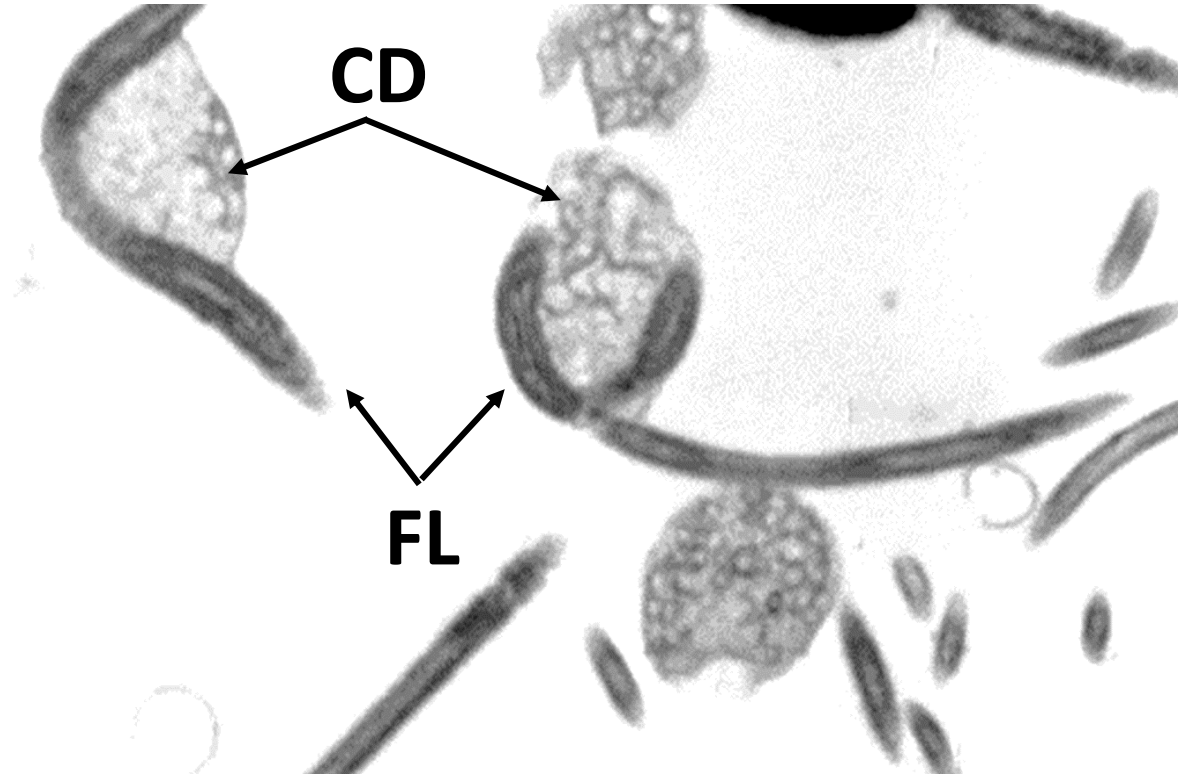


Figure 4.33 Transverse/ oblique section showing the occurrence of bends in flagella (FL) either at the end of the midpiece (midpiece reflex or bent midpiece) or at the principal piece (a coiled principal piece or bent midpiece) with residual cytoplasmic (CD) droplets present.

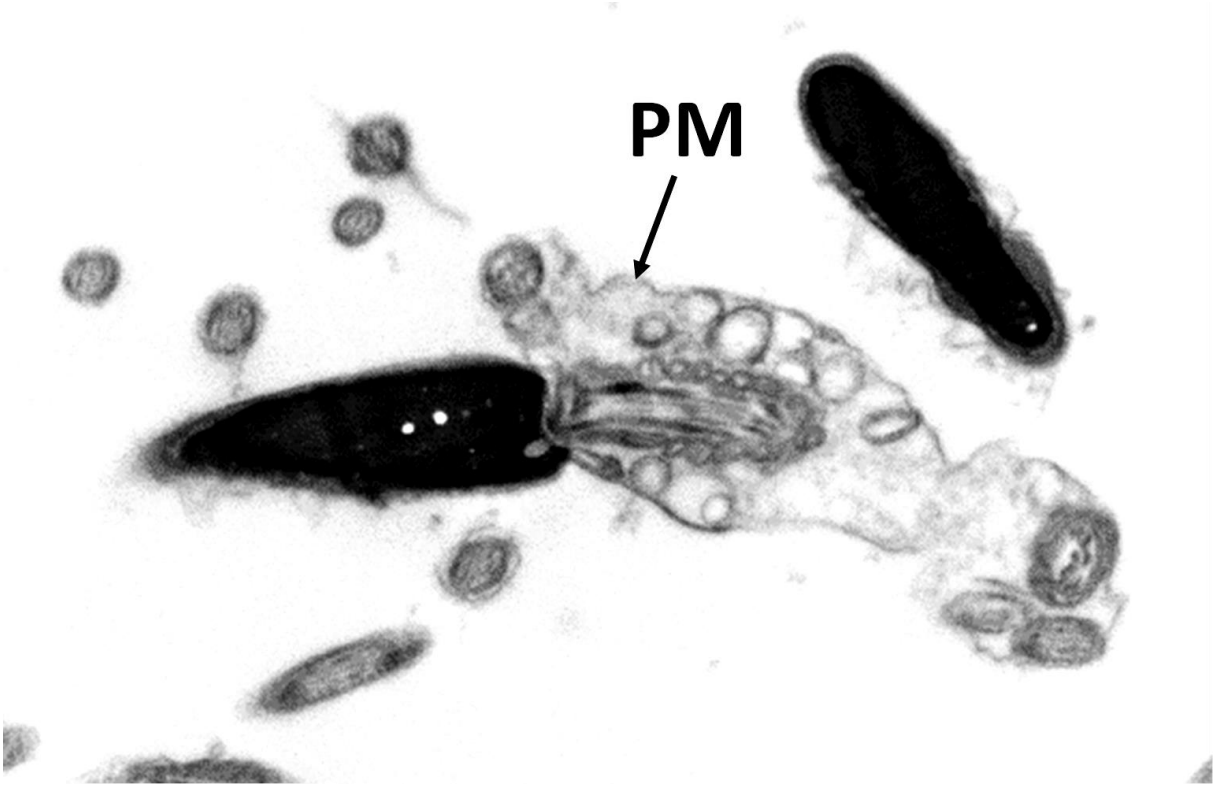


Figure 4.34 Transverse section of a Dag defect; showing the coiling of the flagellum contained within a common plasma membrane (PM).

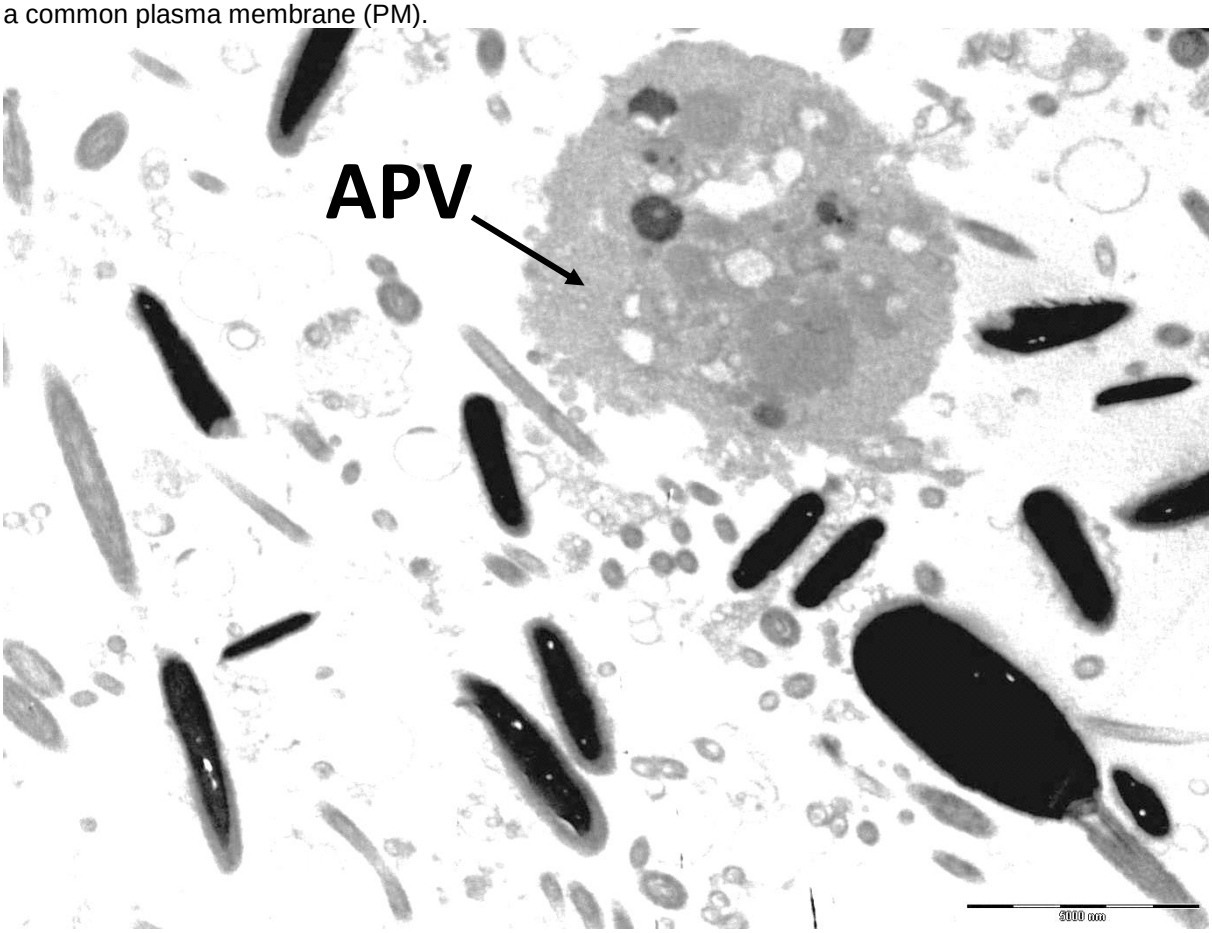


Figure 4.35 Transverse section of apoptotic vesicle (APV) showing blebbing and breakdown of cellular organelle (Scale bar = 5µm).

CHAPTER 5

SOUTHERN WHITE RHINOCEROS (*Ceratotherium simum simum*) SEMEN AND SPERM CHARACTERISTICS

A series of comprehensive evaluation techniques were performed on 10 ejaculates collected from 10 adult rhinoceros bulls older than seven years. The semen samples were collected from two different rhinoceros populations during April 2010. Listed in Table 5.1 is the general ejaculate data per individual from both populations.

Table 5.1 Overview of semen samples collected from 10 Southern white rhinoceros individuals during April 2010, Population A (n = 6) and population B (n = 4). The data represents date and location of collection, ejaculate volume, sperm concentration and total number of spermatozoa per ejaculate collected.

No	Date	Location of Collection	ID	Total Volume (ml)	Concentration ($x10^6$/ml)	Total # sperm per ejaculate ($x10^9$/ml)
1	8/4/2010	Musina	CSS 1a	15	1	0.2
2	8/4/2010	Musina	CSS 3a	37	5	1.7
3	8/4/2010	Musina	CSS 4a	12	30	3.5
4	9/4/2014	Musina	CSS 5a	29	15	4.4
5	9/4/2014	Musina	CSS 7a	19	7	1.3
6	9/4/2014	Musina	CSS 8a	22	8	1.2
7	18/4/2014	Mziki	CSS 10b	26	99	21.3
8	18/4/2014	Mziki	CSS 13b	7	255	17.9
9	18/4/2014	Mziki	CSS 14b	16	220	35.2
10	18/4/2014	Mziki	CSS 15b	51	38	19.1

5.1 MACROSCOPIC SEMEN EVALUATION

5.1.1 EJACULATE COLOUR AND VISCOSITY

Semen samples collected varied considerably amongst individual bulls. Some ejaculate fractions contained singular spermatozoa (CSS 2a), had a distinct odour and yellow colour indicating urine contamination (CSS 9a), or contained no spermatozoa (CSS 1b). Contaminated fractions and other highly viscous fractions were discarded and not analysed for this study.

5.1.2 EJACULATE VOLUME

The average rhinoceros ejaculate volume measured during this study was 24.21 ± 18.99mL. There was no significant difference (p =0.8853) between the two populations when compared (Table 5.2 and Figure 5.1 A). Recorded ejaculate volumes ranged from 7mL (CSS 13b) to 51mL (CSS 15b) (Table 5.1).

Table 5.2 Summary statistics of the volume and concentration data collected from ejaculates evaluated during April 2010. The data is presented as mean, ± standard deviation (SD) and median.

	Population A				Population B				Both populations			
Parameters	N	Mean	±SD	Median	N	Mean	±SD	Median	N	Mean	±SD	Median
Volume (mL)	6	**23**	±9.91	21.54	4	**25**	±18.99	21.05	10	**24**	±13.6	21.54
Concentration (million\mL)	6	**13**[a]	±10.38	7.5	4	**153**[b]	±101.89	159.5	10	**83**	±96.94	30

[a, b] values labelled with different superscript letters were significantly different ($p < 0.05$)

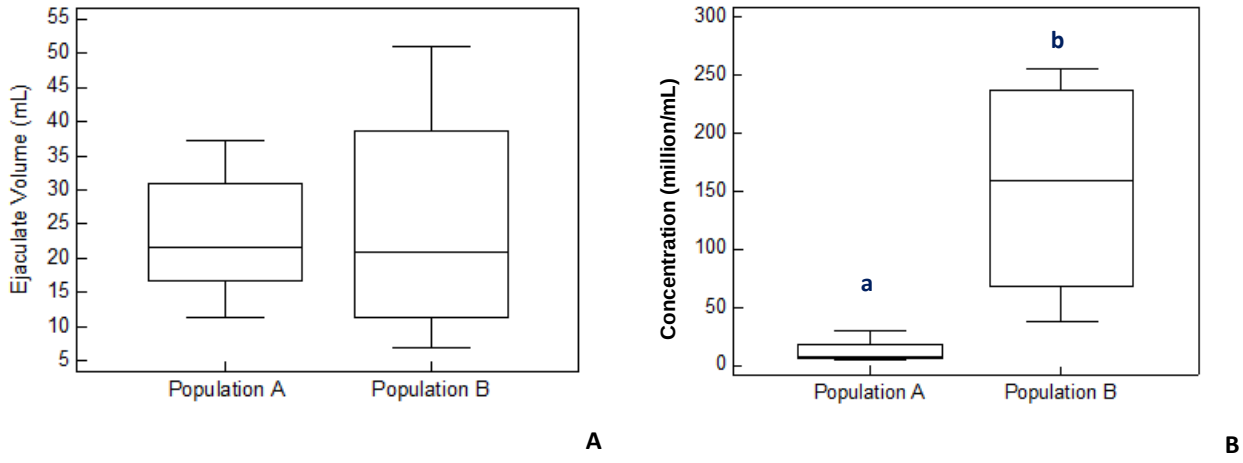


Figure 5.1: Box-and-whisker plots illustrating **(A)** ejaculate volumes (mL) **(B)** and sperm concentrations (10^6/mL) recorded from rhino ejaculates collected during April 2010. Data plots labelled with different letters in the same graph were significantly different ($p<0.05$).

5.2 MICROSCOPIC SPERM EVALUATION

5.2.1 SPERM CONCENTRATION

Sperm concentrations differed significantly (p=0.0007) between the two populations (Table 5.2 and Figure 5.1 B). A higher average sperm concentration per mL was recorded by population B at 152.87 ± 101.89 x 10^6/mL compared to 12.8 ± 10.38 x 10^6/mL, the recorded average for population A. The highest individual total number of sperm per ejaculate was recorded for CSS 15b at 35.2 x 10^9/mL (Table 5.1 and Figure 5.2).

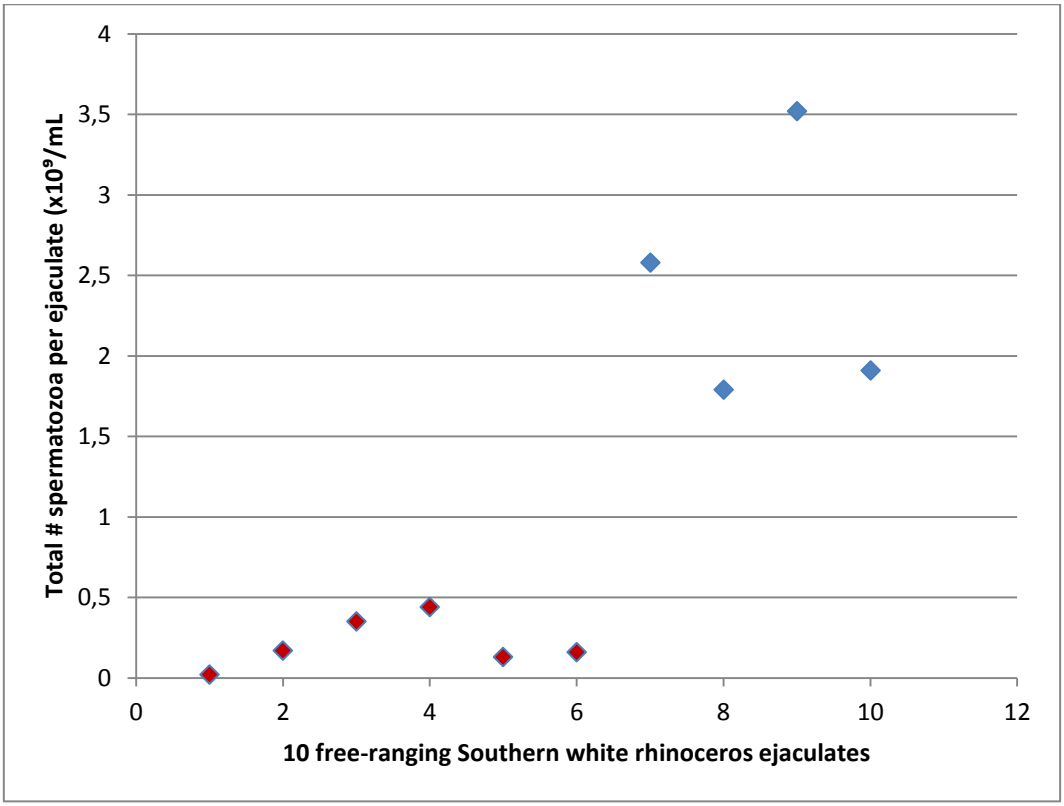


Figure 5.2: Scatter plot representing the total number of spermatozoa per ejaculate (x10^9/mL) collected from both rhinoceros populations (red = Population A, n = 6) and (blue = Population B, n = 4) arranged by the order of collection.

5.2.2 MOTILITY AND KINEMATIC PARAMETERS TO ESTABLISH THE QUALITY OF SPERM SAMPLES

Due to constraints indicated by the conditions in the field and the availability of sufficient sperm samples per ejaculate collected, not all treatments could be conducted simultaneously on all samples in true split-sample fashion. In this study the following semen sample dilutions and evaluation media were compared: EY_BO (Egg Yolk and BO), EY_Ham's (Egg Yolk and Ham's F10), EY_INRA (Egg Yolk and INRA96®), NT_BO (Neat and BO), NT_Hams (Neat and Ham's F10) and NT_INRA (Neat and INRA96®). Inadvertently, the motility and kinematic parameters were evaluated at two different frame rates. Population A was evaluated at 50 frames per second while population B was evaluated at 25 frames per second for sperm motility measurements. Statistical analysis (including ROC curves) recorded no significant differences for any of the motility and kinematic parameters between the two different frame rates (Table 5.3) or media compared (Tables 5.5 and 5.6, Figures 5.3 and 5.4). The results of the combined average motility and kinematic parameters recorded from both populations within the different media is summarised in Table 5.4. No statistical results are given due to a lack of significance. The data recorded per ejaculate collected is listed in Table 5.5. In general, the rhinoceros semen fractions contained a high number of motile spermatozoa.

Total motility (TM) ranged from the highest percentage in EY_INRA at 93.22 ± 4.42% to the lowest percentage in NT_ INRA at 77.52 ± 16.48% (Tables 5.4, Figure 5.3 A). A semen sample collected from CSS 8a recorded the highest individual percentage for TM at 98.1% (in NT_INRA); however, none of these spermatozoa were progressively motile (Table 5.5). During visual analysis, the sperm samples seemed to contain more non-progressive spermatozoa than progressively motile spermatozoa. CASA analysis values confirmed this observation. The percentage of progressively motile spermatozoa amongst all the media compared ranged from EY_Ham's at 27.5 ± 23.89% to 72.35 ± 6.76% in EY_ INRA medium (Figure 5.3 B and C). The highest individual percentage progressive motility was recorded for CSS 3a at 75% in EY_ Ham's (Table 5.4).

Furthermore, not only were the majority of the spermatozoa within the samples non-linear, they also progressed at a low velocity. The average VCL recorded ranged from 103.76 ± 29.31µm/s in NT_BO to 72.04 ± 42.5µm/s in NT_Ham's (Table 5.4 and Figure 5.3 D). The highest individual VCL was recorded for CSS 5a at 158.7µm/s in NT_BO (Table 5.5). Population averages recorded for VSL among all the media evaluated ranged from 30.12 ± 8.88µm/s in EY_BO to 44.48 ± 25.07µm/s in EY_Ham's F10 (Figure 5.3 E). Average VAP velocities ranged from 77.7 ± 16.33µm/s in NT_ INRA to 54.07 ± 5.35µm/s in EY_BO (Table 5.4 and Figure 5.3 F).

However, EY_ Ham's recorded high averages for LIN (50.8 ± 16.64%) and STR (63.2 ± 14.31%), while low averages for these two parameters were recorded in NT_ Ham's (LIN 25.08 ± 14.46% and STR 46.58 ± 22.87%) (Table 5.4 and Figure 5.4 A and B). The parameter for WOB ranged from 66.75 ± 14.2% in NT_BO to 82.01 ± 6.52% in NT_INRA (Table 5.4). The highest ALH measurements were recorded in the EY_BO at 2.95 ± 0.73µm and the lowest in NT_ INRA® at 1.75 ± 0.84µm (Figure 5.4 C). The NT_Ham's samples recorded the lowest BCF frequencies at 10.68 ± 7.48Hz while the highest average was recorded for NT_ BO samples at 16.63 ± 7.48Hz (Figure 5.4 D). The sperm sample collected from CSS 7a in NT_Ham's recorded the lowest VSL at 10 µm/s, while both ALH (µm) and BCF (Hz) measured zero, representing the slow circular motion patterns exhibited by this non-progressive motile sperm population (Table 5.5). Overall, the motility and kinematic parameters confirmed that the collected ejaculates contained a high percentage of non-progressive spermatozoa moving at slow velocities that recorded a non-linear (more circular) trajectory during CASA analyses.

Table 5.3 Statistical analysis of motility and kinematic parameters of the two populations recorded at 25 and 50 frames per second, respectively. There was no significant difference recorded between the different frame rates, thus data was combined.

	Population A & B Combined	
Parameters	**Significance level (p<0.05)****	**F- Ratio**
Total Motility (%)	P = 0.399	F = 1.069
Progressive motility (%)	P = 0.963	F = 1.192
Non-progressive motility (%)	P = 0.848	F = 0.394
VCL (µm/s)	P = 0.588	F = 0.758
VSL (µm/s)	P = 0.923	F = 0.274
VAP (µm/s)	P = 0.731	F = 0.588
LIN (%)	P = 0.163	F = 1.723
WOB (%)	P = 0.449	F = 0.978
STR (%)	P = 0.456	F = 0.966
ALH (µm)	P = 0.124	F = 1.918
BCF (Hz)	P = 0.594	F = 0.749
** ANOVA analysis		
VCL = Curvilinear velocity, VSL = Straight line velocity, VAP = Average path velocity, LIN = Linearity of tract, WOB = Wobble, BCF = Beat cross frequency, ALH = Amplitude of lateral head displacement		

Table 5.4 Summary of the combined motility and kinematic parameters recorded for egg yolk (EY) and neat (NT) semen extended semen samples collected from free-ranging Southern white rhinoceros populations evaluated in BO, Ham's F10 and INRA96® media. The data in the table represent the sample number (N), mean ± standard deviation (SD), median, minimum and maximum values.

	Egg yolk_ Bo (EY_Bo)						Egg yolk_Hams (EY_Hams)						Egg yolk_Inra (EY_Inra)					
Parameters	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.
Total Motility (%)	4	**87.62**	±6.61	85.7	82.3	96.8	5	**81.71**	±8.45	80.7	70.8	93.6	4	**93.22**	±4.42	93.4	89	97.1
Progressive motility (%)	4	**20.57**	±15.94	17.85	4.7	41.9	5	**27.5**	±23.89	19.1	5.1	75	4	**20.87**	±8.85	24.5	7.7	26.8
Non-progressive motility (%)	4	**67.05**	±21.19	67.85	40.4	92.1	5	**54.21**	±25.3	62.5	0	70.7	4	**72.35**	±6.76	71.5	65.1	81.3
VCL (µm/s)	4	**84.62**	±24.2	80.25	60.9	117.1	5	**85.24**	±29.76	73.9	56.8	139.2	4	**96**	±28.21	90.15	70.1	133.6
VSL (µm/s)	4	**30.12**	±8.88	31.45	18.3	39.3	5	**44.48**	±25.07	33.6	20.6	92.9	4	**37.65**	±14.64	36.2	23.6	54.6
VAP (µm/s)	4	**54.07**	±5.35	53.95	48.2	60.2	5	**68.94**	±30.68	52.5	46.3	118.4	4	**76**	±21.12	71.6	55.3	105.5
LIN (%)	4	**39.5**	±18.71	43.65	15.6	55.1	5	**50.81**	±16.64	45.3	30.1	81.5	4	**40.02**	±13.65	39.95	23.4	56.8
WOB (%)	4	**67.1**	±16.19	66.3	51.5	84.3	5	**79.35**	±11.21	82.2	62.5	94.5	4	**79.82**	±8.79	78.95	70	91.4
STR (%)	4	**56.47**	±17.8	63.1	30.3	69.4	5	**63.2**	±14.31	62.7	43.7	86.2	4	**49.2**	±11.87	50.65	33.4	62.1
ALH (µm)	4	**2.95**	±0.73	2.7	2.4	4	5	**2.21**	±0.61	2.5	1.2	2.8	4	**2.15**	±0.4	2.05	1.8	2.7
BCF (Hz)	4	**12.85**	±5.6	13.5	6.7	17.7	5	**15.78**	±6.87	19.6	7.1	23	4	**13.4**	±7.08	13.15	6.9	20.4

VCL = Curvilinear velocity. VSL = Straight line velocity. VAP = Average path velocity. LIN = Linearity of tract. WOB = Wobble. BCF = Beat cross frequency. ALH = Amplitude of lateral head

Table 5.4 Continued.

	Neat_Bo (N_Bo)						Neat_Hams (N_Hams)						Neat_Inra (N_Inra)					
Parameters	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.
Total Motility (%)	6	**88.15**	±6.41	86.85	79.3	97.1	5	**77.52**	±16.48	70.5	58	95.4	7	**79.84**	±20.2	86	39.4	98.1
Progressive motility (%)	6	**26.5**	±18.59	28.3	5.1	47.6	5	**19.22**	±18.57	13.2	0	39	7	**20.51**	±22.03	15	0	67.9
Non-progressive motility (%)	6	**61.65**	±18.97	58.55	41.5	90.8	5	**58.3**	±25.94	57.3	19.2	90.3	7	**59.32**	±27.25	62.6	21.1	98.1
VCL (µm/s)	6	**103.76**	±29.31	92.65	82.2	158.7	5	**72.04**	±42.5	81.5	8.2	125.7	7	**95.54**	±22.81	95	63.7	129.7
VSL (µm/s)	6	**37.11**	±23.27	29.8	17.1	80.5	5	**38.3**	±34.71	30.5	10	97.8	7	**34.04**	±9.67	38.3	15.8	42.4
VAP (µm/s)	6	**70.76**	±32.27	60.6	46.2	132.8	5	**59.18**	±40.55	54.2	6.9	118	7	**77.7**	±16.33	79.1	55.6	98.2
LIN (%)	6	**34.26**	±12.88	35.05	18.5	50.8	5	**25.08**	±14.46	22.1	7.8	42.2	7	**38.55**	±16.25	39.1	12.1	62.8
WOB (%)	6	**66.75**	±14.2	66.75	50.4	83.7	5	**68**	±35.52	82.1	6.1	93.9	7	**82.01**	±6.52	83.3	71.8	88.5
STR (%)	6	**50.11**	±11.58	52.7	36.7	61	5	**46.58**	±22.87	45.5	22.2	82.9	7	**46.62**	±19.22	47.8	16.9	72
ALH (µm)	6	**2.9**	±0.75	2.75	2.2	4	5	**1.94**	±1.35	1.9	0	3.6	7	**1.75**	±0.84	1.9	0	2.6
BCF (Hz)	6	**16.63**	±6.02	19.1	8.3	22.3	5	**10.68**	±7.48	10	0	18.1	7	**10.98**	±7.87	8.9	0	20.2

VCL = Curvilinear velocity. VSL = Straight line velocity. VAP = Average path velocity. LIN = Linearity of tract. WOB = Wobble. BCF = Beat cross frequency. ALH = Amplitude of lateral head

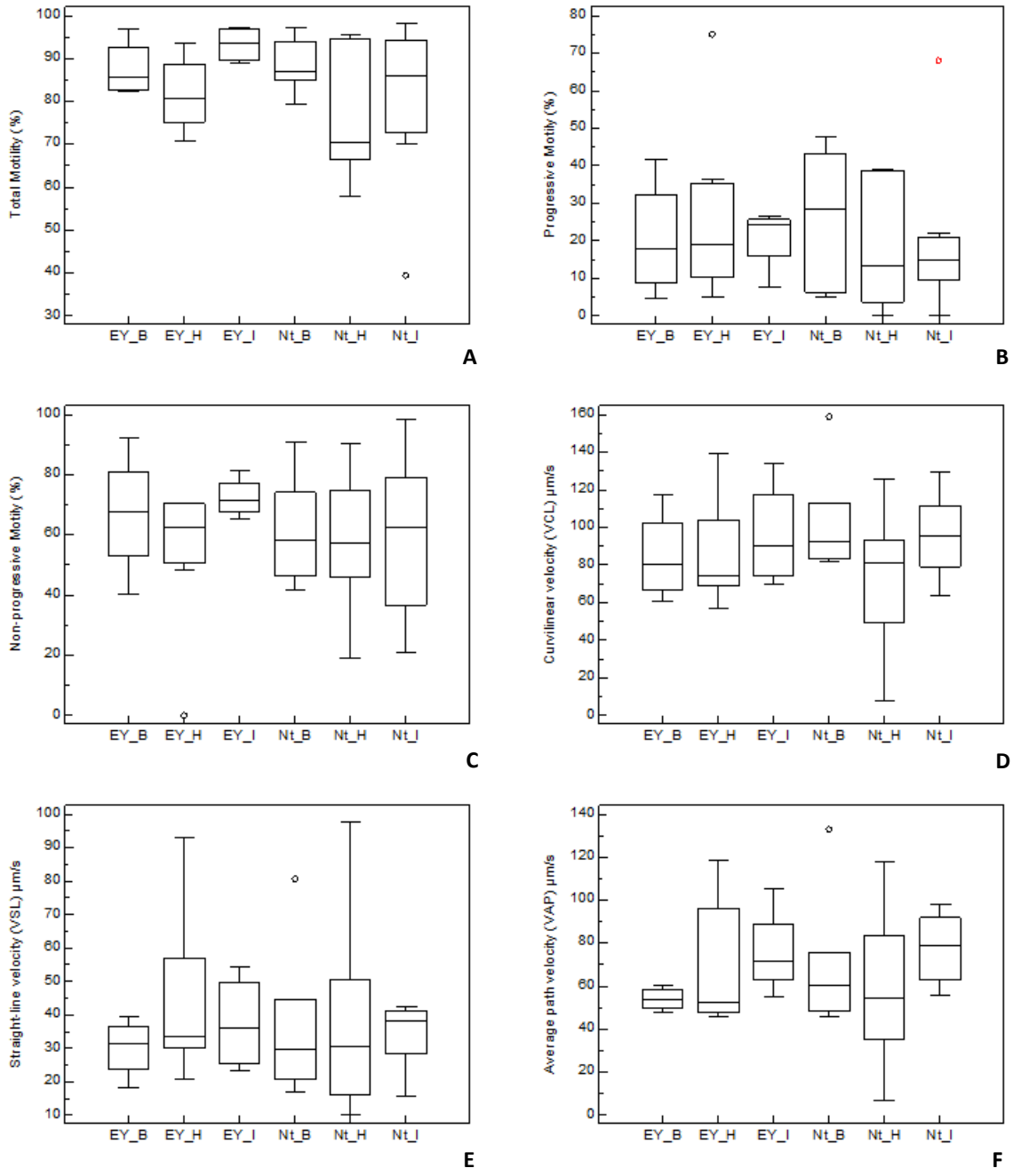


Figure 5.3: Box-and-whisker plots illustrating **(A)** Total Motility (%) **(B)** Progressive Motility (%) **(C)** Non-Progressive Motility (%) **(D)** Curvilinear Velocity (VCL µm/s) **(E)** Straight-line Velocity (VSL µm/s) and **(F)** Average path Velocity (VAP µm/s)**.** These values were recorded from sperm samples in different media: EY_B (Egg Yolk and BO), EY_H (Egg Yolk and Ham's F10), EY_I (Egg Yolk and INRA96®), NT_B (Neat and BO), NT_H (Neat and Ham's F10) and NT_I (Neat and INRA96®).

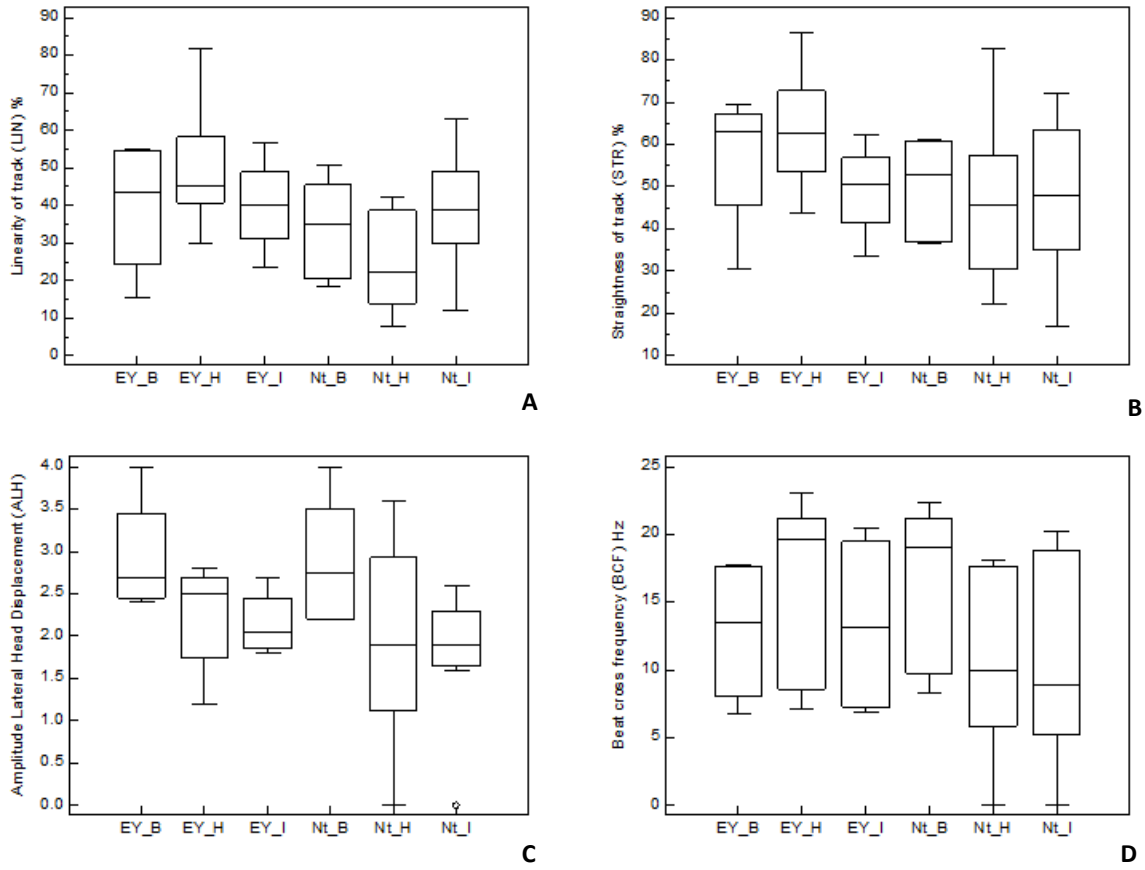


Figure 5.4: Box-and-whisker plots illustrating **(A)** Linearity of the track (LIN %) **(B)** Straightness of track (STR %) **(C)** Amplitude of lateral head displacement (ALH µm) and **(D)** Beat cross frequency (BCF Hz). These values were recorded from sperm samples in different media: EY_B (Egg Yolk and BO), EY_H (Egg Yolk and Ham's F10), EY_I (Egg Yolk and INRA96®), NT_B (Neat and BO), NT_H (Neat and Ham's F10) and NT_I (Neat and INRA96®).

Table 5.5 Individual motility and kinematic parameters for each ejaculate collected and evaluated during April 2010 from two different free-ranging Southern white rhinoceros populations.

Rhino ID	Rx	Total Motile %	Non-progressive Motily %	Proggressive Motily %	VCL µm/s	VSL µm/s	VAP µm/s	LIN %	STR %	WOB %	ALH µm	BCF Hz
CSS 3a	EY_H	75	75	0	114.1	92.9	107.8	81.5	86.2	94.5	1.7	19.6
CSS 4a	EY_H	89.3	19.1	70.2	139.2	63	118.4	45.3	53.2	85.1	2.5	20.6
CCS 7a	EY_H	75.6	5.1	70.5	68.3	20.6	47.1	30.1	43.7	69	1.2	11
CCS 13b	EY_H	93.6	36.4	57.2	73.9	33.2	60.7	44.9	54.6	82.2	2.7	7.1
CCS 14b	Ey_H	87	16.3	70.7	56.8	33.6	49.8	59.2	67.5	87.7	2.7	7.8
CSS 4a	EY_I	97.1	24.3	72.8	133.6	54.6	105.5	40.9	51.8	79	2.2	20.4
CCS 7a	EY_I	89	7.7	81.3	101	23.6	70.7	23.4	33.4	70	1.8	18.6
CCS 13b	EY_I	97	26.8	70.2	70.1	27.4	55.3	39	49.5	78.9	2.7	6.9
CCS 14b	Ey_I	89.8	24.7	65.1	79.3	45	72.5	56.8	62.1	91.4	1.9	7.7
CSS 5a	NT_H	58	38.8	19.2	125.7	97.8	118	7.8	82.9	93.9	1.5	17.5
CCS 7a	NT_H	69.6	0	69.6	62.9	10	45	15.9	22.2	71.6	0	0
CSS 8a	NT_H	95.4	5.1	90.3	81.9	18.1	54.2	22.1	33.4	6.1	1.9	18.1
CCS 13b	NT_H	94.1	39	55.1	81.5	30.5	6.9	37.4	45.5	82.1	3.6	10
CCS 14b	NT_H	70.5	13.2	57.3	8.2	35.1	71.8	42.2	48.9	86.3	2.7	7.8
CSS 5a	NT_I	39.4	8.9	30.5	63.7	40	55.6	62.8	72	87.3	1.6	19.4
CCS 7a	NT_I	80.4	17.8	62.6	108.3	42.4	87.6	39.1	48.3	81	1.8	16.9
CSS 8a	NT_I	98.1	0	98.1	129.7	15.8	93.1	12.1	16.9	71.8	0	0
CSS 10b	NT_I	86	22	64	81	41.5	60.7	51.2	68.3	74.9	2	20.2
CCS 13b	NT_I	96	12	84	95	27	79.1	28.4	34.1	83.3	2.6	8.9
CCS 14b	NT_I	89	67.9	21.1	112.4	38.3	98.2	34	39	87.3	2.4	6.7
CCS 15b	NT_I	70	15	55	78.7	33.3	69.6	42.3	47.8	88.5	1.9	4.8
CSS 5a	EY_BO	88.1	22.4	65.7	73.1	39.3	56.6	53.7	69.4	77.4	2.5	17.7
CCS 7a	EY_BO	96.8	4.7	92.1	117.1	18.3	60.2	15.6	30.3	51.5	2.9	17.5
CCS 13b	EY_BO	82.3	41.9	40.4	87.4	29.3	48.2	33.6	60.8	55.2	4	9.5
CCS 14b	EY_BO	83.3	13.3	70	60.9	33.6	51.3	55.1	65.4	84.3	2.4	6.7
CSS 5a	NT_BO	86.6	36.2	50.4	158.7	80.5	132.8	50.8	60.7	83.7	2.2	21.2
CCS 7a	NT_BO	79.3	5.1	74.2	82.2	17.1	46.2	20.8	36.9	56.3	2.4	22.3
CSS 8a	NT_BO	97.1	6.3	90.8	113.1	20.9	56.9	18.5	36.7	50.4	3.1	18.7
CSS 10b	NT_BO	87.1	20.4	66.7	98.2	44.6	75.9	45.4	58.7	77.3	2.2	19.5
CCS 13b	NT_BO	84.9	43.4	41.5	87.1	29.6	48.5	34	61	55.6	4	9.8
CCS 14b	NT_BO	93.9	47.6	46.3	83.3	30	64.3	36.1	46.7	77.2	3.5	8.3

VCL = Curvilinear velocity. VSL = Straight line velocity. VAP = Average path velocity. LIN = Linearity of tract. WOB = Wobble. BCF = Beat cross frequency. ALH = Amplitude of lateral head displacement

5.2.3 HYPERACTIVATED MOTILITY ANALYSIS IN SPERM POPULATIONS USING CASA

5.2.3.1 Pattern analysis and the classification of motile spermatozoa

Individual spermatozoa in EY or NT in either BO (10mM caffeine) or Ham's F10 media were classified according to the motion pattern displayed. The VCL, VSL, LIN, STR, ALH and BCF parameter values for each individual spermatozoon classified was recorded. As illustrated in screen images A - D (Figure 5.5) the motion patterns of individual spermatozoa were analysed and classified according to Group 1: Straight-line (non-HA) pattern (Figure 5.6), Group 2: Linear (Intermediate) pattern, Group 3: HA Circular pattern (HA C) and Group 4: HA Starspin pattern (HA S) (Figure.5.7).

Extracted parameter values were plotted to analyse the frequency distribution of the data recorded per motion pattern. Distribution based analysis allowed for the graphic illustration of parameter data, recorded per individual spermatozoon, after classification according to the motion pattern displayed. The distribution of the data points for every motion pattern (Non-HA, Intermediate, HA C and HA S) recorded per parameter is illustrated in Figures 5.8 and Figure 5.9. The changes in the progression parameters displayed by spermatozoa during the process of hyperactivation (HA) is clearly exhibited in these scatter plots. A data summary of the parameters recorded per motion pattern group is presented in Table 5.6. Statistical analysis of the above-described motion patterns recorded several similarities and differences in progression parameters between the four pattern groups compared (Figure 5.10).

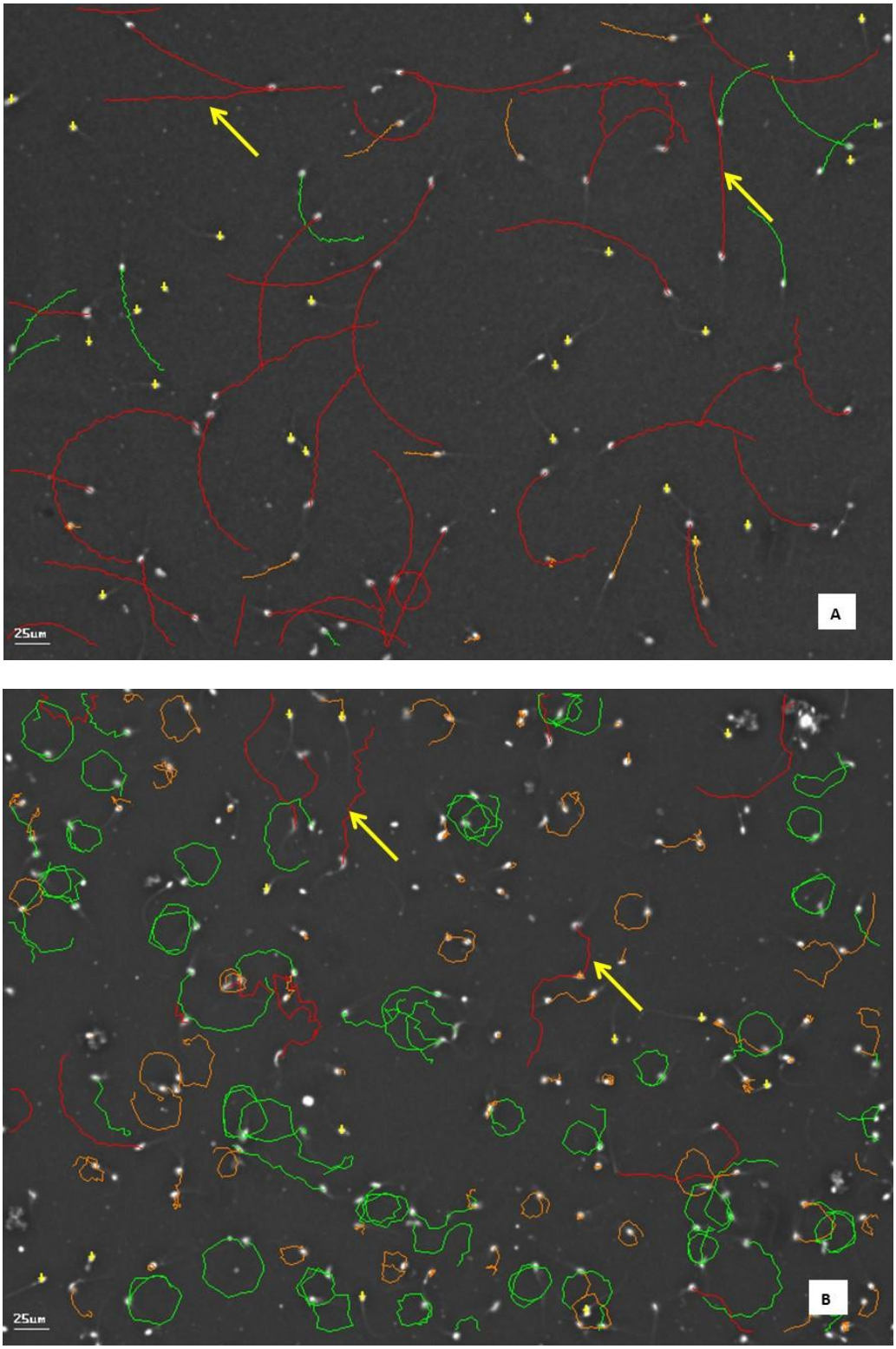


Figure 5.5: A selection of various SCA® screen images showing sperm motion tracks or patterns recorded for various individual Southern white rhinoceros spermatozoa. Specific motion patterns displayed by individual spermatozoa are indicated by yellow arrows, for example in **(A)** a straight-line (non-HA) pattern and **(B)** a linear (intermediate) pattern.

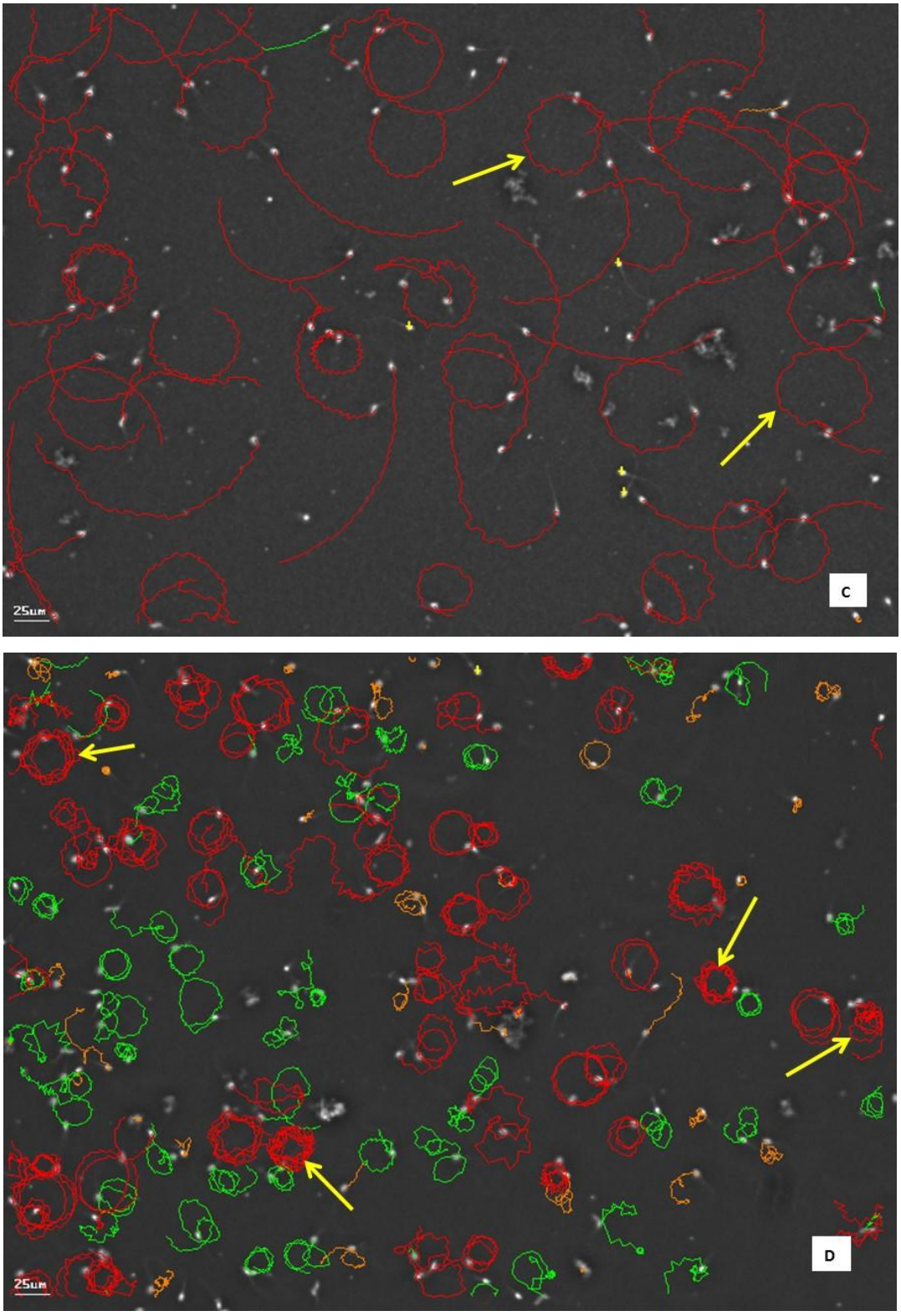


Figure 5.5: (continued) (C) An example of circular motion patterns (HA C) **(D)** and starspin motion patterns (HA S) recorded for Southern white rhinoceros spermatozoa displaying hyperactivated motility.

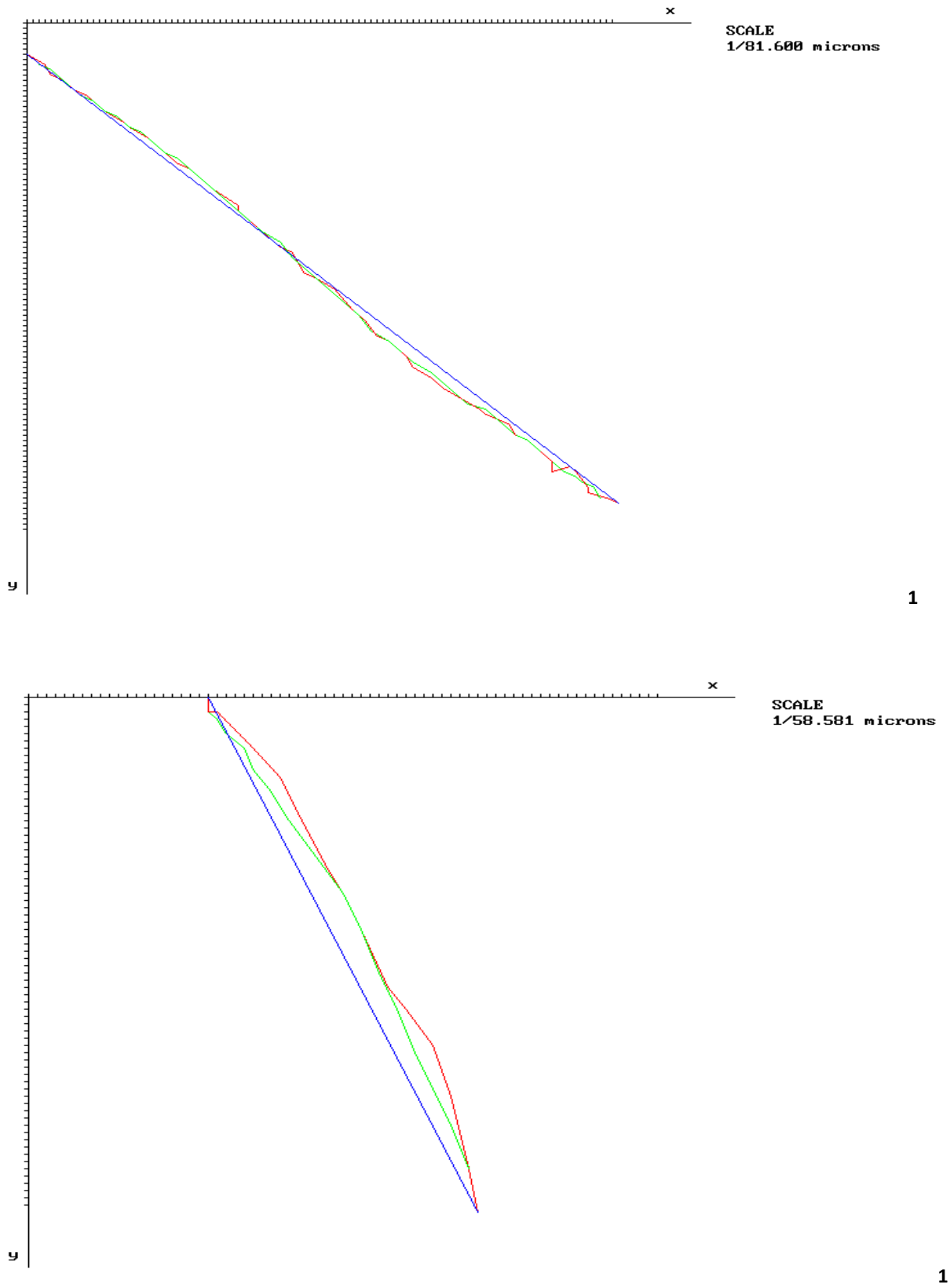


Figure 5.6: Group 1: Spermatozoa exhibiting a straight-line motion pattern or trajectory. The red line represents the actual sperm path also referred to as the curvilinear velocity (VCL µm/s), the green line represents the straight-line velocity (VSL µm/s) and the blue line the average path velocity (VAP µm/s). This visual analysis allowed for the classification of individual motion patterns.

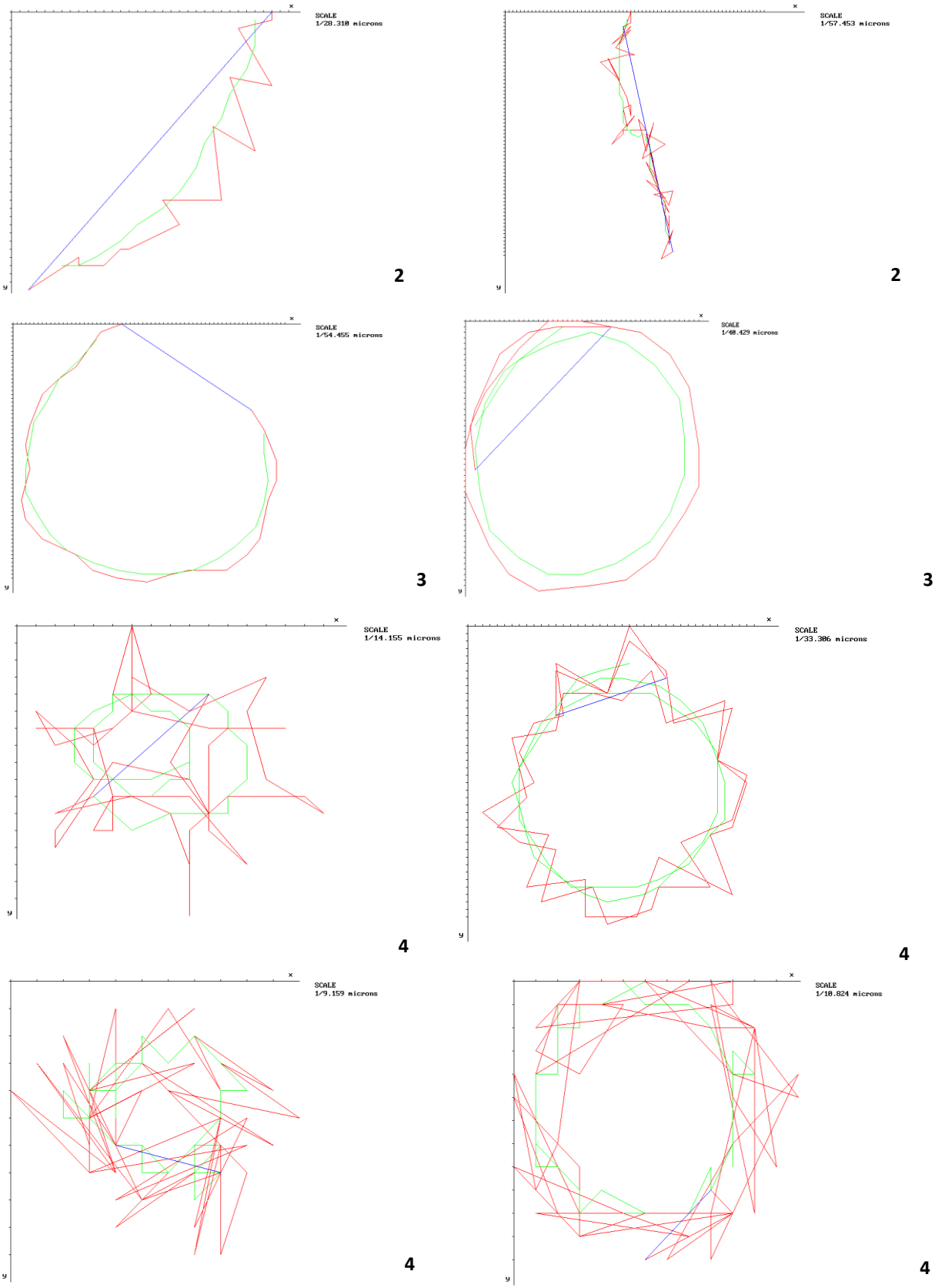


Figure 5.7: Visual identification of Southern white rhinoceros spermatozoa allowed for the classification of individual motion patterns. **(2)** Group 2: Linear pattern. **(3)** Group 3: Circular pattern and **(4)** Group 4: Starspin pattern. Red line = curvilinear velocity (VCL µm/s), green line = straight-line velocity (VSL µm/s) and blue line = average path velocity (VAP µm/s).

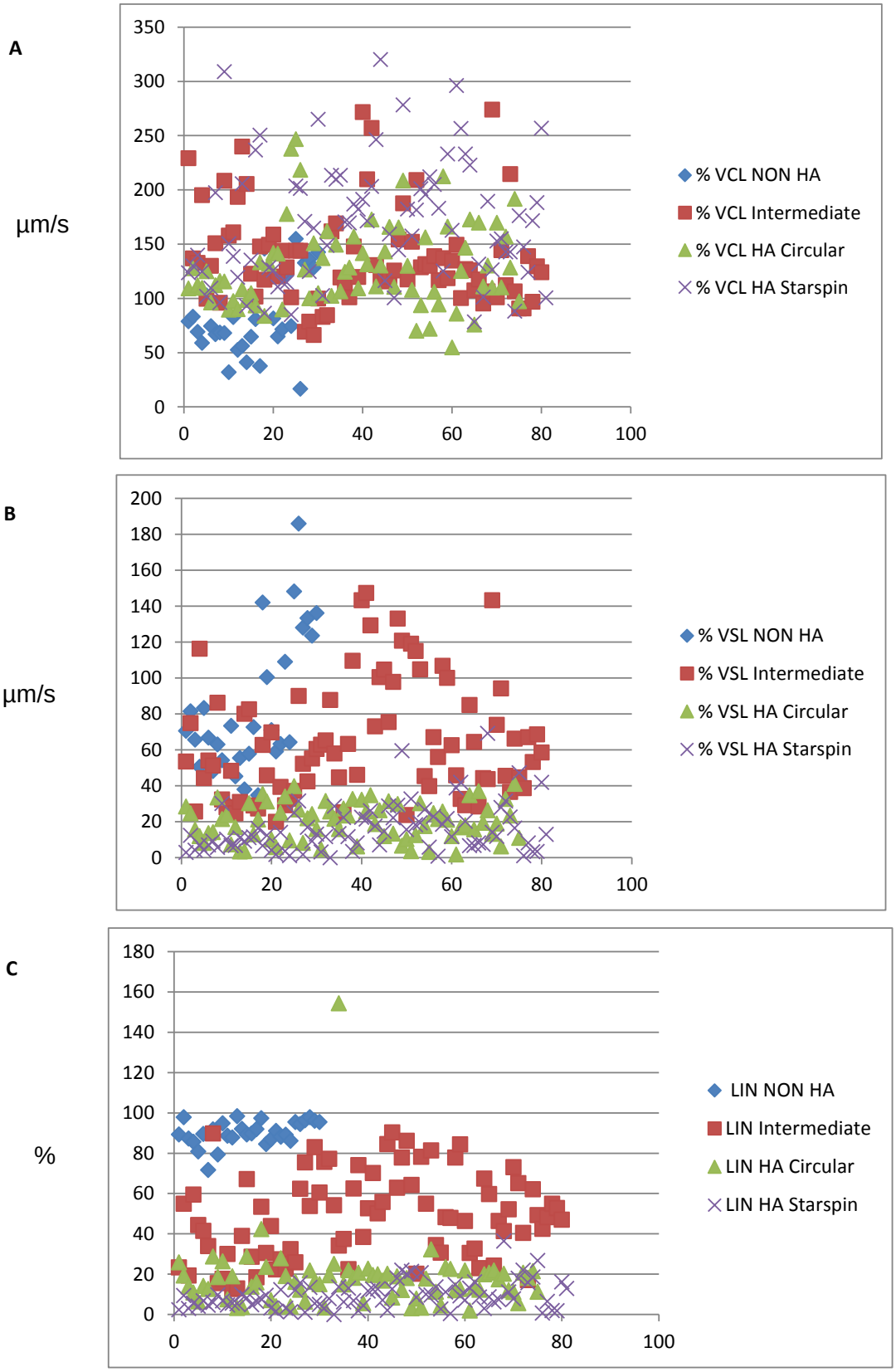


Figure 5.8: Distribution based analysis of progression parameters recorded from individual rhinoceros spermatozoa displaying hyperactive (HA) characteristics. Data dots in scatter graphs represents **(A)** The VCL (curvilinear velocity µm/s) **(B)** The VSL (straight-line velocity µm/s) and **(C)** LIN (Linearity of the track %) recorded for every: 1. Straight-line (NON HA) (n = 30), 2. Linear (Intermediate) (n = 80), 3. Circular (HA C) (n = 74), and 4. Starspin (HA S) (n = 82) motion pattern.

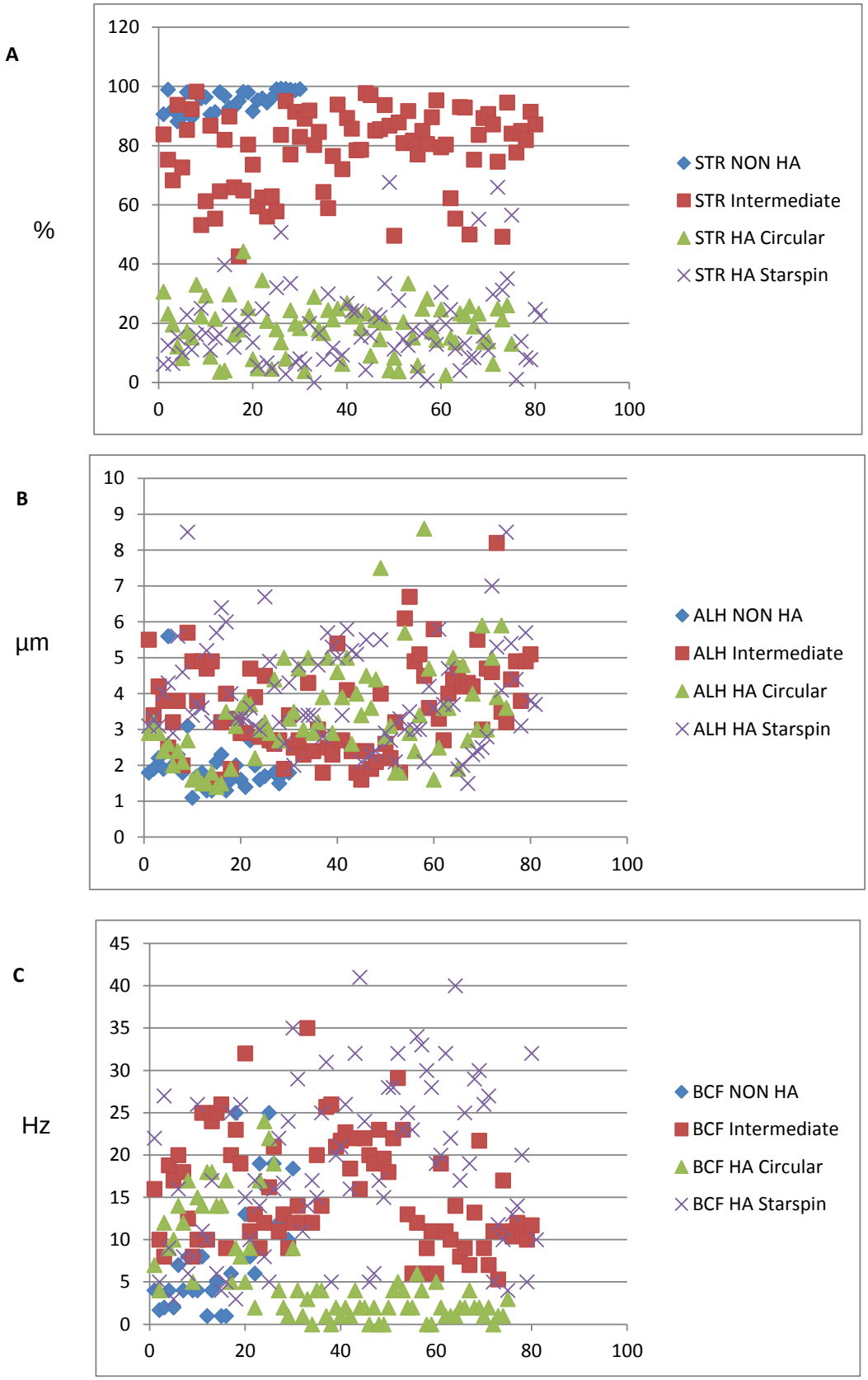


Figure 5.9: Distribution based analysis of progression and vigour parameters recorded from individual spermatozoa displaying hyperactive (HA) characteristics. Data dots in scatter graphs represents **(A)** STR (straightness of track %) **(B)** ALH (amplitude of the lateral head displacement µm) and **(C)** BCF (beat cross frequency Hz) recorded for every 1. Straight-line (NON HA) (n = 30), 2. Linear (Intermediate) (n = 80), 3. Circular (HA C) (n = 74), and 4. Starspin (HA S) (n = 82) motion pattern.

Table 5.6 Summary table for Southern white rhinoceros sperm populations parameters classified according to their motion patterns displayed. The data in the table is presented as sample number (N), mean ± standard deviation (SD), and median, minimum and maximum values for Curvilinear velocity (VCL µm/s), Straight line velocity (VSL µm/s), Linearity of track (LIN %), Straightness of tract (STR %), Amplitude of lateral head displacement (ALH) and Beat cross frequency (BCF Hz) and pattern diameters.

	1. STRAIGHT-LINE (NON-HA)						2. LINEAR PATTERN (INTERMEDIATE*)						3. HA CIRCULAR PATTERN (HA C)						4. HA STARSPIN PATTERN (HA S)					
Parameters	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.
VCL (µm/s)	30	**83.76**^{a}	±36.21	74.6	16.8	155.1	80	**138.7**^{b}	±44.5	129.25	66.4	274.1	74	**129.8**^{b}	±38.56	125.25	55	246.8	82	**167.24**^{b}	±55.81	161.05	78.4	320.3
VSL (µm/s)	30	**81.92**^{a}	±38.99	68.8	30.5	186	80	**65.3**^{b}	±32.48	58.3	20	147.4	74	**20.17**^{c}	±10.17	21.25	1.8	41.1	82	**15.98**^{c}	±13.16	12.15	0	69.3
LIN (%)	30	**90.26**^{a}	±6.04	89.75	71.8	98.4	80	**48.92**^{b}	±21.19	48.3	12.8	90.4	74	**17.85**^{c}	±17.94	17.2	2.1	154.5	82	**9.47**^{d}	±6.64	8.1	0	36.5
STR (%)	30	**95.31**^{a}	±3.46	96.1	88.2	99.3	80	**78.68**^{b}	13.67	81.95	42.6	98.3	74	**18.33**^{c}	±8.785	19.3	2.5	44.3	82	**18.59**^{c}	±13.78	15.55	0	67.6
ALH (µm)	30	**1.98**^{a}	±0.81	1.8	1.1	5.6	80	**3.65**^{b}	±1.31	3.55	1.6	8.2	74	**3.4**^{c}	±1.38	3.15	1.4	8.6	82	**3.96**^{d}	±1.44	3.6	1.5	8.5
BCF (Hz)	30	**9.01**^{a}	±7.26	6.5	1	25	80	**15.57**^{b}	±6.61	13.6	5.3	35	74	**5.74**^{c}	±6.11	4	0	24	82	**18.38**^{b}	±9.89	17	3	41
Diameter (µm)	-	-	-	-	-	-	-	-	-	-	-	-	75	**32.1**	±10.89	30.58	14.7	74.2	75	**24.12**	±10.57	23.29	7.5	54.5

^{a,b,c} values labelled with different superscript letters in the same row were significantly different ($p<0.05$)

* Spermatzoa with straight-line patterns but with increased ALH classified under 2. Linear pattern (intermediate*) group

HA = Hyperactive

In general, the straight-line (non-HA) spermatozoa revealed higher population averages for VSL (81.92 ± 38.99µm/s), LIN (90.26 ± 6.04%) and STR (95.31 ± 3.46%) compared to the other three motion pattern groups (Table 5.6). The highest population average for VCL was recorded at 167.24 ± 55.81µm/s in the hyperactive (HA) starspin group. A lower average VCL was recorded in the non-HA group at 83.76 ± 36.21µm/s (Figure 5.10 A). There was no clear grouping for VCL in any of the four pattern groups when collected data was presented as scatter plots shown in Figure 5.8 A. However, a clear grouping of lower VSL values was found for HA circular and HA starspin motion pattern groups as seen in Figures 5.8 B and 5.10 B. The lowest average VSL was recorded in HA starspin motion group recorded at 15.98 ± 13.16µm/s.

The percentages recorded for the LIN parameter dropped from 90.26 ± 6.04% (in non-HA group) to 9.47 ± 6.64% (in HA S pattern group) as presented in Figure 5.10 C. Similar to the data grouping shown for LIN, STR also revealed a distinct grouping of the HA circular and HA starspin motion pattern groups illustrated in the scatter plot in Figure 5.9 A. The significantly lower population averages for STR were recorded at 18.33 ± 8.79% and 18.59 ± 13.78% for the HA circular and HA starspin motion pattern groups, respectively (Figures 5.10 D). The highest average ALH amongst the motion pattern groups classified was recorded in the HA starspin motion pattern group at 3.96 ± 1.44µm and the lowest average ALH recorded in the non-HA group at 1.98 ± 1.8µm. Average ALH values differed significantly amongst all four motion pattern groups compared (Figures 5.9 B and 5.10 E). Similarities for BCF measurements were recorded in the linear (15.57 ± 6.61Hz) and HA starspin (18.38 ± 9.89Hz) motion patterns groups (Figures 5.9 C and 5.10 F). The lowest BCF was recorded in the HA circular pattern group at 5.74 ± 6.11Hz.

Data collected from the diameters measured for both circular and starspin motion pattern groups (Table 5.6) revealed that these patterns were quite similar in diameter (Figure 5.11).

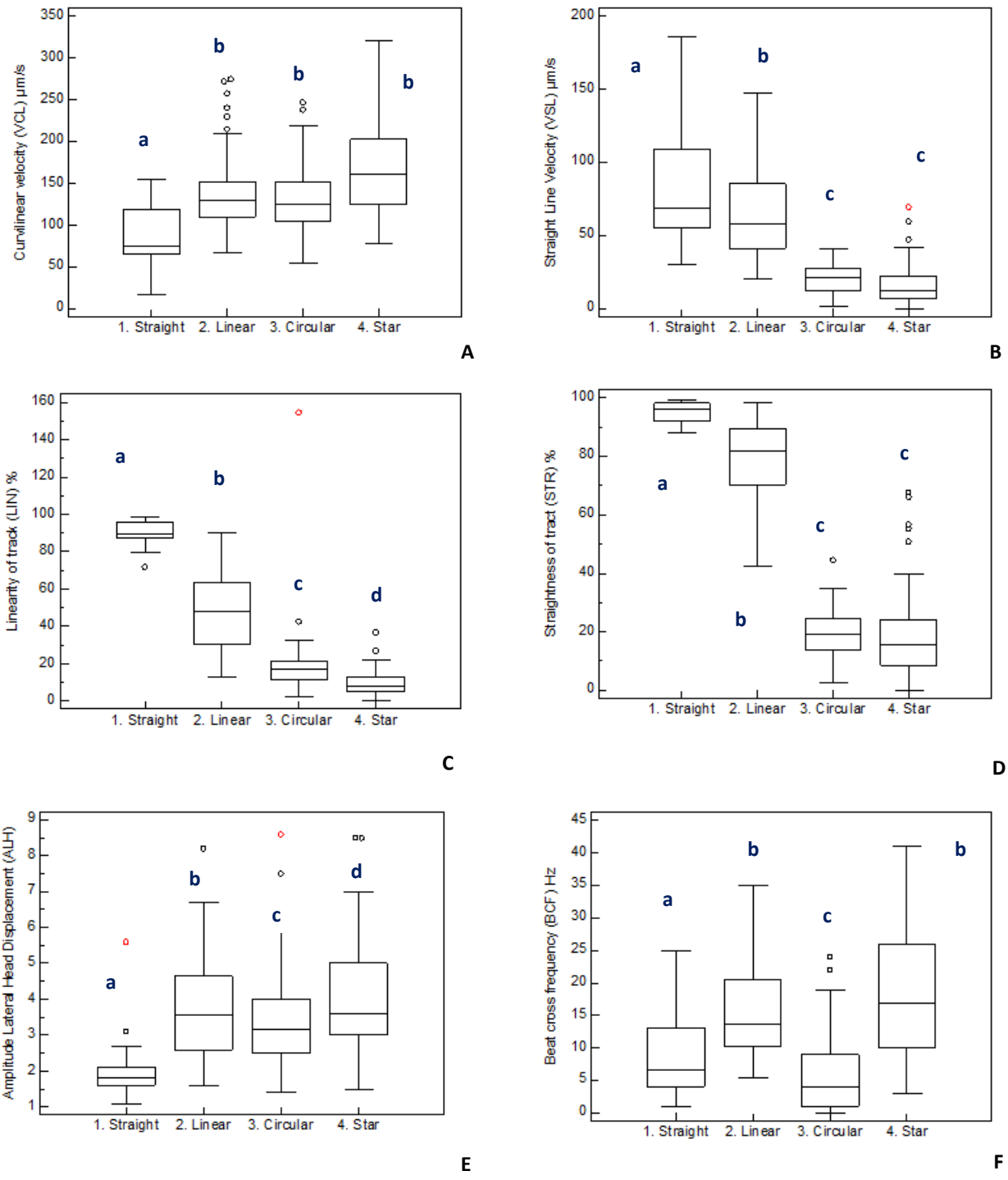
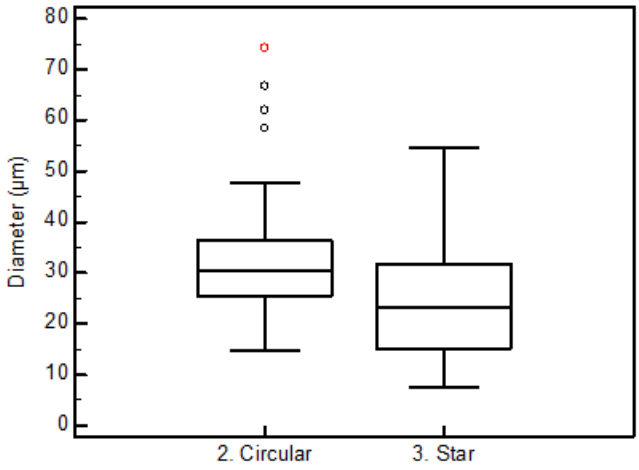


Figure 5.10: Box and whisker plots of the kinematic parameters **(A)** curvilinear velocity (VCL µm/s) **(B)** straight-line velocity (VSL µm/s) **(C)** linearity of track (LIN %) **(D)** Straightness of track (STR %) **(E)** Amplitude of lateral head displacement (ALH µm) and **(F)** beat cross frequency (BCF Hz) of hyperactive patterns or motion tracks. Data plots labelled with different letters in the same graph were significantly different ($p < 0.05$).

A



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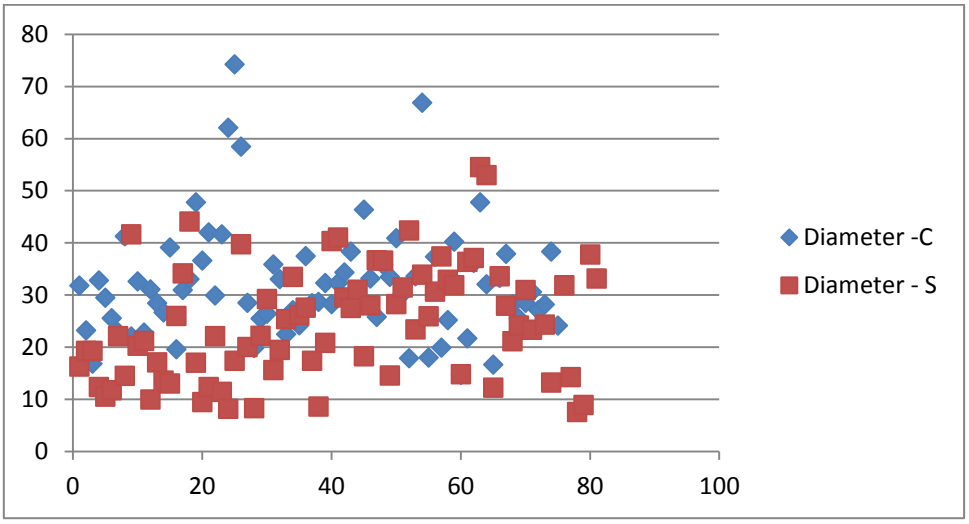


Figure 5.11: Distribution based analysis of the diameter of circular and starspin patterns investigated. **(A)** Box and whisker plot and **(B)** Data dots in the scatter plot represent the diameter (µm) measured of all the spermatozoa evaluated displaying hyperactive (HA) characteristics; C = Circular (n = 74), S = starspin (n = 82).

5.2.3.2 CASA cut-off values for hyperactive motility analysis

The extracted data from each pattern group was arranged for ROC curve analysis. Parameter criteria for CASA were derived from a comparison of straight-line pattern (non-HA) data to HA circular (HA C) and HA starspin (HA S) pattern data, combined during ROC analysis (Table 5.8). For the rhinoceros the cut-off criteria for parameters VCL (Figure 5.12 A) and ALH (Figure 5.12 E) indicated low sensitivity and specificity (> 75 %). While parameters VSL (Figure 5.12 B), STR (Figure 5.12 D), and LIN (Figure 5.12 C) revealed a high sensitivity and specificity (>90%) as cut-off criterions with no data overlapping (AUC = 1) (Table 5.8) when using ROC curve analysis.

5.2.3.3 Hyperactivated motility analysis of sperm samples in different media

CASA derived cut-off values (Figure 5.12 F) were used to discriminate between the motile and hyperactivated rhinoceros spermatozoon populations within every captured NT_Ham's, NT_BO, EY_Ham's and EY_BO samples were re-analysed accordingly. The data collected per sperm sample analysed and sorted is presented in Table 5.9. The type of motility recorded as a result of interaction with the media is depicted as bar graphs in Figures 5.13 and 5.14.

Percentages recorded for overall HA motility ranged from 37% in NT_Ham's compared to 45% recorded for NT_BO media group (used to stimulate HA). Population A recorded a higher percentages range for HA motility at 46% and 57% (in NT_Ham's and NT_BO) compared to Population B at 29% and 33%. The analysed sperm samples of population A (CSS 7 and CSS 8) recorded percentages above > 50% for HA motility, in both NT_Ham's (61.7% and 73.8%) and NT_BO (64.5% and 86.7%) media groups analysed. The highest percentage for HA motility was recorded at 47% in EY_BO and 32% in EY_Ham's. The highest individual percentage for HA motility was recorded for CSS 4 at 79.4% (in EY_BO) compared to 48.7% recorded in EY_Ham's. Percentages for population B (CSS 10, CSS 13 and CSS 14) was recorded below 50% for HA motility in EY_Ham's (16.8%, 37.4 % and 32.7%) and EY_BO (19%, 39.6% and 41%) media groups. The difference recorded for HA motility percentages ranged from 8% - 15% between NT_Ham's and NT_BO and EY_Hams and EY_BO media groups. Results from HA motility analyses of rhinoceros spermatozoa in NT_Ham's, NT_BO, EY_Hams and EY_BO media groups classified a percentage of the motile sperm population as HA.

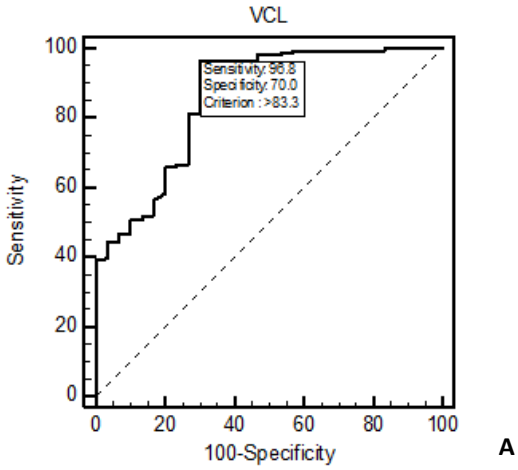
Table 5.8 ROC curve analyses for Southern white rhinoceros sperm parameter data classified according to individual motion patterns displayed. 1) Straight-line non-hyperactive (non-HA), 3) Hyperactive circular (HA C), 4) Hyperactive starspin (HA S) patterns and 5) Total HA (HA C and HA S combined). The data collected is presented in the table as number of samples (N), area under the curve (AUC), minimum (Min.) and maximum (Max.) values for Curvilinear velocity (VCL µm/s), Straight line velocity (VSL µm/s), Linearity of track (LIN %), Straightness of tract (STR %), Amplitude of lateral head displacement (ALH µm) and Beat cross frequency (BCF HZ) parameters.

	1. STRAIGHT-LINE (NON-HA)						3. HA CIRCULAR PATTERN (HA C)						4. HA STARSPIN PATTERN (HA S)						5. TOTAL HA (HA C + HA S)					
Parameters	N	ROC Criterion	% Sensitivity / % Specificity	AUC	Min.	Max.	N	ROC Criterion	% Sensitivity / % Specificity	AUC	Min.	Max.	N	ROC Criterion	% Sensitivity / % Specificity	AUC	Min.	Max.	N	ROC Criterion	% Sensitivity / % Specificity	AUC	Min.	Max.
VCL (µm/s)	30	**> 83.3**	96.2 / 70	0.85	16.8	155.1	74	**> 83.3**	94.6 / 70	0.811	55	246.8	82	**> 169.9**	46.3 / 87.8	0.903	78.4	320.3	156	**> 83.3**	96.8 / 70	0.859	55	320.3
VSL (µm/s)	30	**≤ 44.8**	75.8 / 90	0.86	30.5	186	74	**≤ 37.1**	97.3 / 93.3	0.991	1.8	41.1	82	**≤ 11.9**	50 / 78.4	0.983	0	69.3	156	**≤ 34.1**	92.3 / 96.7	0.987	0	69.3
LIN (%)	30	**≤ 78.3**	96.6 / 96.7	0.988	71.8	98.4	74	**≤ 42.5**	98.6 / 100	0.986	2.1	154.5	82	**≤ 13.7**	81.7 / 62.2	1	0	36.5	156	**≤ 42.5**	99.4 / 100	1	0	154.5
STR (%)	30	**≤ 87.9**	90.7 / 100	0.973	88.2	99.3	74	**≤ 44.3**	100 / 100	1	2.5	44.3	82	**≤ 13.5**	43.9 / 75.7	1	0	67.6	156	**≤ 67.6**	100 / 100	0.994	0	67.6
ALH (µm)	30	**> 2.3**	84.3 / 86.7	0.89	1.1	5.6	74	**> 2.3**	79.7 / 86.7	0.844	1.4	8.6	82	**> 3**	73.2 / 47.3	0.93	1.5	8.5	156	**> 2.3**	84.6 / 86.7	0.889	1.4	8.6
BCF (Hz)	30	**> 8**	65.7 / 63.3	0.637	1	25	74	**≤ 3**	48.6 / 80	0.844	0	24	82	**> 4**	93.9 / 62.2	0.788	3	41	156	**> 13**	43.6 / 76.7	0.767	0	41

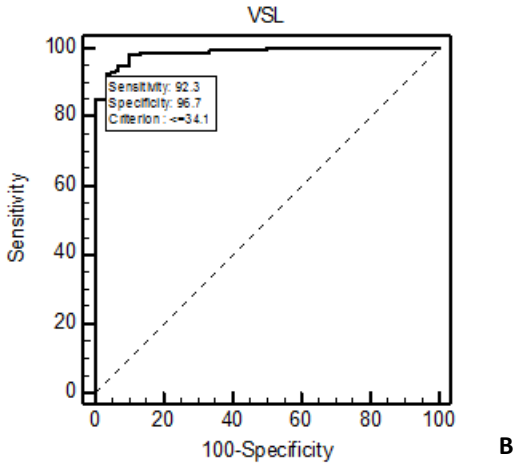
Total % HA : HA C + HA S combined

HA = Hyperactived motility

AUC = Area under ROC curve



A



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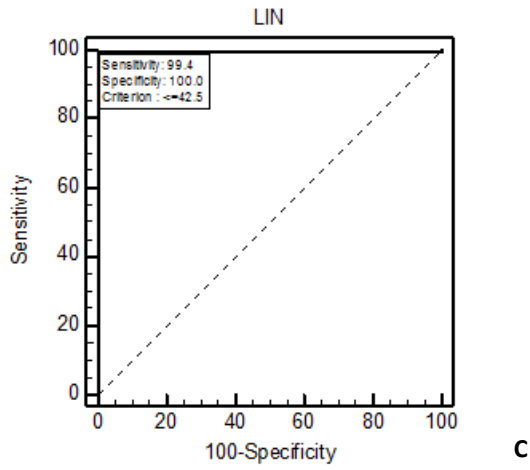
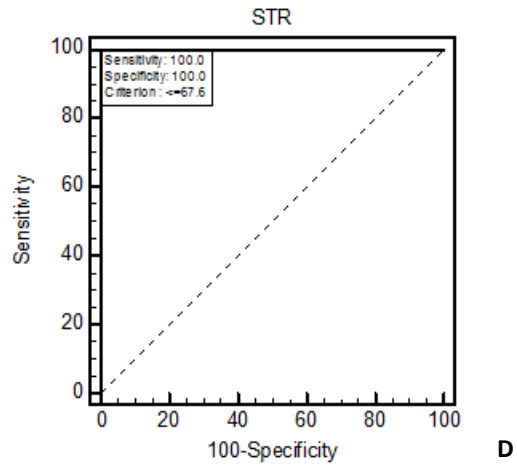


C



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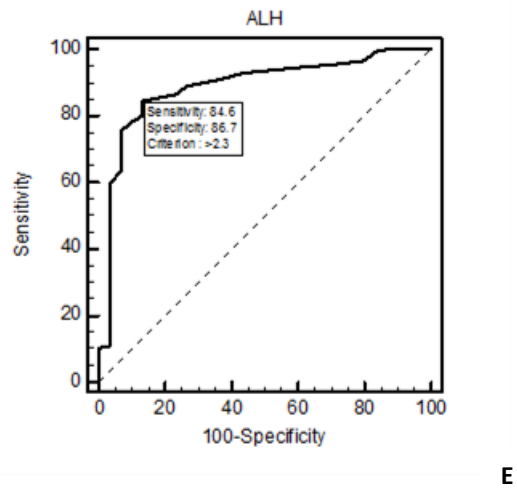

E

Southern White Rhinoceros	
SCA sort	**% HA**
VCL	> 55 and < 500
VSL	> 1 and < 34.1
STR	> 1 and < 67.6
LIN	> 1 and < 42.5
Restriction	
VSL	< 30

F

Figure 5.12 ROC criteria derived to assess hyperactivated motility in the Southern white rhinoceros sperm samples by comparing the straight-line pattern to HA circular (HA C) and HA starspin pattern combined. Cut-off criteria for CASA **(A)** VCL (curvilinear velocity µm/s) **(B)** VSL (straight-line velocity µm/s) **(C)** LIN (linearity of track %) **(D)** STR (straightness of track %) and **(E)** ALH (amplitude of head displacement µm). **(F)** ROC derived cut-off criteria derived by applying the Boolean argument to determine the total percentage of spermatozoa displaying hyperactivated motility in each sample evaluated.

Table 5.9 The total percentage hyperactivated motility (HA) measured per sperm sample analysed using CASA cut-off criterion derived from motion pattern analyses. HA results recorded in either egg yolk (EY) samples with Ham's F10 or BO (10mM caffeine) media (EY_Ham's and EY_BO) or in neat (NT) with Ham's F10 or BO (10mM caffeine) media (NT_Ham's and NT_BO). Results presented as percentage (%) and population average.

ID	Total % HA (NT_Ham's)	Total % HA (NT_BO)	Total % HA (EY_Ham's)	Total % HA (EY_BO)
CSS 4	-	-	48.7	79.4
CSS 5	2.3	17.7	-	
CSS 7	61.1	64.5	-	
CSS 8	73.8	86.7	-	-
CSS 10	16.8	19	-	-
CSS 13	37.4	39.6	36.6	43.4
CSS 14	32.7	41	11	19.2
Population average:	**37 %**	**45 %**	**32 %**	**47 %**

HA = Hyperactivated motility
NT_Ham's (neat semen and Ham's F10), NT_BO (neat semen and BO),
EY_Ham's (egg yolk semen and Ham's F10), EY_BO (egg yolk semen and BO)

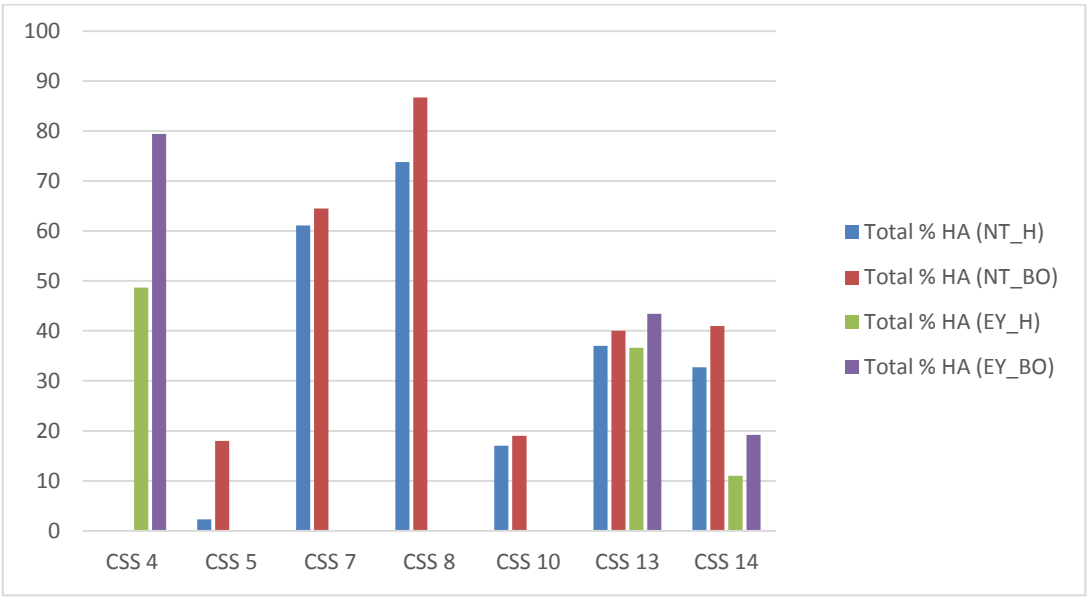

Figure 5.13: A comparison of total percentage hyperactivated motility (HA) measured per sperm sample analysed. Data recorded from either egg yolk (EY) extended samples with Ham's F10 media or BO (10mM caffeine) (EY_H and EY_BO) or from neat (NT) extended samples with Ham's F10 media or BO (10mM caffeine) (NT_H and NT_BO). Samples were measured on CASA with cut-off criteria derived after applied motion path pattern and ROC analyses.

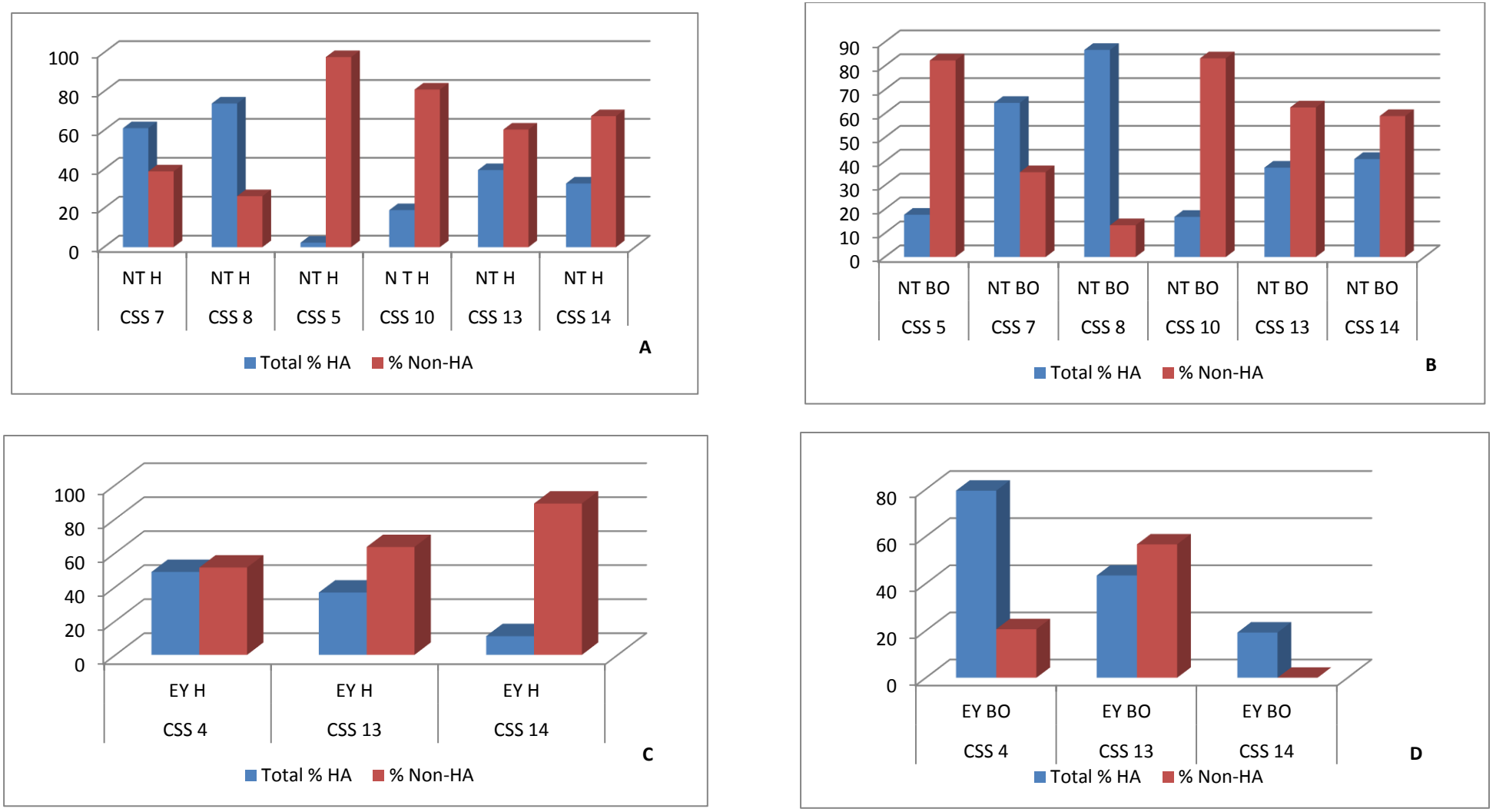

Figure 5.14: Bar graphs representing percentage hyperactivated (HA %) motility vs. percentage non-hyperactivated (Non-HA %) spermatozoa for each sperm sample analysed. Recorded in neat (NT) samples extended with Ham's F10 media or BO (10mM caffeine) **(A)** NT_H and **(B)** NT_BO or in the egg yolk (EY) extended samples with Ham's F10 media or BO (10mM caffeine) **(C)** EY_H **(D)** EY_BO. Measured by CASA after cut-off criterion was derived following applied motion path pattern and ROC analysis.

5.2.4 STRUCTURAL INTEGRITY ANALYSIS OF SPERMATOZOA

5.2.4.1 Plasma membrane integrity (viability) and acrosome integrity

A summary of the statistical data is displayed in Table 5.10 and individual data recorded per ejaculate is presented in Table 5.11. Examples of the classification of spermatozoa according to viability and acrosome integrity are illustrated in Figure 5.15. The statistical analyses of data compared revealed a significant difference for viability ($p = 0.045$, $F = 2.365$) and acrosome integrity ($p = 0.055$, $F = 7.158$) between the two rhinoceros populations' sperm samples (Figure 5.16). Population A recorded a significantly higher average at 80 ± 7.5% for the number of spermatozoa with intact plasma membranes integrity, compared to 65 ± 8.65% recorded for Population B. Overall, 73 ± 10.78% of the rhinoceros spermatozoa analysed were recorded to have intact plasma membranes (Table 5.10). Acrosome integrity analyses of spermatozoa recorded a population average at 75 ± 4.99% for the number of intact acrosomes across both populations. Population A recorded significantly higher averages at 78 ± 2.98% when compared to Population B at 71 ± 4.24% (Table 5.10).

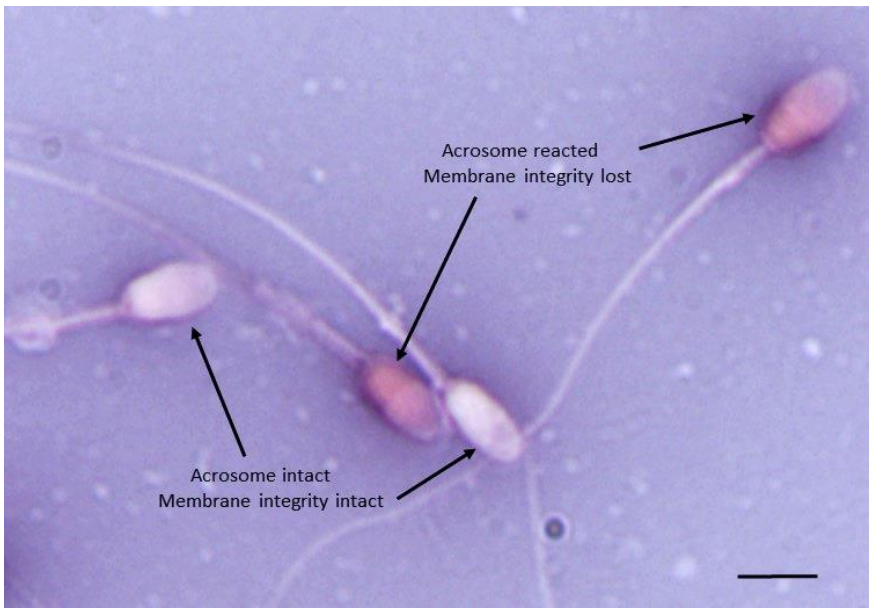

Figure 5.15: Eosin-nigrosin smear of Southern white rhinoceros sperm to illustrate the classification of plasma membrane integrity and acrosome integrity of spermatozoa during microscopic evaluation (Scale bar = 5.5µm).

Table 5.10 Average plasma membrane integrity (viability) and acrosome integrity data recorded for two separate Southern white rhino populations. The data is presented as sample number (N), mean ± standard deviation (SD), median, minimum (min.) and maximum (max.).

	Population A						Population B						Both Populations			
Parameters	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median
Membrane integrity intact (%)	4	**80**[a]	±7.5	80.5	72	88	4	**66**[b]	±8.65	65	56	77	8	**73**	±10.78	74
Membrane integrity lost (%)	4	**20**[a]	±7.5	19.5	12	28	4	**34**[b]	±8.65	35	23	44	8	**27**	±10.78	26
Acrosome intact (%)	4	**78**[a]	±2.98	79	75	82	2	**71**[b]	±4.24	71	68	74	6	**75**	±4.99	76.5
Acrosome reacted (%)	4	**22**[a]	±5.31	22.5	16	28	2	**29**[b]	±4.24	29	26	32	6	**25**	±5.71	25.5

[a, b] values labelled with different superscript letters were significantly different ($p < 0.05$)

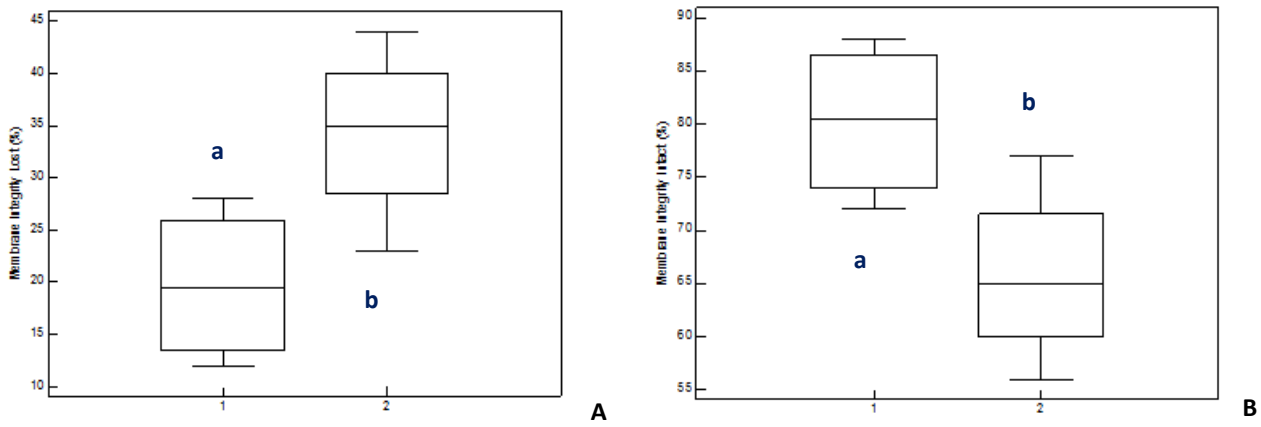

Figure 5.16: Box-And-Whisker Plots of **(A)** percentage spermatozoa with plasma membrane integrity lost (%) and **(B)** percentage spermatozoa where plasma membrane integrity remained intact (%). Data was recorded from eosin-nigrosin smears prepared during April 2010 for population A **(1)** and B **(2)**. Data plots labelled with different letters in the same graph were significantly different ($p < 0.05$).

Table 5.11 Overview of individual viability and acrosome integrity results of all Southern white rhinoceros semen samples evaluated.

Rhino ID	% Viability Live	% Viability Dead	% Acrosome Intact	% Acrosome Reacted
CSS 4	88	12	82	16
CSS 5	85	15	80	20
CSS 7	72	28	78	28
CSS 8	76	24	75	25
CSS 10	66	34	68	32
CSS 13	64	36	74	26
CSS 14	77	23	-	-
CSS 15	56	44	-	-

5.2.4.2 Sperm morphology

The spermatozoon of the Southern white rhinoceros comprises a head and tail (flagellum). The tail is the longest part of the spermatozoon and includes a midpiece, principal piece and end piece. An example of what is considered as a normal rhinoceros spermatozoon is illustrated in Figure 5.17. The morphological evaluation and classification of Southern white rhinoceros spermatozoa for structural abnormalities are listed in Table 5.12 and individual abnormalities are listed in Table 5.13.

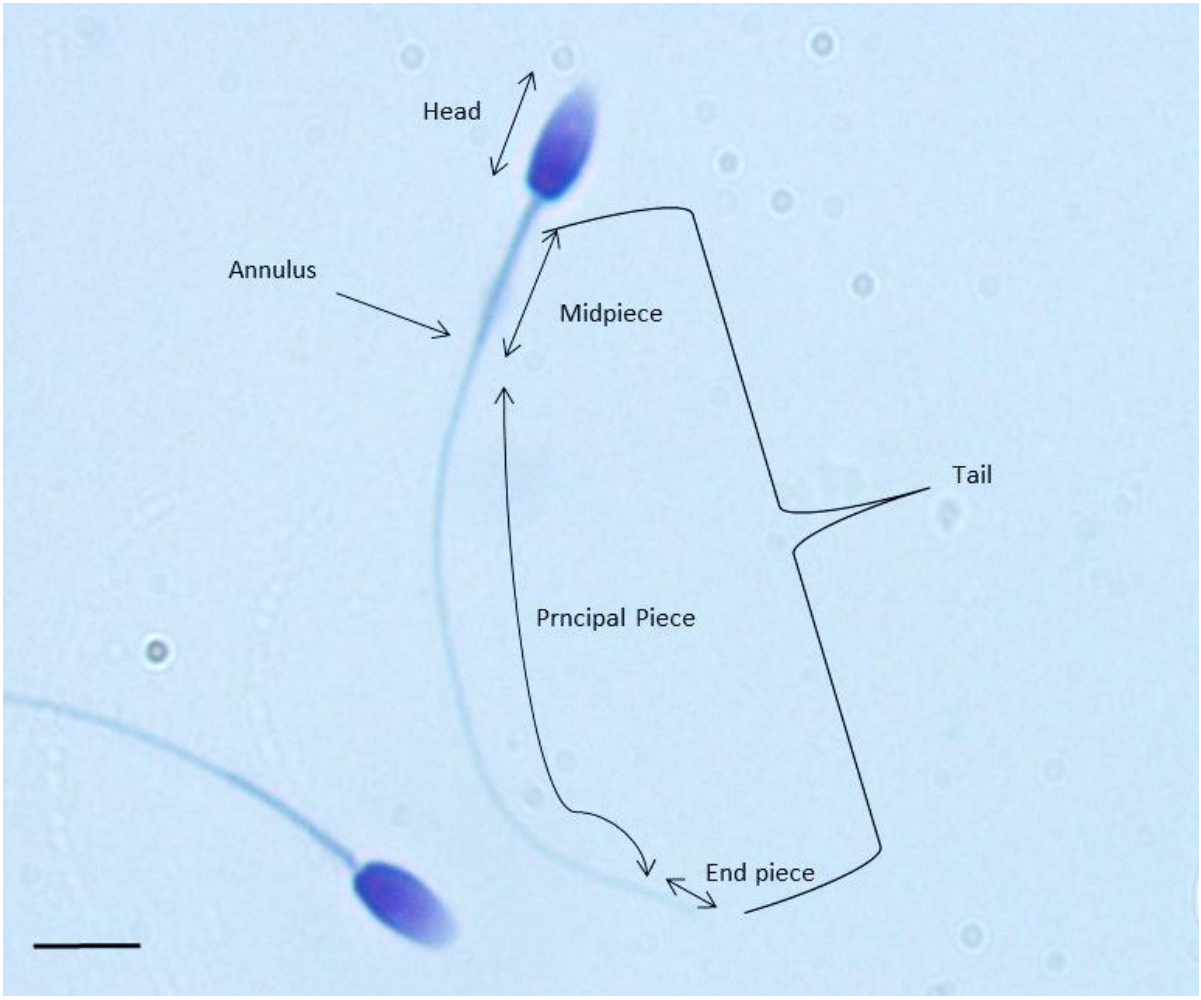

Figure 5.17: Example of a Southern white rhinoceros spermatozoon stained with SpermBlue® in order to microscopically identify and evaluate individual sperm components (Scale bar = 5.5µm).

Analysis of sperm morphological data revealed no significant difference between the two rhinoceros populations for normal ($p = 0.456$; $F = 0.636$) or abnormal ($p = 0.477$; $F = 0.575$) morphology. Furthermore, no statistical difference was found regarding the head ($p = 0.187$; $F = 2.215$) or midpiece ($p = 0.114$; $F = 3.420$) or tail ($p = 0.862$; $F = 0.389$) abnormalities recorded for the two populations.

The most common abnormalities recorded during morphological evaluation were midpiece reflex (Figure 5.18 B) and Dag-like defects (Figure 5.18 C). Other abnormalities recorded included teratogenic spermatozoa, abnormal loose heads, mitochondrial defects (segmental aplasia) (Figure 5.18 F) and proximal and distal droplets. The bent principal piece was the most common tail defect recorded (Figure 5.18 A) amongst all samples evaluated.

In summary, the semen samples consisted on average of 61.75 ± 14.67% morphologically normal spermatozoa (Table 5.12).

Table 5.12 Population averages of sperm morphology parameters recorded for the Southern white rhinoceros. The data is presented as sample number (N), mean ± standard deviation (SD), median, minimum (min.) and maximum (max.).

	Population A						Population B						Both Populations			
Parameters	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median
Normal Morphology (%)	4	**66**	±19.32	72	38	82	4	**57**	±9	55	50	70	8	**62**	±14.67	64
Abnormal Morphology (%)	4	**34**	±19.8	28	18	63	4	**43**	±9	45	30	50	8	**38**	±14.9	36
Head (%)	4	**12**	±6.23	7	6	19	4	**18**	±7.04	16	9	26	8	**15**	±7.2	12
Midpiece (%)	4	**14**	±7.97	10	2	21	4	**19**	±4.57	19,5	14	24	8	**16**	±7.54	15.5
Tail (%)	4	**8**	±3.51	7,5	4	11	4	**6**	±4.08	3,5	2	11	8	**7**	±3.77	4.5

Table 5.13 Individual morphology percentages and classification of defects recorded for Southern white rhinoceros sperm samples.

Rhino ID	Concentration ($x10^6$/ml)	% Normal Morphology	% Head Defects	% Midpiece Defects	% Tail Defects	Comments
CSS 4	30	74	19	2	5	Abnormal heads, loose heads, Dag defect
CSS 5	15	82	6	8	4	Abnormal heads, loose heads, Dag defect, proximal droplets, midpiece reflex
CSS 7	7	38	6	21	11	Abnormal heads, Dag defect, midpiece reflex, proximal & distal droplets, coiled principal piece
CSS 8	7.5	70	8	12	10	Abnormal heads, Dag defect, midpiece reflex, proximal & distal droplets, coiled principal piece
CSS 10	99	58	15	14	3	Loose heads, Dag defect, proximal droplets, midpiece reflex
CSS 13	255	50	26	22	2	Abnormal heads, loose heads, Dag defect, midpiece reflex, proximal & distal droplets
CSS 14	220	52	17	24	11	Abnormal heads, loose heads, midpiece reflex, proximal & distal droplets, coiled principal piece
CSS 15	37.5	70	9	17	4	Loose heads, Dag defect, proximal & distal droplets, midpiece reflex

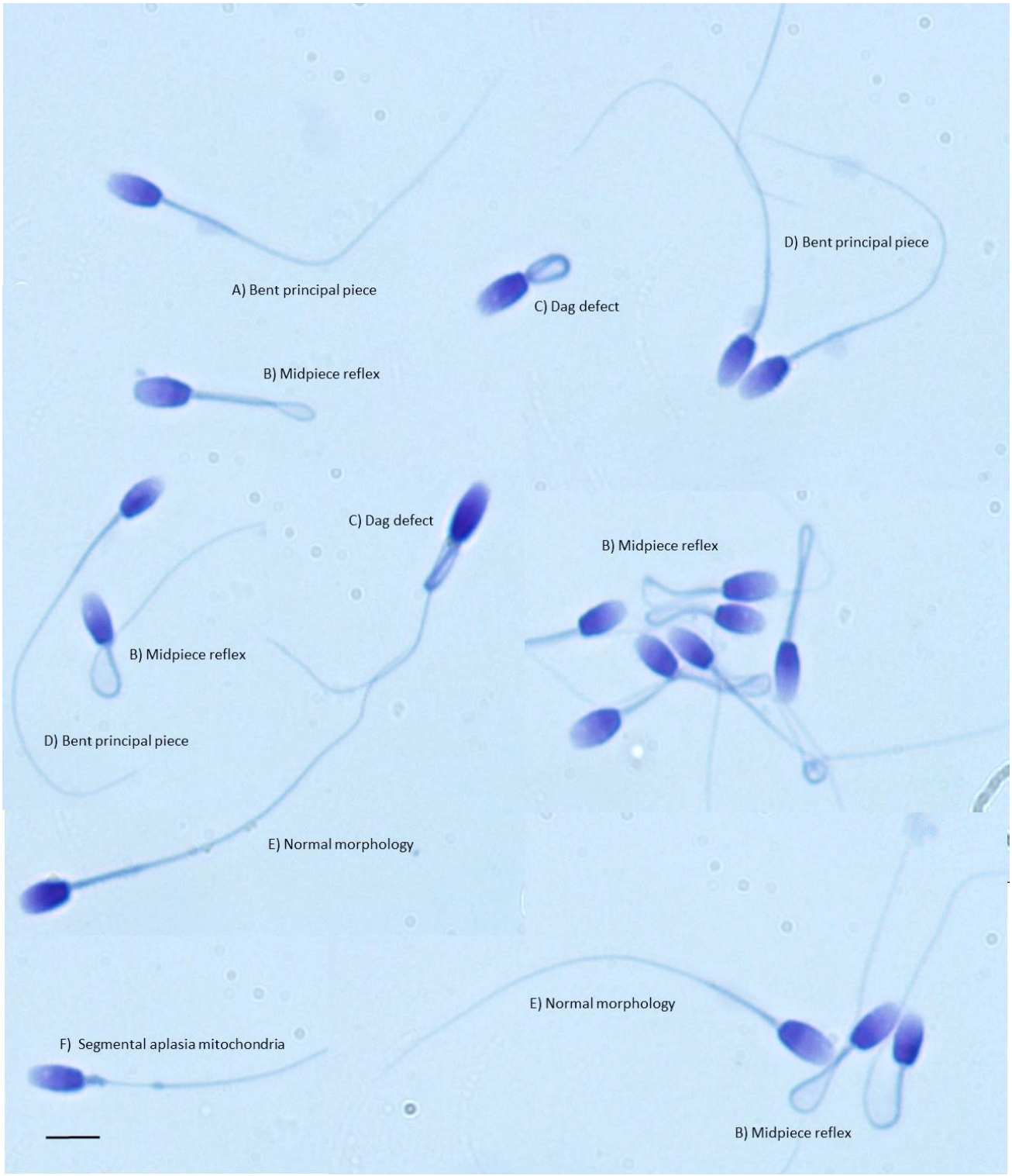

Figure 5.18: A collection of images of the normal and abnormal sperm morphology recorded during evaluation of semen samples from different Southern white rhino individuals. SpermBlue® smear evaluated under oil immersion objective (Scale bar = 5.5µm).

5.2.4.3 Sperm head morphometric analysis

Using the SCA® automated morphology module allowed measurement of eight sperm head morphometric characteristics. Statistical analysis of the sperm head morphometric data of both Southern white rhino populations verified similarities between the two populations compared. A summary of the statistical data is presented Table 5.14 and individual data recorded per ejaculate presented in Table 5.15. There were no significant differences found between the two populations compared for head length (p=0.810; F=0.0631), head width (p=0.474; F=0.582), head area (p=0.663; F=0.210), head ellipticity (p=0.252; F=1.609), head elongation (p=0;246; F=1.653), head perimeter (p=0.942; F=0.0058), head regularity (p=1.0; F=0), head roughness (p=0.569; F=0.364) or acrosome coverage (p=0.340; F=1.075).

These results indicate that, on average, the head of a Southern white rhinoceros spermatozoon measures 5.53 ± 0.17µm in length, 2.85 ± 0.19µm in width with a total head area of 14.75 ± 1.43µm^2. The acrosome covers approximately 36.3 ± 0.59% of this total head area as indicated by the yellow cap on the sperm head in Figure 5.19.

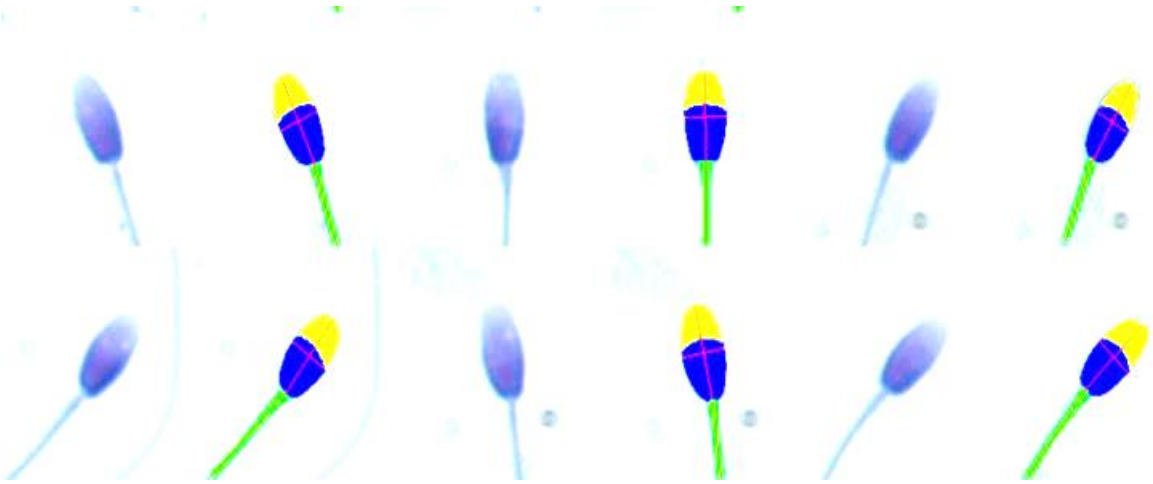

Figure 5.19: Computer-aided morphometric analysis of sperm head analysis of the Southern white rhinoceros. An image of each sperm assessed is shown on the left and its morphometric assessment on the right. Red lines represent head length and width measurements. The yellow shaded area represents the acrosome coverage in a cap-like shape. Green structures represent part of the midpiece as well as the angle of tail insertion.

Table 5.14 Average sperm head morphometric measurements recorded for both populations of Southern white rhinos. Data is presented as sample number (N), mean ± standard deviation (SD), median, minimum (min.) and maximum (max.).

	Population A						Population B						Both Populations		
Parameters	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median	Min.	Max.	N	Mean	±SD	Median
Length (µm)	4	**5.55**	±0.08	5.55	5.46	5.65	4	**5.52**	0.24	5.54	5.21	5.79	8	**5.53**	0.17	5.55
Width (µm)	4	**2.79**	0.16	2.75	2.65	3.02	4	**2.9**	0.23	2.91	2.66	3.13	8	**2.85**	0.19	2.76
Area (µm²)	4	**14.51**	1.27	14.59	12.88	15.98	4	**15**	1.73	15.1	13.17	16.63	8	**14.75**	1.43	14.59
Ellipticity	4	**2**	0.1	2.04	1.84	2.07	4	**1.91**	0.09	1.92	1.8	2	8	**1.95**	0.1	1.98
Elongtion	4	**0.33**	0.02	0.34	0.29	0.35	4	**0.3**	0.02	0.31	0.28	0.33	8	**0.31**	0.025	0.32
Perimeter (µm)	4	**12.14**	0.25	12.21	11.79	12.37	4	**12.17**	0.67	12.26	11.35	12.82	8	**12.16**	0.47	12.21
Regularity	4	**0.85**	0.03	0.84	0.82	0.89	4	**0.85**	0.008	0.85	0.84	0.86	8	**0.85**	0.02	0.85
Roughness	4	**1.25**	0.06	1.26	1.17	1.31	4	**1.27**	0.02	1.28	1.23	1.29	8	**1.26**	0.04	1.28
Acrosome coverage (%)	4	**36.09**	0.43	36.03	35.64	36.66	4	**36.52**	0.71	36.75	35.51	37.09	8	**36.3**	0.59	36.36

Table 5.15 Overview of individual morphometric analysis results for Southern white rhinoceros sperm samples.

Rhino ID	Head Length (µm)	Head Width (µm)	Head Area (µm²)	Head Perimeter (µm)	Head Ellipticity	Head Elongation	Head Roughness	Head Regularity	% Acrosome Coverage
CSS 4	5.53	3.02	15.98	12.37	1.84	0.29	1.31	0.83	35.91
CSS 5	5.65	2.79	14.48	12.13	2.03	0.34	1.24	0.86	36.66
CSS 7	5.58	2.72	14.7	12.3	2.06	0.34	1.28	0.82	36.16
CSS 8	5.46	2.65	12.88	11.79	2.07	0.35	1.17	0.89	35.64
CSS 10	5.21	2.66	13.17	11.35	1.97	0.32	1.28	0.84	37.09
CSS 13	5.47	2.74	13.88	11.91	2	0.33	1.23	0.86	36.94
CSS 14	5.79	3.09	16.63	12.82	1.87	0.3	1.28	0.85	36.56
CSS 15	5.62	3.13	16.33	12.62	1.8	0.28	1.29	0.85	35.51

5.2.4.4 Ultrastructural evaluation of Southern white rhinoceros spermatozoa by means of transmission electron microscopy (TEM)

The aim of this section was to confirm the major ultrastructural features of the spermatozoa from rhinoceros ejaculates. Furthermore, our aim was to confirm and/or describe any other abnormal sperm characteristics not visualized with bright field microscopy. The electron micrographs were representative of ejaculates collected from three bulls from two different populations. Most of the spermatozoon ultrastructural features observed were similar for all the rhinoceroses. Table 4.15 includes some of the major semen and sperm characteristics recorded of these ejaculates processed for TEM analyses.

As indicated in section 5.2.4, the spermatozoon of the Southern white rhinoceros consists of a head and flagellum (tail). The sperm head is composed of the nucleus, acrosome and post-acrosomal region (Figures 5.20 & 5.23). The nucleus was generally uniformly electron dense covered anteriorly by a well-defined acrosome (Figures 5.20 & 5.23). The posterior end of the nucleus formed a concave implantation fossa the basal plate. Immediately beneath it was the connecting piece consisting of the capitulum, the segmented columns and the proximal centriole (Figure 5.21). Extending posteriorly from the capitulum were the segmented columns with their caudal ends overlapping the tapering ends of the outer dense fibres of the flagellum (Figure 5.21). The core of the flagellum consists of the axoneme and the outer dense fibres. The axoneme consists of the characteristic “9 + 2” pattern (Figure 5.21). The outer dense fibres are surrounded by the mitochondrial gyres in the midpiece (Figures 5.20 & 5.21) and by a fibrous sheath in the principal piece of the flagellum (Figure 5.21).

In longitudinal sections of the principal piece the fibrous sheath is characterised by a series of electron dense ribs (Figure 5.21). The plasma membrane surrounds the entire spermatozoon and is usually seen loosely attached around the acrosomal region of the head and around sections of the flagellum (Figures 5.20 & 5.22) and considered a preparation artefact.

Structural abnormalities observed by TEM were the occurrence of small nuclear craters or fragmentation within the nucleus of some of the sperm heads (Figures 5.22 & 5.23). Flagellar defects included the presence of Dag-defects showed by coiling of the flagellum in the midpiece area and transverse sections of the axoneme and associated fibres all contained within a common plasma membrane (Figure 5.22), as well as proximal cytoplasmic droplets (Figure 5.23). Furthermore, apoptotic vesicles with characteristic blebbing of breakdown of cellular organelles were observed (Figures 5.24 & 5.25).

Table 5.16 Characteristics of selected semen samples from Southern white rhinoceros processed for transmission electron microscopic evaluation. Samples processed were collected from Population A (n = 2) and Population B (n = 1).

Semen characteristic of samples processed for electron microscopy									
TEM images Figure #	Rhino ID	Volume ml	Conc. [million/ml]	% Normal Morphology	% Head defect	% Midpiece defect	% Tail defect	% Acrosome intact	% Acrosomal coverage
5.23	CSS 4	37.3	4.5	74	19	2	5	82	35.91
5.25	CSS 7	18.5	7	38	6	21	11	78	36.16
5.26	CSS 13	7	255	50	26	22	2	74	36.94

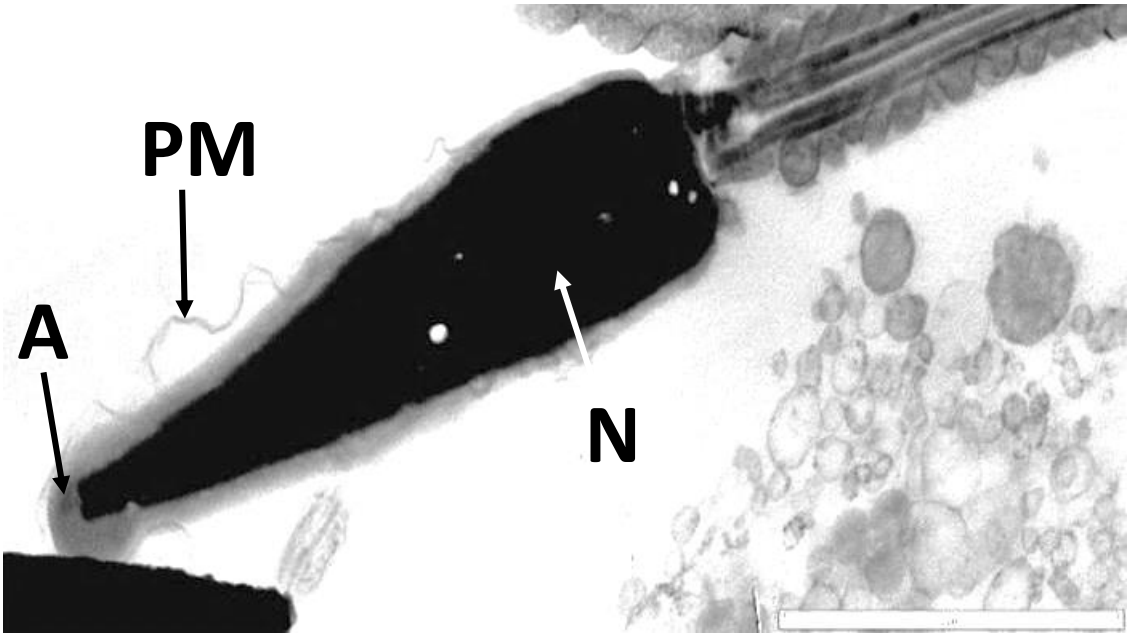

Figure 5.20 Longitudinal section showing the acrosome (A), nucleus (N) and plasma membrane (PM) of a rhinoceros spermatozoon (Scale bar = 2µm).

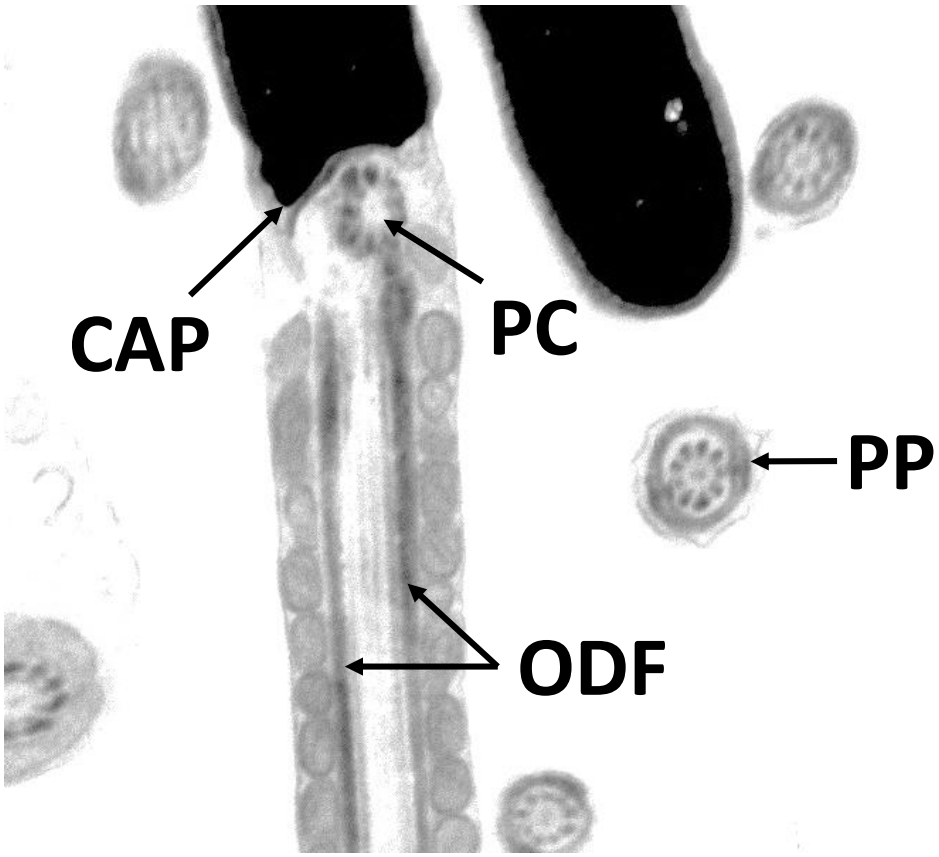

Figure 5.21 Longitudinal section showing the capitulum (CAP), the proximal centriole (PC) and the outer dense fibres (ODF) of the midpiece and a cross section showing the principal piece (PP).

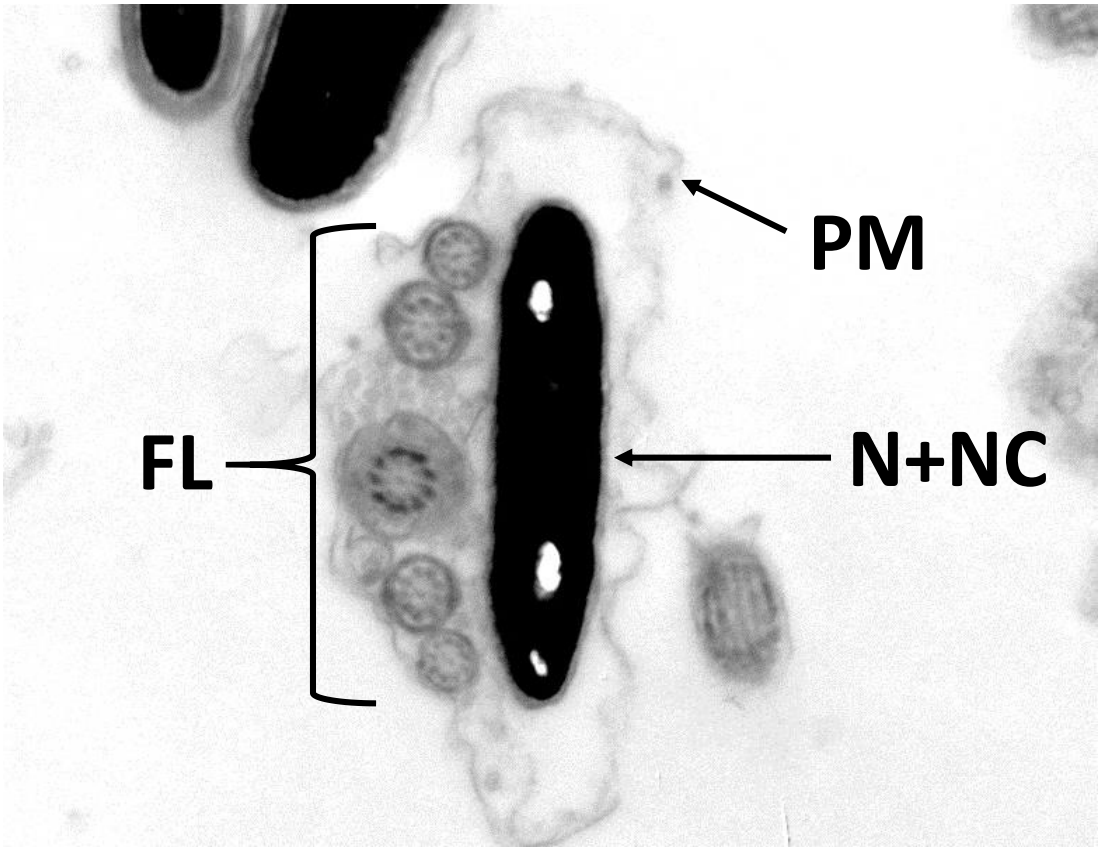

Figure 5.22 Transverse/oblique section showing the coiling of the flagellum (FL) and nucleus with the occurrence of nuclear craters (N+NC) all contained within a common plasma membrane (PM).

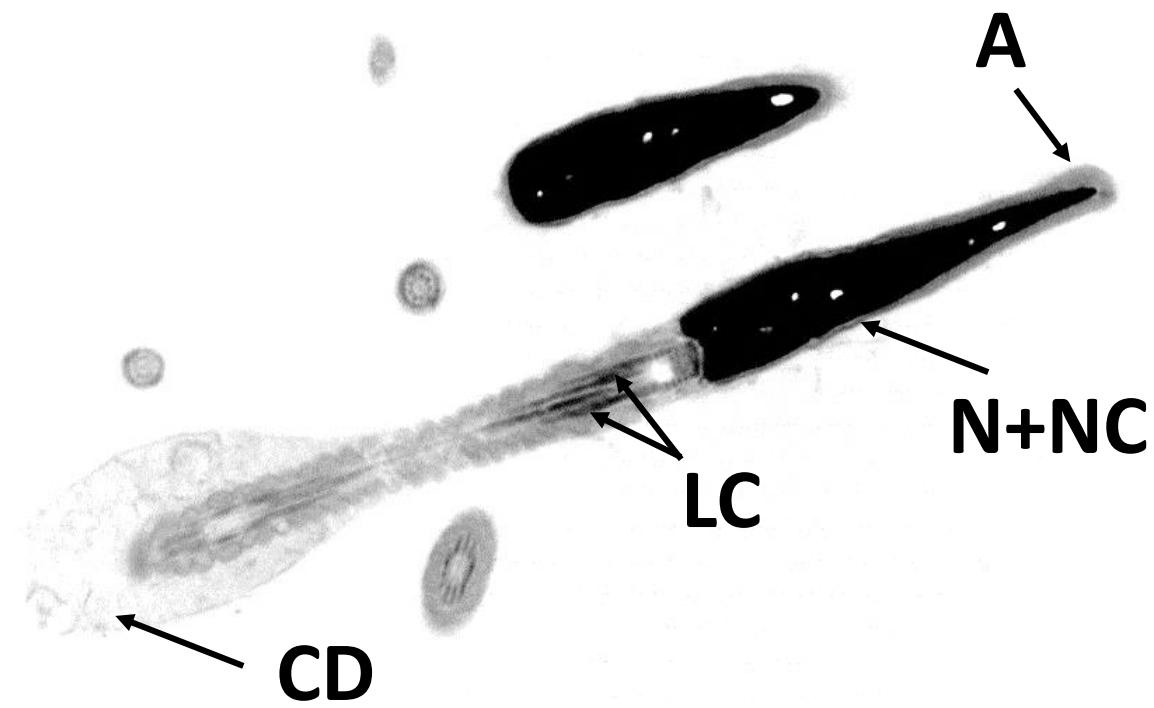

Figure 5.23 Transverse/oblique section showing the acrosome (A) and the nucleus with the occurrence of nuclear craters (N+NC), the longitudinal columns (LC) of the neck/midpiece with a distal cytoplasmic droplet (CD) attached.

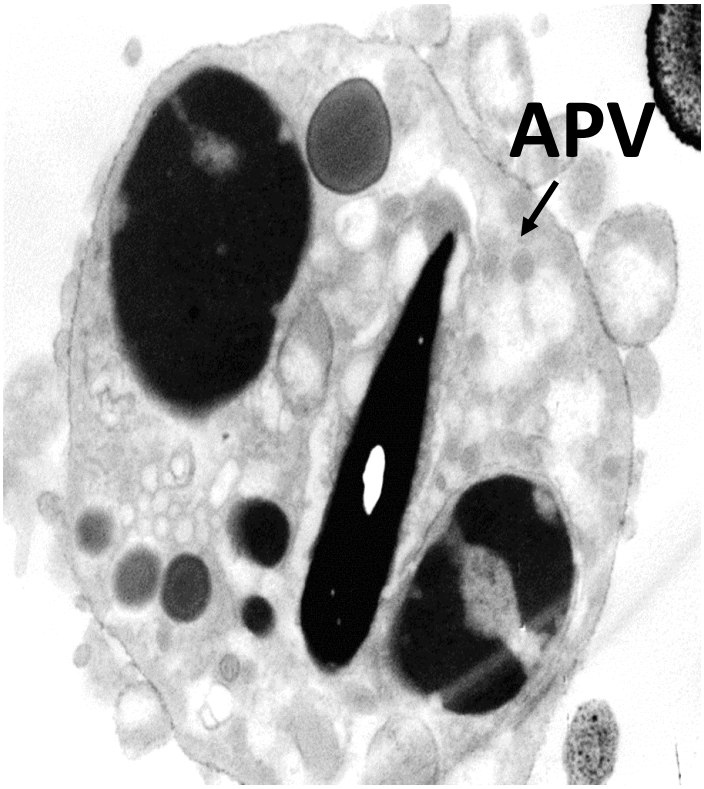

Figure 5.24 Transverse section of apoptotic vesicle (APV).

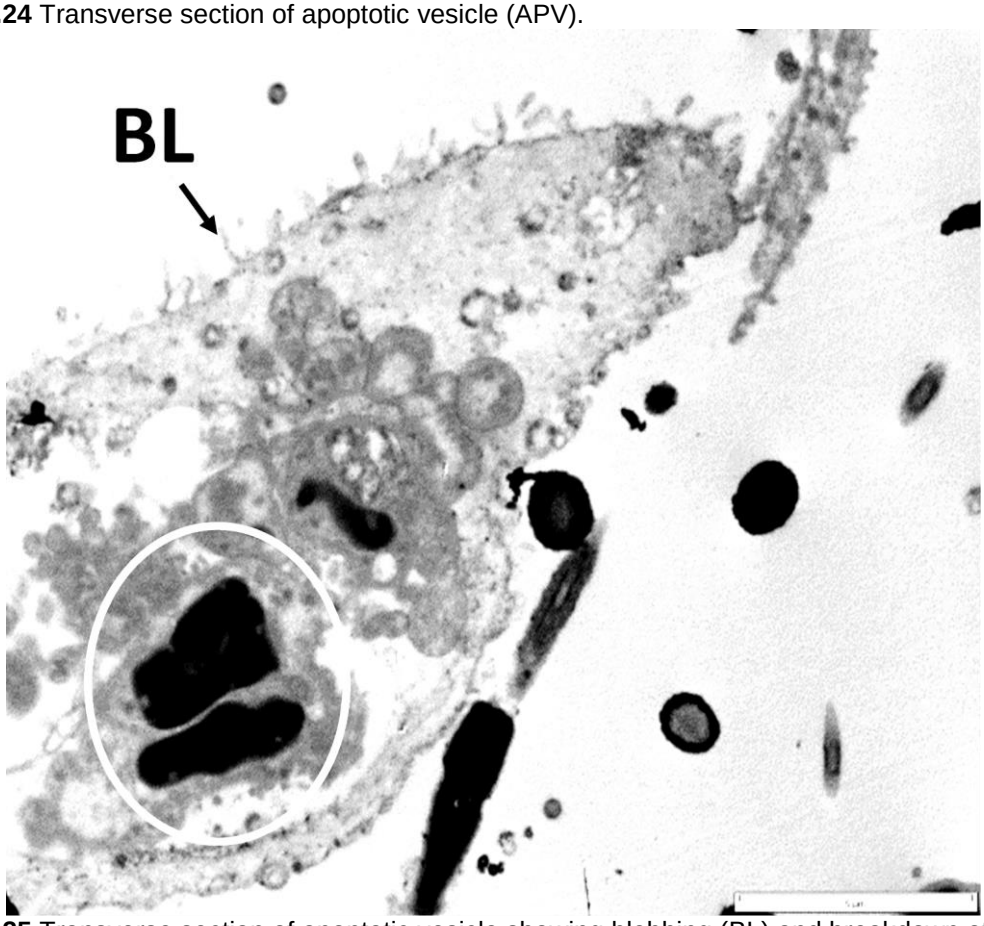

Figure 5.25 Transverse section of apoptotic vesicle showing blebbing (BL) and breakdown of cellular organelles (Scale bar = 5µm).

CHAPTER 6

GENOMICS: SEQUENCING CATSPER1 GENE FOR THE AFRICAN ELEPHANT (*Loxodonta africana)*

6.1 GENERAL INTRODUCTION

The general aim of a genomic study is to gather and provide information written within the genome of an organism and how it interacts and reacts to the environment. The publication of the human genome project in 2001 changed the approach and perspective of life science research forever (Sachidanandam, *et al.*, 2001). The massive amount of data produced from various genomic studies since the start of the human genome project in 1990, has additionally resulted in further advancements being made in the development of bioinformatics analysis methodologies and data mining strategies (Johnson & Koepfli, 2014). To date, the genomes of many organisms have been sequenced and the results annotated, augmented and refined as a result of advancement made in transcriptomics, proteomics and metabolomics that has optimised the characterization of messenger RNA, protein and metabolites (Allendorf, *et al.*, 2010; Johnson & Koepfli, 2014). The fast developing science of genomics has allowed the comparison of sequences of various species, individuals and populations that have expanded our knowledge regarding the similarities and differences amongst them (Allendorf, *et al.*, 2010; Johnson & Koepfli, 2014). Genomics has become a central part of biological sciences and is shifting the mindset towards applying new methodologies to animal breeding and conservation research approaches (Veerkamp & Beerda, 2007; Allendorf, *et al.,* 2010; Johnson & Koepfli, 2014).

Genomics can be defined as a branch of molecular genetics that focuses on the structure, function, evolution and mapping of complete genomes or the DNA sequencing of specific sets of genes within organisms (Merriam-Webster, 2014). Genomic selection allows for the improvement or maintenance of genetic diversity (Veerkamp & Beerda, 2007). Genomic selection is based on the information from the whole genome (tens of thousands SNP per individual) derived from high-throughput single

nucleotide polymorphisms (SNP) technology. The application of this technology can result in improvements of fertility through the addition and screening for individual genes during gene assisted selection (Veerkamp & Beerda, 2007; Allendorf, *et al*., 2010). Genomics combined with bioinformatics offers an exciting starting point for the development of new conservation strategies of isolated or migrated wildlife populations from a molecular point of view. However, before any of this can be realised many genomes from various individuals are required build a more precise pedigree of the captive or migrated population and to determine whether the founders from the wild are relatives (Allendorf, *et al.*, 2010).

In 2008, CatSper orthologs revealed its presence in all the mammals investigated (e.g. human, chimpanzee, dog and rat) (Cai & Clapham, 2008). Evolutionary biologists suggested that during the process of natural selection, species with a thicker zona pellucida surrounding the oocyte (e.g. mouse and human) maintained the CatSper gene functionality within male counterparts (Cai & Clapham, 2008). However, the identity of CatSper orthologs may also differ among species (Cai & Clapham, 2008; Singh & Rajender, 2014). CatSper genes have been reported in reptiles, tunicates, and echinoderms, but not within the genomes of birds, amphibians, insects, fishes, flies, worms or plants (Cai & Clapham, 2008). In the case of species (e.g. bird and fish) with a thinner zona pellucida surrounding the oocytes, it seems that the CatSper genes fundamentally became redundant (Cai & Clapham, 2008; Singh & Rajender, 2014).

6.1.1 CATSPER CHANNEL PROTEINS

The first pore-forming CatSper subunit, CatSper1 was discovered in 2001, is found exclusively in the principal piece of spermatozoa and has shown to be essential for male fertility (Ren, *et al*., 2001; Singh & Rajender, 2014). CatSper cationic channels of spermatozoa are the only calcium channels whose physiological function has been confirmed by gene manipulation (Ren, *et al*., 2001; Qi, *et al.*, 2007). The CatSper channel proteins are exclusively expressed in the testis and are localised in the

principal piece of sperm tail, Figure 6.1 A (Ren, *et al.*, 2001; Ren & Xia, 2010; Zheng, *et al*., 2013; Singh & Rajender, 2014). CatSper is a heterotetrameric calcium (Ca^{2+}) channel, composed out of four independent α (alpha) subunits known as CatSper1, CatSper2, CatSper3 and CatSper4 along with three additional auxiliary subunits: CatSper β (beta), CatSper γ (gamma) and CatSper δ (delta) (Figure 6.1 B) (Singh & Rajender, 2014).

The expression of CatSper1 is associated with the progressive motility of spermatozoa, hyperactivated motility (HA), and progesterone-induced acrosome reaction (AR) (Tamburrino, *et al.*, 2014). These four pore-forming CatSper channel proteins regulate the entry of positively charged calcium (Ca^{2+}) atoms (calcium cations) into spermatozoa (Ren, *et al*., 2001; Nikpoor, *et al*., 2004; Qi, *et al.*, 2007; Ren & Xia, 2010; Zheng, *et al.*, 2013). The rapid change in sperm motility is caused by an influx of calcium (Ho & Saurez, 2001; Suarez, 2008). The CatSper-complex channel has been shown to be essential for hyperactivated motility of spermatozoa, which is necessary for detachment of spermatozoa from the epithelium in the female reproductive tract, and for penetration of the *Cumulus oophorous* and *Zona pellucida* barrier surrounding the oocyte during fertilisation (Ho & Saurez, 2001; Qi, *et al*., 2007; Ren & Xia, 2010)**.** A number of Ca^{2+} channels are suggested to form part of the plasma membrane of mature spermatozoa (Zheng, *et al.*, 2013; Singh & Rajender, 2014; Tamburrino, *et al*., 2014). Well-established Ca^{2+} channels are significant to the key activities of a spermatozoon (Costello, *et al.*, 2009).Previous genomic studies using antibodies or nucleotide probes have labelled several Ca^{2+} permeable channels in spermatocytes or spermatozoa, including voltage-sensitive Ca^{2+} channels (CatSper and CaVs), cyclic nucleotide-gated channels and transient receptor potential channels (Nikpoor, *et al.*, 2004; Jin, *et al.*, 2007; Qi, *et al.*, 2007). Signal initiation simply requires that Ca^{2+} permeable membrane channels are open, allowing ions to flow down their electrochemical gradient (Cooper & Hausman, 2009). A functional, intact plasma membrane is therefore a pre-requisite for sperm function as it maintains the chemical gradient relevant for sperm metabolism and the ability of spermatozoa to interact with their surroundings as well as the oocyte (Rodriques-Martinez & Barth, 2007).

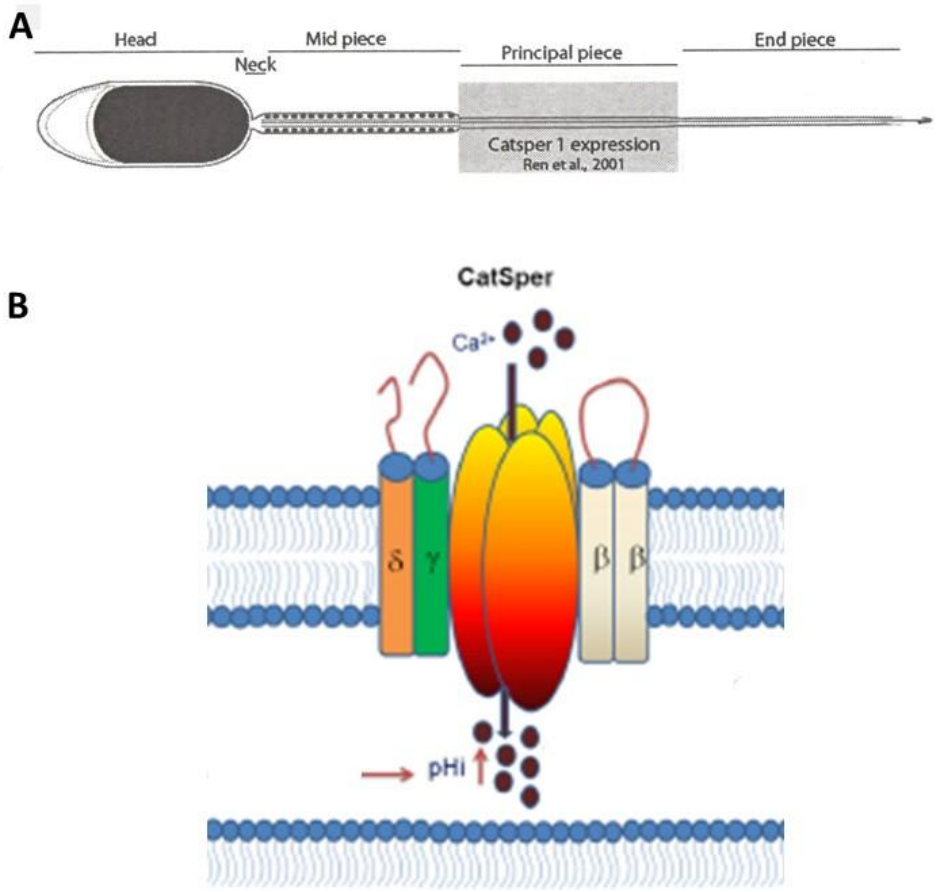

Figure 6.1: (A) The CatSper channel proteins are localized in the principal piece of sperm tail and are essential for male fertility (Avenarius, et al., 2009) **(B)** Schematic representation of the heterotetrameric Ca^{2+} channel composed of four separate α (alpha) subunits, CatSper1-4 and three additional auxiliary subunits: CatSper β (beta), CatSper γ (gamma) and CatSper δ (delta) (Singh & Rajender, 2014).

6.1.2 SPERM GENE EXPRESSION

Regulation of cellular activity occurs at a number of levels in response to the extracellular environment or signals received from other cells; however, gene expression controls long-term regulation (Costello, *et al.*, 2009; Zheng, *et al.*, 2013; Singh & Rajender, 2014). This process occurs through the control of translation and/or transcription as well as the regulation of messenger RNA (mRNA) transcripts and regulation of the protein product. Typically, these effects occur over hours (Costello, *et al.*, 2009). Regulation of cellular activity over a shorter period (minutes or seconds) is accomplished by rapid post-translational modification of functions of proteins already present. Various pathways have been

characterised by which the actions of extracellular signals such as hormones, growth hormones and transmitters transducer, lead to appropriate modification of protein function (Costello, *et al*., 2009). The inclusion of molecular genetic studies is of tremendous importance on various levels, providing a new dimension to the study of gametogenesis and the underlying mechanisms involved. For the genomic component of this study, an attempt was made to verify the presence and sequence of the CatSper1 gene for the African elephant (*Loxodonta africana*). There is enormous scope in undertaking functional genomics experiments aimed at establishing the relationship between hyperactivated motility, CatSper mutations and infertility (Singh & Rajender, 2014). Keeping species specific differences in mind, identification and functional characterization of elephant sperm ion channels would be helpful in the understanding of sperm quality that might indicate fertility or infertility. Sperm specific ion channels may be functionally active in one species, but be functionally absent in another (Singh & Rajender, 2014).

6.2 MATERIALS AND METHODS

6.2.1 BLOOD SAMPLE COLLECTION

During sedation, a minimum of 3mL blood was collected from an ear vein from each individual at the time of semen collection in an EDTA vacutainer® blood tube. Samples collected were kept at 5°C and transported to the laboratory, decanted into Nunc® cryotubes (Sigma-Aldrich, Johannesburg, S.A.), and stored at - 80°C until analyses.

6.2.2 SEMEN SAMPLE COLLECTION, HANDLING, AND TRANSPORT

Semen samples were collected from sedated elephant bulls by means of electroejaculation. After collection a 2mL aliquot of neat semen was removed and pipetted into a clearly labelled Nunc® cryotube (Sigma-Aldrich, Johannesburg, S.A.). During transport the samples were placed in a shipping box containing a water bag, heated to 35°C, in order to minimise temperature shock to spermatozoa. All the collected sperm samples were transported by means of a helicopter to the field laboratory. All sperm samples were processed within 30 minutes of collection.

6.2.3 mRNA EXTRACTION FROM COLLECTED SPERM SAMPLES UNDER FIELD CONDITIONS

Sperm samples collected from five (n = 5) elephant bulls were processed for mRNA extraction under field conditions. Upon arrival in the field laboratory, within 30 minutes after collection, sperm samples were immediately processed for mRNA extraction by removing a 500µl aliquot of neat semen which was then washed with Pure Sperm® (Highveld Biological, Kelvin, S.A.). This double density gradient technique allowed for the separation of progressively motile spermatozoa from immotile spermatozoa as well as any contaminants and debris in the collected sample. To create a density gradient, 500µl of Pure Sperm 80® (Highveld Biological, Kelvin, S.A.) was pipetted into a 2.5mL Eppendorf tube (Whitehead Scientific, Johannesburg, S.A.) and then overlaid with 500µl of Pure Sperm 40® (Highveld Biological, Kelvin, S.A.). Then the neat semen was carefully overlaid on the Pure Sperm 40® before the preparation was centrifuged at 300*g* for 10 minutes. After centrifugation, the supernatant was removed and discarded. Special care was taken not to disturb the pellet. For RNA extraction, 1mL cold Tripure RNA extraction reagent (Roche Diagnostics, Whitehead Scientific, Johannesburg, S.A.) was added to wash the cell pellet (5 – 10 x 10^6 cells) which was then homogenised on a vortex for 15 seconds. Each mL of homogenate was transferred to a separate 2mL Eppendorf tube (Whitehead Scientific, Johannesburg, S.A.) and incubated for 5 minutes at room temperature.

After incubation, 0.2mL chloroform (Merck, Johannesburg, S.A.) was added per mL of Tripure and vortexed for another 15 seconds. Thereafter the sample was incubated for 5-10 minutes at room temperature. The sample was then centrifuged at 12000*g* for 15 minutes at 4ºC, which allows separation of the solution into three phases. The colourless upper aqueous phase (350-500µl) was transferred to a 1.5mL Eppendorf tube, and 0.5mL isopropanol (Sigma-Aldrich, S.A.) was added per mL of Tripure and mixed thoroughly. Precipitation of the RNA occurred overnight at -20ºC.

The following day the sample was centrifuged at 12000*g* for 10 minutes at 4ºC and the supernatant removed. One mL of 75 – 80% ethanol (Sigma-Aldrich, Johannesburg, S.A.) was added to the RNA pellet, vortexed for 15 minutes and centrifuged at 7500*g* for 5 minutes at 4ºC. The supernatant was

removed without allowing the pellet to dry completely and re-suspended in 100 µl denuclease-free water. The suspended pellet was stored in 5-10µl aliquots at -80 ºC until further processing. The purity and yield of the total RNA were measured by means of a spectrophotometer (NanoDrop 1000, Thermo Scientific, USA.) at 260 and 230nm. All samples were screened for sample quality with a Nanodrop 2000 Spectrophotometer (Vacutec, Cape Town, S.A.). For control samples, 2µl nuclease-free H_2O was measured as blank with a measurement value of 0, measured at 260/280nm. A 2µl aliquot of each extracted RNA sample was used for Nanodrop measurements. The closer the measurement is to an optical density (OD) value of 2, the higher the extracted RNA quality (Ing, *et al*., 2014). Previous reports indicated good quality RNA integrity values of around 2.6 (Ing, *et al*., 2014).

Reverse transcription (RT) of the RNA was done using an RT kit (Promega, USA.) according to standard packaging procedure. The total RNA (1µg) was reverse transcribed to copy DNA (cDNA). All RNA samples were diluted with nuclease-free H_2O to 1µg and 1µl RNA was added to 1µl of 0.5 1µg/µl Oligo (dT) primer and then made up to a final total volume of 5µl with nuclease-free H_2O. The sample was heated to 70°C for 5 minutes in a Thermal cycler (Mastercycler®, Eppendorf, S.A.) and snap cooled on ice for 5 minutes. Five µl of sample was added to 15µl of Reverse transcription (RT) mix that consisted of 8µl Nuclease-free H_20, 4µl Transcriptase Reaction Buffer, 0.5µl Protector RNAse inhibitor, 2µl Deoxynucleotide mix and 0.5µl Transcriptor reverse transcriptase. Annealing occured at 25°C for 5 minutes and first strand synthesis at 42ºC for 60 minutes after which the reaction was held at 70°C for 15 minutes.

6.2.4 CATSPER1 PRIMER DESIGN

The CatSper 1 gene sequence, including intron and exon junctions, was predicted utilising CLC genomic workbench V6.5 (CLC Bio, Denmark), the published CatSper 1 gene sequence (derived from mRNA accession number XM_003419414) together with the African elephant genome sequence (Genome 224). The predicted gene model was then used to design primers for the amplification and

sequencing of the entire gene from genomic DNA. Primers were designed using the CLC genomic workbench primer module with maximum primer length set at 35bp, minimum primer length at 16bp, maximum melting temperature set @ 70°C and the minimum melting temperature set at 50°C. Primers were synthesised by Inqaba biotechnical industries Pty. Ltd. (S.A.) (Table 6.1).

Table 6.1 Primers designed for *Loxodonta africana* CatSper1 gene sequencing.

Primer combinations:	1. Elle-66-F and Elle-2156-Rev
	2. Elle-808-F and Elle-2955-Rev
	3. Elle-2455-F and Elle-5000-Rev
	4. Elle-3108-F and Elle-6081-Rev
	5. Elle-4868-F and Elle-7341-R
	6. Elle-5946-F and Elle-End-Rw
	7. Elle-Ex12-F and Elle-Ex12-R

6.2.5 DNA EXTRACTION AND PCR

Blood samples collected from seven African elephants listed in Table 6.2 was used during this study. Genomic DNA was extracted from 100µL blood using the ZR Genomic DNA™- Tissue MiniPrep kit (Zymo® Research) according to the manufacturer's instructions. Resulting DNA was measured using a spectrophotometer (NanoDrop 1000, Thermo Scientific, USA) and if required the concentration was adjusted to 50ng/µl with nuclease free water (Thermo Scientific, USA). Polymerase chain reaction (PCR) amplification was performed using DreamTaq® (Thermo Scientific, S.A.) in a Thermal cycler (Mastercycler®, Eppendorf, S.A.). Primer combination used for each PCR is given in Table 6.1. The amplification conditions for initial denaturation were 95°C for 5 minutes, followed by 45 cycles at 95°C for 30 seconds, 53°C for 30 seconds and 72°C for 3 seconds, with a final extension of 72°C for 10 minutes. Thereafter PCR products were kept at 4°C.

The PCR products were separated on a 1% agarose gel (containing 0.5µg/ µl Ethidium bromide) by gel electrophoresis running at 100V for 25 minutes. To visualise the fragments, UV trans-illumination was used. A positive result was indicated by a fragment of the appropriate base pair (bp) size visible on the gel in comparison with molecular size standard.

Table 6.2 List of seven African elephant blood samples sequenced during analyses. Information includes whether semen samples were collected as well as the total percentage hyperactive motility (HA) recorded.

#	Sample ID	Elephant ID	Name		Total HA* motility (%)
1	13999	LA 5a	Mbondwe	No semen samples collected, major pathology	-
2	14002	LA 8a/ LA 1b/ LA 8b	Righthook	Semen samples collected	24%
3	14003	LA 9a	Looney	Semen samples collected, during season 1	-
4	14004	LA 2b/ La 10b	Bump	Semen samples collected	46%
5	14005	LA 14b	Rex	Semen samples collected	13%
6	15293	LA 1b/ LA 9b	Young 1	Semen samples collected	21%
7	15294	LA 7b	Smiley	No semen samples collected	-
*HA = Hyperactive motility					
a = Season 1, September 2009					
b = Season 2, April 2010					

6.2.6 SEQUENCING CATSPER1 GENE FOR THE AFRICAN ELEPHANT

Sanger sequencing of the resulting amplicons was conducted by Inqaba biotechnical industries PTY. Ltd (S.A.) utilising Big Dye® version 3.1 (Applied Biosystem, C.A.) and the ABI 3500 XL Genetic Analyser (Applied Biosystem, C.A.). The primers used for sequencing are listed in Table 6.3.

Table 6.3 Primers utilised to sequence the CatSper1 gene for *Loxodonta africana.*

Primer Name	Size of the amplified fragment
Elle-3445	GTCTCTGGGTGGCTGTTT
Int 6541-Reverse	CTGGGTGAATTCAAACCACC
Int 3851 Forward	GTAGTCACATAGAGCAG
Int 981 Reverse	GGTATTCACTATCAGTGTG

6.3 RESULTS

6.3.1 mRNA EXTRACTION FROM COLLECTED SEMEN SAMPLES UNDER FIELD CONDITIONS

An attempt to extract pure and good quality RNA from the 500µL aliquot of the neat semen samples proved to be unsuccessful under field conditions. Nanodrop measurements recorded of the total RNA yield of each ejaculate processed are listed in Table 6.4. The total RNA yield was transcribed to cDNA, and then PCR was performed using the designed primers, thereafter the PCR product was loaded on a gel for visualisation, and then sequenced.

Unfortunately, sequencing of these PCR products was unsuccessful. The quality of mRNA extracted from the collected semen samples was suboptimal and no results were obtained. Rapid degradation of the RNA was suspected as a result of the field extraction method used during this study.

Table 6.4 Semen quality parameters recorded for five African elephant semen samples including total RNA concentration and quality measurement (OD) values.

Elephant ID	Name	Volume (mL)	Concentration ($x10^6$/ml)	Tot. Motile (%)	Prog. Motile (%)	Non-prog. Motile (%)	Total HA* motility (%)	Normal Morphology (%)	Nanodrop RNA (ng/µl)	OD 260/230
LA 1b	Righthook	227	80	98	74	24	8	65	98.3	1.5
LA 5b	Young 1	57	2025	95	68	27	13,5	-	439.8	1.74
LA 6b	No 3	68	472	100	80	20	43,6	60	335.8	1.75
LA 8b	Righthook	67	712	99	72	27	24,4	37	140.5	1.62
LA 12b	No Name	55	1815	91	78	13	20,6	56	410.5	1.54

*HA = Hyperactive motility

6.3.2 DNA EXTRACTED FROM WHOLE BLOOD FOR CATSPER1 GENE SEQUENCING

DNA extracted from whole blood was used to produce a PCR product that could be visualised in gel images (Figure 6.2). The gel image contains the molecular size standard (Fast Ruler® (R), Thermo Scientific, S.A.) representing from top to bottom; 5000; 2000; 850; 400; 100 base pairs (bp) sizes, as well as three samples and a negative control sample. Each sample group comprises, 1) Mbondwe (LA 5 A, 13999) 2) Righthook (LA 8a, 1b and LA 8b, 14002) and 3) Looney (LA 9a, 14003). Visualised on the gel image are the primer combinations used (Table 6.1). C represents the negative control in each group.

The entire CatSper1 gene (8357 bp) sequencing was achieved in this study by overlapping Sanger DNA sequences. Primer sets 1-6, as listed in Table 6.1, were used to amplify exons 1 – 10 and primer set 7 was used to amplify exon 11 and 12.

The entire CatSper 1 gene was sequenced for seven individuals (listed in Table 6.2). All seven sequences were aligned with one another utilising CLC genomic workbench V6.5 (CLC Bio, Denmark) to screen for potential variation between individuals. These sequencing results are depicted in Figure 6.3. Five single nucleotide polymorphisms (SNP) or single gene mutations (change in base pair positions) were recorded in exon 1, exon 6, exon 9 and 11 of the sequence from LA 7b (15294). The base pair change consisted of a cytosine/ thymine (C/ T) in the intron between exon 10 and 11. There were no mutations recorded for the other six individuals.

The sequence of the CatSper1 gene for *Loxodonta africana* was submitted to NCBI and accepted for publication on the Genbank database (Table 6.5 and Appendix 1).

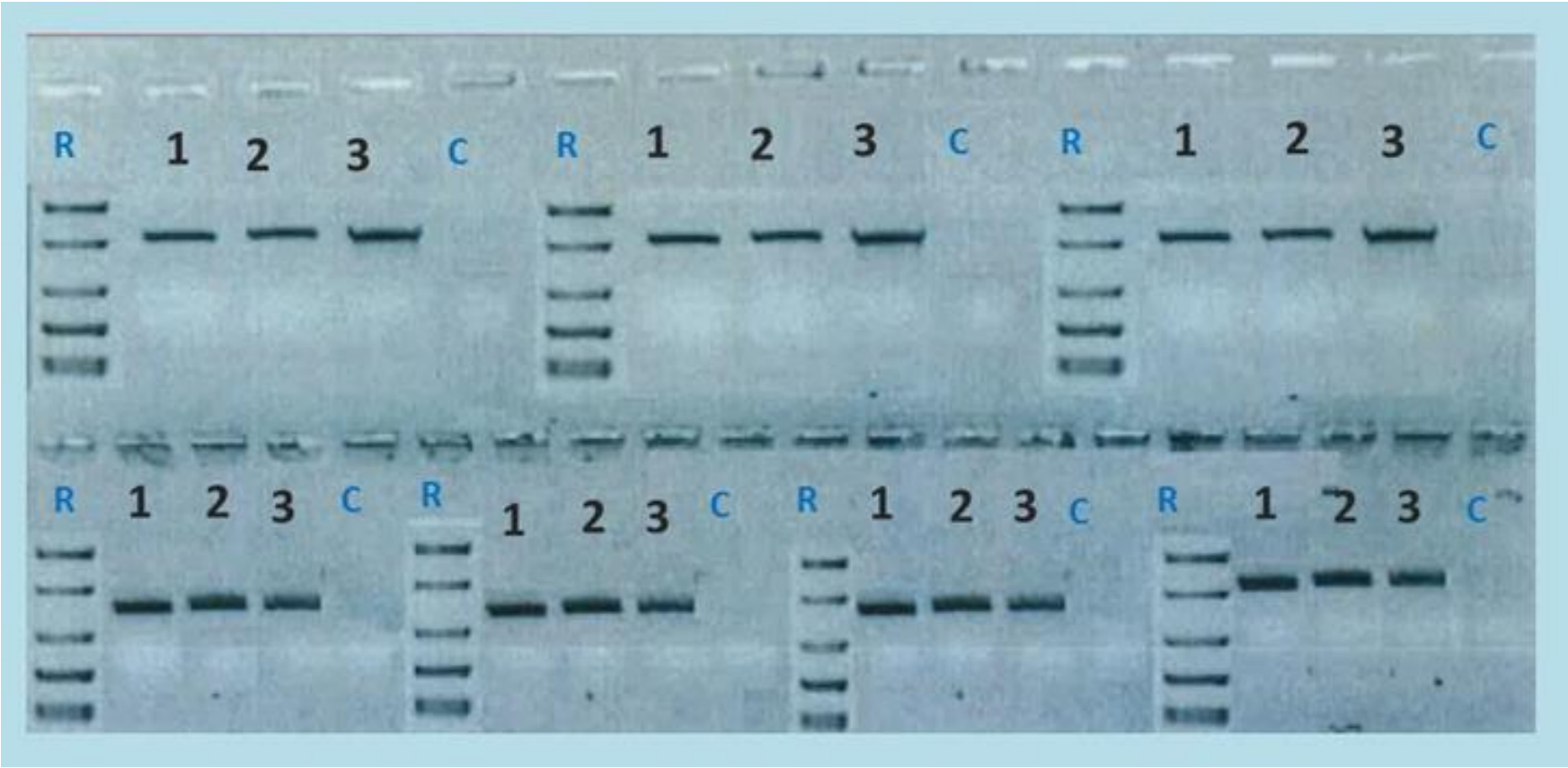

Figure 6.2: This gel image represents the PCR product of samples 1) Mbondwe 2) Righthook and 3) Looney (R = ruler and C = negative control sample) for seven sets of primer combinations used to cover exons 1 – 12 of CatSper1 gene for *Loxodonta africana*.

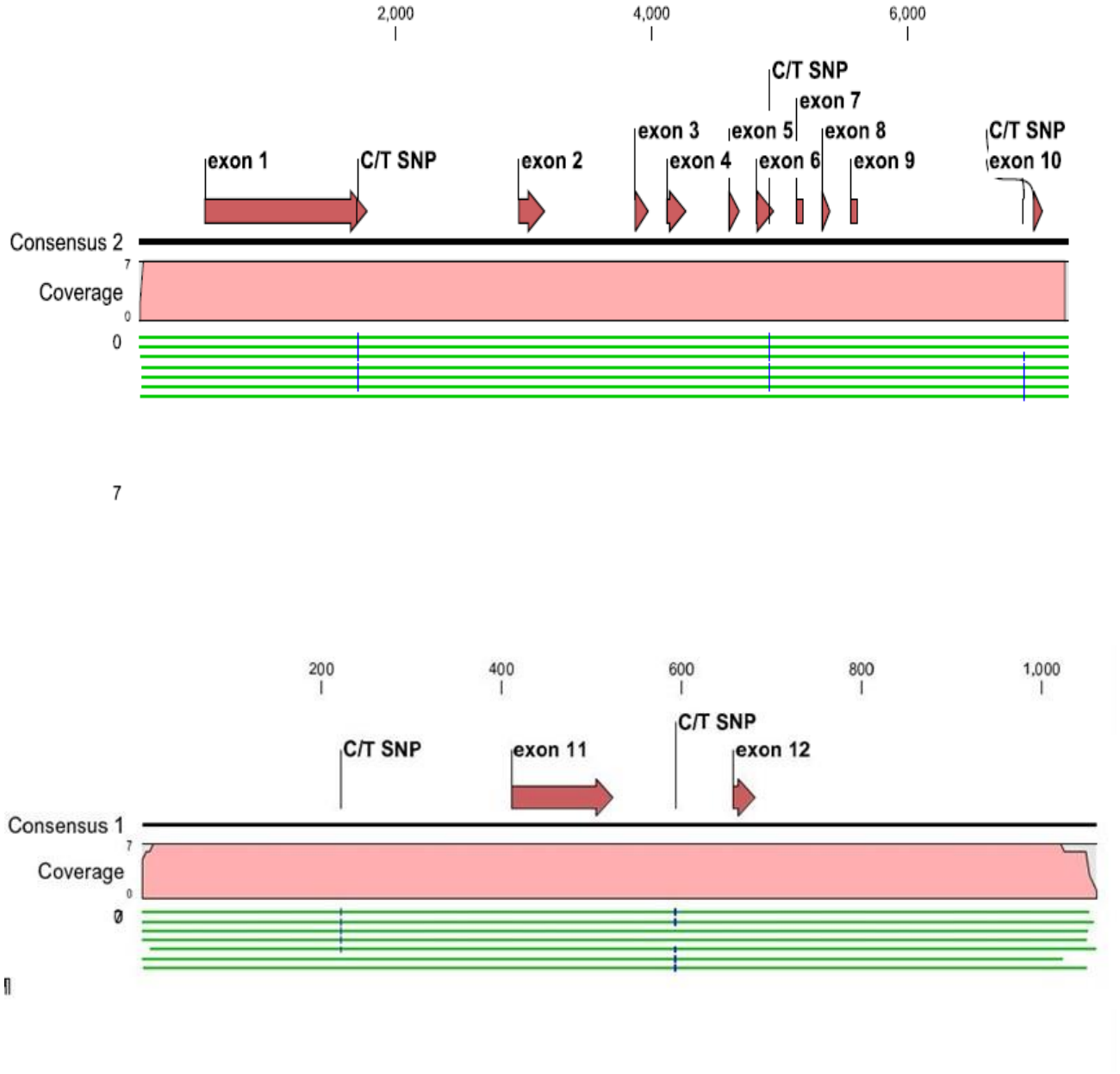

Figure 6.3: Sequence alignment depicting the entire CatSper 1 gene for seven African elephants, with annotations indicating exons as well as 5 single nucleotide polymorphisms (SNP's) and their locations.

Table 6.5 CatSper1 (*Loxodonta africana*) genome sequence accession number per isolate (individual) and information available from the U.S. National Centre for Biotechnology (NCIB).

1	NCBI KR 856273	>Seq1 [organism=*Loxodonta africana*] [isolate=13999] [dev-stage=adult, 26 years] [note=free-ranging animal] [sex=male] [country=South Africa: Kwazulu-Natal, Phinda Private Game Reserve] [lat-lon= 27.95S 32.60E] cation channel, sperm associated 1 (CATSPER1) gene
2	NCBI KR 856274	>Seq2 [organism=*Loxodonta africana*] [isolate=14002] [dev-stage=adult, 34 years] [note=free-ranging animal] [sex=male] [country=South Africa: Kwazulu-Natal, Phinda Private Game Reserve] [lat-lon=27.95S 32.60E] cation channel, sperm associated 1 (CATSPER1) gene
3	NCBI KR 856275	>Seq3 [organism=*Loxodonta africana*] [isolate=14003] [dev-stage=adult, 26 years] [note=free-ranging animal] [sex=male] [country=South Africa: Kwazulu-Natal, Phinda Private Game Reserve] [lat-lon=27.95S 32.60E] cation channel, sperm associated 1 (CATSPER1) gene
4	NCBI KR 856276	>Seq4 [organism=*Loxodonta africana*] [isolate=14004] [dev-stage=adult, 32 years] [note=free-ranging animal] [sex=male] [country=South Africa: Kwazulu-Natal, Phinda Private Game Reserve] [lat-lon=27.95S 32.60E] cation channel, sperm associated 1 (CATSPER1) gene
5	NCBI KR 856277	>Seq5 [organism=*Loxodonta africana*] [isolate=14005] [dev-stage=adult, 25 years] [note=free-ranging animal] [sex=male] [country=South Africa: Kwazulu-Natal, Phinda Private Game Reserve] [lat-lon=27.95S 32.60E] cation channel, sperm associated 1 (CATSPER1) gene

6	NCBI KR 856278	>Seq6 [organism=*Loxodonta africana*] [isolate=15293] [dev-stage=adult, unknown years] [note=free-ranging animal] [sex=male] [country=South Africa: Kwazulu-Natal, Phinda Private Game Reserve] [lat-lon=27.95S 32.60E] cation channel, sperm associated 1 (CATSPER1) gene
7	NCBI KR 856279	>Seq7 [organism=*Loxodonta africana]* [isolate=15294] [dev-stage=adult, 24 years] [note=free-ranging animal] [sex=male] [country=South Africa: Kwazulu-Natal, Phinda Private Game Reserve] [lat-lon=27.95S 32.60E] cation channel, sperm associated 1 (CATSPER1) gene

6.4 DISCUSSION

During this investigation the gene sequence of CatSper1 (*Loxodonta africana)* for free-ranging African elephant bulls was identified, sequenced and annotated, the data derived from sequencing obtained during this study was submitted to a public database NCBI. The presence of the CatSper1 (*Loxodonta africana)* gene subsequently verifies the presence of the sperm specific CatSper ion channel, located in the principal piece of the African elephant sperm flagellum.

The presence of the ion channel is further implied by the rapid response of the African elephant sperm populations after five-minute incubation in BO media containing caffeine, used to induce hyperactivated (HA) motility in spermatozoa during this study. The rapid response and change from linear to HA motion patterns ranged from 4% to 5% in Ham's F10 medium compared to 20% - 29% in the BO (caffeine) medium in the samples analysed. The physiological change that occurs during hyperactivation is an important endpoint of capacitation and together with the acrosome reaction, are vital for a spermatozoon to acquire fertilisation potential (Mortimer, *et al*., 1998). It is widely accepted that calcium is a requirement for hyperactivated motility (Ren & Xia, 2010; Hinrichs & Louw, 2012).

Furthermore, the individual Catsper1 gene sequences obtained during this study were aligned and 5 SNP mutations encountered in one of seven free-ranging African elephant individuals screened. Currently, the CatSper ion channel is recognised as the most important calcium channel required for the fertility of male mammals (Zheng, *et al.*, 2013; Singh & Rajender, 2014). Insights from knock-out studies have demonstrated that the sperm specific ion channel, CatSper, may cause infertility in man without affecting normal sperm production (Singh & Rajender, 2014).

6.4.1 SPERMATOGENIC RNA EXTRACTION

Unfortunateno success was achieved in an attempt to extract RNA from sperm samples collected under field conditions. The extraction of good quality RNA in other than optimal conditions proved to be difficult. RNA is known to be very sensitive to degradation or RNase activity, and the field processing methodologies should be adapted accordingly to limit the rate of RNA degradation during sampling handling and extraction. The RNA field extracted method used during this study is directly related to the quality of the RNA yield obtained. Possible factors include the time frame of transport to the laboratory, the equipment and disposables used (was not dedicated to RNA use only), as well as the ambient temperature (during South African spring and autumn months) in the uncontrolled environment of the field laboratory. Even though stringent clinical cleanliness and sterility were applied and the environmental conditions controlled as much as possible, mimicking true RNA laboratory conditions was not achieved in the field during this study.

A previous study has suggested that a good quality yield of RNA should record an optical density (OD) reading of approximately 2.6 (Ing, *et al*., 2014). The highest OD value recorded in this study was 1.75 in the sample collected from LA 5b (Young 1) that reflected 95% total motility with a sperm concentration of 2 x 10^9/ml and 56 % morphologically normal spermatozoa (Table 4.1 & 4.11). A study in 2014 reported on the successful yield of good quality RNA extracted from horse ejaculates (Ing, *et al.*, 2014). The purified spermatozoa were immediately snap-frozen in liquid nitrogen before being stored at -80°C until further processing (Ing, *et al.*, 2014). Spermatozoa have a greater ratio of nuclear to cytoplasmic volume and genomic DNA to RNA when compared to somatic cells; therefore contamination of RNA preparations with genomic DNA can be a concern (Ing, *et al.*, 2014). Further optimisation of this protocol would be the inclusion of RNase inactivators for RNA preservation before samples are snap-frozen in liquid nitrogen for extraction under RNA laboratory conditions.

6.4.2 GENOMICS DNA AND CATSPER1 GENE SEQUENCING

The initial approach aimed to sequence CatSper1 by overlapping sequences obtained from both genomic DNA (blood samples) and cDNA transcribed from mRNA (sperm samples). Since mRNA isolation from sperm was problematic, the information for CatSper1 sequencing was derived from genomic DNA. Fortunately, high-quality genomic DNA was extracted from the collected EDTA whole blood samples. DNA is not as sensitive to degradation as RNA; however, great care was taken in the collection, transportation and processing of the blood samples to ensure that sample integrity was maintained.

Five single nucleotide polymorphisms (SNP) or single gene mutations (change in base pair positions) were recorded in exon 1, exon 6, exon 9 and 11 of the sequence from LA 7b (15294 - Smiley). The base pair change consisted of a cytosine/ thymine (C/ T) in the intron between exon 10 and 11. There were no mutations recorded for the other six individuals. The SNP's were located in an intron and exons. However, this work was done with genomic DNA where gene exons and introns are unprocessed, compared to mRNA where the exons are processed and ready for protein synthesis. Disruption of CatSper genes results in a significant reduction in specific motility parameters (Ren, *et al.*, 2001). Computer-aided sperm analysis (CASA) shows that parameters such as path velocity, progressive motility, and track speed are significantly compromised in mutant sperm (Ren, *et al.*, 2001). Spermatozoa from all African elephant samples sequenced recorded high values for these CASA kinematic parameters. Unfortunately, the ejaculate collected from LA 7b consisted only of seminal plasma and contained no spermatozoa in any of the fractions (azoospermia).

However, a lack of spermatozoa in the fractions collected for LA 7b could be due to technical issues (unsuccessful stimulation during electroejaculation) and may not reflect biological differences. Mutations in CatSper1 and 2 have been identified in infertile individuals (man and mice); however,

CatSper3 and 4 have not been further investigated (Singh & Rajender, 2014). Since the CatSper channel is regulated by changes in intracellular pH, all four of these subunits are required for alkalinization-activation to induce hyperactivation (HA) (Jin, *et al.*, 2007; Qi, *et al*., 2007; Ren & Xia, 2010; Chenoweth & Kastelic, 2007). CatSper mutant sperm are unable to move beyond the oviductal sperm reservoir and can maintain initial motility consisting only of symmetrical flagellar beats. Any mutations (genetic disruptions) within any of the four pore-forming CatSper subunits may results in infertility (Ho & Saurez, 2001; Singh & Rajender, 2014). The absence of HA motility affects the physiological adaptability of a spermatozoon within the female reproductive tract. If a spermatozoon fails to undergo the process of hyperactivation, it will fail to pass through the viscous fluid within the oviduct or to penetrate the *Cumulus oophorous* and *Zona pellucida* barriers that surround the oocyte (Ren & Xia, 2010; Singh & Rajender, 2014). Currently, CatSper is recognised as the most important calcium channel required for mammalian male fertility (Zheng, *et al*., 2013; Singh & Rajender, 2014). Besides responding to pH change, the CatSper channel complex may additionally mediate other important sperm function regulators (Zheng, *et al*., 2013).

6.4.3 CONCLUSION

To study the molecular processes underlying hyperactivation, it is ideal to verify and sequence the complete CatSper gene complex. Further studies of CatSper 1-4, including the subunits, are needed to determine the physiological basis that controls hyperactivated motility in both elephant and rhinoceros spermatozoa. Future studies should include improving field sperm samples processing methodologies to ensure the quality of the sample. The availability of the primers (Table 6.1 and 6.3) as well as CatSper1 sequence data provided by this study may aid future CatSper gene sequencing studies. The future addition of real-time PCR may assist to quantify the relationship between the percentage of hyperactivated motility and the actual expression of the CatSper1 gene within ejaculates collected from various individuals and between populations (*in situ* and *ex situ*).

The functional importance of this CatSper complex ion channel for sperm motility and male fertility is highlighted for further research on elephant fertility and infertility. The importance of genetic maintenance or improvement is obvious during conservation efforts of wildlife species. The inclusion of genetic studies is of tremendous importance on different levels as they offer a new alternative to study gametogenesis and the underlying mechanisms involved, in turn not only allowing the identification of genes involved in spermatogenesis, but also the attendant risk factors (Inati, *et al.*, 2012).

Genomic tools may assist in the management of *ex-situ* populations and reintroductions by providing increased precision and accuracy of estimates of neutral population genetic parameters and by identifying specific genes of importance, which is essential for selecting founder individuals (Veerkamp & Beerda, 2007; Allendorf, *et al.,* 2010). Genomics will make it possible to provide estimates of functional genetic variation and fitness of individuals and populations based on data mining genome information available from individuals, populations and species (Allendorf, *et al*., 2010). The inclusion of genetic screening allows for the identification of individual bulls for any mutations that might occur within the CatSper gene family that might affect sperm functionality.

CHAPTER 7

MULTI-VARIATE ANALYSIS AS A TOOL TO INVESTIGATE POTENTIAL RELATIONSHIPS AMONG RECORDED SEMEN AND SPERM CHARACTERISTICS OF THE AFRICAN ELEPHANT (*Loxodonta africana)*

7.1 INTRODUCTION

Male fertility is a complex subject. The theory behind estimating male fertility is based on using the assessment of semen and sperm parameters to define the probable potential of fertilisation to occur, as well as the relative fecundity of the male population (Salisbury, *et al.*, 1978). However, semen quality and fertility are not synonymous (Holt & Van Look, 2004; Rodriques-Martinez & Barth, 2007). Furthermore, ejaculates of mammals are heterogeneous and none of the spermatozoa are equal neither in terms of haploid information nor in attributes for fertilisation (Holt & Van Look, 2004; Rodriques-Martinez & Barth, 2007; Holt, *et al.*, 2007). Holt (2005) suggested that future investigations need to concentrate on interpreting the heterogeneity and subpopulation structures of ejaculates and that data should be expressed not as simple measures such as 'percentage normal forms' but as proportions of sperm with the multiple attributes needed for fertilisation (Holt, 2005).

Assuming that the functionality of an individual spermatozoon depends ultimately on the reliability of all steps during spermatogenesis and sperm maturation, there are many possible aspects that might influence its future fertilising ability (Holt, 2005). A spermatozoon is expected to contain quality DNA to support embryonic development, a functional delivery mechanism (flagellum, mitochondria, acrosome, etc.), and its surface characteristics must not attract phagocytes from within the female reproductive tract (Holt, 2005). Additionally, spermatozoa are laden with molecular receptors to which they must respond appropriately (Meizel, 2004 cited by Holt, 2005).

Laboratory assessment techniques should include the analysis of several sperm attributes that are relevant to both fertilisation potential and normal embryo development. Currently, an array of evaluation methods are available, from simple visual assessment to detailed molecular analysis, but no single useful method is able to determine sperm fertility (Gordon, 2003). During semen and sperm evaluation, a series of analytical techniques are applied in an attempt to determine which parameters will best describe the semen and sperm as an indication of quality. Additionally, the identification of possible relationships between the measured parameters could assist in determining the fertilisation potential of the sperm population within the evaluated semen sample (Holt & Van Look, 2004; Miro, *et al.*, 2005).

Manual semen/sperm evaluation methods can often provide an estimate of the quality of a sperm sample (Abaiger, et al., 2001; Holt & Van Look, 2004; Rodriques-Martinez & Barth, 2007). It is clear that computer-aided sperm analysis (CASA) technology provides sophisticated and quantitative analysis regarding sperm motility and kinematic parameters, as well as sperm morphometric. However, CASA data can have even more significance when considering the behaviour of individual spermatozoa (Abaigar, *et al.*, 1999; Abaiger, *et al*., 2001; Holt & Van Look, 2004; Miro, *et al*., 2005; Holt, *et al.*, 2007; Maree & Van der Horst, 2013). The combination of CASA and multivariate statistical analysis may yield more comprehensive biological information (Abaigar, *et al*., 1999; Holt & Van Look, 2004) and will be further elaborated on in this chapter. In this context, exploratory factor analysis (EFA) is one of the several multivariate statistical methods applied to uncover the relationships of a relatively large data set consisting of various parameters. EFA is applied when attempting to develop a scale or a collection of questions or methods used to measure a particular research topic. In the case of this study, to investigate the potential relationship between semen and sperm characteristics in ejaculates collected from free-ranging African elephants.

Methods to analyse and quantify ejaculate heterogeneity (i.e. within-male variability or within-population variability) allow the analyses of complex relations between sperm motility, sperm velocity, sperm morphology and sperm integrity. These factors could possibly be utilised to indicate sperm quality that can be directly correlated to fertility potential (Abaigar, *et al.*, 1999; Cancel, *et al*., 2000; Holt & Van Look, 2004; Holt, *et al*., 2007; Abaigar, *et al.*, 2011). The ideal is the identification of readily, measurable sperm parameters that positively correlate with fertilisation potential. If a series of functional tests were available to distinguish and quantify spermatozoa within an ejaculate that are actually capable of fertilisation, the value of reproductive biology investigations would most likely be transformed (Holt, 2005; Abaigar, *et al.*, 2011). It has been suggested that it may be possible to define and suggest a minimum set of parameters that can be applied to evaluate for maximum functional coverage (Holt, 2005; Abaigar, *et al.*, 2011).

The main aim of the current chapter was to determine possible correlations or relationships that exist among semen and sperm parameters (volume, sperm concentration, motility, kinematics, hyperactivation, sperm head morphometric, sperm morphology, viability and acrosome integrity) in order to assess the quality of ejaculates collected from the same population of free-ranging African elephant bulls over two seasons.

7.2. MATERIALS AND METHODS

7.2.1 DATA INCLUDED FOR ANALYSIS

Due to restrictions of the analytical methods only the African elephant's dataset presented in chapter 4 was used to perform various multivariate statistical analysis during this study. The datasets presented in the current chapter originated from 16 ejaculates collected from the same population of free-ranging African elephants during two different seasons. The absolute and relative values of semen volume and sperm concentration (Table 4.1), motility and kinematic values (Table 4.2), percentage hyperactivated motility (Table 4.8), vitality and acrosome integrity (Table 4.9), sperm

morphology (Table 4.11), and sperm head morphometric parameters (Table 4.13) wereincluded in the statistical analysis. Due to constraints indicated by the conditions in the field and the availability of sufficient samples, not all assessments were conducted simultaneously on all samples in true split-sample fashion. Three datasets containing different scenarios of parameters were analysed: Dataset 1: Parameters (n=13) with no missing data from 16 ejaculates, Dataset 2: Parameters (n=26) with missing data from 11 ejaculates, and Dataset 3: All parameters (n=29) with no missing data from six ejaculates. This resulted in complete and incomplete datasets used for multivariate statistical analyses.

7.2.2 STATISTICAL ANALYSIS

All statistical analyses, as discussed below, were performed using the Statistica® data analysis software system, version 10 (StatSoft Inc.).

7.2.2.1 Random Correlations

The relationships between 31 semen and sperm parameters were determined by making use of Pearson's ($p < 0.05$, parametric data) and Spearman ($p < 0.05$, non-parametric data) rank correlation coefficient (r) analyses. These parameters included ejaculate volume, sperm concentration, total motility (Tot. motile), progressive motility (Prog. motile), non-progressive motility (Non-prog motile), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), normal morphology, abnormal morphology, head defects, midpiece defects, tail defects, acrosome intact, acrosome reacted, viability intact (LIVE), viability lost (DEAD), head length, head width, head area, head perimeter, head ellipticity, head elongation, head roughness, head regularity and percentage acrosome coverage.

7.2.2.2. Exploratory Factor Analysis (EFA):

EFA is based on the common factor model that includes 1) function of common factors, 2) unique factors and 3) errors extracted from measured parameters. Common factors influence two or more measured parameters while each unique factor influences only one measured variable and it does not explain the correlations amongst the measured semen and sperm parameters. EFA assumes that any indicator/ measured semen or sperm parameter may be associated with any factor that it is measured against.

a) Factor Selection for EFA:

When selecting how many factors to include in a model it is important to balance 1) caution, a model with relatively few factors and 2) plausibility, ensuring that there are enough factors that adequately account for variance and correlations amongst measured semen and sperm parameters of the individual ejaculates collected. There are a number of procedures designed to determine the optimal number of factors to make available in EFA, including the eigenvalue-greater-than-one rule (or K1-rule). The eigenvalue represents the amount of variance of parameters accounted for by that factor. The lower the eigenvalue, the less that factor contributes to the explanation of variances in the parameters.

b) Parallel Analysis (PA):

A PA test determines the eigenvalues for the correlation matrix and plots the values from largest to smallest against a random set of eigenvalues. The number of eigenvalues before/above the intersection points of the line graphs indicates how many factors to include in the analysis model. This procedure is somewhat arbitrary as a factor just meeting the cut-off will be included, and one just below excluded. As a result, this method is very sensitive to sample size, thus for PA many datasets with larger sample sizes is suggested.

c) Factor Interpretation:

Factor loadings are numerical values that indicate strength and direction of a factor on a measured variable. Factor loadings indicate how strongly the factor influences the measured variable. In order to label the factors in a model, the factor pattern is examined to see which parameters load highly on which factors and determine what they have in common. Whatever the parameters have in common will indicate the meaning of the particular factor.

d) Biplot:

Principal component analysis (PCA) biplots were constructed for the three datasets created during analyses of the African elephant ejaculates. Biplots are a type of exploratory graph used in statistics that allows information on both individuals (ejaculates) and parameters (semen and sperm parameters) of the data matrix to be displayed graphically. Biplots determine the distribution of the selected semen and sperm parameters from the different ejaculates collected as well as an indication of the relationship among these parameters.

In the biplots constructed for this study, ejaculates are displayed as points while parameters are displayed as linear axes. The arrangements of the points in the biplot result in the recognition of ejaculates containing similar values for the parameters portrayed. The angles between the projected axes in the biplot are an indication (not exact estimation) of the relationships among different parameters. For example, smaller angles between axes imply higher correlation. The direction of the axis is also an indication of whether two parameters are positively (axes point in the same direction) or negatively (axes point in opposite direction) correlated.

7.3 RESULTS

7.3.1 RANDOM CORRELATIONS

During random correlation coefficient analysis, a total number of 465 different correlations were found among the various motility, kinematic, vitality, morphology and morphometric parameters analysed. The p-values obtained using the Pearson's ($p < 0.05$, parametric data) and Spearman ($p < 0.05$, non-parametric data) analyses highlighted various parameters with a strong tendency to correlate. Ninety-one (20 %) significant correlations were categorised among the parameters. All correlations between semen and sperm parameters, either negative or positive, are listed in Table 7.1.

The volume of the ejaculates collected during this study indicated a significant positive (+) association with VCL and BCF, whereas for sperm concentration a significant negative (-) correlation was found with tail defects, head length, head width, head area, and head perimeter. Amongst the significant correlations, the progressive sperm motility parameters, VCL, VSL, VAP, LIN, STR and BCF were positively (+) associated with sperm head morphometric parameters such as head regularity, head perimeter, head elongation, head length and head area.

Normal morphology correlated positively (+) with head length and negatively (-) with midpiece defects. Abnormal morphology correlated negatively (-) with head length; however, it was positively (+) associated with the head area and head defects. Intact acrosomes correlated positively (+) with acrosomes reacted, which in turn correlated negatively (-) with the number of viable (LIVE) spermatozoa as well as head width and head area. The morphometric parameter, percentage acrosome coverage, correlated positively (+) with total motility, head length, head width, head area, head perimeter and head roughness. However, percentage acrosome coverage correlated negatively (-) with ejaculate concentration, STR, abnormal morphology, head defects, tail defects, acrosome intact, DEAD (viability lost), head ellipticity and head elongation. Another morphometric parameter, head regularity, indicated a significant negative (-) association with progression kinematics total motility, progressive motility and VCL. Additionally, although not significant, head regularity was negatively (-) linked to VSL, VAP, LIN, STR, WOB, ALH, and BCF.

Table 7.1 Correlations indicated between the semen and sperm characteristics of African elephant ejaculates analysed.

Random Correlation Analysis:	
Volume	Non-progressive motility (-), VSL (-), **VCL ****, VAP (-), WOB (-), ***BCF**** , Normal morphology (-), Midpiece defect (-), Acrosome intact (-), LIVE (-), Head width (-), Head area (-), Head roughness (-)
Concentration	Non-progressive motility (-), WOB (-), **ALH***, Normal morphology (-), Midpiece defect (-), **Tail defect*** (-), Acrosome reacted (-), DEAD (-), ***Head length* (-)*** , ***Head Width* (-)*** , ***Head area* (-)*** , **Head perimeter* (-)**, Head roughness (-), % Acrosome coverage (-)
Total Motility	***Non-progressive motility**** , ***Progressive motility**** , ***VCL**** , ***VSL**** , ***VAP**** , ***LIN**** , ***ALH**** , Abnormal morphology (-), Head defects (-), Acrosome intact (-), LIVE (-), **Head area***, Head ellipticity (-), **Head elongation****, ***Head roughness*, Head regularity* (-)*** , ***% Acrosome coverage****
Non-progressive motility	STR (-), BCF (-), Abnormal morphology (-), ***Head defects* (-)*** , Midpiece defects (-), ***Acrosome intact* (-)*** , ***Acrosome reacted**** , ***LIVE* (-)*** , DEAD (-), ***Head length**** , Head regularity (-)
Progressive motility	***VCL**** , ***VSL**** , ***VAP**** , ***LIN**** , ***ALH**** , ***BCF**** , Abnormal morphology (-), Head defect (-), Acrosome reacted (-), DEAD (-), Head ellipticity (-), ***Head regularity* (-)***
VCL	***VSL**** , ***VAP**** , ***LIN**** , **STR****, **ALH***, ***BCF**** , Abnormal morphology (-), Head defect (-), Acrosome intact (-), LIVE (-), Head lenght (-), **Head perimeter****, Head ellipticity (-), Head elongation (-), ***Head regularity* (-)***
VSL	***VAP**** , ***LIN**** , ***STR**** , ***WOB*, ALH**** , Normal morphology (-), Abnormal morphology (-), Head defect (-), Acrosome reacted (-), DEAD (-), Head lenght (-), Head ellipticity (-), Head elongation (-), Head regularity (-)
VAP	***LIN**** , ***WOB**** , ***ALH**** , Abnormal morphology (-), Head defect (-), Acrosome intact (-), LIVE (-), **Head length****, Head ellipticity (-), Head elongation (-), Head regularity (-)
LIN	***ALH**** , Normal morphology (-), Abnormal morphology (-), Head defect (-), Acrosome reacted (-), DEAD (-), Head width (-), Head regularity, **Head elongation****
STR	Normal morphology (-), Head defect (-), Acrosome reacted (-), DEAD (-), Head length (-), Head width (-), Head area (-), **Head perimeter****, Head ellipticity (-), Head regularity (-), % Acrosome coverage (-)
WOB	ALH (-), BCF (-), Abnormal morphology (-), Head defect (-), Tail defect (-), Acrosome intact (-), LIVE (-), **Head area****, **Head perimeter****, Head regularity (-)
ALH	Midpiece defect (-), Acrosome intact (-), LIVE (-), Head length (-), Head width (-), **Head perimeter****, Head regularity (-), **Head elongation****
BCF	Normal morphology (-), Acrosome reacted (-), DEAD (-), **Head defects****, **Head length****, Head ellipticity (-), Head elongation (-), Head regularity (-)
Normal morphology	Abnormal morphology (-), Head defect (-), ***Midpiece defect* (-)*** , Tail defect (-), Acrosome intact (-), LIVE (-), ***Head length**** , Head roughness (-)
Abnormal morphology	***Head defects**** , Acrosome reacted (-), DEAD (-), ***Head length* (-)*** , Head width (-), **Head area****, Head perimeter (-), Head ellipticity (-), Head elongation (-), Head regularity (-), % Acrosome coverage (-)
Head defects	Tail defects (-), Acrosome reacted (-), LIVE (-), Head length (-),Head width (-), ***Head area* (-)*** , ***Head perimeter* (-)*** , % Acrosome coverage (-)
Midpiece defects	Tail defects (-), Acrosome reacted (-), Head length (-), Head ellipticity (-), Head elongation (-), Head regularity (-)
Tail defects	Acrosome reacted (-), LIVE (-), Head length (-), Head width (-), Head area (-), Head perimeter (-), Head ellipticity (-), Head elongation (-), Head regularity (-), % Acrosome coverage (-)
Acrosome Intact	***Acrosome reacted* (-)*** , DEAD (-), Head length (-), Head perimeter (-), Head ellipticity (-), Head elongation (-), Head regularity (-), % Acrosome coverage (-)
Acrosome Reacted	***LIVE* (-)*** , ***DEAD**** , Head width (-), Head area (-), Head roughness (-)
LIVE	***DEAD* (-)*** , **Head length****, Head ellipticity (-), Head elongation (-), Head regularity (-)
DEAD	Head width (-), **Head length****, Head perimeter (-), Head roughness (-), % Acrosome coverage (-)
Head length	***Head perimeter**** , Head regularity (-), **Head roughtness**(-)**, ***% Acrosome coverage****
Head width	***Head area**** , ***Head perimeter**** , ***Head ellipticity* (-)*** , ***Head elongation* (-)*** , ***Head roughness**** , ***Head regularity**** , ***% Acrosome coverage****
Head area	***Head perimeter**** , Head ellipticity (-), Head elongation (-), ***Head roughness**** , ***Head regularity* (-), % Acrosome coverage****
Head perimeter	Head ellipticity (-), Head elongation (-), ***Head roughness**** , ***% Acrosome coverage****
Head ellipticity	***Head elongation*, Head roughness* (-)*** , % Acrosome coverage (-)
Head elongation	***Head roughness* (-)*** , % Acrosome coverage (-)
Head roughness	***Head regularity* (-)*** , ***% Acrosome coverage****
Head regularity	***% Acrosome coverage* (-)***
* Pearson's p-value (p < 0.05)	
** Spearman's p-value (p < 0.05)	
(-) Negative correlation	
BCF = Beat cross frequency, ALH = Amplitude of lateral head displacement, VCL = Curvilinear velocity, VSL = Straight line velocity, VAP = Average path velocity, LIN = Linearity of tract, WOB = Wobble,	

7.3.2 EXPLORATORY FACTOR ANALYSIS: DATASET 1 - PARAMETERS WITH NO DATA MISSING

Parallel analysis of 13 semen and sperm parameters (macroscopic, motility and kinematic) of 16 ejaculates collected over two seasons (parameters with no data missing) loaded three factors above the line plot intersection to be included for further interpretation, as illustrated in Figure 7.1. These three factors explained 79.19% of the variance recorded amongst the 13 parameters included of 16 ejaculates collected over two seasons (Table 7.2).

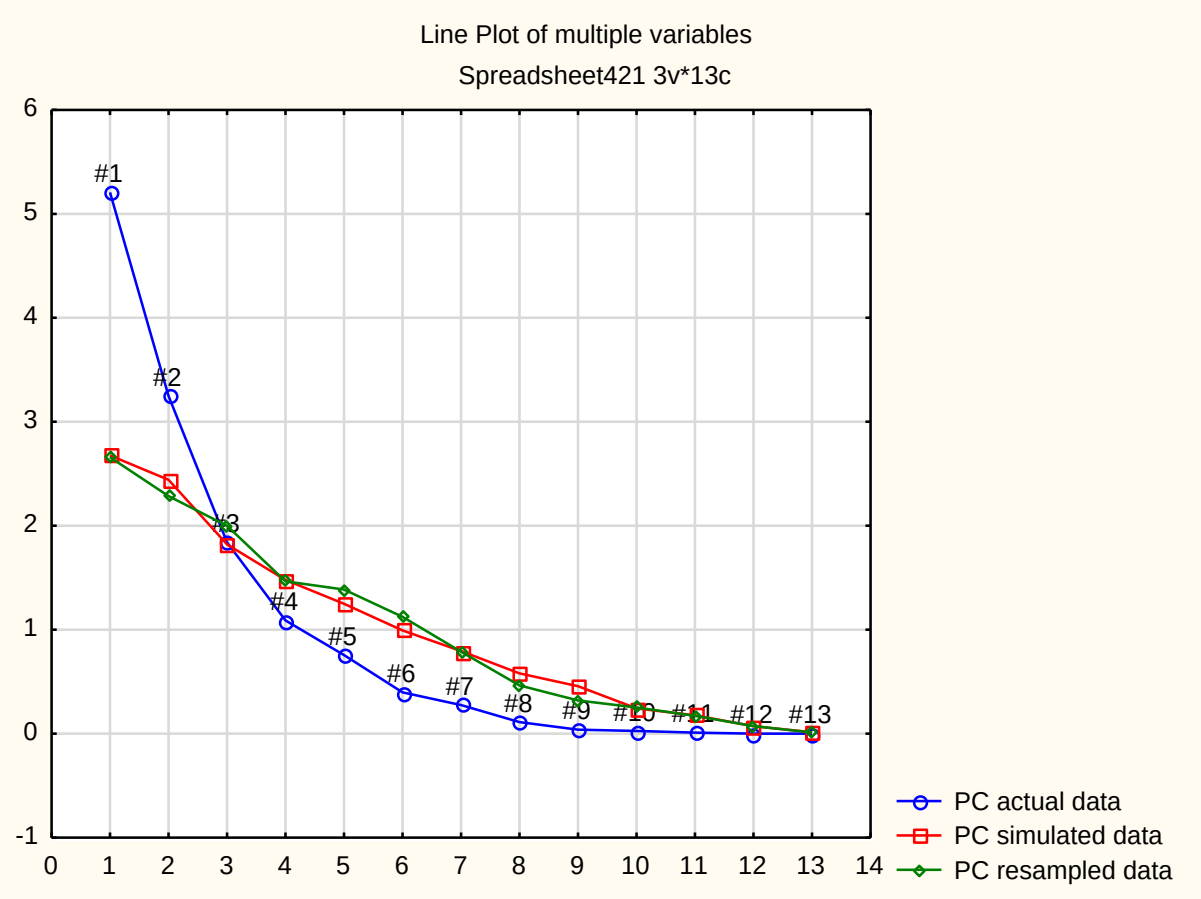

Figure 7.1: Line plot for determining eigenvalues (y – axis) and a variable number (x-axis) to consider for subsequent factor loadings (13 macroscopic, motility, and kinematic parameters of 16 ejaculates collected over two seasons, parameters with no data missing).

Factor 1 accounted for a total variance of 40.05 % (Eigenvalue 5.21). The factors loaded for Factor 1 labelled sperm progressive kinematics included sperm concentration, total motility, and progressive motility, VCL, VSL, VAP, LIN and ALH (Table 7.3).

Table 7.2 Eigenvalues of three selected factors and cumulative percentage of variance accounted for amongst the parameters of dataset 1.

Value	Eigenvalue	% Total Variance	Cumulative Eigenvalue	Cumulative %
1	5.207128	40.05483	5.20713	40.05483
2	3.248295	24.98688	8.45542	65.04171
3	1.840141	14.15493	10.29556	79.19664

Table 7.3: EFA analysis of the three selected factors for 13 semen and sperm parameters from 16 ejaculates collected over two seasons.

Factor 1 of 3 (Dataset 1)	**Factor 2 of 3** (Dataset 1)	**Factor 3 of 3** (Dataset 1)
Sperm Progressive Kinematics	**Sperm Kinematics**	**Sperm Volume/Kinematics**
Concentration	Total Motile (-)	Volume (-)
Total Motile	Non-progressive Motile (-)	LIN
Progressive Motile	LIN	WOB
VCL	WOB	ALH (-)
VSL	ALH (-)	
VAP		
LIN		
ALH		

BCF = Beat cross frequency, ALH = Amplitude of lateral head displacement, VCL = Curvilinear velocity, VSL = Straight line velocity, VAP = Average path velocity, LIN = Linearity of tract, WOB = Wobble

Factor 2 accounted for 24.98% (Eigenvalue 3.24) of the total variance. The factors loaded for Factor 2 (Table 7.3) labelled sperm kinematics and included two positively correlated parameters (LIN and WOB). Total motility (-) and non-progressive motility (-) correlated negatively with parameters loaded for Factor 2. Factor 3 accounted for 14.15% (Eigenvalue 1.84) of the total variance.

The factors loaded for Factor 3 (Table 7.3) labelled sperm volume\ kinematics: positively (+) correlated parameters, LIN and WOB correlated negatively with volume (-) and ALH (-). The PC analysis provided a good discrimination between the 13 macroscopic, motility and kinematic parameters of 16 ejaculates collected over two seasons, accounting for 65% of the total variance (Figure 7.2).

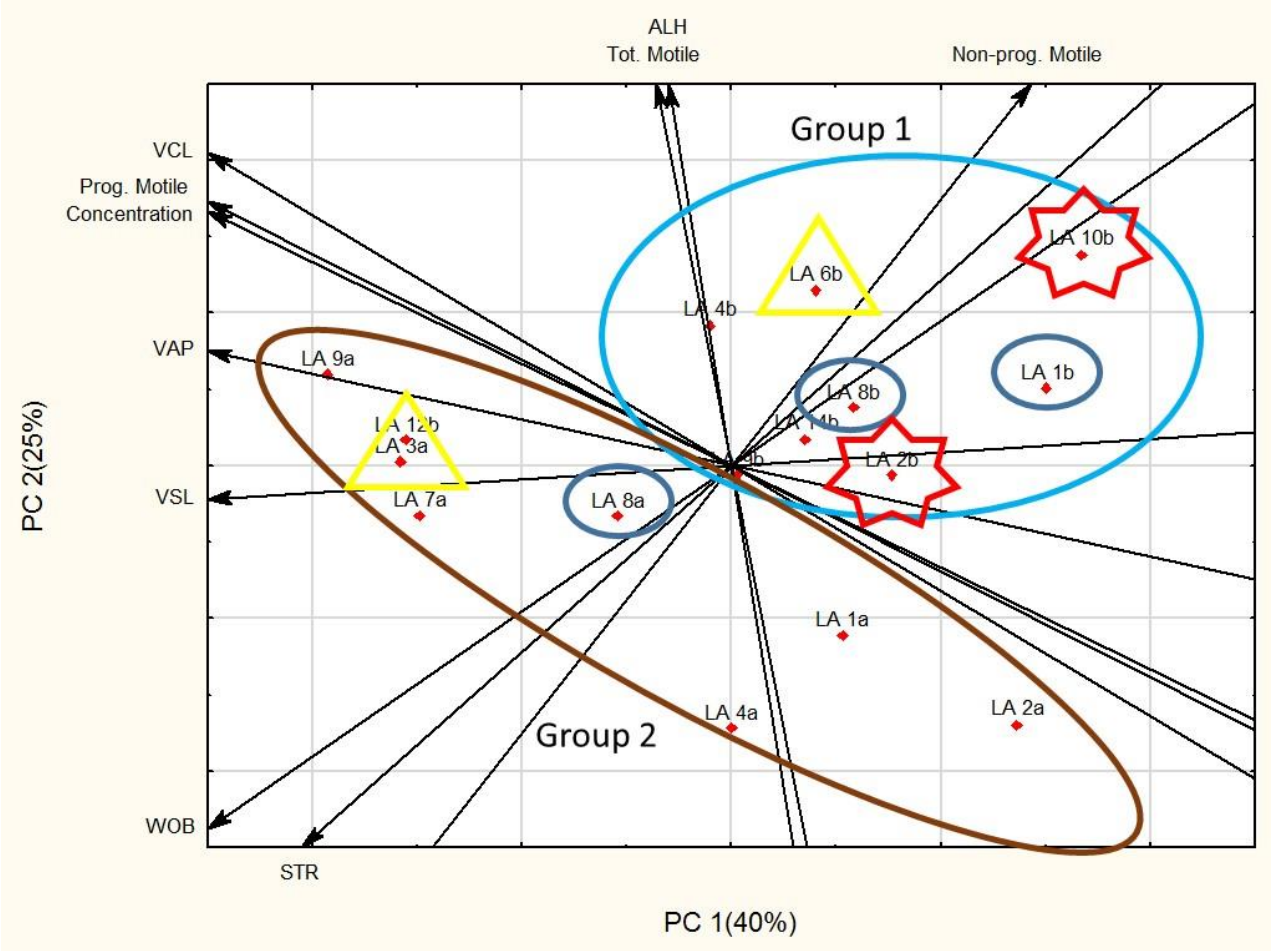

Figure 7.2: PCA biplot used to determine the distribution of the selected semen and sperm parameters and the association between 13 semen and sperm parameters from 16 ejaculates, explaining 65 % of the total variance. There is a clear discrimination between Group 1: season 2 (light blue), and Group 2: majority season 1 (brown). Series of ejaculates collected during the same or over two seasons. Blue: Righthook (LA 8a, LA 1b, LA 8b), Yellow: Number 3 (LA 3a, LA 6b), and Red: Bump (LA 2b, LA 10b). VCL = Curvilinear velocity, VSL = Straight-line velocity, VAP = Average path velocity, LIN = Linearity of track, WOB = Wobble, ALH = Amplitude of lateral head displacement, Tot. Motile = Total motile, Prog. = Progressive, Non-prog. = non-progressive.

The biplot illustrates some of the associations/factor loadings for Factor 1 (sperm progressive kinematics) on the left-hand side and top right-hand side, as well as a mixture of the Factor 1 (sperm progressive kinematics) and Factor 2 (sperm kinematics) factor loadings, including factors loaded negatively (WOB and STR). The fact that the factor loadings for Factor 3 (sperm volume/ kinematics) are not clear on the biplot might be an indication that this factor is not so important (lowest eigenvalue).

The elephant ejaculates plotted positions give a picture of the variation recorded between and among ejaculates from the same free-ranging elephant population over two seasons (Figure 7.2). Ejaculates plotted indicated an inter-individual variation between ejaculates collected from the same bull. Consecutive ejaculates were collected from Righthook (LA 8a, LA 1b, and LA 8b), and Number 3 (LA 3a, LA 6b). The ejaculates collected from Bump (LA 2b, LA 10b) during the same season recorded a decrease in progressive motility, VCL, VSL, and VAP values in the second ejaculate compared to the first (Figure 7.2).

The ejaculates of Group 1 (encircled in light blue) are generally clustered within relatively close proximity to other, compared to the disperse plotting of ejaculates in Group 2 (encircled in brown) due to large variations recorded in concentration, progressive motility, VCL, , VSL and VAP parameter values between and among the two seasons. There is a clear discrimination in the grouping amongst the ejaculates from Group 1: (encircled light blue) collected during season 2, and ejaculates in Group 2: (encircled brown) collected during season 1, except LA 12b from season 2.

7.3.3 EXPLORATORY FACTOR ANALYSIS: DATASET 2 - PARAMETERS WITH MISSING DATA

Parallel analysis of 26 semen and sperm parameters (macroscopic, kinematic, morphology and sperm head morphometric) of 11 ejaculates (with missing data) over two seasons loaded four factors above the line plot intersection to be included for further interpretation, as illustrated in Figure 7.3. These four factors explained 76.61% of the variance (Table 7.4). Factor 1 explained a total variance of 28.5% (Eigenvalue 7.41).

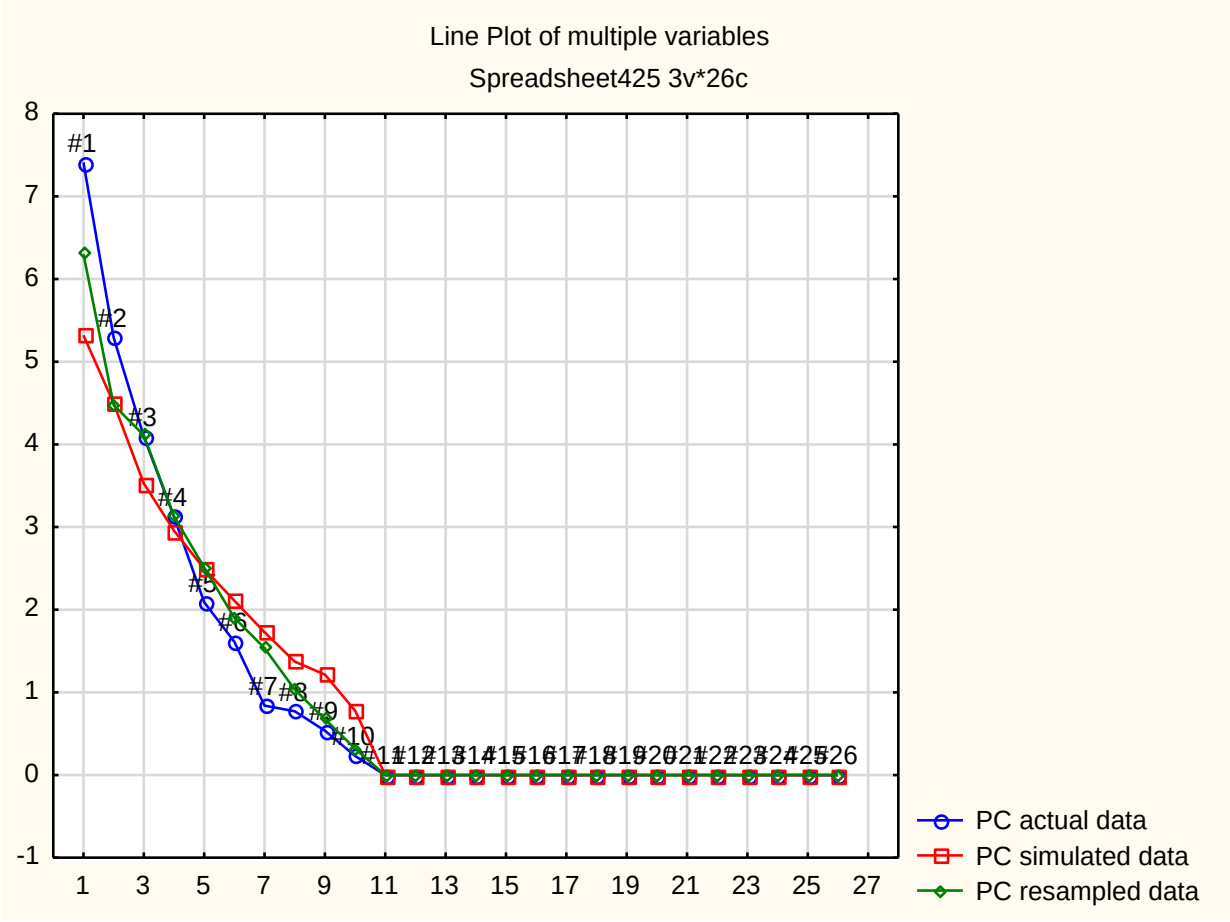

Figure 7.3: Line plot for determining eigenvalues (y – axis) and a variable number (x-axis) to consider for subsequent factor loadings (26 macroscopic, kinematic, morphology and sperm head morphometric parameters of 11 ejaculates collected over two seasons, a few of the variables had data missing).

Factor 1 (Table 7.5) labelled sperm progressive kinematics loaded factors that included concentration, total motility, progressive motility, and kinematic parameters VCL, VSL, VAP, LIN and ALH. The inclusion of morphology and sperm head morphometric parameters additionally loaded head regularity (-), normal morphology (-), head defects and sperm concentration to Factor 1 during EFA of 26 semen and sperm parameters.

Table 7.4: Eigenvalues of four selected factors and the cumulative percentage of variance accounted for amongst the parameters analysed in dataset 2.

Value	Eigenvalue	% Total Variance	Cumulative Eigenvalue	Cumulative %
1	7.410317	28.50122	7.41032	28.50122
2	5.292305	20.35502	12.70262	48.85624
3	4.093855	15.74560	16.79648	64.60183
4	3.123803	12.01463	19.92028	76.61646

Table 7.5: EFA analysis of the four selected factors for 26 semen and sperm parameters from 11 ejaculates collected over two seasons.

Factor 1 of 4 (Dataset 2)	**Factor 2 of 4** (Dataset 2)	**Factor 3 of 4** (Dataset 2)	**Factor 4 of 4** (Dataset 2)
Sperm Progressive Kinematics	**Sperm Morphometric/Volume**	**Sperm Morphometrics/Morphology**	**Sperm Morphology/Kinematics**
Head Regularity (-)	Head Length	Head Width	Head Regularity
Normal Morphology (-)	Head Area	Head Area	Acrosome Coverage (-)
Head Defects	Head Perimeter	Head Ellipticity (-)	Tail Defects (-)
Concentration	Acrosome Coverage	Head Elongation (-)	Total Motile (-)
Total Motile	Midpiece Defects	Head Roughness	Non-progressive Motile (-)
Progressive Motile	Tail Defects (-)	Head Regularity (-)	STR
VCL	Volume	Midpiece Defects	BCF
VSL	WOB (-)	Normal Morphology (-)	
VAP	ALH	LIN	
LIN			
ALH			

BCF = Beat cross frequency, ALH = Amplitude of lateral head displacement, VCL = Curvilinear velocity, VSL = Straight line velocity, VAP = Average path velocity, LIN = Linearity of tract, WOB = Wobble

Factor 2 accounted for 20.36% (Eigenvalue 5.29) of the total variance. The factors loaded onto Factor 2 (Table 7.5) mainly represent sperm morphometric parameters and semen volume. Positively correlated parameters included head length, head area, head perimeter, acrosome coverage, midpiece defects, and ALH while tail defects (-) and WOB (-) correlated negatively with factors loaded onto Factor 2.

Factor 3 accounted for 15.75% (Eigenvalue 4.10) of the total variance. The factors loaded onto Factor 3 (Table 7.5) seemed to represent sperm morphometric and morphology. Positively correlated parameters included head width, head area, head roughness, midpiece defects, and LIN. Head ellipticity (-), head elongation (-), head regularity (-), and normal morphology (-) associated negatively with factors loaded onto Factor 3. Factor 4 explained 12.01% (Eigenvalue 3.12) of the total variance. The factors loaded onto Factor 4 (Table 7.5) seemed to represent sperm morphology and kinematics: positively correlated parameters included head regularity, STR and BCF. Acrosome coverage (-), tail defects (-), total motility (-) and non-progressive motility (-) correlated negatively with factors loaded onto Factor 4.

The PC analysis provided good discrimination between the 26 macroscopic, kinematic, morphology, and sperm head morphometric parameters, accounting for 49 % of the total variance (Figure 7.4). The biplot illustrates some of the associations/factor loadings for Factor 1 (sperm progressive kinematics) on the top right-hand side of the biplot. On the left-hand side of the biplot is a mixture of Factor 2 (sperm morphometric/volume) and Factor 3 (sperm morphometric/morphology) factor loadings, including the negative factor loadings (head ellipticity, elongation and regularity) reported for Factor 3 (bottom of biplot). The fact that the factor loadings for Factor 4 (sperm morphology/kinematics) are not distinct on the biplot might be an indication that this factor is not so important (lowest eigenvalue).

The biplot positions the 11 ejaculates in multidimensional form to discriminate between elephant ejaculates based on semen and sperm parameter values (Figure 7.4). The plotted positions illustrate intra– and inter-individual variation in semen and sperm parameter values (macroscopic, motility, kinematic, morphology and morphometric parameters) recorded amongst the ejaculates collected during both seasons. The inclusion of morphology and morphometric data per ejaculate changed the distribution frequency of ejaculates into the general group, unrelated to season collected around or within close proximity to the spatial centre of the biplot.

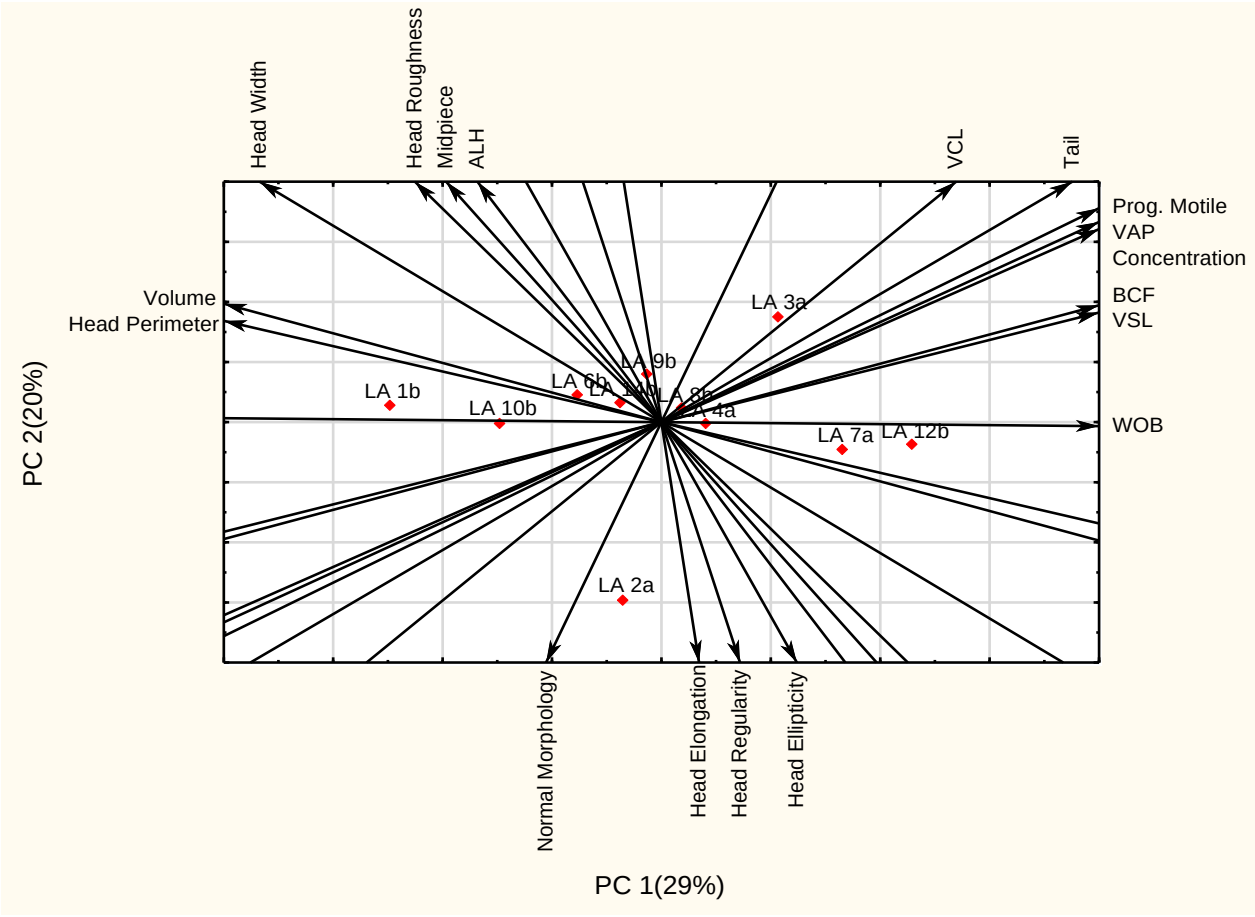

Figure 7.4: PCA biplot used to determine the distribution of the selected semen and sperm parameters and the association between 26 semen and sperm parameters from 11 ejaculates collected during season 1 (LA a) and season 2 (LA b), explaining 49% of the total variance. Tot. Motile = Total motility, Prog. = Progressive motility, Non-prog. = non-progressive motility, VCL = Curvilinear velocity, VSL = Straight-line velocity, VAP = Average path velocity, WOB = Wobble, BCF = Beat cross frequency, ALH = Amplitude of lateral head displacement, Head = Head defect, Midpiece = Midpiece defect, Tail = Tail defect.

7.3.4 EXPLORATORY FACTOR ANALYSIS: DATASET 3 - ALL PARAMETERS, NO DATA MISSING

Parallel analysis of 29 semen and sperm parameters (macroscopic, motility, kinematic, viability, acrosome integrity, morphology, sperm head morphometric) collected from six ejaculates during season 2 indicated two factors above the line plot intersection to be included for further interpretation, as illustrated in Figure 7.5. These two factors explained 62.84% of the variance recorded (Table 7.6).

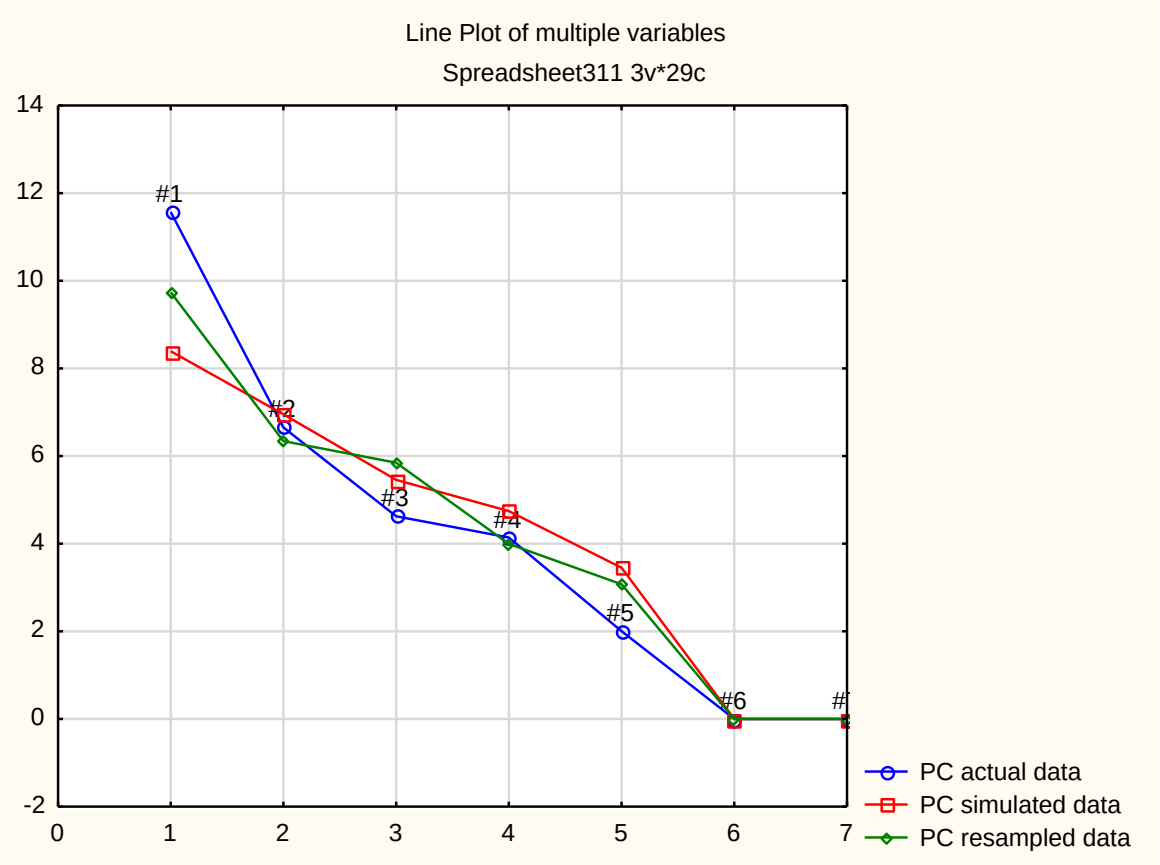

Figure 7.5: Line plot for determining eigenvalues (y – axis) and a variable number (x-axis) to consider for subsequent factor loadings (29 macroscopic, motility, kinematic, viability, acrosome integrity, morphology, sperm head morphometric parameters collected from six ejaculates).

Factor 1 accounted for 39.89% (Eigenvalue 11.56) of the total variance. The factors loaded onto Factor 1 (Table 7.7) seemed to represent sperm kinematics and morphology: positively correlated parameters included sperm concentration, progressive motile, VSL, LIN, STR and WOB, acrosome intactness and tail defects, that loaded negatively with non-progressive motility (-), ALH (-), Total %

HA (-), Midpiece defects, head length (-) and head perimeter (-).Factor 2 accounted for 22.95% (Eigenvalue 6.65) of the total variance. The factors loaded onto Factor 2 (Table 7.7) seemed to represent sperm velocity and sperm head morphometric: positively correlated sperm parameters included VCL, VSL, VAP, head ellipticity, head elongation and head regularity, which correlated negatively with head width (-), head area (-), head perimeter (-), and head roughness (-).

Table 7.6 The Eigenvalues of two selected factors and the cumulative percentage of variance accounted for amongst the parameters of dataset 3.

Value	Eigenvalue	% Total Variance	Cumulative Eigenvalue	Cumulative %
1	11.56980	39.89585	11.56980	39.89585
2	6.65547	22.94989	18.2256	62.84573

Table 7.7 EFA analysis of the two selected factors for 29 parameters collected from six ejaculates collected over two seasons.

Factor 1 of 2 (Dataset 3)	Factor 2 of 2 (Dataset 3)
Sperm Progressive Kinematics/Morphology	**Sperm Velocity/ Morphometrics**
Concentration	VCL
Progressive Motile	VSL
Non-progressive Motile (-)	VAP
VSL	Head Width (-)
LIN	Head Area (-)
STR	Head Perimeter (-)
WOB	Head Ellipticity
ALH (-)	Head Elongation
Total % HA (-)	Head Roughness (-)
Midpiece Defects (-)	Head Regularity
Tail Defects	
Acrosome Intact	
Head Length (-)	
Head Perimeter (-)	

BCF = Beat cross frequency, ALH = Amplitude of lateral head displacement, VCL = Curvilinear velocity, VSL = Straight line velocity, VAP = Average path velocity, LIN = Linearity of tract, WOB = Wobble, Total % HA = Total percentage hyperactivated motility

The PC analysis provided good discrimination between 29 macroscopic, motility, kinematic, acrosome integrity, morphology, sperm head morphometric parameters collected from six ejaculates parameters during season 2, accounting for 62.8% of the total variance (Figure 7.6). The biplot clearly illustrates the associations/factor loadings for Factor 1 (sperm progressive/ kinematics/morphology) on the top right-hand side and opposite to that, the negative factors loaded at the bottom left-hand side. Similarly, associations/factor loadings for Factor 2 (sperm velocity/morphometric) are located on the top left-hand side, and factors that loaded negatively at the bottom right-hand side. The plotted positions also illustrate intra– and inter-individual variation in semen and sperm parameter values (macroscopic, motility, kinematic, acrosome integrity, and morphology and morphometric parameters) recorded amongst the ejaculates collected during season 2 (Figure 7.6).

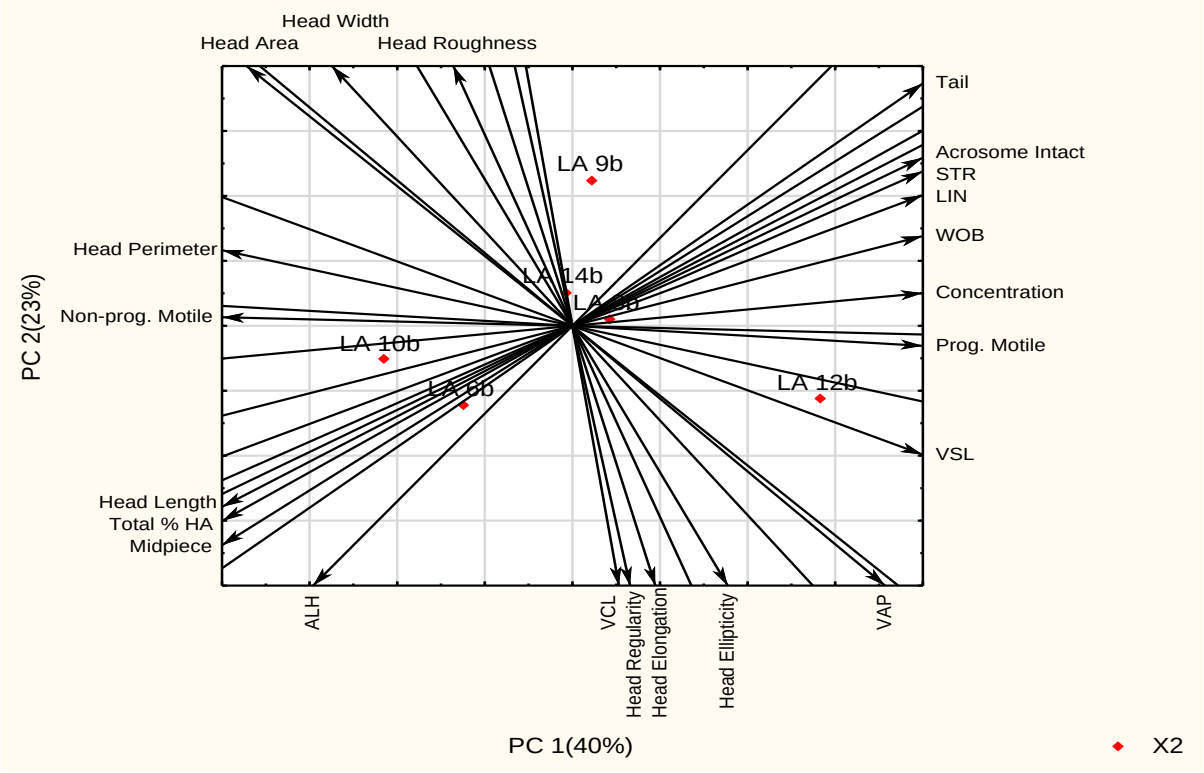

Figure 7.6: PCA biplot used to determine the distribution of the selected semen and sperm parameters and the association between 29 semen and sperm parameters from six ejaculates collected during season 2 (LA b), explaining 63% of the total variance. VCL = Curvilinear velocity, VSL = Straight-line velocity, VAP = Average path velocity, LIN = Linearity of track, WOB =Wobble, ALH = Amplitude of lateral head displacement, HA = Hyperactivated, Prog. = Progressive, Non-prog. = non-progressive.

7.4 DISCUSSION

Multivariate analysis has been successfully employed during this study in determining associations among several of the semen and sperm parameters analysed between and amongst ejaculates. Significant relationships were highlighted between standard/routine semen and sperm parameters (sperm concentration, normal morphology, head defects, midpiece defects, tail defects, acrosome intact), CASA parameters (total motility, progressive motility, VCL, VSL, LIN, ALH) and CASMA parameters (head length, head regularity and head perimeter). In this chapter clear associations were found between sperm concentration, progressive motility, VSL, VAP and BCF. The seasonal variation recorded in sperm head dimensions (head length, head regularity and head perimeter) and their relationship with VCL was of particular interest as it contradicts previous studies of human and stallion spermatozoa. The relationship between normal morphology and sperm head dimensions (head elongation, head regularity and head ellipticity) also become apparent. Head ellipticity is a function of head width and length and associated with linear and progressive motility (LIN and STR) (Gil, *et al.*, 2009).

Associations amongst total motility, progressive motility, VCL, VSL, VAP, LIN, STR, and ALH were also apparent. These findings suggests that in natural elephant populations high-quality ejaculates have both high sperm numbers and spermatozoa that swim at high speeds in straight-line trajectories. The parameters VSL, LIN, and STR are used to measure the forward and linear progression of spermatozoa, a significant parameter used to predict the fertility potential of mammalian spermatozoa (Holt, 2000; Malo, *et al*., 2005; Vutyavanich, *et al.*, 2009). These parameters have been shown to be sensitive indicators used to detect any adverse changes in sperm motion.

Sperm concentration, velocity and the proportion of morphologically normal sperm has been shown to correlate with human fertility under *in vivo* conditions of conception, and within natural populations

of Iberian red deer (Eggert-Kruse, *et al.,* 1996; Malo, *et al.*, 2005). The number of spermatozoa transferred to the female is currently understood to be positively correlated to fertility (Holt, 2005; Malo, *et al.*, 2005). However, it should be taken into consideration that any possible dysfunction during the process of spermatogenesis may not affect sperm number, despite the fact that affected spermatozoon might be misshapen or dysfunctional (Yan, 2009). Analysis indicated that the proportion of motile spermatozoa and the quality of motility are associated closely with the proportion of normal spermatozoa. This finding suggests that semen samples with high sperm concentrations also contain a sperm population that varies in normal morphology and sperm head morphometric. VCL signifies swimming speed, and is determined by the total distance that the sperm head covers during the observation period (Gil, *et al.*, 2009). BCF, along with ALH and VCL, are parameters indicative of spermatic vigour (Gil, *et al*., 2009). The amplitude of lateral head displacement (ALH) is an indicator of the power exerted by the flagellum. ALH has been identified as being predictive of fertility, an important parameter of motility, and vital to a spermatozoon's ability to penetrate the cervical mucus (Holt, 2000; Gil, *et al.*, 2009).

Furthermore, acrosome integrity associated with the parameters STR and LIN, suggests that spermatozoa with normal acrosomes maintain a higher straightness and linearity in their trajectories. Results from previous fertility based studies in natural deer populations insinuated that the parameters viability and acrosome integrity are stabilised at high values within natural populations. Since all these parameters play an essential part in the process of fertilisation, it is suggested that males with lower values for these parameters have been intensively selected against during natural selection processes within natural populations (Malo, *et al.*, 2005). Small geometric differences in sperm head morphometric have been reported to cause large differences in sperm hydrodynamics (Saacke, 2008). A seasonal variation in sperm head size and shape was confirmed between the two seasons compared in this study. This study revealed that the ejaculates collected during season 2 (end of summer months) had larger head morphometries and travelled at faster velocities compared to

smaller and slower values recorded for ejaculates collected during season 1 (end of winter months). These findings indicated that increased elephant sperm head size associated positively with increased elephant sperm velocities within a natural population. These findings contradict to previous reports that correlated a decrease in average linear and progressive motility with an increase in average sperm head length and head area in humans (Gil, *et al.*, 2009). Likewise, fertility studies in stallions indicated that sperm head morphometric values (head length, width and area) are higher in subfertile stallions compared to fertile stallions (de Paz, *et al.*, 2011). Sperm head morphometric in relation to fertility has been analysed in man, boar, stallion, goat and bull (de Paz, *et al.*, 2011). Previous studies have indicated that the shape of the sperm head, not sperm concentration, correlates positively with male fertility (Yan, 2009).

Fertility data was only available for 1 out of the 16 ejaculates analysed during this study. Hildebrandt, *et al.,* (2012) reported the birth of the first female elephant calf following post-thaw artificial insemination, in a captive female elephant at Schoënbrunn zoo in Vienna, Austria, sired from a free-ranging elephant bull, LA 1a (Steve), also evaluated during this study. Considering the position plotted of ejaculate LA 1a (with proven fertility, post-thaw) in relation to the positions plotted of the other ejaculates it appears that sperm and semen quality is generally high within natural elephant populations (Figure 7.2). It is considered that sperm traits such as concentration, motility, velocity, proportion of normal spermatozoa and sperm head morphometric, all play an important role in the context of sperm competition, also shown during this study, as males within natural populations with lower values for these parameters will have been intensively selected against due to decreased chances of progeny being born to transfer these genetic traits. This investigation demonstrates that it is possible to identify relationships between semen and sperm traits recorded among the ejaculates collected from a free-ranging (natural) elephant population. It is generally presumed that the reproductive success of an elephant bull (among polygamous mammals) is mainly as a result of its ability to win agonistic encounters and to defend females from rival bulls.

7.5 CONCLUSION

The exploratory factor analysis (EFA), principal component analyses (PCA) and biplots presented in this chapter indicated the associations between various semen and sperm parameters. These statistical tests can therefore be regarded as valuable exploratory data analysis tools and should be employed in similar comparative studies to identify associations among semen and sperm parameters in free-ranging populations. The findings suggest that the variation recorded in semen and sperm quality between two different seasons should be taken into consideration when collecting elephant semen samples for either artificial insemination or cryopreservation studies, as the differences in quality that emerged may contribute significantly to the fertility potential of the spermatozoa. As multivariate techniques for the analysis of semen and sperm quality continuously develop, more information will become available in an attempt to identify correlations amongst parameters that can be used to predict fertility potential amongst ejaculates collected (Holt, 2000).

CHAPTER 8

DISCUSSION OF FREE-RANGING AFRICAN ELEPHANT AND SOUTHERN WHITE RHINOCEROS SEMEN AND SPERM CHARACTERISTICS

This study aimed to typify the semen and sperm parameters of ejaculates collected from free-ranging (natural) African elephant and Southern white rhinoceros populations. An intra- and interdisciplinary approach was used to analyse the samples that included routine semen analysis (macroscopic and microscopic) combined with Computer-aided sperm analysis (CASA), Computer-aided sperm morphology analysis (CASMA), Transmission electron microscopy (TEM), genomics and multivariate analysis of the comprehensive datasets was employed to identify current natural ranges and trends in free-ranging elephant and rhinoceros reproduction biology. The results presented in this investigation are based on the evaluation and classification of ≈ 45 000 individual African elephant spermatozoa and ≈ 18 000 individual Southern white rhinoceros spermatozoa.

8.1 THE AFRICAN ELEPHANT (*Loxodonta africana*)

The information gathered during this study substantially adds to the currently available database for elephant (both African and Asian) semen and sperm parameters (Table 8.1). The data collected during this study represents free-ranging (natural) African elephant (*Loxodonta africana*) populations and was compared to available literature (Jainudeen, *et al*., 1971; Howard, *et al.,* 1984; Schmitt & Hildebrandt, 1998: Kitiyanant, *et al*., 2000; Graham, *et al.,* 2004; Thongtip, *et al.,* 2006, Thongtip, *et al.,* 2008, Rittem *et al*., 2010; Buranaamnuay, *et al*., 2013, Imrat, *et al*., 2013; O'Brien *et al.,* 2013, Hermes, *et al*., 2013).

Table 8.1 Summary table for data comparison of semen and sperm parameter recorded during this study with available literature on semen characteristics of both African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants.

	African elephant Free-ranging EE CASA, Manual, CASMA	African elephant Free-ranging EE Manual	African elephant Captive MS (24h post-collection) CASA, Manual	Asian elephant Captive MS Manual	Asian elephant Captive MS Manual	Asian elephant Captive MS CASMA	Asian elephant Captive MS Manual	Asian elephant Captive MS CASA	Asian elephant Captive MS Manual	Asian elephant Captive AV CASA, Manual	Asian elephant Captive MS Manual	African elephant Free-ranging EE Manual	Asian elephant Captive MS Manual
	This study	Hermes *et al.*, 2013	O'Brien *et al.*, 2013	Imrat, *et al.*, 2013	Buranaamnuay *et al.*, 2013	Rittem *et al.*, 2010	Thongtip *et al.*, 2008	Thongtip *et al.*, 2006	Graham *et al.*, 2004	Kitiyanant *et al.*, 2000	Schmitt *et al.*, 1998	Howard *et al.*, 1984	Jainudeen *et al.*, 1971
	n = 19 of 12 individuals	n = 19 of 12 individuals	n = 6 of 1 individual	n = 9 of 3 individuals	n = 65 of 10 individuals	n = 24 of 2 individuals	n = 118 of 8 individuals	n = 1 of 1 individual	n = 4 of 2 individuals	n = 7 of 1 individual	n = 250 of 1 individual	n = 8 of 8 individuals	n = 7 of 1 individual
Parameters:	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**	**Values**	**Mean ± SEM**	**Range**	**Mean ± SD**	**Mean ± SEM**	**Mean (Range)**
Volume (mL)	56 ± 38	56 ± 38	11 ± 5	14 ± 6		27 ± 5	24 ± 1			40 - 73	28 ± 4	93 ± 48	9 (5 - 25)
Concentration (x 10^6/mL)	818 ± 750	794 ± 640	1734 ± 90	1137 ± 200		1158 ± 183	1502 ± 91	173	830 ± 220	1000 - 3000	1610 ± 4	2409 ± 521	1200 (600 - 2000)
Total # sperm per ejaculate (x 10^9)	47.17		17 ± 16										
Total motility (%)	81 ± 29.7	86 ± 9	66 ± 11	38 ± 9	65 ± 2			90		50 - 90		70 ± 6	50 (40 - 70)
Progressive motility (%)	62 ± 26.9		35 ± 10			14 ± 4	31 ± 2	50					
Non-progressive motility (%)	19 ± 9.2												
VCL (µm/sec)	241 ± 58.5		221 ±36					132					
VSL (µm/sec)	173 ± 181.6		84 ± 13					110					
VAP (µm/sec)	201 ± 54.5		112 ± 13					167					
LIN (%)	67 ± 16.4		39 ± 8					63					
WOB (%)	83 ± 6.6												
STR (%)	86 ± 85.7		73 ± 5					80					
ALH (µm)	4 ± 0.8		10 ± 2					5.7					
BCF (Hz)	21 ± 3.1		30 ± 2					27.5					
Membrane integrity intact (LIVE) (%)	68 ± 11.9		73 ± 11	68 ± 4	71 ± 1	47 ± 5	33 ± 3		87 ± 2.7				83 (62 - 90)
Normal morphology (%)	55 ± 14.2	73 ± 15	69 ± 9	85 ± 3		75 ± 4	84 ± 2			64 - 70		78 ± 23	84 (79 -90)
Number of head defects (%)	15 ± 17.1		13 ± 6*	5 ± 1*		4 ± 1							
Number of midpiece defects (%)	14 ± 9.7			1 ± 0.3		9 ± 2							
Number of tail defects (%)	16 ± 1			8 ± 2		12 ± 3							
Acrosome intact (%)	77 ± 11.3	80 ± 12	70 ± 10		51 ± 5								
Head length (µm)	6.8 ± 0.3					7.8 ± 0.1				7 - 8			7.5
Head width (µm)	3.3 ± 0.2					3.8 ± 0.4				4 - 4.5			4.5
Head area (µm²)	20.2 ± 1.2					23.9 ± 0.4							
Head ellipticity	2.1 ± 0.1												
Head elongation	0.4 ± 0.02												
Head perimeter	14.8 ± 0.6					19.6 ± 0.2							
Head regularity	0.9 ± 0.02												
Head roughness	1.2 ± 1.2												
Acrosomal coverage (%)	38.95 ± 0.9												

* detached heads

EE = Electro-ejaculation, MS = Manual stimulation, AV = Artificial Vagina

CASA = Computer-aided sperm analysis, Manual = Phase contrast microscope , CASMA = Computer-aided sperm morphology analysis

VCL = Curvilinear velocity, VSL = Straight line velocity, VAP = Average path velocity, LIN = Linearity of tract, STR = Straightness of tract, WOB = Wobble, BCF = Beat cross frequency, ALH = Amplitude of lateral head displacement

8.1.1. MACROSCOPIC SEMEN EVALUATION OF THE AFRICAN ELEPHANT

8.1.1.1 Ejaculate colour and viscosity

The sperm samples analysed during this study were collected by means of electroejaculation (EE). The majority of the elephant semen fractions evaluated consisted of viscous seminal plasma and had a grey-milky colour. This suggested that the majority of the ejaculates were comprised of high ratios of seminal plasma components compared to a sperm-rich fraction. Previous reports on elephant EE ejaculates attributed the high viscosity due to the over-stimulation of the accessory glands during EE (Howard, et al., 1984). The bulbourethral gland of the elephant produces a very viscous component and the seminal vesicles excrete a more watery secretion (Perry, 1953; Short, *et al.*, 1967).

Additionally, some of the fractions collected during this study were either too viscous to process, or had a distinct urine odour and yellow colour indicating urine contamination, or contained no spermatozoa (azoospermia). These fractions were not further analysed and discarded. In domestic species a creamy white colour is usually considered as an indication of the good semen quality (Howard, et al., 1984; Schmitt & Hildebrant, 1998). However, semen and sperm quality still seem to vary considerably, as well as the protocols used for semen collection (Schmitt & Hildebrant, 1998; Kitiyanant, *et al.*, 2000; Thongtip, *et al.*, 2008; Hildebrandt, *et al.,* 2012; Imrat, *et al.*, 2013; O'Brien, *et al*., 2013). The correct placement of the probe electrodes is crucial to avoid overstimulation of any particular gland during EE stimulation. Excessive seminal plasma can have a negative influence on the quality of both fresh and frozen sperm samples, possibly due to the change in semen pH, volume and sperm concentration (Howard, et al., 1984; Schmitt & Hildebrant, 1998; Thongtip, et al., 2008; Imrat, et al., 2013). The combined efforts of continuous development and improvement of anaesthesia protocols, the utilisation of ultrasound technology, optimal stimulation protocols for MS or EE, and the design and positioning of species-specific probes for EE are vital for the collection of good quality semen fractions (Schmitt & Hildebrant, 1998; Kitiyanant, *et al.*, 2000; Hildebrandt, *et al.,* 2012; Hermes, *et al.*, 2013; Imrat, *et al.*, 2013; O'Brien, *et al.*, 2013).

8.1.1.2 Ejaculate Volume

In this study, a mean ejaculate volume of 56 ± 38mL (range: 6 – 133mL) was recorded from 19 ejaculates collected over two seasons from 12 free-ranging African elephant bulls, aged 17 to 35 years. In domestic bulls ejaculates are mainly collected by means of an artificial vagina (AV) and the volumes recorded reflect the production potential of those bulls (Hafez, 1987; Barth, 2007). The volumes attained from electroejaculation (EE) and manual stimulation (MS) depend on the duration of stimulation and selection of the various fractions collected (ejaculates collected through EE are usually larger) and are not representative of the true ejaculate volumes collected during natural ejaculation (Hafez, 1987; Barth, 2007).

Kitiyanant (2000) studied the semen and sperm parameters of seven ejaculates collected by means of an artificial vagina (AV) from one trained Asian (*Elephas maximus)* elephant, and here the ejaculate volumes ranged from 40 – 73ml (Kitiyanant, et al., 2000). The ejaculate volumes reported during this study seem comparable to the AV volume range reported by Kitiyanant (2000) and EE volumes range of 93.3 ± 48.4mL in free-ranging African elephants (Howard, et al., 1984). However, the volumes reported for EE and AV ejaculates appear to be generally higher when compared to MS ejaculate volumes reported in captive Asian and African elephant populations (Schmitt & Hildebrant, 1998; Rittem, *et al.*, 2010; O'Brien, *et al.*, 2013) (Table 8.1).

8.1.2 MICROSCOPIC SPERM EVALUATION OF THE AFRICAN ELEPHANT

8.1.2.1 Sperm concentration

Free-ranging African elephant ejaculates obtained by EE contained an average of 47 x 10^9 spermatozoa (volume of 56 ± 38mL x concentration of 818 ± 750 x 10^6/mL). Amongst the 21 elephant sperm samples analysed, sperm concentrations ranged from 0 – 2025 x 10^6/mL and recorded an average of 818 x 10^6 spermatozoa/mL.

The EE sperm concentration recorded during this study was less than the 2409 x 10^6/mL (Howard, et al., 1984) but higher compared to 4.8 x 10^6/mL (Jones, 1973) and 0.8 x 10^6/mL (Reudi, *et al.*, 1981) reported for sperm concentrations in free-ranging elephants. When the sperm concentrations across all the collection techniques are compared (AV, MS and EE) it appears that ejaculates from MS records on average higher sperm concentrations compared to EE collection methods (except for Howard, *et al.;* 1984: 2409 x 10^6/mL) and collections by AV (Kitiyanant, *et al*., 2000: 500 x 10^6/mL) (Table 8.1). Studies reporting on sperm concentrations for MS ejaculates averaged around 2050 x 10^6/mL (Schmitt & Hildebrant, 1998), 1502.03 x 10^6/mL (Thongtip, *et al.,* 2008), 1153 x 10^6/mL (Rittem, *et al.*, 2010), 1137.8 x 10^6/mL (Imrat, *et al.*, 2013), and 1734 x 10^6/mL (O'Brien, *et al.,* 2013).

8.1.2.2 Motility and kinematic parameter assessment using computer-aided sperm analysis (CASA)

For this study, the CASA motility and kinematic parameter values recorded in egg-yolk/Ham's F10 (EY_Ham's F10) sperm samples were used to estimate the motility component of semen quality. The data represents 19 of the 21 ejaculates collected from 12 free-ranging African elephant bulls (17 to 35 years old). Motility estimation is an essential step in determining semen quality, and can be considered a functional test because of its relationship with the energy status of a spermatozoon (Contri, *et al.,* 2010). It is generally accepted that motility and kinematic parameters cannot be considered as a reliable marker for fertilisation; however it is reasonable to assume that the higher the number of progressively motile spermatozoa, the higher the potential to reach the site for fertilisation (Holt, 2000; Contri, *et al.*, 2010). During this investigation the average sample contained 81 ± 29% motile spermatozoa (Thongtip *et al.*, 2008: 90%) (O'Brien *et al.*, 2013: 66.3%) (Hermes, *et al.*; 2014: 86%) of which 62 ± 26% were progressively motile (Thongtip *et al.*, 2008: 50%) (O'Brien *et al.*, 2013: 34.7%). The total motility (TM) percentage recorded during this study was comparable to that of Howard, *et al.* (1984) (TM range: 60 – 95%) for EE samples from free-ranging African elephants, as well as to that of Kitiyanant, *et al.* (2000) (TM range: 50 – 90%) for AV samples from captive Asian elephants. However, these values were higher than those reported by (Thongtip, *et al.*,

(2008): (26.5%), Imrat, *et al.*, (2013) (38.2%), Buranaamnuay, *et al.*, (2013) (65%) and O'Brien, *et al.*, (2013) (66.3%) for MS collected samples, with the exception of Tongtip, *et al.*, 2008: 90%). Studies by Thongtip *et al.* (2008) and O'Brien *et al.* (2013) reported on CASA values for captive Asian and African elephants. Thongtip (2008) reported on CASA values of one MS ejaculate collected from a captive Asian elephant during a cryopreservation study, while more recently O'Brien (2013) reported on CASA values for 19 MS ejaculates collected from two captive African elephant bulls (O'Brien, *et al*., 2013). Both these studies reported that egg yolk (EY) based extenders were added to the collected semen samples immediately after collection. As these studies did not state otherwise, it is presumed that CASA values were recorded forthese egg yolk based extenders. During the current study, evaluation of sperm samples took place within 30 minutes after collection, similar to the methodology reported by Thongtip *et al.* (2008).

This can possibly explain why CASA kinematic values reported during this study was more similar to the values reported by Thongtip *et al.* (2008) than to those of O'Brien *et al.* (2013). Values reported by O'Brien *et al.* (2013) were recorded 24 hours post collection. The VCL, LIN, STR and high ALH reported by O'Brien *et al.* (2013) indicated that sperm were swimming at low velocities while displaying non-linear motion patterns. CASA parameters values recorded during this study were higher when compared to Thongtip *et al.* (2008) and O'Brien *et al.* (2013) (Table 8.1). During this study CASA recorded velocities for VCL (241 ± 58µm/s), VSL (173 ± 181µm/s) and VAP (201 ± 54µm/s), and kinematics at STR (86 ± 85%), LIN (67 ±16%), ALH (4 ± 0.75µm) and BCF (21 ± 3Hz). Progressive (linear) motility (STR and LIN) and sperm velocity (VCL, VSL, and VAP) parameters are useful and sensitive for detecting any adverse effects on sperm motion (Vutyavanich, *et al.*, 2009; Contri, *et al*., 2010). Sperm velocity may determine the ability of a sperm to overcome physical barriers within the female reproductive tract when entering and leaving the sperm reservoir, leading to penetration of the oocyte. In relation to semen and sperm quality when rival males compete, the sperm population with the highest velocities will reach the oocyte first (Thurston, *et al.*, 1999; Malo, *et al.*, 2005).

8.1.3 STRUCTURAL ANALYSES OF AFRICAN ELEPHANT SPERMATOZOA

8.1.3.1 Plasma membrane integrity (viability) and acrosome integrity

During this study, plasma membrane integrity analyses revealed that 68 ± 11% of the spermatozoa were viable (intact plasma membrane). The findings from this study are comparable to the 73 ± 11% (O'Brien *et al.*, 2013), 68 ± 4% (Imrat *et al.*, 2013), and 71 ± 1% (Buranaamnuay, et al., 2013), higher than the 47 ± 5% (Rittem, *et al.,* 2010) and 27 ± 3% (Thongtip, *et al*., 2008), but lower when compared to the 87 ± 3% (Graham, *et al.*, 2004) and 83 ± 3% (Jainudeen, *et al.*, 1971) recorded in other studies (Table 8.1). During this study elephant spermatozoa that stained red from the post-acrosomal region (dead) with a white acrosomal cap (intact) were classified as dead. These results indicate the percentage of viable spermatozoa irrespective of their motile capabilities at the time of motility analysis. It has been previously reported that the loss of sperm viability is mainly as a result of osmotic or cold shock stressors (Khan, *et al.,* 2009). The integrity of the plasma membrane gives a good indication of sperm quality. Only a functional intact plasma membrane is able to maintain the chemical gradient relevant for sperm metabolism and the ability required to interact with the surrounding environment; if there is no interaction, there will be no fertilisation (Rodriques-Martinez & Barth, 2007).

Our findings recorded that 77 ± 11.3% of elephant spermatozoa within all the samples collected had retained their acrosomal integrity. These findings are comparable to the 80 ± 12% (Hermes, *et al.*, 2013) and 70 ± 10% (O'Brien, *et al.*, 2013), and higher when compared to the 51 ± 5% (Buranaamnuay, *et al.*, 2013) previously reported (Table 8.1). Abnormalities of the acrosome have been associated with abnormal spermiogenesis, sub- and even infertility in stallions, bulls, boars, rams and man (Pesch & Bergmann, 2006). Reasons for the occurrence of the abnormalities are various e.g. prolonged periods of sexual rest, cell death, or could even be an artefact caused during smear preparation (Pesch & Bergmann, 2006). Only viable (plasma membrane intact) sperm can undergo capacitation and the acrosome reaction (Yanagimachi, 1981; Pesch & Bergmann, 2006).

Capacitation/acrosome reaction is a progressive membrane destabilisation that eventually results in cell death, thus damage to the acrosomal membranes decreases the viability of a spermatozoon (Chenoweth & Kastelic, 2007). Spermatozoa with abnormal acrosomes seemed to have an impaired ability to attach to the ovum *in vitro* and are believed to not participate in fertilisation (Pesch & Bergmann, 2006; Saacke, 2008).

8.1.3.2 Sperm morphology

The morphological structure of 1700 individual spermatozoa within 17 ejaculates collected from 11 free-ranging African elephant bulls was analysed during this study. On average, an ejaculate contained 55 ± 14% morphologically normal spermatozoa. This is less compared to the 73 ± 15% (Hermes, *et al.*, 2013), 78 ± 23% (Howard, et al., 1984), and 69 ± 9% (O'Brien, *et al.*, 2013) previously reported for African elephants, and less compared to reports for Asian elephants of 84% (Jainudeen, *et al.*, 1971), 86 ± 2% (Thongtip, *et al*., 2008), and 75 ± 4% (Rittem, *et al*., 2010) (Table 8.1). The data capture sheet used during this study organised defects according to the stage of spermatogenesis during which defects originate as reported in domestic bulls (Nöthling & Irons, 2008). Stringent criteria have been developed for the domestic bull (selected for fertility traits over decades) and were applied in this study to evaluate sperm morphology and abnormalities observed in free-ranging elephant populations (under natural selection). The pathogenesis and effects of specific sperm defects have been studied in great detail in the domestic bull and are discussed where relevant below.

During this study the use of strict criteria to classify morphological defects as either head-, midpiece- or tail defects and recorded averages of 15 ± 17%, 14 ± 9% and 16 ± 11%, respectively. This is higher when compared to previous reports of 4 ± 1%, 9 ± 2%, 12 ± 3% (Rittem, *et al.*, 2010), and 5 ± 1%, 1 ± 0.3%, 8 ± 2% (Imrat, *et al*., 2013) (Table 8.1). The strict classification criteria used during this study recorded noticeably less 'normal' spermatozoa compared to standard procedures applied in previous

investigations. However the types of abnormalities observed during this study were similar to those previously reported for elephant spermatozoa (Jainudeen, *et al.*, 1971; Howard, *et al.*, 1984; Kitiyanant, *et al*., 2000; Imrat, *et al.*, 2013; O'Brien, *et al.*, 2013). The most prevalent sperm defects recorded during this study in season 1 (end of the dry season) were diadem (nuclear crater), Dag-defect, cytoplasmic droplets, midpiece reflex, and coiled principal piece. Similar defects were prevalent during season 2 (end of wet season) but additionally included teratoid and abnormal loose head defects. The proportion of all other sperm defects recorded was low (double head, diadem, pyriform, stump tail, mitochondrial segment aplasia). However, the ejaculates collected during season 2 showed a higher number of cytoplasmic droplets (proximal and distal) and tail defects compared to season 1.

The occurrence of nuclear craters (diadem defect) suggests possible exposure to a causative stressor (e.g. change in nutrition) during cellular division. These defects originate during spermiogenesis and are of a testicular origin, yet further modifications may occur during epididymal transfer (Barth & Oko, 1989; Nöthling & Irons, 2008). Spermatozoa with nuclear craters and otherwise normal heads are able to reach and bind to the oocyte as do normal spermatozoa; however, they result in lower embryo quality and fertility (Nöthling & Irons, 2008; Saacke, 2008). Spermatozoa with abnormal heads or proximal droplets are unable to attach or penetrate the zona pellucida barrier of the ovum in domestic bulls (Saacke, 2008; Nöthling & Irons, 2008). The occurrence of loose heads has been reported in previous elephant studies (Jainudeen, *et al.*, 1971; Kitiyanant, *et al.*, 2000; Imrat, *et al.*, 2013; O'Brien, *et al.*, 2013). The presence of loose heads in high numbers may be due to sperm accumulation in the epididymis, abnormal spermiogenesis, or sperm senescence in the epididymis (Barth, 2007; Nöthling & Irons, 2008). Spermatozoa that die and start to degenerate are passively voided in the urine between ejaculations (Nöthling & Irons, 2008). However the occurrence of detached sperm heads may also be the result of urine contamination of sperm fractions during collection. A positive correlation between pH of semen and the proportion of detached head post-thaw has been previously reported in Asian elephant ejaculates (Imrat, *et al*., 2013). The excessive alkalinity of both bull and

ram semen has been related to poor fertility or motility (Imrat, *et al.*, 2013). However, these defects may also originate during storage in the epididymal cauda, or within the ampulla, or during ejaculation, whereas manipulations and robust mechanical handling may cause detached heads (Nöthling & Irons, 2008).

Spermatozoa in which head shape is relatively normal (indicating normal spermiogenesis and manchette formation) but which display residual cytoplasm around the flagella, would be suggestive of defects in the spermiation process (O'Donnel, 2011). “Dag” defect is named after the first bull that was identified with this sperm defect which can be described as strong bending/coiling and fracture of the distal part of the midpiece within a common surrounding plasma membrane (Barth & Oko, 1989; Pesch & Bergmann, 2006; Brito, 2007). Aberrations in sperm morphology associated with impaired sperm function include tail abnormalities and poorly developed midpieces that contain the mitochondria for electron transfer and ATP production via oxidative phosphorylation. The coordinated movement of the sperm tail requires energy (e.g. ATP) and signalling (e.g. Ca^{2+}) from their surroundings, and disruption in energy supply or signal transduction can alter motility (Yan, 2009). Cytoplasmic droplets are the most common defect of the neck region or midpiece and indicate testicular or epididymal maturation malfunction (Pesch & Bergmann, 2006). A defect originating during spermiogenesis may take 30 days or more for the prevalence to decrease if the cause of the malfunction is rectified (Barth & Oko, 1989; Nöthling & Irons, 2008). Seasonal variation in the proportion of normal sperm and the occurrence of bent midpieces and retained cytoplasmic droplets has been reported for domestic goats, cattle, and African buffalo (Herrick, *et al.*, 2004). Analysis of flagellar defects is an important and relatively straight-forward component of semen assessment as a high incidence may cause problems with several aspects of sperm motility, transport through the female reproductive tract and penetration of the zona pellucida (Holt, 2005; Saacke, 2008).

During this study, bent midpiece and bent principal pieces were prevalent in the majority of the semen smears analysed. When spermatozoa are exposed to an environment after ejaculation that is hypotonic or too cold it can induces bent flagella (Barth, 2007; Nöthling & Irons, 2008; Saacke, 2008). Depending on the conditions that spermatozoa are exposed to after ejaculation, bent midpiece and bent principal piece defects may be artefacts (Barth & Oko, 1989; Nöthling & Irons, 2008). During this study the manifestation of bent midpiece and bent principal pieces might possibly be the result of the increased ratio of seminal plasma within the semen samples collected as previously discussed in this section. Studies on sperm morphology are important for understanding the reproductive biology of the species investigated. Morphologically abnormal spermatozoa in semen collected from domestic bulls have been associated with subfertility and sterility (Saacke, 2008). Fertilisation failure or the failure in development of a fertilised oocyte is considered to result from the inability of a spermatozoon to maintain its biological functionality (Nöthling & Irons, 2008; Saacke, 2008). Sperm morphology provides valuable information about sperm quality and is therefore useful for semen and sperm quality investigations as it contributes to calculations of the probability of natural conception (Eggert-Kruse, *et al.*, 1996). The theory behind sperm morphology analysis with regards to sperm competition is that it indicates the subpopulation of spermatozoa within ejaculates that could gain a competitive edge. Previously suggested ideas are that specific subpopulations within ejaculates have fertility advantages for one animal over another (Thurston, *et al.,* 1999).

8.1.3.3 Sperm head morphometric analyses

During this study sperm head morphometric of 1600 individual spermatozoa from 8 elephant semen samples was analysed. CASMA determined the average elephant sperm head length at 6.83 ± 0.26µm and width 3.32 ± 0.18µm, covering a total head area of 20.17 ± 1.96µm^2, of which 38.95 ± 0.92% was covered by the acrosomal cap. These CASMA sperm head length and width measurements from this study are slightly greater than the 6µm by 2.5 – 3µm (measured with eyepiece slide and micrometre) reported for African elephant by Jainudeen, *et al.,* (1971). However,

this was slightly less than the CASMA dimensions of 7.8µm and 3.8µm, covering a total head area of 23.9µm^2 by Rittem, *et al.* (2010) for Asian elephants. Computer-aided sperm morphometric analysis (CASMA) has been shown to be an accurate, precise and repeatable analytical method during this study (Table 8.2). Small geometric differences in sperm head morphometric have been reported to cause large differences in sperm hydrodynamics (Saacke, 2008). A variation in sperm head size and shape was confirmed in this study between the two seasons compared. Subtle sperm head morphometric differences were recorded between ejaculates and between seasons. This investigation revealed that spermatozoa analysed during season 2 (end of summer months) had larger head morphometries and travelled at faster velocities compared to the smaller and slower values recorded for spermatozoa analysed during season 1 (end of winter months). These findings suggest a relationship between elephant sperm morphometric and kinematics.

8.1.4 SEASONAL VARIATION IN AFRICAN ELEPHANT SEMEN AND SPERM QUALITY

Previous reports were of the opinion that seasonal effects were not evident in the semen quality of free-ranging African elephants (Howard, et al., 1984). Recent studies confirmed a seasonal variation in the semen quality of captive Asian elephants (Thongtip, *et al.*, 2008; Saragusty, *et al*., 2009). Hermes, (2013) suspected a seasonal difference in the proportion of morphologically normal and acrosome intact spermatozoa between seasons, reported for the same ejaculates evaluated during this study. However, the statistical and multivariate analyses findings from this study confirmed a variation in semen and sperm quality between the ejaculates collected over two seasons from the same free-ranging (natural) African elephant population. During this study 32 semen and sperm parameters were analysed and recorded from ejaculates collected during season 1 (September 2009, n = 7) and during season 2 (April 2010, n = 14) (Table 8.2). Samples analysed during season 2 recorded significantly higher motilities (progressive and total motility), significant faster velocities (VCL, VSL, VAP) and higher kinematics (LIN and ALH) compared to season 1.

The present reported no difference in the the proportion of morphological normal spermatozoa between the two seasons. However, using strict criteria (Nöthling & Irons, 2008) to classify sperm morphology a seasonal difference in the type of morphological defects (origin of defect) was recorded within the free-ranging African elephants. Overall, the proportion of head defects recorded during season 1 was significantly higher compared to season 2, while the proportion of tail defects was significantly higher during season 2 compared to season 1. Additionally CASMA analysis recorded increased sperm head measurements during season 2 for head regularity, head roughness and for the total percentage acrosomal coverage compared to season 1 (Table 8.2).

The suggested breeding season for African elephants is primarily during the rainy season, from November to April, in the Southern African region (Skinner & Smithers, 1990; Spinage, 1994). During this time, females are more likely to come into estrus. The ejaculation frequency of a male is directly related to the number of reproductive opportunities with estrus females. Estrus refers to a period in time when behaviourally females are most likely to mate with a male, and physiologically where mating is most likely to results in successful fertilisation. The increased proportion of sperm head defects and high sperm concentration recorded during season 1, may possibly be an indication of decreased ejaculation frequency and the storage of spermatozoa over a prolonged period. The epididymis/ampulla stores a high concentration of spermatozoa on a continuous basis. Prolonged storage or extended periods between ejaculations will result in senescence; the degeneration of spermatozoa prior to ejaculation or passive voiding into the urethra (Nöthling & Irons, 2008). Ejaculates collected during season two recorded increased sperm tail defects and cytoplasmic droplets, possibly due to increased ejaculation frequency resulting in less mature spermatozoa being ejaculated. The occurrence of residual bodies appeared to be more prevalent in the micrographs from ejaculates collected during season 2 (end of wet season) compared to those of season 1 (end of the dry season) and suggests a possible seasonal relationship.

In many wildlife species seasonal fluctuation in body fat has been suggested as an adaptation to survive food scarcity (Pond, *et al*., 2004). Whitetail deer accumulates body fat as the days grow shorter. As they become less active during winter they use body fat to meet their energy needs (Pond, *et al.,* 2004). This evolutionary adaptation is so strong that even in captivity, with *ad libitum* food supply, deer eat less in summer than in winter (Pond, *et al.*, 2004). During winter months natural vegetation deteriorates and food sources become scarce. The search for food may result in greater energy expenditure than nutrients and energy acquired; additionally there might be a dominance hierarchy that puts submissive animals at a disadvantage (Pond, *et al.*, 2004).

Mammalian and avian sperm contain large proportions of polyunsaturated fatty acids (Dierenfeld & Clauss, 2001). The polyunsaturated fatty acid composition of sperm is, to a certain degree, influenced by the polyunsaturated fatty acids composition of the diet (Dierenfeld & Clauss, 2001). A difference in the fatty acid composition of the sperm of captive African and Asian elephants has been detected (Dierenfeld & Clauss, 2001). Spermatozoa from Asian elephants are known to be difficult to cryopreserve, and have been shown to contain a lower proportion of omega-3 fatty acids compared to African elephants (Dierenfeld & Clauss, 2001; Saragusty, *et al.*, 2009). Several studies have confirmed that elephants forced to live in national parks consisting mainly of open grassland suffer severly largely from arterial disease while animals living in forested areas are virtually free of this condition (Dierenfeld & Clauss, 2001). Analyses of the diets have shown that the vegetation in the forests provides considerably larger quantities of polyunsaturated fatty acids than the grassland vegetation (Dierenfeld & Clauss, 2001). If the integrity of arteries can be affected by vegetation deterioration, surely the complex process of spermatogenesis can also be affected. Essential fatty acid deficiency has been shown to impair spermatogenic development, as well as survival of the foetus and newborn (Dierenfeld & Clauss, 2001).

Most mammalian species breed seasonally. The proximate stimuli for the onset of breeding may include changes in rainfall, temperature, chemical stimuli or increased day length (Dewsbury, 1996). It is generally considered that reproductive patterns have evolved so that animals breed at a time of year that allows the young to be born when the environmental conditions are most favourable for their growth and survival (Dewsbury, 1996). During the winter month's energy is diverted to vital organs, thus the possibility exists that the quality of spermatogenesis between seasons may vary due to seasonal changes and environmental stressors that include vegetation deterioration. This promotes considerable interest in the possibility that the structural variabilities observed among sperm subpopulation reflect directly on individual functional and adaptive differences to seasonal changes and environmental stressors. Variability among subgroups may relate to adaptive and functional differences within the ejaculate possibly providing some sperm subpopulations with a selective fertility advantage (Abaigar, *et al.,* 1999; Abaiger, *et al.*, 2001; Holt & Van Look, 2004; Miro, *et al*., 2005; Holt, *et al*., 2007; Maree & Van der Horst, 2013).

Table 8.2 Summary table of 32 semen and sperm parameter analysed during season 1 (September 2009, n = 7) and season 2 (April 2010, n = 14). The overall population average (n = 21) represents the semen and sperm quality of free-ranging African elephants analysed during this study.

Summary of African elephant (*Loxodonta africana*) semen and sperm characteristics recorded:			
Parameters	**Population Average Season 1**	**Population Average Season 2**	**Overall Population Average**
Colour	watery-grey to milky	watery-grey to milky	**watery-grey to milky**
Volume (mL)	46 ± 47	61 ± 58	**56 ± 38**
Concentration (x 10^6/mL)	976 ± 784	739 ± 655	**818 ± 750**
Total # sperm per ejaculate (x 10^9)	64.54	38.49	**47.17**
Total motility (%)	77 ± 25.3[a]	97 ± 3.01[b]	**81 ± 29.7**
Progressive motility (%)	58 ± 25.3[a]	78 ± 7.5[b]	**62 ± 26.9**
Non-progressive motility (%)	17 ± 11.1	20 ± 8.1	**19 ± 9.2**
VCL (µm/sec)	193 ± 70.1[a]	270 ± 20.4[b]	**241 ± 58.5**
VSL (µm/sec)	133 ± 55.3[a]	196 ± 25.9[b]	**173 ± 181.6**
VAP (µm/sec)	158 ± 63.9[a]	226 ± 26.9[b]	**201 ± 54.5**
LIN (%)	57 ± 22.6[a]	73 ± 7.9[b]	**67 ± 16.4**
WOB (%)	81 ± 8.7	84 ± 5.1	**83 ± 6.6**
STR (%)	84 ± 5.7	86 ± 5.5	**86 ± 85.7**
ALH (µm)	3.2 ± 0.9[a]	3.9 ± 0.4[b]	**4 ± 0.8**
BCF (Hz)	20 ± 3.2	21. ± 2.9	**21 ± 3.1**
% Hyperactive motility (Ham's F10)	-	5%	**5%**
% Hyperactive motility induced (BO)	-	29%	**29%**
Membrane integrity intact (LIVE) (%)	-	68 ± 11.9	**68 ± 11.9**
Normal morphology (%)	58 ± 21.8	54 ± 11.3	**55 ± 14.2**
Number of head defects (%)	24 ± 24.1[a]	9 ± 6.8[b]	**15 ± 17.1**
Number of midpiece defects (%)	12 ± 7.8	16 ± 10.7	**14 ± 9.7**
Number of tail defects (%)	9 ± 4.9[a]	21 ± 11.3[b]	**16 ± 11**
Acrosome intact (%)	-	77 ± 11.3	**77 ± 11.3**
Head length (µm)	6.73 ± 0.45	6.85 ± 0.18	**6.83 ± 0.26**
Head width (µm)	3.23 ± 0.23	3.35 ± 0.15	**3.32 ± 0.18**
Head area (µm²)	18.84 ± 2.59	20.76 ± 1.39	**20.17 ± 1.96**
Head ellipticity	2.09 ± 0.13	2.06 ± 0.09	**2.1 ± 0.1**
Head elongation	0.35 ± 0.03	0.34 ± 0.03	**0.4 ± 0.02**
Head perimeter	14.5 ± 0.78	14.9 ± 0.46	**14.8 ± 0.58**
Head regularity	0.91 ± 0.03[a]	0.88 ± 0.01[b]	**0.9 ± 0.02**
Head roughness	1.11 ± 0.05[a]	1.17 ± 0.03[b]	**1.2 ± 1.15**
Acrosomal coverage (%)	38.11 ± 0.66[a]	39.32 ± 0.77[b]	**38.95 ± 0.92**
Motility and kinematic parameters recorded in EY_Ham's F10 diluted samples			
Data presented as mean ± standard deviation (±SD)			
[a, b] values in rows labelled with different superscript letters indicate significantly difference between seasons ($p<0.05$)			
VCL = Curv+A14:D38ilinear velocity, VSL = Straight line velocity, VAP = Average path velocity, LIN = Linearity of tract, STR = Straightness of tract, WOB = Wobble, BCF = Beat cross frequency, ALH = Amplitude of lateral head displacement			

8.2 THE SOUTHERN WHITE RHINOCEROS (*Ceratotherium simum simum)*

The information gathered during this study substantially adds to the currently available database for rhinoceros semen and sperm parameters, as tabled in Table 8.3. The data represents free-ranging (natural) Southern white rhinoceros (*Ceratotherium simum simum)* populations and was compared to available literature on captive rhinoceros populations (O'Brien & Roth, 2000; Roth, *et al.*, 2005; Hermes, *et al.*, 2005; O' Brien, *et al*., 2009; Hermes, *et al*., 2009; Behr, *et al.*, 2009).

8.2.1 MACROSCOPIC SEMEN EVALUATION OF THE SOUTHERN WHITE RHINOCEROS

8.2.1.1 Ejaculate colour and viscosity

During April 2010, ten semen samples were successfully collected by means of electroejaculation (EE) from ten free-roaming Southern white rhinoceros. The semen fractions collected generally appeared watery-grey with minor variation in viscosity. Previous studies have reported that EE ejaculates especially those collected from White rhinoceros have an extremely viscous gel fraction that seems to entrap the spermatozoa (Behr, *et al.*, 2009). Not all fractions collected during this study contained spermatozoa and some were urine-contaminated and thus discarded. It is generally accepted that electroejaculation can affect the viscosity of semen samples produced and that results are usually technique related. Anatomically, African rhinoceros have three male accessory glands, the bulbourethral glands, the prostate and the vesicular glands, which may all contribute to the high viscosity of fresh rhinoceros ejaculates (Schaffer, *et al*., 2001; Behr, *et al.*, 2009). However, rhinoceros do not appear to ejaculate distinctly different gel and sperm-rich fractions, compared to their equid relatives (Roth, *et al.*, 2005). During the semen collection procedure, it can only be speculated which fraction contains only glandular fluid and which fraction is sperm rich, until confirmed by microscopy (Roth, 2001).

One challenge of electroejaculation in many species is contamination of the ejaculate with urine. The same appears to be true for the rhinoceros. However, whereas some fractions of the ejaculate might contain urine, those fractions collected before and after the urine-contaminated sample could contain high-quality spermatozoa (Roth, *et al.*, 2005; Behr, *et al.*, 2009). The frequent changing of collection condoms allowed for the collection of sperm rich fractions during this study.

8.2.1.2 Ejaculate volume

The average rhinoceros ejaculate collected by means of EE during this study recorded a volume of 24 ± 24mL (range: 7 - 51mL). These results were either comparable (Behr, *et al.*, 2009; Reid, *et al.,* 2009; Hermes, *et al.*, 2009) or less (Hermes, *et al.*, 2005; Roth, *et al.*, 2005) when compared to previous EE volume reports, and less than post-coital recovered ejaculate from a female Sumatran rhinoceros after mating (O'Brien & Roth, 2000) (Table 8.3). Unfortunately, how close this relates to natural ejaculates is unknown because attempts at training individual to ejaculate into artificial vaginas (AV) have not been successful (Schaffer, *et al.*, 1990; Roth, 2006; Pesch & Bergmann, 2006; Brito, 2007; Amann & Waberski, 2014). Nevertheless, several methods of collecting semen have been useful in gaining some insight into rhinoceros ejaculate and sperm characteristics. Further optimisation of the electroejaculation technique and protocol is still required for the repeatable collection of good quality semen samples from rhinoceros. For technologies such as AI or IVF, good quality semen is required. Even though progress over the years has been slow, future advancements in developing assisted reproduction technologies for the rhinoceros taxon seem promising

Table 8.3 Summary table for data comparison of semen and sperm parameter recorded during this study with available literature on semen characteristics of Rhinoceros population.

	White Rhinoceros Free-ranging EE CASA, Manual, CASMA	White Rhinoceros Captive EE Manual	White Rhinoceros Captive EE Manual	White Rhinoceros Captive EE Manual	White Rhinoceros Captive EE Manual	White Rhinoceros Captive EE Manual	One-horned, Black and White Rhinoceros Captive EE Manual	Sumatran Rhinoceros Captive Post-coital Manual
	This study	Behr *et al.*, 2009	Hermes *et al.*, 2009	Reid *et al.*, 2009	O'Brien *et al.*, 2009	Hermes *et al.*, 2005	Roth *et al.*, 2005	O'Brien & Roth, 2000
	n = 10 of 10 individuals	n = 19 of 13 individuals	n = 2 of 1 individuals	n = 37 of 13 individuals	n = 2 of 1 individuals	n = 34 of 21 individuals	n = 7 of 7 individuals	n = 5 of 1 individuals
Parameters:	**Mean ± SD**	**Mean ± SD**	**Average**	**Mean ± SD**	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**
Volume (mL)	24 ± 24	21 ± 16	42	30 ± 5		67 ± 16	98 ± 22	104 ± 9
Concentration (x 10^6/mL)	83 ± 96	117 ± 112	80	164 ± 31		76 ± 16		25 ± 8
Total # sperm per ejaculate (x 10^9)	1.1					2.8 ± 0.8	37 ± 12	2.5 ± 1
Total motility (%)	82 ± 8.5	74 ± 16	90	83 ± 2	83 ± 1.4	86 ± 1.3	81 ± 5	60 ± 3
Progressive motility (%)	28 ± 23.9	39 ± 24	4	4 ± 1	51 ± 6		3 ± 0.2	3 ± 0.1
Non-progressive motility (%)	54 ± 25.3							
VCL (µm/sec)	85 ± 29.8							
VSL (µm/sec)	44 ± 25.1							
VAP (µm/sec)	69 ± 30.7				113 ± 15			
LIN (%)	51 ± 16.7							
WOB (%)	79 ± 11.2							
STR (%)	63 ± 14.3							
ALH (µm)	2 ± 0.6							
BCF (Hz)	16 ± 6.9							
Membrane integrity intact (LIVE) (%)	73 ± 10.8	72.5 ± 4	84			76 ± 3.2		72 ± 3
Normal morphology (%)	62 ± 14.7		74				42 ± 6	40 ± 6
Number of head defects (%)	13 ± 7.2							33 ± 2
Number of midpiece defects (%)	15 ± 7.5							
Number of tail defects (%)	6 ± 3.8							
Acrosome intact (%)	76 ± 4.9	70 ± 5						80 ± 7
Head length (µm)	5.5 ± 0.2							5.2 ± 0.1
Head width (µm)	2.9 ± 0.2							3 ± 0.02
Head area (µm²)	14.8 ± 1.4							
Head ellipticity	1.9 ± 0.1							
Head elongation	0.3 ± 0.03							
Head perimeter	12.2 ± 0.5							
Head regularity	0.9 ± 0.02							
Head roughness	1.3 ± 0.04							
Acrosomal coverage (%)	36.3 ± 0.6							

EE = Electro-ejaculation, MS = Manual stimulation

CASA = Computer-aided sperm analysis, Manual = Phase contrast microscope , CASMA = Computer-aided sperm morphology analysis

VCL = Curvilinear velocity, VSL = Straight line velocity, VAP = Average path velocity, LIN = Linearity of tract, STR = Straightness of tract, WOB = Wobble, BCF = Beat cross frequency, ALH = Amplitude of lateral head displacement

8.2.2 MICROSCOPIC SPERM EVALUATION OF THE SOUTHERN WHITE RHINOCEROS

8.2.2.1 Sperm concentration

The average rhinoceros ejaculate evaluated during this study contained a total number of 1.1 x 10^9 spermatozoa (volume of 24 ± 24mL x concentration of 83 ± 96 x 10^6/mL). Amongst the 10 rhinoceros sperm samples analysed, recorded sperm concentrations ranged between 1 – 225 x 10^6/mL and recorded an average of 83 ± 96 x 10^6 spermatozoa/mL. The results recorded during this study were similar to other reported EE ejaculate concentrations (Hermes, *et al*., 2005; Hermes, *et al*., 2009) and higher than that of a post-coital recovered ejaculate (O'Brien & Roth, 2000). However, the concentrations recorded during this study was less than EE ejaculates of captive White rhinoceros (Behr, *et al.*, 2009; Reid, *et al.*, 2009) (Table 8.3). The electroejaculation procedure does not always result in a good-quality fraction despite concerted efforts with modified rectal probes that have been improved to conform to rhinoceros anatomy, placement of the probe visualised by ultrasound, and frequently changing collection condoms during the procedure (Hermes, *et al*., 2005; Roth, 2006).

8.2.2.2 Motility and kinematic parameter assessment using computer-aided sperm analysis (CASA)

During this study CASA kinematic parameters of 3 500 rhinoceros spermatozoa were analysed from seven semen samples collected from free-ranging (natural) rhinoceros populations. The rhinoceros sperm samples recorded an average total motility (TM) at 82 ± 8% of which 28 ± 23% were progressive motility (PM). These results seem comparable to sperm motilities reported for captive rhinoceros of 86.6 ± 1.4% (Hermes, *et al*., 2005), TM: 81 ± 5%, PM: 3 ± 0.2% (Roth, *et al.*, 2005), TM: 38.5 ± 24.4% (Behr, *et al.*, 2009), TM: 83 ± 2%, PM: 4 ± 1% (Reid, et al., 2009) and 60 ± 3% (O'Brien & Roth, 2000). CASA recorded average velocities for VCL (85 ± 29µm/s), VSL (44 ± 25µm/s) and VAP (69 ± 30µm/s, and kinematics at STR (63 ± 14%), LIN (51 ± 16%), ALH (2 ± 0.16µm) and BCF (16 ± 6Hz). The only comparative data available in the literature for rhinoceros CASA kinematic parameters is from a sperm sorting study that reported on VAP values of fresh rhinoceros spermatozoa by O'Brien (2009) which

recorded total motility at 83 ± 2%, progressive motility at 51 ± 6% and VAP of 113 ± 14.7µm/s (O' Brien, *et al.*, 2009) (Table 8.3). Within the fresh rhinoceros semen samples evaluated during this study a high percentage of non-progressive spermatozoa displayed a circular motion pattern, usually associated with hyperactivated motility. This circular motion pattern has been subsequently observed during analyses of other freshly ejaculated sperm samples collected by EE from other free-ranging Southern white rhinoceros individuals (Luther, unpublished data). This occurrence of circular trajectories repeatedly observed within fresh rhinoceros ejaculates might be normal for rhinoceros spermatozoa. Alternatively, the circular motion pattern observed might also be a response to possible urine contamination during the electroejaculation stimulation procedure and positioning of the probe. Conclusions made from the available literature for captive rhinoceros populations suggest that in general the semen and sperm quality may not be particularly high in these species (Roth, 2006); however, all available data is derived from captive animals that are subjected to various factors and incomparable to the free-ranging population. The rhinoceros sperm velocities recorded during this study were generally below <100µm/s. However, further investigation is required to assess this behaviour in motility displayed by rhinoceros spermatozoa as well as its implication on sperm quality and successful fertilisation.

8.2.3 STRUCTURAL ANALYSES OF SOUTHERN WHITE RHINOCEROS SPERMATOZOA

8.2.3.1 Plasma membrane integrity (viability) and acrosome integrity

Within all the rhinoceros sperm samples collected during this study an average of 73 ± 11% spermatozoa maintained viability and these results are in agreement with intact sperm plasma membrane reports in the literature of 72 ± 3% (O'Brien & Roth, 2000), 75 ± 3% (Hermes, *et al.*, 2005), and 74 ± 16% (Behr, *et al*., 2009). During this study the rhinoceros spermatozoa that stained red from the post-acrosomal region (dead) with a white acrosomal cap (intact) were classified as dead. These results indicate the percentage of viable spermatozoa irrespective of their motile capabilities at the time of motility analysis. It has been previously reported that the loss of sperm viability is mainly a

result of osmotic or cold shock stressors (Khan, *et al.*, 2009). Acrosome integrity analyses recorded a 76 ± 5% average for intact acrosomes and did not indicate a difference between the two rhinoceros populations. The findings of this study are similar when compared to the 80 ± 7% (O'Brien & Roth, 2000), and 73 ± 12% (Behr, *et al.*, 2009) of previous studies, and higher compared to the 58 ± 2% Reid, *et al.,* (2009) (Table 8.3).

8.2.3.2 Sperm morphology

Rhinoceros ejaculates analysed during this study contained on average of 62 ± 14% morphologically normal spermatozoa. Sperm morphological abnormalities were common across all rhinoceros ejaculates analysed during this study. There was no difference recorded for sperm morphology between the two free-ranging rhinoceros populations evaluated, neither in the number or type of abnormalities. Slightly higher percentages for “normal” sperm was reported by (Reid, *et al.*, 2009) compared to this study. However, the percentage of normal spermatozoa in the current investigation was lower than previous reports for epididymal and post-coital collected semen (O'Brien & Roth, 2000). The structural abnormalities recorded during this study (34 ± 15%) was further classified and the occurrence of head defects (13 ± 7%) and midpiece defects (15 ± 8%) were higher that tail defects (6 ± 4%) within both populations (Table 8.3).

The most prevalent sperm defects recorded during this study was abnormal head shapes, nuclear craters, loose heads, Dag-defect (bent/coiled tails within a common surrounding plasma membrane), cytoplasmic droplets, midpiece reflex, and coiled principal piece. The proportion of all other sperm defects recorded was relatively low (pyriform, mitochondrial segment aplasia). The majority of these defects originate during the process of spermiogenesis and are of a testicular origin, and further modifications may occur during epididymal transfer (Barth & Oko, 1989; Nöthling & Irons, 2008). Roth *et al*., (2005) discussed the presence of structurally abnormal spermatozoa and reported that bent

midpieces and bent or coiled tails are some of the most prevalent morphological abnormalities within rhinoceros species (Roth, *et al.*, 2005). Hermes *et al*., (2005) reported that separated (loose) sperm heads and tails were abundant and frequently observed within captive rhinoceros populations (n = 34). The results suggest that the evaluation of sperm morphology using strict classification criteria (Nöthling & Irons, 2008) provided valuable information during a basic rhinoceros semen and sperm quality investigation.

8.2.3.3 Sperm head morphometric analyses

This study applied CASMA technology to analyse 800 individual Southern white rhinoceros spermatozoa collected from two free-ranging populations (n = 8). CASMA measured head length at 5.5 ± 0.17µm and width of 2.9 ± 0.19µm (total head area of 14.8 ± 1.43µm^2) of which 36.3 ± 0.59% is covered by the acrosomal cap. Very little data is available for rhinoceros sperm head morphometric in the literature. A study by O'Brien & Roth (2000) that reported on sperm head morphometric analyses of epididymal Black rhinoceros (n = 1) and post-coital Sumatran rhinoceros (n = 5) spermatozoa measured by means of an eyepiece graticule and light microscopy. This study revealed a head length of 6 ± 0.06µm and head width of 3 ± 0.02µm for the Black rhinoceros, and a head length of 5.2 ± 0.07µm and head width of 3 ± 0.02µm for the Sumatran rhinoceros (O'Brien & Roth, 2000) (Table 8.3). Computer-aided sperm morphometric analysis (CASMA) has been shown to be an accurate, precise and repeatable method to analyse sperm morphometric (Davis, 1992; Gil, *et al.*, 2009). Subtle differences between individuals, undetected with subjective, manual evaluation methods (Gil, *et al.*, 2009; Maree, *et al.*, 2010) were detected with CASMA during this study.

8.2.4 VARIATION IN SEMEN AND SPERM QUALITY BETWEEN POPULATIONS

The information presented in this study substantially adds to the current physiological database for reproductive parameters recorded for semen samples collected from free-ranging Southern white rhinoceros (*Ceratotherium simum simum*) populations by means of electroejaculation, within South Africa. During this study 32 semen and sperm parameters were analysed and recorded from ejaculates collected from Population A (n = 6) and Population B (n = 4) (Table 8.4). Sperm concentration and viability were the only two parameters that recorded a significant difference between the two populations compared. Population A was a highly dense population (>5 individuals per 100 hectares) that recorded lower sperm concentrations compared to the less dense (<0.5 animals per 100 hectares). It is recommended that rhinoceros stocking density should not exceed the recommended 0.5 animals per 100 hectares for savannah regions (du Toit, 2005). Plasma membrane integrity (viability) was higher in Population A (80.25 ± 7.5%) that mainly fed on hay or teff (*Eragrostis tef*) and lucerne, whereas Population B (65.75 ± 8.65%) only fed on natural vegetation (had no access to additional feed e.g. hay teff or lucerne).

White rhinoceros usually occur in small family groups that can consist of cows, calves and sub-adults. Dominant adult bulls are generally solitary and fiercely protect their territory from other dominant males, while not allowing subordinate bulls in their territory (du Toit, 2005). During sampling from Population A, semen from solitary bulls was collected; however it was unknown whether these males were dominant or subdominant in the hierarchy dynamics of the population A. During sampling of population A, fighting was observed between two bulls during the daily feed regime, thus the competition for food could be a possible factor, combined with the hierarchy dynamics of the population, that accounts for the variation in sperm concentration recorded between the two rhinoceros populations. This suggests that sperm concentration might have been suppressed by dominant bulls in population A compared to the more natural population number of population B that recorded significantly higher sperm concentrations in the ejaculates collected.

The white rhinoceros is a low-selective grazer that feeds almost exclusively on grass (du Toit, 2005). Rhinoceros prefer more palatable broad-leaved grass species with a leaf height less than 400mm, and can graze down to 30mm above the ground (du Toit, 2005). Less data is available on the impact of diet and nutrition on the grazing rhinoceros, but it appears that the browsing species (black rhinos, *Diceros bicornis*, and Sumatran rhinos, *Dicerorhinus sumatrensis*) may be particularly susceptible to dietary fatty acid influences (Dierenfeld & Clauss, 2001). Nutrients are defined as chemical elements or compounds in a diet that are responsible for supporting normal reproduction, growth, lactation or maintenance of life processes (Pond, *et al.*, 2004). Nutrients support cellular needs for water, fuel, structural constituents (muscle, skin, bone, nerve, fat) and metabolic regulation (Pond, *et al.,* 2004). The basic structure of the plasma membrane consists of the phospholipid bilayer, which is impermeable to most water-soluble molecules. The passage of ions and most biological molecules across the plasma membrane is therefore mediated by proteins, which are responsible for the selective traffic of molecules into and out of the cell (Fawcett, 1970; Weiss & Greep, 1977; Cooper & Hausman, 2009). This significant difference recorded for rhinoceros sperm concentrations and viability during this study might be as a result of the difference in nutrition and the general social structure between the two populations compared. However, further investigation and correlations between nutrition, social structure and sperm quality should be included for future conservation studies on the breeding of rhinoceros.

Table 8.4 Summary table of the overall population averages recorded from ten free-ranging Southern white rhinoceros ejaculates collected during April 2010 from population A (n = 6) and population B (n = 4).

Summary of Southern white rhinoceros (Ceratotherium simum simum) semen and sperm characteristics recorded:			
Parameters	**Population A Average**	**Population B Average**	**Overall Population Average**
Colour	watery-grey	watery-grey	**watery-grey**
Volume (mL)	24 ± 9.9	25 ± 18.9	**24 ± 24**
Concentration (x 10^6/mL)	13 ± 10.38[a]	153 ± 101.9[b]	**83 ± 96**
Total # sperm per ejaculate (x 10^9)	0.21	2.45	**1.1**
Total motility (%)	80 ± 8.1	90 ± 4.7	**82 ± 8.5**
Progressive motility (%)	21 ± 15.9	28 ± 23.9	**28 ± 23.9**
Non-progressive motility (%)	67 ± 21.2	54 ± 25.3	**54 ± 25.3**
VCL (µm/sec)	85 ± 24.2	85 ± 29.7	**85 ± 29.8**
VSL (µm/sec)	30 ± 8.9	44 ± 25.1	**44 ± 25.1**
VAP (µm/sec)	54 ± 5.4	69 ± 30.7	**69 ± 30.7**
LIN (%)	40 ± 18.7	51 ± 16	**51 ± 16.6**
WOB (%)	67 ± 16.2	51 ± 16	**79 ± 11.2**
STR (%)	67 ± 16.2	79 ± 11.2	**63 ± 14.3**
ALH (µm)	3 ± 5.6	2 ± 0.6	**2 ± 0.6**
BCF (Hz)	13 ± 5.6	16 ± 6.9	**16 ± 6.9**
% Hyperactive motility (NT_Ham's F10)	46%	29%	**37%**
% Hyperactive motility induced (NT_BO)	57%	33%	**45%**
Membrane integrity intact (LIVE) (%)	80 ± 7.5[a]	66± 8.7[b]	**73 ± 10.8**
Normal morphology (%)	66 ± 19.3	58 ± 9	**62 ± 14.7**
Number of head defects (%)	10 ± 6.2	17 ± 7	**13 ± 7.2**
Number of midpiece defects (%)	11 ± 8	19 ± 4.6	**15 ± 7.5**
Number of tail defects (%)	8 ± 3.5	5 ± 4.1	**6 ± 3.7**
Acrosome intact (%)	79 ± 3	71 ± 4.2	**76 ± 4.9**
Head length (µm)	6 ± 0.1	6 ± 0.2	**5.5 ± 0.17**
Head width (µm)	3 ± 0.2	2 ± 0.2	**2.9 ± 0.19**
Head area (µm²)	15 ± 1.3	15 ± 1.7	**14.8 ± 1.43**
Head ellipticity	2 ± 0.1	2 ± 0.1	**1.9 ± 0.1**
Head elongation	0.3 ± 0.02	0.3 ± 0.02	**0.3 ± 0.03**
Head perimeter	12 ± 0.3	12 ± 0.7	**12.2 ± 0.5**
Head regularity	0.9 ± 0.03	0.9 ± 0.008	**0.9 ± 0.02**
Head roughness	1.3 ± 0.06	1.3 ± 0.02	**1.3 ± 0.04**
Acrosomal coverage (%)	36.1 ± 0.4	36.5 ± 0.7	**36.3 ± 0.6**
Motility and kinematic parameters recorded in EY_Ham's F10 diluted samples			
Hyperactivated motility analyses recorded in neat (NT) sperm samples in Ham's F10 (NT_Ham's) and BO (NT_BO) media			
Data presented as mean ± standard deviation (±SD)			
[a, b] values in rows labelled with different superscript letters indicate significantly different between populations ($p<0.05$)			
VCL = Curvilinear velocity, VSL = Straight line velocity, VAP = Average path velocity, LIN = Linearity of tract, STR = Straightness of tract, WOB = Wobble, BCF = Beat cross frequency, ALH = Amplitude of lateral head displacement			

8.3 MOTILITY ANALYSIS IN AFRICAN ELEPHANT AND WHITE RHINOCEROS SPERM POPULATIONS USING CASA TECHNOLOGY

The application of CASA technology during this study proved to be a reliable and field-friendly diagnostic tool. The inclusion of CASA allowed for the comprehensive and standardised analysis of sperm populations under *in vitro* conditions within 21 elephants and 10 rhinoceros ejaculates. Functional analysis permitted the testing of elephant and rhinoceros spermatozoa under *in vitro* conditions. Furthermore, the availability of the various CASA derived datasets from the same ejaculate additionally allowed further exploration of the CASA data, based on using logical sets of values by the concepts that they represent (Boolean argument).

Many advances have been made to make CASA instruments more user-friendly for both the human clinical and domestic animal fields (Mortimer, *et al.*, 2015). The development of the CASA system over the last forty years has come a long way for the objective evaluation of spermatozoa motion characteristics. (Contri, *et al.*, 2010; Amann & Waberski, 2014; Mortimer, *et al.,* 2015). The evaluation of thousands of sperm tracks allows for very accurate and repeatable quantification of numerous kinematic parameters (Davis & Katz, 1993; Holt, *et al.*, 2007; Mortimer, *et al.,* 2015). Furthermore the development and cost-effective availability of higher resolution digitizers with greater computing power have corrected many of the previously reported image analysis issues (Amann & Waberski, 2014; Mortimer, *et al.*, 2015). These improvements combined with advanced CASA software features progressed to the development of automated illumination control, Brownian motion filtering, smart sperm collision tracking and adaptive smoothing to derive the average path (Mortimer, *et al.*, 2015). It is also important to acknowledge that biological and technical limitations can affect the ability of CASA systems to provide accurate quantification during analyses of sperm concentration and between motile and progressively motile spermatozoa (Davis & Katz, 1993; Holt, *et al.*, 2007; Contri, *et al.*, 2010; Amann & Waberski, 2014; Mortimer, *et al.*, 2015). The technical limitations of digital image analysis do not allow CASA instruments to directly analyse and classify spermatozoa according to flagellar beating displayed, as is done during visual sperm motility observations, whereas CASA

rely on tracking the movement of the sperm head (Mortimer, *et al.*, 2015). Continuous validation and development of species-specific CASA software settings are essential to avoid the generation of meaningless CASA data (Holt, *et al.*, 2007; Contri, *et al.*, 2010; Amann & Waberski, 2014; Mortimer, *et al.*, 2015).

8.3.1 COMPARISON OF CASA RECORDED MOTILITY AND KINEMATIC PARAMETERS OF ALL SPERM SAMPLES EVALUATED IN DIFFERENT MEDIA

The aim of this part of the study was to examine the influence of extender composition by comparing the types of motility displayed by elephant and rhinoceros spermatozoa (including individual parameter values) mainly due to media interaction. Subtle differences amongst sperm populations within the different evaluation media was identified during this study. The CASA motility and kinematic traits of all available neat (NT) or egg yolk (EY) extended sperm samples, further diluted in either Ham's F10, INRA96® or BO (10mM caffeine) media were recorded.

All three evaluation media used during this study, Ham's F10, INRA96® and BO (10mM caffeine) allowed for clear optical backgrounds during CASA evaluation. The neat semen samples required the minimal removal of particles or debris manually during the reanalyses of recorded CASA fields. The semen samples extended with egg yolk required more frequent manual removal of particles within the CASA fields analysed. During this study the neat (NT) samples groups recorded higher percentages for total and progressive motilities compared to the egg yolk (EY) sample groups. Overall the lowest total motility was recorded in EY extended samples since the kinetics of sperm motility is ruled by their surrounding medium (Ren, et al., 2001). The viscosity of the surrounding environment of elephant and rhinoceros spermatozoa analysed during this study consisted of egg yolk (15.6% v/v) and fructose/lactose TES/Tris buffer (8.59% w/v) solution. For the elephant sperm samples CASA data recorded similarities and significant differences amongst the seven media groups compared. The rhinoceros ejaculates recorded no statistical difference between any of the 33 different egg yolk

(EY) or neat (NT) semen samples, diluted with either Ham's F10 or INRA96® during CASA analyses. However both Ham's F10, INRA96® and BO (10mM caffeine) media allowed the functional evaluation of elephant and rhinoceros spermatozoon populations during CASA analyses. The inclusion of caffeine in BO changes the motion patterns significantly when compared to Ham's F10 and INRA96®. McPartlin *et al.*, (2009) confirmed that procaine-induced hyperactivation of equine spermatozoa could be defined by an increase in ALH and a decrease in the straightness parameters (LIN and STR). In previous studies on bovine, equine and horse spermatozoa, increases in VCL and ALH also defined hyperactivated motility (Marguez & Saurez, 2009; McPartlin, et al., 2009; Olson, et al., 2011). During this study it was concludeD that Ham's F10 medium provided the optimal environment for both elephant and rhinoceros spermatozoa and recorded the highest velocities. The only drawback of Ham's F10 media is its sensitivity to pH changes and needs for frequent monitoring under field conditions. Sperm velocities recorded in INRA96® diluted samples were comparable to Ham's F10 values. Accordingly INRA96® could be considered for the transport of fresh-chilled elephant or rhinoceros semen between and across continents for the use in timed AI's in isolated and fragmented populations.

Ham's F10 medium, is routinely used to evaluate spermatozoa and is known to maintain sperm function over a long period in a vast number of species (Brinders, 1994; Van der Horst, 1995; Mahadevan, *et al.*, 1997; Maree & Van der Horst, 2013). Both Ham's F10 and INRA96® are widely available and affordable, promote high sperm motility in domestic species, are particle free media and have a low viscosity (Aurich, 2008; Imrat, *et al.*, 2013). Comparison of CASA data between activating substances such as bicarbonate (Ham's F10) and fructose (INRA96®) used during this study considerately activated motility of "quiescent spermatozoa" in the egg yolk-only extended sperm samples. Previous reports also indicated that Ham's F10 and INRA96® are sufficient for the activation of sperm motility in other mammals (Holt & Harrison, 2002; Graham, *et al.*, 2004; Buranaamnuay, *et al.*, 2013).

Semen extenders are primarily based either on milk or egg yolk (Aurich, 2008). Semen extenders or diluents used during CASA analysis can cause changes in kinematic parameters (Davis & Katz, 1993). Extender components can affect the nature and extent to which spermatozoa interact and function (Graham, *et al.*, 2004; Aurich, 2008). If spermatozoa are to be cooled or cryopreserved, as is done during most investigations, the recommended protocol includes that the collected samples should be extended with a protective substance such as egg yolk. Egg yolk in semen extender protects sperm against cold-shock and the low-density lipoproteins in egg yolk acts as protective agents by directly infiltrating the sperm plasma membrane, possibly stabilising it (Graham, *et al.*, 2004; Aurich, 2008). The contact between egg yolk and sperm plasma membrane ultimately increases sperm tolerance to temperature changes (Graham, *et al.*, 2004; Buranaamnuay, *et al.*, 2013). It is generally accepted that the function of semen extenders includes stabilising the sperm plasma membrane.

Commercially, INRA96® and other milk-based extenders are mainly used for equine fresh semen processing and storage (Aurich, 2008). The development of better-defined milk based extenders containing only the beneficial milk ingredients has made the quality of milk based extenders more consistent and reliable than egg yolk based extenders (Aurich, 2008). Jones (1973) reported on the activation of sperm motility in phosphate buffered saline (PBS), noting that the addition of potassium and fructose could further increase motility (Jones, 1973). Studies by Graham, *et al.,* (2004) and Imrat, *et al.,* (2013) both reported that egg yolk had a positive effect on maintaining the viability and motility of Asian elephant spermatozoa over time (Graham, *et al*., 2004; Imrat, *et al.*, 2013). Recent studies have indicated that milk-based powder is an equally effective or slightly better non-permeating cryoprotectant for Asian elephant spermatozoa compared to egg yolk extenders (Saragusty, *et al.*, 2009; Imrat, *et al.*, 2013).

However, contact between the egg yolk and sperm plasma membrane may have a negative impact on a spermatozoon's response to activating motility. It is suggested that the membranes associated with the tail, and mitochondrial- and axonemal systems that are responsible for motility, are susceptible to cold-induced damage, therefore benefit from the membrane protection provided by the egg yolk components within semen extenders (Comizzoli, *et al.,* 2000; Chenoweth & Kastelic, 2007). However, this may have a negative impact on sperm functionality that could affect the fertility potential of spermatozoa, since it may decrease the ability of a spermatozoon to initiate and maintain the capacitation process and the acrosome reaction (Pommer, *et al.*, 2002). The information provided during this study significantly adds to the currently available elephant and rhinoceros semen characteristic database. Previous studies have advised that interpreting CASA results should be done with caution until the effect of extender and evaluation media is better understood (Davis & Katz, 1993). Future CASA studies should include investigating the possible influence of egg yolk and skim milk-based media on the functional fresh and post-thaw fertility potential of spermatozoa during cryopreservation studies, under *in vitro* conditions. This is important so as to discriminate between fertile and subfertile bulls during sperm quality assessment based on motility and kinematic parameters values recorded. Further investigations are required to determine the effects caused by all extenders or cryopreservation media used in different species.

8.3.2 SPERM MOTILITY PATTERN ANALYSIS AND THE CLASSIFICATION OF MOTILE SPERMATOZOA

During this study, frequency distribution and statistical analysis of data collected per sperm motion group were used to derive CASA cut-off values (based on Boolean argument) to determine the percentage of hyperactivated (HA) spermatozoa within ejaculates collected from free-ranging African elephants and Southern white rhinoceros. Four distinct motion patterns were identified 1) straight-line (non-HA), 2) linear (intermediate), 3) HA circular (HA C), and 4) HA starspin (HA S). A total of 326 individual African elephant spermatozoa and 266 individual Southern white rhinoceros spermatozoa

were classified accordingly and the measured descriptors were recorded. The approach of pattern analysis assists to define sperm subpopulations based on motion characteristics (Abaigar, *et al.,* 1999; Thurston, *et al.*, 1999; Abaiger, *et al.*, 2001). Pattern analysis thus provides information of the heterogeneity of sperm motion by allowing exploration of CASA data (Abaigar, *et al*., 1999). The outcome of the motion pattern analysis, and the statistical analysis and frequency distribution of parameter data per motion pattern, derived accurate cut-off values for CASA to define hyperactivated sperm subpopulations within African elephant and Southern white rhinoceros ejaculates. Quantitative differences between activated and hyperactivated motile elephant and rhinoceros spermatozoa are now documented, based on motion patterns displayed. These values can now be used in future studies to further quantify and define different motility patterns in different wildlife species.

Sperm subpopulation investigations demonstrated that significant information can be explained by the incorporation of histograms to analyse sperm subpopulation structures within human ejaculates (Holt *et al.,* 2007). Pattern analysis of CASA datasets simplifies the structure of complete datasets by reducing the number of individual observations and measured descriptors without losing their information content (Abaigar, *et al.*, 1999). In the scatter diagrams the general grouping of data plotted per motion pattern indicated that parameters VSL, LIN and STR were sensitive descriptors to sort between straight line progressive motility and HA motility of both elephant and rhinoceros sperm populations. ROC curve analysis also recorded high sensitivity/specificity (>90 percentage) for VSL, LIN and STR when used to establish cut-off values. VCL and ALH have been reported to be indicators of a change in motility to hyperactivated motility. Results obtained during ROC analyses recorded low sensitivity/specificity for VCL, ALH and BCF. This development of a robust definition for HA should be independent of ALH during HA assessment as ALH values are not consistent between instruments and so cannot be standardised across CASA platforms (Mortimer, *et al.*, 2015). CASA parameter cut-offs are generally used to classify hyperactivated motility and increased values for VCL and ALH and a decrease in values for VSL, LIN and STR parameters are used for most species (Saurez, et al., 1993; Mortimer, et al., 1998; Ho & Saurez, 2001; McPartlin, et al., 2009; Olson, et al., 2011; Hinrichs

& Louw, 2012). During this study CASA parameter cut-off criteria was derived after motion pattern and ROC analysis. The frequency distribution of the descriptors VSL, LIN and STR, combined with ROC analysis made it possible to derive species-specific cut-off criteria for CASA to sort between activated and hyperactivated motility, based on a Boolean argument. The derived parameter values wascompared to the available literature (Saurez & Dai, 1992; Mortimer, *et al.*, 1998; Abaigar, *et al.,* 1999; Marquez, *et al.*, 2007; Loux, *et al*., 2010) (Table 8.5). Progressive motility is characterised by linear forward trajectory and a symmetrical waveform. Directional progressive motility of spermatozoa increases on entering the female reproductive tract (Suarez, 2008; Ren & Xia, 2010; Singh & Rajender, 2014). Hyperactivated motility occurs when the flagellum develops an asymmetrical waveform (whip-like motion) that results in high amplitude of lateral head movement in a medium of standard viscosity characterised by highly nonlinear (starspin) or circle trajectories (Olson, *et al.*, 2011; Hinrichs & Louw, 2012) as observed in fresh ejaculates during this study.

The purpose of a spermatozoon during natural fertilisation is to fuse with an oocyte and deliver the genetic material, regardless of the obstacles within the female reproductive tract (Suarez, 2008). In theory, spermatozoa undergo progressive forward motility or activated motility and hyperactivated motility (Olson, *et al*., 2011). In the fallopian tubes, a majority of the sperm attach to the epithelial cells to form a sperm reservoir. Deeper within the fallopian tubes where a more alkaline environment is present spermatozoa undergo a process called hyperactivation (Suarez, 2008; Ren & Xia, 2010; Singh & Rajender, 2014).

Table 8.5 CASA parameter cut-off criteria derived after motion pattern and ROC analysis and compared to available literature.

	African elephant		White Rhinoceros		Stallion		Mouse		Boar		Human		Mouse	
	This study		This study		Loux *et al* ., 2010		Marques *et al.*, 2007		Abaigar, et al., 1999		Mortimer *et al.* , 1998		Saurez & Dai, 1992	
Parameters:	**Activated**	**HA**	**Activated**	**HA**	**Activated**	**HA**	**Activated**	**HA**	**Activated**	**HA**	**Activated**	**HA**	**Activated**	**HA**
VCL	245.8	**244**	83.3	**83.3**	225	**318**	330	**393**	139	**178.4**	-	**150**	200	**260**
VSL	152.4	**70.2**	44.8	**34.1**	-	-	-	-	44.6	**104.5**	-	-	85	**55-60**
STR	93.9	**26.5**	87.9	**67.6**	-	-	-	-	-	-	-	-	-	-
LIN	73.7	**45.2**	78.3	**42.5**	-	-	-	-	-	-	-	**50**	65.9	**37.6**
ALH	-	-	-	-	7.7	**12.4**	-	-	10	**7.8**	-	**7**	-	-

HA = Hyperactive motility

VCL = Curvilinear velocity, VSL = Straight line velocity, VAP = Average path velocity, LIN = Linearity of tract, STR = Straightness of tract, ALH = Amplitude of lateral head displacement

8.3.3 HYPERACTIVATED MOTILITY ANALYSIS

The aim of this study was to establish quantitative differences between hyperactivated and non-hyperactivated spermatozoa which could then be used for CASA (sort function). Establishing cut-off points for such tracks is relatively simple, involving comparing their kinematic parameters to those of the relatively straight swimming sperm tracks using receiver operating characteristic (ROC) curve analysis: those kinematic parameters that show high levels of specificity and sensitivity (>90%) are then used to construct a Boolean argument. This approach has been successfully used in conjunction with the Sort function of the SCA to establish the percentage HA and non-HA within a sperm sample. During this study, three successive elephant ejaculates collected from Young 1 was analysed for HA motility during season 2. HA percentages recorded increased when the first (LA 5 at 4.7% and 13.5% in EY_Ham's and EY_BO) ejaculate was compared to the second (LA 9 at 4 % and 21.5 % in EY_Ham's and EY_BO) and third ejaculates (LA 11 at 0.5% and 20.6% in EY_Ham's and EY_BO). These results suggest variation in functional sperm populations between ejaculates collected from the same individual. The data also suggests that potentially the second ejaculate contained more functional sperm based on their ability to become hyperactivated. Hyperactivation is considered an important endpoint of capacitation and is positively correlated to live birth outcome (McPartlin, et al., 2009; Hinrichs & Louw, 2012). Hyperactivated motility provides the spermatozoa with the necessary

force required to free themselves from the oviduct reservoir and penetration of the cumulus oophorous and zona pellucida surrounding the oocyte (Yanagimachi, 1969; Yanagimachi, 1994; Saurez, *et al*., 1993; Ho & Saurez, 2001; Ren, *et al*., 2001; McPartlin, *et al.*, 2009; Ren & Xia, 2010). As there is currently no information available on hyperactivated motility for the African elephant or Southern white rhinoceros sperm, this data can serve as new baseline data to define HA for these species. We can assume that the type of motility recorded is mainly as a result of interaction with the media. In this study, pattern analysis and classification of spermatozoa within different media allowed for the functional assessment of the motile sperm population's ability to become hyperactivated. This may indicate the ability/ competence of sperm to reach the site of fertilisation and penetrate the zona pellucida of an oocyte (Mortimer, *et al.*, 1998).

8.4 TRANSMISSION ELECTRON MICROSCOPY (TEM) FOR THE AFRICAN ELEPHANT AND SOUTHERN WHITE RHINOCEROS

The aim of the combined use of bright field and electron microscopy was not to re-describe the elephant and rhinoceros spermatozoon at the micro and ultrastructural level but to confirm the occurrence of the major structural features expected from elephant and rhinoceros spermatozoa as presented in the literature review. Furthermore, we aimed to confirm and/or describe any other abnormal sperm or characteristics that were not visualized during bright field microscopy. The inclusion of TEM analyses during this study allowed the verification of the morphological anomalies observed during light microscopy at the ultrastructural level. In a few micrographs, the overlying plasma membrane in the region of the sperm nucleus sometimes seemed loose fitting and wavy in contour. As previously reported, due to an osmotic effect during processing this portion of the membrane has a tendency to balloon out from adjacent structures. This was initially interpreted as a defect although it is generally accepted to be a normal processing artefact (Fawcett, 1970; Jones, 1973). In general, the occurrence of nuclear craters within the micrographs was limited. The chromatin appeared to be densely packed within the sperm nucleus and seems that the occurrence of nuclear

craters is not common in free-ranging elephant and rhinoceros spermatozoa. It has been proposed that granulation of nuclear chromatin can be observed in infertile domestic bulls, and by default will result from the faulty maturation of spermatids (McCosker, 1969). Ruminant spermatozoa have highly condensed chromatin and optimal sperm DNA packaging seems to be essential for full expression of male fertility potential (Rodriques-Martinez & Barth, 2007).

Apical vesiculation (acrosome reaction) of African elephant sperm heads was also observed. In Asian elephant spermatozoa apical vesiculation is regarded as a critical sign indicative of the acrosome reaction (Kitiyanant, *et al.,* 2000). The fusion of the plasma membrane and the outer acrosomal membrane results in vesiculation and causes leakage of the acrosomal content around the apical region of the sperm head (Kitiyanant, *et al.*, 2000). In this study various subpopulations of spermatozoa, which differed in acrosome status, co-occurred within an ejaculate. The acrosome reaction of a dead elephant spermatozoon can be distinguished from a live spermatozoon. In a dead elephant spermatozoon random breakdown (random vesiculation) of the plasma and outer acrosomal membrane occurs over all the areas of the sperm head (Kitiyanant, *et al*., 2000). In a live elephant spermatozoon random, vesiculation occurs only around the acrosomal cap region (Kitiyanant, *et al*., 2000). Spermatozoa with acrosome defects have a reduced ability to bind and penetrate the zona pellucida, and are predisposed for premature capacitation and spontaneous acrosome reaction compared to normal acrosomes (Pesch & Bergmann, 2006).

The occurrence of the redundant nuclear envelope (RNE) at the base of the elephant and rhinoceros sperm heads were observed. RNE is formed from the remaining nuclear membrane (Weiss & Greep, 1977; Pesch & Bergmann, 2006). It is suspected that either hyperactivation motility (HA) is induced by the direct influx of calcium, or HA may trigger calcium release from internal calcium stores through either second messenger-induced or calcium-induced calcium release (Hinrichs & Louw, 2012). The basis of the latter theory is that sperm in at least some species have a redundant nuclear envelope,

which is suspected to function as an internal calcium store (Ho & Suarez, 2003). The presence of the RNE might suggest that an internal Ca^{2+} store is required by elephant and rhinoceros spermatozoa for optimal functionality. The occurrence of spermatozoa with a head shape which is relatively normal (indicating normal spermiogenesis and structural formation), yet there is residual cytoplasm around the tail (flagellum), would be suggestive of defects in the spermiogenesis process (Nöthling & Irons, 2008; O'Donnel, 2011). Flagellae with deformities (E.g. bent flagellae with residual cytoplasmic droplets present, and Dag defect or structural defects in the mitochondria) can result in abnormal or terminated motility. Abnormal sperm morphology may be a reflection of poor testicular physiology (Eggert-Kruse, *et al.*, 1996). Careful assessment of when a sperm abnormality arises within the testis (e.g., early in spermiogenesis or at the end of maturation) can provide important insights regarding testicular functionality of an individual (Nöthling & Irons, 2008; O'Donnel, 2011).

Classification of midpiece and tail defects during this study included bent flagellae with cytoplasmic droplets, the Dag-defect, flagellae with abnormal 9 + 2 configurations, and structural defects of the mitochondrial or fibrous sheath components. The micrographs clearly illustrated the bending of the sperm tail with cytoplasmic droplets attached. Dag-defects were demonstrated as bending or coiling of the entire tail, usually accompanied by fractured mitochondria or disrupted axonemal fibres, all surrounded by a common plasma membrane. The defects render spermatozoa mostly immotile thus they are unable to reach the oocyte. Additionally, this TEM study revealed structural anomalies of elephant and rhinoceros spermatozoa such as distortion of the axonemal doublets and disorganisation of the fibrous sheath of the principal piece. These defects have been associated with sterility in bull, boar, man and mouse and are thought to result from underlying genetic mutations (Pesch & Bergmann, 2006). If a flagellum lacks a central pair of microtubules it renders a spermatozoon immotile, identified as the “9+0” syndrome caused by gene mutations in mice and in man (Pesch & Bergmann, 2006).

The current TEM study provided additional information regarding the seminal content of free-ranging elephant and rhinoceros ejaculates collected by electroejaculation during this study. Residual bodies (free cytoplasmic droplets) and apoptotic vesicles (small spheres containing membranous organelles within the cytoplasm) were evident in some of the elephant and rhinoceros samples studied. Within the semen samples collected from the elephant bulls, Steve (35 years) and No. 3 (17 years) numerous residual bodies (free cytoplasmic droplets) were present. The occurrence of residual bodies appeared to be more prevalent in the micrographs from ejaculates collected during season 2 (end of wet season) compared to those of season 1 (end of the dry season) and suggests a possible seasonal relationship. Interestingly, the ejaculates from season 2 scored slightly higher percentages for normal morphology and contained more residual bodies when compared to ejaculates collected during season 1.

Additionally apoptotic vesicles (small spheres containing membranous organelles within the cytoplasm) were also evident in elephant and rhinoceros samples. These apoptotic vesicles were particularly noticeable in the elephant bull Young 1, (age unknown, estimated <15 years). These ejaculates also recorded a high percentage of spermatozoa with midpiece reflex and cytoplasmic droplets anomalies. The normal process of apoptosis discard cells that are no longer functional or when functionality has been altered (Polanski & Kubiak, 1999; Giampietri, *et al.*, 2005). Mature spermatozoa also undergo apoptosis, during which phosphatidylserine is released on the outside of the plasma membrane and recognised as a chemotactic factor for leukocytes recognising phosphatidylserine receptors present on the surface of the phagocyte (Ricci, *et al*., 2002). This process allows leukocytes to remove apoptotic spermatozoa without causing an inflammatory reaction within the epididymis (Ricci, *et al.*, 2002). The apoptotic vesicles were not identified in this study using bright field microscopy as proper identification of these structures require a different staining techniques e.g. Giemsa of Diff-Quick® stains. These stains should be combined with TEM analyses during future investigations.

Light microscopy has been a reliable routine tool and suitable in most cases to detect sperm structural abnormalities however, during this study the inclusion of electron microscopy proved to be a valuable diagnostic tool to identify the occurrence of sperm normality, abnormalities and seminal contents on an ultrastructural level. The information obtained during this study contributes to the current information available for elephant and rhinoceros reproductive biology and confirms similarities to the ultrastructure previously described (Jones, 1973). Future investigations should consider the biological processes involved in removing redundant sperm and their organelles within the male reproductive system.

8.5 GENERAL CONCLUSION

The main novelty of this study is the detailed sperm assessment in relatively large numbers of wild African elephants and Southern white rhinoceros, using sophisticated computer-aided semen analysis technology, electron microscopy and genomics. To study sperm function objective techniques was used to measure various aspects of sperm motility, and investigated the outcome using different extenders and dilutions on sperm activity. By making use of CASA during this study, the motility and kinematic parameters exhibited by individual elephant and rhinoceros spermatozoa could be quantified. A methodology was defined to detect different methods of sperm swimming, including hyperactivation, and then used the method to see whether the addition of caffeine to sperm diluents would enhance the development of hyperactivation. The main objective of semen evaluation is to distinguish between males exhibiting traits of fertility, subfertility and infertility. The analysis of sperm motility, functionality, viability, acrosome integrity and morphology are important sperm characteristics since abnormalities in one or more of these parameters may result in the complete or partial elimination of sperm at several natural barriers in the female reproductive tract (Nöthling & Irons, 2008; Saacke, 2008). The availability of baseline information representing the reproductive characteristics of natural free-ranging populations is minimal. The majority of current available elephant and rhinoceros reproductive biology data is based on captive populations. When considering captive populations, it is important to take into consideration that the environmental impact

experienced by parents may induce epigenetic effects in the captive offspring which may not be a true reflection of a free-ranging population (Milligan, *et al.*, 2009). Additionally, it has been reported that male infertility contributes significantly to reproductive failure in many captive wildlife populations (Wildt, 1996). The elephants and rhinoceros in this study were from natural populations with no prior information available regarding their fertility or social structure. The social mating systems of these species involves bulls defending oestrus females or territories with a great deal of ferocity and physical dominance, and therefore the chances of sexual selection based on sperm selection is suspected to be very low. Motility and morphology analysis form part of the routine list of practices in animal production laboratories used for selecting high quality stud bulls, stallions, boars and rams, where sperm function and shape have been moulded over generations of artificial selection. The rationale behind using a domestic morphology classification system allowed for the identification of the types of abnormalities that occur within these animals under natural selection strategies. Seasonal differences were recorded for the elephant population, and for the rhinoceros similar defects were recorded between the two populations. This approach is a starting block to identify the types of defects that occur within the same population over two seasons. There is a possibility that sperm morphology in elephants may be influenced by age, however, Spearman rank indicated no significant correlation to the R-value and the P-value.

CASMA technology was used to discriminate different sperm head morphometric objectively. A seasonal difference in particular head size and acrosome coverage was recorded for the elephant. This could be because there is little advantage in deploying resources to maximise sperm function, also considering the seasonal cost of energetics required for reproduction. The two rhinoceros populations recorded similar head measurements allowing for a current reference value. The use of transmission electron microscopy (TEM) allowed for the ultrastructural assessment of elephant and rhinoceros spermatozoa. The main focus was to establish the presence of any abnormalities or sperm features that might not have been picked up by light microscopy, and to establish that most of the microscopic features were similar for all the elephant and the rhinoceros.

There is considerable potential for understanding the relationships between semen and sperm parameters analysed and fertility potential. Multivariate analysis (MVA) proved to be a powerful data exploration tool, compared to typical mean values. MVA allowed the additional identification of association amongst semen and sperm parameters analysed, showed the relation between elephant sperm kinematics and head morphology, demonstrating the clear seasonal grouping of ejaculates, as well as highlighting general semen and sperm quality amongst all the ejaculates. Significant relationships were identified between standard/routine semen and sperm parameters (sperm concentration, normal morphology, head defects, midpiece defects, tail defects, acrosome intact), CASA parameters (total motility, progressive motility, VCL, VSL, LIN, ALH) and CASMA parameters (head length, head regularity and head perimeter). Analysis indicates that the proportion of motile spermatozoa and the quality of motility are associated closely with the proportion of normal spermatozoa. This finding suggests that the semen samples with high sperm concentrations also contained a sperm population that varied in morphology and sperm head morphometric.

In an attempt to understand species-specific reproductive biology it is important to take into consideration the normal morphology and function of the species' reproductive system. This includes the social structure and influences affecting social behaviour such as stimulation or suppression as well as the environment in which they evolve. Since reproduction is correlated with naturally occurring variation in environmental factors, a meaningful linkage should be aimed at between ecological and physiological considerations in our quest to understand the energetic of reproduction. The gene sequence of CatSper1 (*Loxodonta africana)* for free-ranging African elephant bulls was identified, sequenced and annotated during this study. The presence of the CatSper1 (*Loxodonta africana)* gene subsequently verifies the presence of the sperm specific CatSper ion channel, located in the principal piece of the African elephant's sperm flagellum. It has become beneficial to identify any possible genes that are involved in these underlying physiological processes, including any mutations that may occur within this gene amongst different individuals screened. Genomics technologies combined with bioinformatic tools that support data analysis and interpretation of gene functioning and protein

expression facilitates an exciting new development in conservation strategies and tools. Genomic investigations generate holistic information, are high-output generating and resource generating techniques that allow for further research and development (Allendorf, *et al*., 2010).

The current rate at which wildlife species are becoming extinct is far greater and faster than ever expected. In a short time-frame from about 2012 to present, an estimated 100 000 elephants and approximately 3900 rhinoceros have been poached. This is over well 100 000 individual contributors lost to the genetic diversity of future elephant and rhinoceros generations in a period of only three years, threatening the vulnerability of these mega land species even more. Currently flagship populations are being targeted by poaching syndicates. The One Plan approach to species conservation, supported by the IUCN was initiated in 2013, aimed to develop conservation management strategies and actions plans. The responsible parties include all custodians of populations of a species, whether inside or outside their natural range (CBSG, 2013). To ensure the future success of sustaining isolated or fragmented elephant and rhinoceros populations combined with ART requires innovation and the gathering of basic reproductive data without delay. The implementation of active collaboration between biologists, wildlife owners and hunters presents realistic possibilities of frequent data and sample collection opportunities from various species that should include post mortem rescue of superior and population representative genetics.

It is important to understanding the characteristics of a healthy and functional male reproductive system. This is a precondition before these traits can be differentiated. The problem with semen evaluation in general is that semen samples with very high or low semen and sperm quality fertility potential may be predictable, yet not so much for semen samples of medium quality, since no single sperm attribute has been identified that highly and accurately correlates with fertility *in vivo* (Pesch & Bergmann, 2006). The use of appropriate data analysis methods to further increase our understanding of the effect of sperm subpopulation selection for applied ART. The ability to detect the slightest changes in motion/morphometric can become critically important to identify or monitor any

environmental/external stressors on sperm. A more detailed understanding regarding fundamental differences among wildlife species is required (e.g. genetic health, reproduction anatomy and physiology, nutritional plane and requirements, social structure and environmental) when ART is considered to form part of the conservation management action plans aimed to assist in the long-term maintenance and successful breeding of endangered, captive, isolated of fragmented wildlife populations. To date many advancements have been made; however, only singular successes have been achieved during ART interventions in captive elephant and rhinoceros population that resulted in the birth of live offspring. The routine application of these technologies is still not possible, mainly due to the non-existence or lack of basic information.

The future success of conservation breeding and applied reproductive technologies requires the immediate collection of all baseline information and biomaterial (specifically gametes) as and when any opportunity of sampling arises. More importantly these biomaterial and data sampling opportunities should be shared amongst innovation research groups to ensure the maximum inclusion of all possible factors that are involved in the growth and development (environment), per genetic superior individual (genetics), per location and under the current environmental circumstances (epigenetics). The most important finding from this study is the comprehensive semen and sperm parameter data presented for the free-ranging African elephant and Southern white rhinoceros. The data from this study, largely empirical, provides innovative strategies to study the reproductive biology of various male wildlife species. Furthermore, this study provides confirmation that a considerable amount of biological information can be yielded by a multidisciplinary approach and can be applied during semen and sperm quality investigations (routine semen analysis, CASA, CASMA, TEM and genomics) under field conditions. The semen and sperm characteristics recorded during this study varied amongst the ejaculates, individuals, seasons and populations. These factors should all be taken into consideration for future semen collection projects. The knowledge generated from extensive reproductive research represents a starting point for technology and application development through the understanding of species-specific reproductive biology.

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>Seq1 [organism=Loxodonta africana] [isolate=13999] [dev-stage=adult, 26 years] [note=free-ranging animal] [sex=male] [country=South Africa: Kwazulu-Natal, Phinda Private Game Reserve] [lat-lon= 27.95S 32.60E] cation channel, sperm associated 1 (CATSPER1) gene

ACCCGCTCATGGTAGAGGCAGTAAGTCTTAAATGCAGAGCCAGCTGGTATCTAGGTGGGACTGGGGCTTGACTGCTTGGCCTCTTAAAGTTCTTTCCAACAACAGAACTAAAG
CAACTGATGGGAGCCCATGGTGTCGTCCCGGACTTCTTCACCCAGCTTTGCCATTGACCCACATTGACACCTTGAGTCAGTCCCTTACCTTTTTTTTTTTTTTTTGTCCTCTATTTC
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TCCAGCCACAA

NCBI KR 856274

>Seq2 [organism=Loxodonta africana] [isolate=14002] [dev-stage=adult, 34 years] [note=free-ranging animal] [sex=male] [country=South Africa: Kwazulu-Natal, Phinda Private Game Reserve] [lat-lon=27.95S 32.60E] cation channel, sperm associated 1 (CATSPER1) gene

ACCCGCTCATGGTAGAGGCAGTAAGTCTTAAATGCAGAGCCAGCTGGTATCTAGGTGGGACTGGGGCTTGACTGCTTGGCCTCTTAAAGTTCTTTCCAACAACAGAACTAAAG
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NCBI KR 856275

>Seq3 [organism=Loxodonta africana] [isolate=14003] [dev-stage=adult, 26 years] [note=free-ranging animal] [sex=male] [country=South Africa: Kwazulu-Natal, Phinda Private Game Reserve] [lat-lon=27.95S 32.60E] cation channel, sperm associated 1 (CATSPER1) gene

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NCBI KR 856276

>Seq4 [organism=Loxodonta africana] [isolate=14004] [dev-stage=adult, 32 years] [note=free-ranging animal] [sex=male] [country=South Africa: Kwazulu-Natal, Phinda Private Game Reserve] [lat-lon=27.95S 32.60E] cation channel, sperm associated 1 (CATSPER1) gene

ACCCGCTCATGGTAGAGGCAGTAAGTCTTAAATGCAGAGCCAGCTGGTATCTAGGTGGGACTGGGGCTTGACTGCTTGGCCTCTTAAAGTTCTTTCCAACAACAGAACTAAAG
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NCBI KR 856277

>Seq5 [organism=Loxodonta africana] [isolate=14005] [dev-stage=adult, 25 years] [note=free-ranging animal] [sex=male] [country=South Africa: Kwazulu-Natal, Phinda Private Game Reserve] [lat-lon=27.95S 32.60E] cation channel, sperm associated 1 (CATSPER1) gene

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NCBI KR 856278

>Seq6 [organism=Loxodonta africana] [isolate=15293] [dev-stage=adult, unknown years] [note=free-ranging animal] [sex=male] [country=South Africa: Kwazulu-Natal, Phinda Private Game Reserve] [lat-lon=27.95S 32.60E] cation channel, sperm associated 1 (CATSPER1) gene

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NCBI KR 856279

>Seq7 [organism=Loxodonta africana] [isolate=15294] [dev-stage=adult, 24 years] [note=free-ranging animal] [sex=male] [country=South Africa: Kwazulu-Natal, Phinda Private Game Reserve] [lat-lon=27.95S 32.60E] cation channel, sperm associated 1 (CATSPER1) gene

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