

SEASONAL DIFFERENCES IN SEMEN CHARACTERISTICS AND SPERM FUNCTIONALITY IN TANKWA GOATS

BY

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2018

Declaration

I hereby declare that “**Seasonal differences in semen characteristics and sperm functionality in Tankwa goats**” is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated or acknowledged by the references.

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Date: 09 October 2018

Signed:.....



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List of abbreviations

ALH	: Amplitude of Lateral Head Displacement
BCF	: Beat Cross Frequency
BO medium	: Brackett and Oliphant medium
CASA	: Computer-Aided Sperm Analysis
CASMA	: Computer-Aided Sperm Morphology Analysis
DAFF	: Department of Agriculture, Forestry and Fisheries
FAO	: Food and Agricultural Organization
FAOSTAT	: Food and Agricultural Organization Statistics
IVF	: <i>In vitro</i> fertilization
LIN	: Linearity
mtDNA	: Mitochondrial DNA
PBS	: Phosphate Buffered Saline
PNA	: <i>Arachis hypogea</i> agglutinin
PSA	: <i>Pisum sativum</i> agglutinin
SD	: Standard Deviation
SCA	: Sperm Class Analyzer
STR	: Straightness
VAP	: Average Path Velocity
VCL	: Curvilinear Velocity
VSL	: Straight-Line Velocity
WHO	: World Health Organization



WOB : Wobble



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Abstract

Tankwa goats have been free-ranging in the Tankwa Karoo National Park in the Northern Cape for more than 80 years. A genetic study concluded that these feral goats are a unique genetic resource compared to other goat breeds in South Africa and should be conserved as a distinctive population. A decision taken by the South African National Parks who is the managing authority in the park, was to remove all alien species, which included the Tankwa goats. Several animals were translocated to the Carnarvon Research Station by the Northern Cape Department of Agriculture, Land Reform & Rural Development, where the Tankwa goat population has grown to a few hundred individuals. Currently, sound scientific decisions including the application of a wide range of technologies and approaches are applied to conserve the population, such as an informed understanding of the reproductive biology of these goats. The aim of this study was to define sperm quality in Tankwa goats using various macroscopic and microscopic evaluation techniques. Ejaculates (n=108) were collected over a two-year period (August 2015, February 2016, August 2016 and February 2017) using electro-ejaculation. Semen was macroscopically evaluated for macro movement, pH and volume. Sperm concentration, morphology, motility, kinematic parameters and hyperactivation status were assessed using the Sperm Class Analyzer CASA system. Sperm morphology and vitality analysis involved SpermBlue and nigrosin-eosin staining techniques, respectively. Hyperactivation was induced with 5 mM procaine hydrochloride. The acrosome status was assessed by FITC-PNA assay and fluorescence microscopy. Semen volume (summer: 1.1 ml; winter: 1.3 ml), pH (summer: 6.3; winter: 5.9), macro movement (summer: 4.1; winter: 3.6), sperm concentration (summer: $2611.2 \times 10^6/\text{ml}$; winter: $2240.8 \times 10^6/\text{ml}$) and total motility (summer: 82.4%; winter: 73.1%) were obtained and summer had higher averages than winter except for volume. Although no differences were found in percentage total motility (summer: 82.4%; winter: 73.1%), several other motility and kinematic parameters (percentage progressive motility, percentage rapid swimming spermatozoa, VCL, ALH) were significantly higher in summer compared to winter semen samples. Normal sperm morphology had an average of 68.9% in summer, and 76.7% in winter, but was not significantly different, possibly due to large variations encountered in the February 2016 samples. Sperm vitality was significantly higher in

summer (74.9%) as compared to winter (68.1%). Hyperactivation could be induced in most of the Tankwa goats tested during February 2016, August 2016 and February 2017. Procaine induced an average of 22.9% hyperactivation as compared to only 0.8% in phosphate buffered saline. Acrosome intactness (n=6) varied from 80 to 95%. The results obtained were higher when compared to British goat breeds for mass movement and total motility. Percentage progressive motility, sperm morphology and vitality were lower when compared to the results obtained in Zairabi goats from Egypt during the same seasons (summer and winter). The results of this study contributes to baseline information on semen characteristics of the Tankwa goat and provides guidance as to when it is best to collect quality semen for future cryopreservation and artificial breeding.

Keywords: Tankwa indigenous goat, sperm motility, sperm morphology, sperm vitality, hyperactivation, acrosome intactness, computer-aided sperm analysis (CASA)



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Chapter 1: General Introduction

1.1 Introduction

The effective conservation of animal genetic resources in South Africa, especially of endangered as well as relatively unknown indigenous domesticated species, requires us to expand and improve efforts to maintain healthy, viable populations (Cousins *et al.*, 2008). Moreover, with the ever changing climate and scarcity of resources, there is a need to fully utilise farm animal genetic resources in order to maximise profits (Mohlalole *et al.*, 2015). Although improvement of production traits in cattle breeding is well-defined, more research is required on other domesticated species (e.g. sheep and goats). The smallholder sector of the South African goat industry is still lagging behind in terms of improvement of traits such as growth, reproduction and health particularly for the indigenous veld ecotypes (Morrison, 2007).

South African indigenous goat breeds are known to be naturally bred for functional efficiency and are antelope-like with longer legs for ease of movement and walking long distances to either graze or browse on a variety of plant species. These goats are said to be tolerant to diseases, have the ability to survive under harsh conditions such as extreme temperatures and poor vegetation (Donkin and Boyazoglu, 2000; FAOSTAT, 2016). These goats are considered to be highly fertile even from a young age and can produce offspring throughout the year. The indigenous goats have commercial potential in products such as their meat, which has a good flavour and is lower in cholesterol than beef (López-Aliaga, 2005) and has milk that is lower in lactose than beef (McBean and Miller, 1998). Furthermore, these goats can be used for breeding purposes to transfer their desirable traits in order to improve commercial breeds.

Selection and breeding of indigenous goats resulted in meat goat breeds namely the Boer, Kalahari Red and Savannah goats (Snyman, 2014).

Tankwa goats are thought to have been free-roaming for more than 80 years without any human interference. Kotze *et al.* (2014) compared the Tankwa goat to indigenous breeds such as the Boer, Angora and Saanen goats and concluded that the Tankwa goat is a unique genetic resource that is genetically different. The study also concluded that this population needs to be conserved and optimally managed to maintain genetic

variation. Understanding the reproductive biology of the Tankwa goats can add valuable information to goat conservation and breeding in South Africa.

The use of computer-aided sperm analysis (CASA) in determining sperm quality has been well documented in numerous mammalian and domesticated species.

Gündoğan and Demirci (2003) reported that semen quality differs according to season in Akkaraman and Awassi rams under continental climate conditions. Karagiannidis *et al.* (2000) reported that semen quantity and quality in Chios and Friesian rams were significantly different, with the best semen being produced mainly during autumn and the worst during spring.

1.2 Aims

The main aim of this study was to investigate semen characteristics and sperm functionality in Tankwa goats over different seasons using various macroscopic and microscopic evaluation techniques.

1.3 Objectives

In order to achieve the aim listed above, the objectives of the study were to:

1. Assess semen characteristics over a two-year period to include semen volume, pH, macro-movement, sperm concentration, total motility and morphology.
2. Evaluate sperm functionality over a two-year period by assessing detailed sperm motility and kinematics, vitality, hyperactivation and acrosome intactness.
3. Compare the summer vs winter to establish any differences in semen characteristic and sperm function.

1.4 Overview of thesis chapters

Chapter 2 reviews the advent of domestic goats, the global goat statistics and the Southern African indigenous goat breeds. It further puts emphasis on the Tankwa goats and their potential commercialisation. The chapter also includes justification of the assessment of sperm quality, focusing on sperm motility, morphology, vitality, hyperactivation and acrosome intactness.

Chapter 3 describes detailed methodologies employed as well as statistical analysis performed. Results obtained for the study are portrayed in Chapter 4, including tables

and figures. Significant differences between seasons are shown ($p < 0.05$) and comparison of the different seasons is included. Chapter 5 focuses on the discussion of the study's findings which incorporates basic semen parameters and sperm functionality with findings from other goat breeds in South Africa and other countries. Chapter 6 summarises the study's main findings.

1.5 Research output

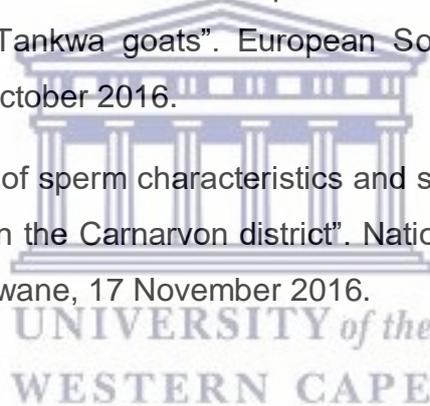
Presentation: "CASA analysis of sperm morphology and motility in Tankwa goats".

National Zoological Gardens 6th Student Research Symposium, Tshwane. 18 November 2015.

Presentation: "Sperm quality in Tankwa goats over two seasons". 49th SASAS Congress, Stellenbosch. 4 July 2016.

Part of work presented by Prof van der Horst: "Sperm Functionality in domestic animals with special reference to Tankwa goats". European Society for Domestic Animal Reproduction, Lisbon. 27 October 2016.

Presentation: "Assessment of sperm characteristics and sperm functionality over two seasons of Tankwa goats in the Carnarvon district". National Zoological Gardens 7th Research Symposium, Tshwane, 17 November 2016.



Chapter 2: Literature Review

2.1 Background

Goats are classified under the family *Bovidae* and are part of the goatantelope subfamily *Caprinae*. Worldwide there are over 500 distinct breeds of goat. Goats are one of the oldest domesticated species, and have been used for their milk, meat, hair, and skins. The origin of goat domestication is believed to date back to 10 000 years ago in the Zagros Mountains of the Fertile Crescent in Iran (Zeder and Hesse, 2000). The bezoar (*Capra aegagnus*) is believed to be one of the ancestors of the wild goat (Manceau *et al.*, 1999). Phylogeographic mitochondrial DNA (mtDNA) analysis revealed that there are three different goat descendent lineages (estimated deviation >200 000 years ago) and suggested three different main origins of domestic goats (Luikart *et al.*, 2001). Naderi *et al.* (2008) reported that all mtDNA found in domestic goats are also found in bezoars.

In 2016, there were more than 1022.8 million live goats around the globe, according to the United Nations Food and Agriculture Organization (FAOSTAT, 2016). The largest number of goats is found in Asia, followed by Africa, representing about 55.4% and 38.7% of the global number, respectively. The lowest number of goats is found in Oceania, accounting for 0.4% of the global number (Table 2.1). The registered number of goat breeds globally is 579, while Africa has 97 indigenous breeds. Nineteen breeds are listed as extinct of which only one is from Africa.

Table 2.1. The number of live goats (millions) in different parts of the world and their proportion (%) globally (data extracted from FAOSTAT, 2016).

Continent	Number (million)	Percentage of world total (%)
Asia	556.0	55.4
Africa	387.6	38.7
Americas	36.3	3.8
Europe	17.1	1.7
Oceania	4.0	0.4

As of 2016, China (mainland) contributed 14.3% and South Africa only 0.5% to the global goat population (Table 2.2).

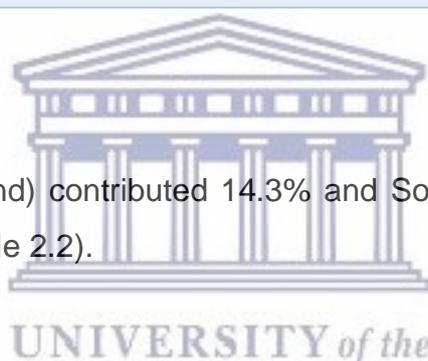


Table 2.2. The five countries with the highest number of live goats (million) together with South Africa and their percentage (%) of the global number (data extracted from FAOSTAT, 2016).

Country	Number (million)	Percentage of world's total (%)
China, mainland	148.9	14.3
India	133.8	13.3
Nigeria	73.8	7.3
Pakistan	70.3	7.0
Bangladesh	56.0	5.5
South Africa	5.6	0.5

In the past, exotic breeds of goat have been introduced for improvement of local breeds and economic importance of many poor families that survive by breeding goats. For instance, in Sudan, Saanen and Toggenburg goat breeds were imported because of their high milk yield. Nubian goats were unregulatedly crossbred with these exotic goat breeds, which resulted in undesirable reproductive and productive traits due to lack of information on the reproductive ability of the goats (Elsheik and Elhammali, 2015).

2.2 South African indigenous goats

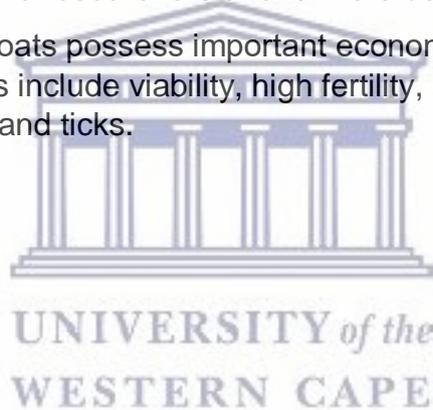
Indigenous goats arrived in South Africa with migrating tribes and are found in the specific areas where these different ethnic groups settled. Their expansion, which occurred around 2000 BC, was a major event in African history and was thought to be responsible for the adoption of pastoralism by the Khoisan people of the Southern African region (Hanotte *et al.*, 2002). The general appearance of these goats tends to support theories that they originated in different ecosystems and specific types have been described fairly accurately (Epstein, 1971).

Indigenous goats refer to various goat types that are kept and bred by small-scale producers and contribute primarily to family needs for meat and to a lesser extent for milk. Indigenous goats represent approximately 63% of the goats found in South Africa and in the past they were regarded as unimproved (not subjected to any selection process), and used for the crossbreeding of the improved goats like the Boer goat, the Kalahari Red and the Savanna goat (DAFF, 2012). The indigenous goats are mainly found in the Eastern Cape (in the former Transkei and Ciskei), Limpopo, North West and KwaZulu–Natal Provinces, with only small numbers in the other provinces of South Africa. The distribution of the indigenous goats in southern African is illustrated in Fig 2.2.1.

Apart from the Tankwa goat, four other indigenous goat breeds (Fig 2.2.2) are found in southern Africa and their general characteristics are summarised in Table 2.2.1. The Xhosa Lobed-ear goat is mainly found in the Eastern Cape region, while the Boer goat

and the Kalahari Red were developed out of the Xhosa Lobed-ear goat (Ramsay *et al.*, 2000). The Cape Skilder (Northern Cape Speckled) goat originates from the dry Northern Cape, Karoo area, stretching from Sutherland to Upington. These goats have excellent pigmentation with good colouration on the most vulnerable parts of the body (muzzle, eyes, ears, top of the back, lower legs and front of the neck). The Mbuzi or Nguni goat, the most abundant indigenous goat. They occur specifically in the higher rainfall area, stretching from Eastern Cape, KwaZulu-Natal, Swaziland, Mpumalanga, Northern Province, Botswana, the Caprivi, and the extreme northern, high rainfall area of Namibia. The Kunene type goat (Kaokoland) of the Himba people is found in the mountainous areas in north-western Namibia. These goats are adapted to walking long distances.

Campbell (2003) points out that the indigenous goat breeds in South Africa have almost been bred to extinction. Some researchers and farmers believe that the pure “unimproved” indigenous goats possess important economic traits, which should not be disregarded. These traits include viability, high fertility, good mothering ability, and tolerance against diseases and ticks.



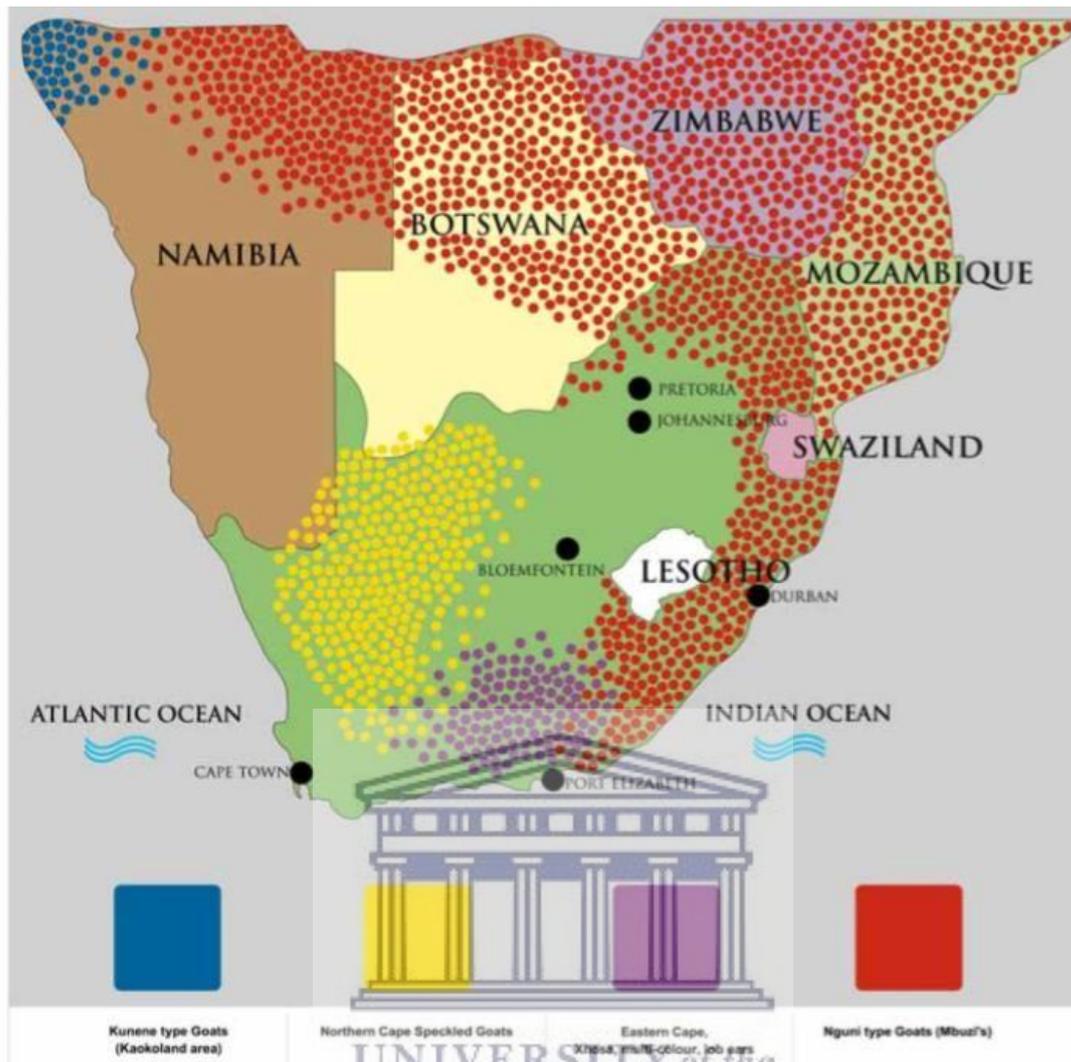


Fig 2.2.1. Map showing distribution of the veld indigenous goats in Southern Africa. (Indigenous Veld Goat Club, 2007). (Blue: Kunene type, Yellow: Northern Cape Speckled, Purple: Xhosa Lobed-ear, Red: Nguni type).



Fig 2.2.2. The phenotypic appearance of four South African indigenous goat breeds (A-Xhosa Lobed-ear, B-Kunene type, C-Nguni type, D- Cape Skilder. (Indigenous Veld Goat Club, 2007).)

Table 2.2.1. Phenotypic and habitat characteristics of the southern African indigenous goat breeds. (Indigenous Veld Goat Club, 2007)

Characteristics	Xhosa	Lobed- Cape Skilder	Nguni	Kunene	Tankwa
Body size	Medium to large, well-muscled	Medium to large, wellmuscled	Small to medium	Lanky, large	Medium to large
Body coat	uniform colours	Wide variety of red, red-brown or black spots	Multi-coloured breed, with a wide variety of colours	Multi-coloured breed, with a wide variety of uniform colours	Multi-coloured, with a variety of uniform colours
Face	Long, flat or slightly convex	Long, flat or slightly convex, with a slight dip in front of the eyes	Flat or slightly concave, although some males exhibit a slight Roman nose	Long narrow faces with a flat to slightly convex profile	Flat, some individuals exhibit a slight Roman nose
Horns	Present in both sexes. Toggles are present	Present in both sexes. Toggles are present	Present in both sexes. Toggles are present	Present in both sexes. Toggles are absent	Present in both sexes. Toggles are mostly absent
Climate	Medium to lower rainfall area	Low rainfall area	Higher rainfall area	Low and very variable rainfall area	Low rainfall area

2.3 Tankwa goat

Tankwa goats (Fig 2.3.1) originate from an isolated population of feral goats found in the arid Karoo district, mainly the Tankwa Karoo National Park, where it is thought to have been free-roaming for more than 80 years with no human interference. Accordingly they can be considered as a feral population. A number of these goats were translocated from the park to the Carnarvon Research Station (Department of Agriculture, Land Reform and Rural Development) due to the threat they posed to indigenous plants found in the park (Kotze *et al.*, 2014). A previous genetic study concluded that these goats are a unique breed due to their genetic differentiation from other goat breeds, such as the Boer, Angora and Saanen goats. The same study also concluded that this breed is at risk of extinction with a population size estimated to vary between 100 and 300 (Kotze *et al.*, 2014).

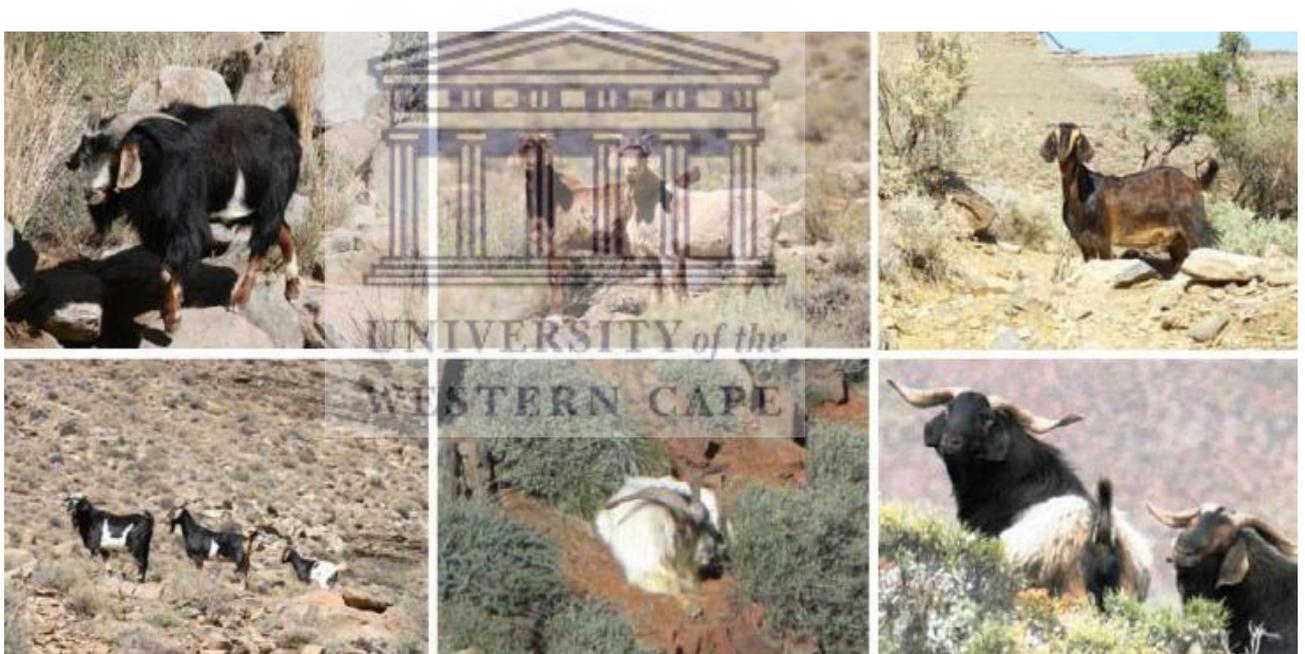


Fig 2.3.1. Phenotypic variations observed in the Tankwa goat (Kotze *et al.*, 2014)

The ability of Tankwa goats to survive in the harsh environment and climatic gradients of the Karoo region could likely be due to natural selection and accordingly to their unique genetic make-up. Previous studies have shown that there is greater resistance to diseases in this and other indigenous goat populations compared to exotic breeds (Donkin and Boyazoglu, 2004; Kotze *et al.*, 2014). Donkin and Boyazoglu (2004) found less mortality due to disease in indigenous goats (4%) than in Saanen goats (15%). Kotze *et al.* (2014) stated

that the Tankwa goats have adapted to surviving under harsh conditions and are parasite resilient. The same study further reported on genetic uniqueness of the Tankwa goats based on eight microsatellite markers. It was concluded that since the breed is highly adapted to living without human intervention, it might be harbouring unexploited genetic characteristics that could be of potential value to commercial goat production.

2.4 Possible commercialisation of the Tankwa goat

Goats are critical to smallholder farmers, being easier (than bulls) to acquire, maintain, and act as scavengers in sparse pasture. Indigenous goats have undergone generations of adaptation and genetic isolation that have led to great phenotypic variation. These indigenous goats serve as a genetic reservoir for the identification of genes important to environmental adaptation, disease resistance, and improved productivity under local conditions (Mohlatlole *et al.*, 2015).

The Boer goat, Savanna and Kalahari Red are currently recognized as commercial goat breeds for the production of meat and skins and small quantities of cashmere.

Saanen, Toggenburg and Alpine goats are mainly kept for milk production. Goat's milk is highly priced because of its quality and is less prone to causing allergies in humans than cow's milk. These breeds are believed to originate from indigenous goat types found in South Africa (Ramsay *et al.*, 2000) and are well-known for their fast growth rate and good carcass traits (Casey and Webb, 2010).

It is assumed that the Tankwa goat and some uncharacterised village goats have qualities that are lacking in the commercialised breeds that could be used for development of breeds to optimise production, even when faced with challenges such as poor nutrition and adverse climatic conditions. There is potential for selecting for production and robustness in the untapped village and feral goats, such as the Nguni and Tankwa goats.

To address the lack of information on reproductive biology of these goat species and in order to select males for future breeding purposes and smallholder farming, assessment of male fertility should be investigated. Assessment of male fertility is primarily based on post-thaw semen evaluation using conventional parameters such as sperm motility, morphology, viability, biochemical estimations of enzyme release, membrane and acrosome integrity, as performed on male Angora goats (Ritar *et al.*, 1992).

2.5 Reproductive system of the buck

2.5.1 Macro-anatomy

The anatomy of the reproductive system of the buck is similar to that of the ram and other ruminants. The male reproductive system consists of testicles, which produce spermatozoa and sex hormones, a duct system for sperm transport, accessory sex glands, and the penis, or male organ of copulation, which deposits semen into the female. The major function of the testes is to produce high quality spermatozoa and these primary sex organs are housed and protected by a pouch of skin called the scrotum. The scrotum plays a part in regulation of the testes temperature by raising them closer to the body or lowering them away from the body. Semen and urine are carried out of the body by the urethra. Bucks have a full complement of the accessory sex glands. Located caudally on either side of the pelvic urethra and can be palpated rectally are small bulbo-urethral glands. Bucks also have a disseminate prostate, lobulated seminal vesicles and a widening of the vas deferens known as the ampulla (Ashdown and Hancock, 1980). The accessory glands, including the seminal vesicles, prostate and bulbo-urethral glands, secrete fluids into the urethra during ejaculation. These fluids contain sugar to nourish the spermatozoa, buffers to maintain a physiological environment and other chemicals (such as sulphate which prevent sperm cells from swelling) that serve to protect and propel spermatozoa out of the urethra. From the testis to the urethra, a duct called the vas deferens is found. Ampullae are situated at the terminus of the vas deferens. Furthermore, the vas deferens is connected to the tail of a large winding tubule (that can be felt on the side of the testes) called the epididymis. The epididymis is the site of sperm maturation and their storage thereafter. The penis is the organ of copulation which allows deposition of semen into the female genital tract. It is normally held in an S-shaped bend (the sigmoid flexure) by retractor penis muscles except during erection and ejaculation (Beckett and Wolfe, 1998). The penis is covered and protected by a fold of skin (prepuce). Fig 2.5.1.1 illustrates the components and location of the male buck's reproductive system

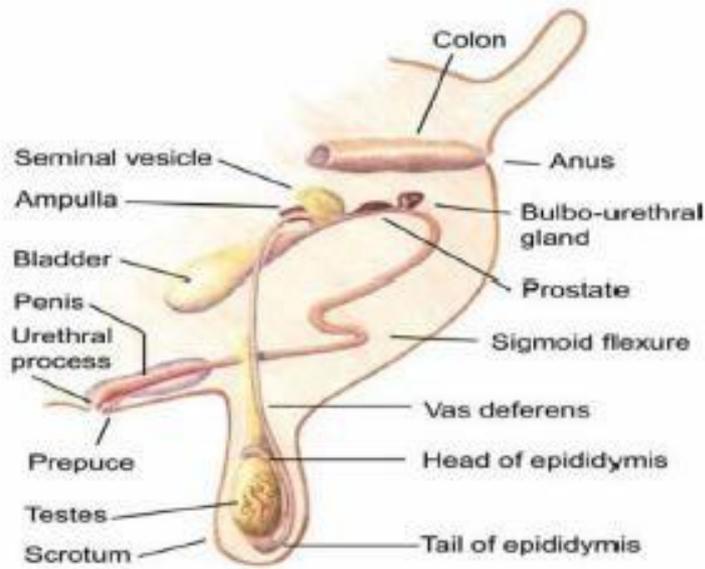


Fig 2.5.1.1. Components of the male reproductive system of the buck and their location in relation to the gastrointestinal and renal systems. (Adapted from Vikaspedia, 2018)

2.5.2 Sperm production and structure

Spermatogenesis of bucks takes approximately 49-60 days from inception of germ cell division until the spermatozoa are released from the seminiferous tubules (Pineda and Faulkner, 1980). The production of the spermatozoa occurs within the walls of seminiferous tubules in the testis, where after spermatozoa are then transported through the caput and corpus regions of the epididymis and stored in the proximal cauda epididymis. Randomly scattered cells called Sertoli cells are also found on the walls of the tubules. The function of these cells is to support and provide nutrients to the spermatogonia as well as transporting them from the outer surface of the seminiferous tubule to the central channel of the tubule. Spermatogonia are derived from stem cells from the outer wall of the seminiferous tubules. The number of stem cells is duplicated by mitotic division, where after the half of the duplicated cells become future spermatozoa and the other half remain as stem cells as to ensure constant source of additional germ cells. Spermatogonia that are selected to become mature spermatozoa are known as primary spermatocytes. Primary spermatocytes move from the outer portion of the seminiferous tubules to a more central location and attach themselves around the Sertoli cells. Primary spermatocytes undergo meiotic divisions to become as secondary spermatocytes. The secondary spermatocytes, then undergo the

second meiotic division to produce four haploid cells, the spermatids (Nuti, 2002; Sharma *et al.*, 2009).

During epididymal transit and storage, spermatozoa (spermatids) acquire functional competence due to a series of morphological, biochemical and physiological changes (reviewed in Mortimer, 1997). This process is known as spermiogenesis. The epididymis functions as a maturing station and spermatozoa are held there for up to 14 days before entering the vas deferens. Acquisition of the ability to move when they come into contact with seminal plasma or with physiological media is an indication of maturational change in spermatozoa. Alterations occur in the sperm plasma membrane during epididymal maturation, such as the distribution of intramembranous particles, increased net negative surface charge, adsorption of antigens, glycoproteins and sialic acid, incorporation of cholesterol, as well as reduction in surface sulfhydryl groups (reviewed in Yanagimachi, 1981). These alterations in the sperm plasma membrane occur sequentially along the epididymis, as the chemical composition of epididymal fluids is region-specific (Yanagimachi, 1994). Also in the epididymis, chromatin condensation and stabilization of the spermatozoon occurs, and the acrosome acquires its final shape (reviewed in Mortimer, 1994).

Smooth muscle contractions assist spermatozoa to move from the testes via the epididymis into the vas deferens. From there they travel through the abdominal cavity in the vas deferens to the urethra where it exits the body through the penis as mature spermatozoa. During ejaculation, spermatozoa are transported from storage in the caudae epididymides and are mixed with prostatic fluid and seminal vesicle fluid before passage along the penile urethra. The bulbourethral gland produces an alkaline mucous secretion (which helps protect spermatozoa during their passage in urethra at ejaculation) called the pre-ejaculate. The first fraction of the ejaculate contains most of the spermatozoa, suspended in epididymal and prostatic fluid, with subsequent fractions containing both prostatic and vesicular fluid (Mortimer, 1994).

Matured mammalian spermatozoa consist of a head, partially covered by an acrosome, a neck, a midpiece and a flagellar-like tail (Fig 2.5.2.1).

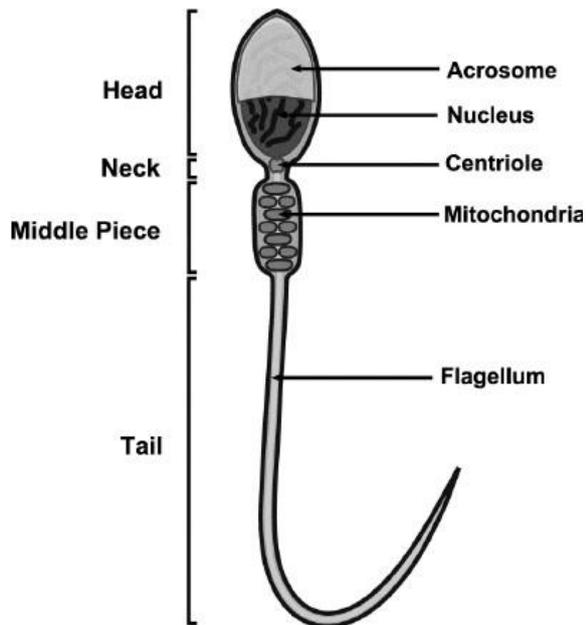


Fig 2.5.2.1. Basic structural components of a mammalian spermatozoon (Shirazi *et al.*, 2014)

The sperm head is subdivided into the nucleus, nuclear envelope, acrosome, equatorial segment, post-acrosomal region and posterior ring (Varner and Johnson, 2007). The acrosome is a Golgi-derived, membrane-bound vesicle formed during an early stage of spermiogenesis and is subdivided into inner and outer acrosomal membranes enclosing the acrosomal matrix (Abou-Haila and Tulsiani, 2000; Toshimori and Ito, 2003). Phospholipids, proteins and cholesterol are composed within these acrosomal membranes. The cap-like acrosome contains hydrolytic enzymes which are essential in penetration of the zona pellucida during fertilization (Abou-Haila and Tulsiani, 2000). The tail consists of the neck, the midpiece, the principal piece and the end piece. The midpiece contains a variable number of mitochondria, essential for adenosine triphosphate (ATP) production (Ramalho-Santos *et al.*, 2007). The internal structure of the tail consists of an axoneme comprising of a 9+2 micro-tubular arrangement of nine doublet pairs of peripheral microtubules, arranged in a circle around the central pair (Ramalho-Santos *et al.*, 2007). Motility in the female genital tract and penetration of the zona pellucida (together with the acrosome) are the main functions of the tail (Gardner and Hafez, 2004).

2.6 Sexual maturation of the buck

A buck's age, breed and nutrition all contribute to the onset of its sexual maturation. The age of sexual maturity can for example range from two to three months in pygmy breeds and up to four to five months in Nubian and Boer goat breeds. In most breeds from temperate environments in the Northern hemisphere, spermatozoa are present in the ejaculate at four to five months. However, the spermatozoa produced at this age are of poor quality and the animals are usually not suitable for breeding. Nubian and Boer goats are observed to exhibit *libido* behaviours at 10 to 12 weeks and only begin to produce good quality semen at approximately 8 months (Smith and Sherman, 1994; Goyal and Meman, 2007).

The natural adhesion of the urethral process and glans penis to the prepuce make the immature buck incapable of copulation. This attachment begins to separate at three months and fertile mating is possible at four to five months. Fast growing, well-fed and well-managed kids are able to breed sooner than starved males of the same age (Smith and Sherman, 1994; Goyal and Meman, 2007). Goyal and Mermon (2007) further reported that the sexual behaviour of bucks includes actively seeking does in estrus, courtship, mounting intromission and ejaculation. It was also reported that the strong thrust with a rapid backward movement of the buck's head is a characteristic of ejaculation and this occurs spontaneously.

Nutrition is considered to be a significant factor impacting on reproductive performance in Payoya bucks. Zarazaga *et al.* (2009) reported that these bucks had better sexual behaviour when given higher level feeding. In young Boer buck and mixed breed bucks, the effect of winter field hay on semen characteristics was studied and both Almeida *et al.* (2007) and Santiago *et al.* (2018) found it had a detrimental effect on semen characteristics such as sperm cell abnormalities. Administration of testosterone to sexual inactive bucks caused the bucks to have a defined sexual activity but this benefit was overridden by underfeeding of bucks (Santiago *et al.*, 2018). The effect of season and artificial photoperiod on semen and seminal characteristics was studied on two Spanish breeds (Murciano-Gonadina and Payoya bucks) and significant differences were observed between the two breeds (Arrebola-Molina and Abecia, 2017). Sperm concentration was found to be higher in spring and summer than in autumn and winter.

2.7 Seasonal breeding in Bovidae

The family Bovidae consist of seasonal and aseasonal breeders depending upon on the number of times they breed during a year. Seasonal breeders have specific periods of time in a year during which they actively breed. All bovids mate at least once a year, and smaller species may even mate twice. Mating seasons occur typically during the rainy months for most bovids. As such, breeding might peak twice in the equatorial regions. Sheep and goats exhibit remarkable seasonality of reproduction, in which the annual cycle of daily photoperiod plays a pivotal role. Other factors that have a significant influence on this cycle include the temperature of the surroundings, nutritional status, social interactions, the date of parturition and the lactation period (reviewed by Vasantha, 2016). Rosa and Bryant (2003) concluded that goats and sheep are short-day breeders. Mating in most sheep breeds begins in summer or early autumn and is also affected by melatonin, which advances the onset of the breeding season.

Zamira *et al.* (2010) investigated the seasonal effect on various semen characteristics, testicular size and plasma testosterone concentration in Chios and Frisian rams (Greece) and concluded that the best semen quality is produced in autumn. In Argentine and Corriedale rams, Aller *et al.* (2012) observed that sexual behaviour, scrotal circumference and plasma testosterone exhibited seasonal variations. Semen of superior quality and quantity of the mentioned rams was collected during summer and autumn. Both these studies are in agreement with the seasonal breeding of rams proposed by Rosa and Bryant (2003).

Snoj *et al.* (2013) investigated seasonal effects on semen characteristics in *Bos taurus* bull breeds; it was reported that summer had a more favourable quality and quantity of semen. The *Bos taurus* is not normally considered a seasonal breeder. A study conducted on two different breeds of bull namely Corriente and European-breed bulls concluded that season had no effect on the semen characteristics in Corriente bulls while European-breed bulls had lower sperm quality during the hotter season (Quezada-Casasola *et al.*, 2016). Seasonal variations in semen characteristics were investigated in Nili-Ravi buffalo breeding bull and semen quality was reported to be superior in autumn and spring (Hameed *et al.*, 2017).

2.8. Assessment of semen and sperm quality

The assessment of sperm quality is important in predicting the fertility potential of males and it is a useful tool in the clinical diagnosis of subfertile males. Conventional semen evaluation techniques have been based on the subjective assessment of parameters such as semen volume, sperm motility, morphology and concentration (Verstegen *et al.*, 2002). One of the primary reasons for the development of commercial computer-aided sperm analysis (CASA) systems was to promote standardized evaluation of semen samples and prevent biased analysis. CASA allows for faster, accurate and more detailed results along with eliminating the subjective biases inherent in manual semen analysis (Coetzee *et al.*, 2001, van der Horst *et al.* 2018).

CASA has been proposed for semen evaluation of several mammalian species and is invaluable in detecting changes in sperm motion. It allows for the accurate and rapid calculation of semen parameters such as total motility and progressive motility, as well as several sperm kinematic parameters (Lu *et al.*, 2014). The system can also assess both individual spermatozoa and subpopulations in sperm head motion and sperm morphology. A CASA system functions by receiving an optical signal from a camera connected to a microscope while analysing a prepared slide. This signal will be converted to electric pulse digital signals and is subsequently processed with digitalisation and automatically converted to corresponding semen parameters. Spermatozoa are normally perceived using a microscope equipped with darkfield, negative phase contrast or fluorescent optics. In darkfield and negative phase contrast microscopy, white sperm heads are visualized on a dark background and the brightness of sperm head is utilized to establish centroid positions in successive fields (Mortimer, 2000). In fluorescent microscopy, the sperm head is identified by staining with a fluorescent probe that binds to the sperm DNA.

With major advances made in CASA and the assistance of cutting edge software programs such as the Sperm Class Analyzer (SCA®) (Microptic, S.L., Barcelona), it has become possible to analyse and therefore predict the capability of sperm to fertilize an oocyte (Henkel *et al.*, 2005; van der Horst, 2014; Mortimer *et al.*, 2015).

SCA® contains several modules, making it possible to measure and analyse various essential processes and parameters required by spermatozoa to maximise its fertilizing

potential. These parameters include sperm concentration, motility, morphology, vitality, DNA fragmentation and acrosome status (van der Horst, 2014). One of the constraints faced when dealing with CASA systems is that it is not a “ready to use robot”, and therefore a user needs training and understanding to correctly utilize the software (Kraemer *et al.*, 1998). Parameter settings for each module need to be set according to the specific species being investigated and one still needs to manually check for any error trapping or lost spermatozoa not detected (Lu *et al.*, 2014).

2.8.1. Sperm motility

Motility is an important feature for spermatozoa to be capable of reaching and fertilizing the oocyte. Spermatozoa will only become motile and capable of fertilization once they have matured and undergone several morphological and physiological changes within the epididymis. The mammalian sperm flagellum, producing the driving force for motility, contains microtubular-based machinery which generates the flagellar beat needed to travel the long distance to the ovum. Spermatozoa generally have two phases of motility, namely active and hyperactivated motility. Active motility of spermatozoa occurs in a fresh ejaculate and displays a symmetrical, low amplitude waveform, which assists the spermatozoa to swim in a straight line (Henkel *et al.*, 2005; Maree, 2011), while during hyperactivation, the pattern and vigour of sperm movement undergo dramatic changes, characterized by wide amplitude, marked lateral movements of head and tail, coupled with a fast, non-progressive motility and ‘star-spin’ movement (Yanagimachi, 1994; Ho & Suarez, 2001; Jin, 2017).

Sperm motility analysis is an essential parameter for evaluation of sperm quality due to the close correlation it has with other parameters such as morphology and membrane integrity, and thus fertilization (Abu Hassan Abu *et al.*, 2012). Sperm motility also remains the parameter of choice to determine the degree of sperm damage inflicted by the cryopreservation procedure. Sperm motility is widely evaluated by visual assessment on a microscope equipped with phase contrast optics (Kathiravan *et al.*, 2011). Due to the availability of CASA, sperm motility characteristics and various kinematics can now be accurately measured in a short period of time; however, evaluations still needs to be verified

to make sure of accurate analysis (Nieschlag *et al.*, 1998; Larsen *et al.*, 2000; Maree, 2011; Van der Horst, 2014).

Sperm motion velocity values include curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) (Lu *et al.*, 2014), and are associated with the progression of the spermatozoa along the female genital tract (Mortimer, 1999). VCL, the average speed between adjacent time points, refers to the time-averaged velocity of a sperm head along its actual curvilinear path and is always the highest among the three sperm velocity parameters (Dunson *et al.*, 1999; Lu *et al.*, 2014). VSL, the distance from the first point/time, refers to the time-averaged velocity of the sperm head along the straight line between its first detected position and its last. Thus, reflecting the net space of forward motility during the observed time, VSL will always be the lowest value among the three sperm velocity parameters (Dunson *et al.*, 1999; Lu *et al.*, 2014). VAP refers to the time-averaged velocity of the sperm head along its average path. This average path is determined by the smoothing of the curvilinear trajectory in the CASA instrumentation. Therefore, if the sperm motion trajectory happens to be very regular and linear, the VAP will be almost identical to VSL, but if irregular, the VAP will be much higher than VSL (Dunson *et al.*, 1999; Lu *et al.*, 2014). The three velocity ratios include linearity (LIN), straightness (STR) and wobble (WOB). LIN, expressed as VSL/VCL , refers to the linearity of a curvilinear path, whereas STR, expressed as VSL/VAP , refers to the linearity of the average path. Furthermore, WOB the final velocity ratio refers to the oscillation of the actual path about the average path and therefore is expressed as VAP/VCL (Dunson *et al.*, 1999; Lu *et al.*, 2014).

The two parameters representing the wobbling index characteristics include the amplitude of lateral head displacement (ALH), and lastly the beat cross frequency (BCF). ALH refers to the magnitude of the lateral head displacement of the sperm head, thus the deviation of the track about its average path, and can therefore be expressed either as a maximum or an average of such displacements (Dunson *et al.*, 1999; Lu *et al.*, 2014). ALH indicates the force of flagellar beating along with the frequency of cell rotation (Kraemer *et al.*, 1998). BCF refers to the average rate at which the curvilinear path crosses the average path, a useful parameter when assessing the changes in wobble of the sperm flagellum (Dunson *et al.*, 1999; Lu *et al.*, 2014). This parameter together with an increase in ALH is associated with cervical mucus penetration. Characteristics of spermatozoa motility parameters measured by CASA systems are shown in Fig 2.8.1.1.

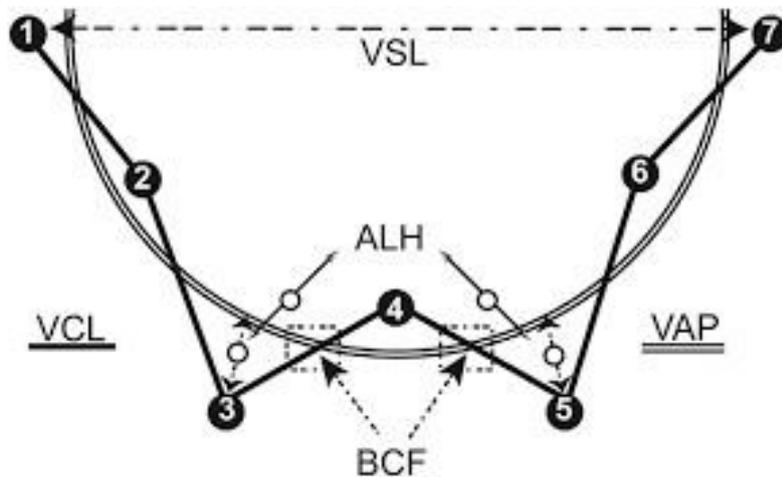


Fig.2.8.1.1. Schematic diagram of computer-aided sperm analysis (CASA) kinematic parameters. Numbers 1 to 7 represent the actual path of the sperm head captured (Gray *et al.*, 2015). VCL = curvilinear velocity, VSL= straight-line velocity, VAP = average path velocity, BCF= beat cross frequency, ALH = amplitude of later head displacement.

2.8.2. Sperm morphology

Poor sperm morphology is an important indicator of decreased fertility in goats, stallions, bulls and men. However, the subjective assessment of sperm morphology based on visual observation has led to widely varying intra- and inter-laboratory results (Cooper *et al.*, 1999). These variations make it difficult to accurately interpret data, highlighting the need for techniques which are objective, precise and repeatable.

The introduction of computer-aided sperm morphometry analysis (CASMA) systems attempted to overcome the problem of the subjectivity of visually based methods of assessment. Although this technology was originally designed for human spermatozoa (Kruger *et al.*, 1993; de Monseraat *et al.*, 1996), it has been progressively adapted for some animal species. These systems are capable of detecting subtle differences that conventional methods were unable to identify, such as the relationship between sperm morphometry and fertility (Casey *et al.*, 1997).

The accuracy of CASMA systems requires the standardization of each species for a number of analytical variables, such as staining and sampling methods (Davis & Gravance, 1993; Gravance, 1995; Gago *et al.*, 1998; Foote, 2003; Hildago, *et al.*, 2006; Maree *et al.*, 2010;

Van der Horst *et al.*, 2018). In addition to the variations inherent to the evaluation process, errors are often the result of differences between CASMA systems or the fact that an insufficient number of spermatozoa, which are not representative of the sample, are analysed (Hildago *et al.*, 2006).

Spermatozoa are very complex cells having undergone an extreme morphological evolution and which subsequently results in large variations in structure (Maree, 2011). Variations in individual sperm morphologies, such as size and dimensions of flagellar, midpiece and principal piece components, could possibly be contributing to morphological adaptations for assisting sperm with travelling and surviving in the female genital tract (Maree, 2011).

In humans, it was shown that morphology significantly correlates with various sperm functions (Menkveld *et al.*, 2011). Defects within the sperm midpiece, which contains mitochondria for energy production, along with defects in the sperm tail, severely affect sperm motility and speed. Defects within the head and abnormal acrosomes have been shown to significantly affect the ability of spermatozoa to bind to the zona pellucida, along with their ability to undergo the acrosome reaction (Sherman, 1989; Menkveld *et al.*, 2011; Abu Hassan Abu *et al.*, 2012).

Sperm morphology may be analysed using Diff-Quik stain and/or Papanicolaou, as recommended by the World Health Organization (WHO, 2010), or SpermBlue[®], which has been developed for the evaluation of human and animal sperm morphology (Van der Horst & Maree, 2009; Maree *et al.*, 2010). According to Björndahl *et al.*, (2003), eosin-nigrosin staining developed for “live-dead” staining of spermatozoa has also been used to assess sperm morphology for many animal species, but it does not clearly differentiate between the various components of the spermatozoa (Van der Horst *et al.*, 2009). Fig2.8.2.1 illustrates the accurate staining of sperm components for morphology assessments of different species of mammals using SpermBlue[®].

With the use of CASA and SCA[®], rapid and accurate, automatic detection of acrosome, head and midpiece of the spermatozoa can be made. SCA[®] accurately measures several head dimensions, namely head length, width, perimeter, surface area, ellipticity, elongation, regularity and the percentage acrosome coverage over the head. CASA systems further makes an automatic assessment of the percentage normal sperm and the various defects

present within the sperm population (van der Horst & Maree, 2009; Maree *et al.*, 2010; Van der Horst *et al.*, 2018).

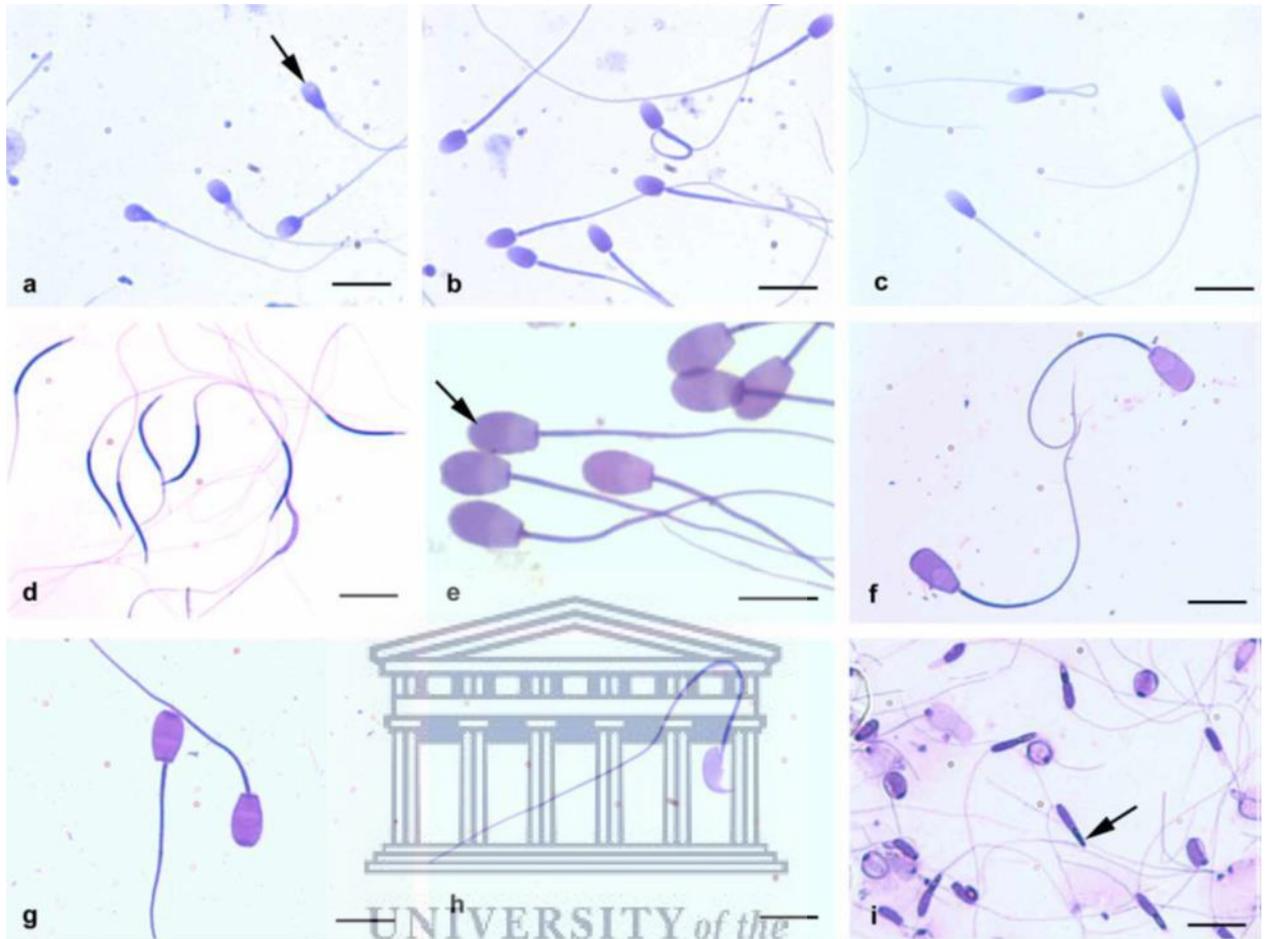


Fig. 2.8.2.1. Sperm morphology of human and eight representative species stained with SpermBlue®. a) Human b) Vervet monkey c) Horse d) Chicken e) Ram f) Boar g) Bull h) Mouse i) Abalone. Each scale bar represents 10 μ m and the large arrows show the acrosome in three representative examples (van der Horst and Maree, 2009).

2.8.3. Sperm vitality

Sperm vitality is an important parameter to evaluate, especially for samples with less than 40% progressively motile spermatozoa. It is clinically important to know whether immotile spermatozoa are alive or dead. Vitality results should be assessed in conjunction with

motility results from the same semen sample. The presence of a large proportion of vital but immotile cells may be indicative of structural defects in the flagellum. A high percentage of immotile and non-viable cells (necrozoospermia) may indicate epididymal pathology (Björndahl *et al.*, 2003).

Vitality is measured as a percentage and mainly involves examining the permeability and integrity of the sperm cell membrane (Björndahl *et al.*, 2003; Cooper and Hellenkemper, 2009). The percentage live spermatozoa is assessed by identifying those spermatozoa with an intact cell membrane, by checking for dye exclusion and/or hypotonic swelling. The dye exclusion method is based on the principle that damaged plasma membranes, such as those found in non-vital (dead) cells, allow entry of membrane-impermeant stains. The hypo-osmotic swelling (HOS) test presumes that only cells with intact membranes (live cells) will swell in hypotonic solutions. The (HOS) test is widely used in various domestic animals and it is based on the swelling ability when functional spermatozoa are placed in hypo-osmotic solutions. It is an important parameter for the evaluation of semen due to its strong correlation with other semen evaluation parameters such as motility (as reviewed by Zubair *et al.*, 2015). The HOS test seems to be more appropriate for predicting the fertilizing capacity of frozen-thawed than fresh semen, because membrane damage is a more important limiting factor than in the former (Colenbrander *et al.*, 2003).

The majority of studies make use of the standard eosin-nigrosin technique to stain slides for vitality assessment. The principal of this technique is based on the fact that eosin will penetrate “dead” cells due to their compromised membranes and therefore stains them red/pink. The nigrosin acts as a background stain which allows the detection of the viable cells which remained unstained, therefore appearing white. The staining analysis can be done totally automatic with CASA, provided that the staining techniques were correctly applied (Cooper and Hellenkemper, 2009).

2.8.4. Sperm hyperactivation

Mammalian spermatozoa cannot fertilize oocytes immediately upon ejaculation nor upon retrieval from the epididymis. Despite these spermatozoa having the functional competence conferred by activation, a series of metabolic and physiological changes must occur before they acquire the ability to penetrate the zona pellucida and bind to the oocyte. In nature,

these changes occur during transit through the female reproductive tract (Austin, 1951; Chang, 1951), and are collectively termed 'capacitation'. The original definition was the observation that a 'sperm must undergo some form of physiological change or capacitation before it is capable of penetrating the egg' (Austin, 1952).

Yanagimachi (1970) and Gwatkin *et al.* (1972) independently reported hyperactivated motility in golden hamster undergoing capacitation *in vitro*. The high amplitude flagellar beats and vigorous movement were first described as 'activation'. In the same study, it was observed that similar movement patterns had been reported *in situ* through the walls of the oviductal ampulla of golden hamsters, indicating a potential physiological role in fertilization. This motility pattern was renamed hyperactivation (Yanagimachi, 1981) to reduce the incidence of confusion with the alternative meaning of activation, whereby immature spermatozoa in the male tract acquire motility upon contact with seminal plasma or culture medium.

Hyperactivation has since been observed *in vitro* for all eutherian spermatozoa studied, including rabbit (Johnson *et al.*, 1981), guinea pig (Yanagimachi and Mahi, 1976), rhesus monkey (Boatman and Bavister, 1984), chimpanzee (Gould *et al.*, 1988), mouse (Fraser, 1977), dolphin (Fleming *et al.*, 1981; Van der Horst *et al.*, 2018), bat (Lambert, 1981), dog (Mahi and Yanagimachi, 1978), rat (Shalgi and Phillips, 1988), bull (Singh *et al.*, 1983; Blottner *et al.*, 1989), ram (Cummins, 1982), boar (Blottner *et al.*, 1989), lion and tiger (Blottner *et al.*, 1989), and human (Mortimer *et al.*, 1984; Burkman, 1984). A number of similarities have been observed in the hyperactivated movement patterns of mammalian spermatozoa, although interspecies differences have also been distinguished. Flagellar bending has been observed to be the main mechanism behind the motility patterns of hyperactivated spermatozoa. This was observed in headless guinea pig and hamster spermatozoa where it was initially thought to be the viscous drag associated with acrosomal loss (Katz *et al.*, 1978).

A number of physiological factors, such as Ca^{2+} , cAMP, bicarbonate and metabolic substrates, are essential for the initiation and maintenance of hyperactivated motility *in vitro* (Ho and Suarez, 2001). Ca^{2+} plays a major role in regulating hyperactivated motility. Extracellular Ca^{2+} is required to maintain hyperactivation in hamster spermatozoa *in vitro* (Yanagimachi, 1994), and treatment of mouse spermatozoa with Ca^{2+} ionophore A23187 rapidly induces hyperactivated motility (Suarez *et al.*, 1987). The most convincing evidence

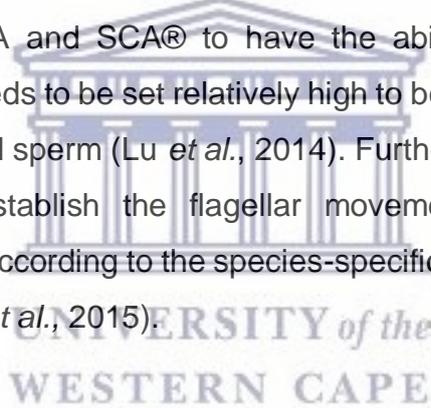
for the requirement of calcium ions for hyperactivated motility has come from studies of intracellular calcium emission patterns in intact hamster spermatozoa (Suarez *et al.*, 1993; Suarez and Dai, 1995). The concentration of calcium ions was increased in the head and midpiece of hyperactivated spermatozoa, and even more so in acrosome-reacted spermatozoa. It was also observed that cytoplasmic Ca^{2+} concentrations within the flagella of hyperactivated hamster spermatozoa were higher than those in activated spermatozoa (Suarez *et al.*, 1993; Suarez and Dai, 1995). Several physiological and chemical factors have been used to stimulate hyperactivation *in vitro*, such as bovine bicarbonate (Neill and Olds-Clarke, 1987), progesterone (Mbizvo *et al.*, 1990), bicarbonate (Suarez *et al.*, 1993) and glucose (Williams and Ford, 2001).

In mice, hyperactivated sperm motility in response to capacitation is dependent upon the presence of sperm-specific pH-gated cation (CatSper) channels (Ren *et al.*, 2003; Carlson *et al.*, 2003) and in humans, mutations in the CATSPER genes are associated with infertility and abnormal motility (Avidan *et al.*, 2003; Nikpoor *et al.*, 2004; Avenarius *et al.*, 2009). The CatSper channels are localised to the principal piece of mouse sperm and can be opened in response to increased intracellular pH, which is usually associated with sperm capacitation (Ren *et al.*, 2003). Torres-Flores *et al.* (2011) observed that in the absence of environmental calcium, the CatSper channel will transport sodium, resulting in sperm depolarization and loss of motility. Mechanisms for CatSper-mediated hyperactivation have been investigated. Progesterone and prostaglandin E_1 directly stimulate CatSper activation in human sperm but not in murine sperm (Lishko *et al.*, 2011; Olson *et al.*, 2011; Strunker *et al.*, 2011).

Capacitation is associated with hyperactivated motility and the spermatozoon's ability to undergo the acrosome reaction (Bedford, 1983). Because hyperactivation is brought about by changes in the flagellar beat pattern, capacitation probably involves changes in the physical and chemical properties of the tail plasma membrane as well as the head plasma membrane (Yanagimachi, 1988). Spermatozoa encounter different environments as they pass through the female genital tract. Capacitated mammalian spermatozoa exhibit hyperactivation shortly before or about the time of acrosome reaction, as they progress through the female oviduct. During hyperactivation, the pattern and vigour of the sperm track undergo dramatic changes, characterized by wide amplitude, marked lateral movements of head and tail, coupled with a slow or non-progressive motility and 'star-spin' movement.

Hyperactivation is thought to provide strong thrusting power to the spermatozoa while passing through the egg membranes, particularly the zona pellucida (Yanagimachi, 1984). Capacitating spermatozoa shed proteins that bind them to the mucosal epithelium, while hyperactivation assist the spermatozoa in detaching off the epithelium pockets and in penetrating the cumulus matrix (Suarez, 2008). In previous studies, it has been shown that if spermatozoa are unable to portray hyperactivated motility, they are unable to fertilize the oocytes *in vitro* (Fleming and Yanagimachi, 1982; Fleming and Kuehl, 1985). The objective measure of this physiological process can serve as a biological marker to evaluate the functional capabilities of spermatozoa.

With the aid of CASA and Sperm Class Analyzer (SCA®), rapid and objective analysis of sperm hyperactivation and kinematic parameters, based on the physiological processes essential for capacitation, can be performed (Nieschlag *et al.*, 1998). The dynamics of hyperactivated spermatozoa are very different from those of fresh ejaculate and non-capacitated sperm. For CASA and SCA® to have the ability to determine and detect hyperactivation, frame rate needs to be set relatively high to be able to accurately detect the true track of the hyperactivated sperm (Lu *et al.*, 2014). Furthermore, SCA® and CASA will detect the head track to establish the flagellar movement and determine whether hyperactivation has occurred according to the species-specific cut off values set by the user for hyperactivation (Mortimer *et al.*, 2015).



2.8.5. Acrosome reaction

The binding of the spermatozoon to the zona pellucida triggers the release of hydrolysing enzymes from the acrosome, known as the acrosome reaction. The acrosome reaction is an exocytosis process and a prerequisite for fertilization, since only spermatozoa that have undergone the acrosome reaction will pass through the zona pellucida, bind to the oocyte plasma membrane and fuse with the oocyte (Yanagimachi, 1994).

The acrosome reaction is a pre-requisite for mammalian fertilization. It involves localized fusions of the plasma membrane and the outer acrosomal membrane over the anterior portion of the sperm head. The acrosome reaction of the fertilizing spermatozoon occurs on the surface of the zona pellucida and is generally believed to be stimulated by ZP3, a glycoprotein component of the zona pellucida (reviewed by Wassarman, 1995). Following

completion of the acrosome reaction, the spermatozoon penetrates the zona pellucida. Opposing mechanisms of zona penetration have been postulated: either that it is a purely chemical process with the acrosomal enzymes digesting the glycoproteins of the zona pellucida, requiring only moderate flagellar movement (the 'binding–release' hypothesis); or that it is a purely mechanical process reliant upon vigorous flagellar movement to force the sperm head through the zona pellucida (reviewed by Yanagimachi, 1994). In either case, it is important that the fertilizing spermatozoon be tightly bound by the zona pellucida prior to zona penetration as the flagellum is still beating at that time and so otherwise could pull the sperm head free from the zona pellucida (Baltz *et al.*, 1988). After successful penetration of the zona pellucida, the spermatozoon enters the perivitelline space where it comes into contact with the oocyte and the post-acrosomal region of the sperm head binds to the oolemma. Flagellar motility ceases at this time, and fusion is initiated between the oolemma and the equatorial segment of the spermatozoon. The whole spermatozoon is then engulfed by the oocyte. The nucleus of the sperm head decondenses to form the male pronucleus. This can then fuse with the female pronucleus which was formed following the resumption of oocyte meiosis triggered by sperm–oolemma contact. The fertilized oocyte is referred to as a zygote, and as an embryo following the first cleavage (Yanagimachi, 1994).

The ability of spermatozoa to undergo the acrosome reaction or the integrity of the acrosome can be detected via fluorescence microscopy in order to determine its fertilizing potential (Tulsiani *et al.*, 2007; van der Horst, 2014). Acrosomal integrity can be assessed by using fluorescently labelled plant lectins (Cross and Meizel, 1989; De Jonge and Barrat, 2013). These lectins are proteins that recognise and bind glucosidic residues in different parts of the acrosomal membrane, such as *Pisum sativum* agglutinin (PSA) derived from the pea plant and *Arachis hypogaea* agglutinin (PNA) derived from the peanut plant. PNA is the most commonly used due to its high affinity and strong specificity. When viewing the spermatozoa using fluorescence microscopy, spermatozoa with intact acrosomes will fluoresce bright neon green due to the labelling of the acrosomal contents (PSA) or acrosomal membrane (PNA), whereas sperm having undergone acrosome reaction will have lost their acrosomal contents and thus do not fluoresce (Jaiswal *et al.*, 1999).

2.9 Conclusion

Numerous studies have reported on the reproductive aspects and seasonal effects on semen and sperm characteristics in other indigenous goat breeds and bovids. Employing mainly CASA, Tankwa goat semen and various structural and functional sperm parameters were compared between summer and winter over a two-year period. The findings of the present study were compared to previous studies on other indigenous goat breeds to establish a correlation.



Chapter 3: Materials and methods

3.1 Study animals and area

Randomly selected and sexually mature male Tankwa goats from the Carnarvon Research Station (Department of Agriculture, Land Reform and Rural Development) were used for the study. The study area is located at 30° 95' S and 22° 13' E in Carnarvon, Northern Cape, South Africa and situated at an altitude of 1234 m above sea level.

The animals were kept in two camps together with females (not limiting ejaculation prior to sampling) and had free access to water and natural vegetation. This study has been ethically approved by the University of the Western Cape (AR17/5/2) and the National Zoological Gardens of South Africa (NZG/RES/P17/34).

3.2 Sample collection

Semen samples (n=108) were collected over different season (August 2015; n=26, February 2016; n=30, August 2016; n=25 and February 2017; n=27) by means of periprostatic electrostimulation applied per rectum by veterinarians or animal health technicians. Rectal probe electro-ejaculation is an accepted procedure for semen collection in domesticated species (Shiple *et al.*, 2007).

The ejaculates were collected using an Eltoro 3 (Electronic Research Group, Midrand, South Africa) rectal probe electro-ejaculator, according to a method adapted from Malejane *et al.* (2014). Prior to stimulation, each buck was placed in a recumbent position and the penis gently extracted from the sheath. The penis was held gently in position with a piece of sterile gauze. Lubricant (liquid paraffin) was applied to the probe and to the anal sphincter before insertion of the probe. The bucks were allowed to acclimatise to the sensation, while the operator gently massaged the accessory sex gland area. Electrical stimulation (5-15 V, 500 mA) was applied at intervals of three to five seconds, and altered with rest periods of three to five seconds, for a maximum of six times. During each stimulation, the current was gradually increased until an ejaculate (0.5-1.5 ml) was obtained within 1-2 minutes (Noakes *et al.*, 2009). All ejaculates were collected in pre-warmed, graded 50 ml plastic tubes. Any sample contaminated with urine was discarded. The freshly ejaculated semen was kept at 37°C immediately after collection and for the duration of all experimental procedures.

3.3 Standard semen analysis

A basic semen analysis was performed to evaluate the quantity and quality of each sample by assessing semen volume, pH, macro-movement, sperm concentration and total sperm motility.

3.3.1 Volume and pH

The ejaculate volume was determined by transferring the semen sample into a preheated, graded 15 ml plastic test tube.

Normal pH was measured immediately after determination of the volume, using Panpeha pH paper strip (Roche, Cape Town, South Africa) which has a range between pH 1 and 14 which has been impregnated with a mixture of several indicators. The pH was assessed via pipetting 300 μ l of semen onto the pH paper strip and evenly spreading the sample over the indicator colour blocks. By immediately comparing the colour of the strip against a colour comparison table, the pH of the semen was determined.

3.3.2 Macro-movement

Macro-movement of the semen samples was recorded using a rating system of 1-5 (1 - poor quality and 5 - best quality) as described by Chemineau *et al.* (1991). A drop of semen was placed on a slide using the pipette, and covered with a cover slip. The slide was then examined under the microscope at a magnification of 100x. A number of rapidly "swarming" spermatozoa were considered best quality sperm while numerous weakly motile spermatozoa, or spermatozoa merely trembling on the spot, together with cells which cannot be differentiated were considered poor quality.

3.3.3 Sperm concentration and motility

Sperm concentration and motility were evaluated by placing 3 μ l of semen into a preheated (37°C) Leja 4 chamber slide (Leja Products B.V., Nieuw-Vennep, The Netherlands). Slides were analysed on a CASA system, using the Motility/Concentration module of the Sperm Class Analyzer (SCA®), version 6.0 (Microptic S.L., Barcelona, Spain). Additionally, a Basler A312fc digital camera (Microptic S.L., Barcelona, Spain), was used for season 1 and 2 whereas a Basler aCa 1300-200uc digital camera (Fig 3.3.3.1) (Microptic S.L., Barcelona, Spain), was used for season 3 and 4. Both digital cameras were mounted onto a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa) and were equipped with a 10x objective, phase contrast optics and a heated stage (37°C). At least two fields,

including a minimum of 200 motile spermatozoa in total, were captured for each sample and analysed accordingly.

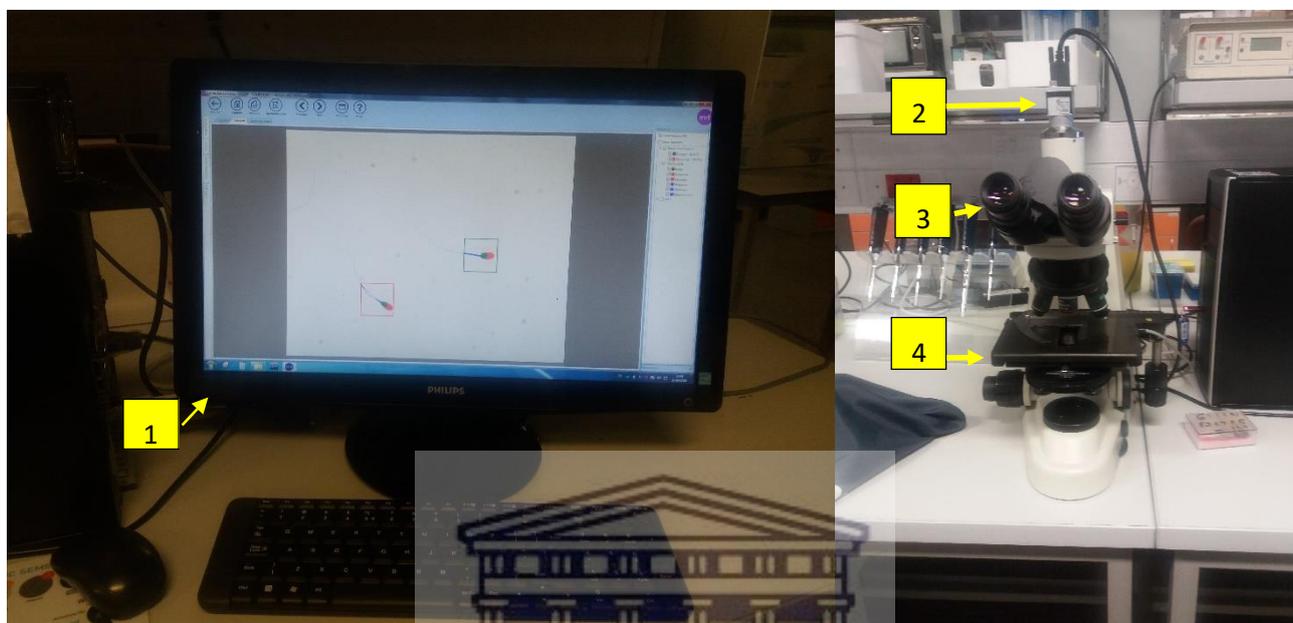


Fig 3.3.3.1. Equipment used for assessment of semen parameters as well as CASA analysis of sperm morphometry, motility, concentration, vitality and hyperactivation. The setup consists of a PC with SCA® software for CASA analysis (1), Basler aCa 1300-200uc digital camera (2) attached to a Nikon Eclipse 50i microscope (3) and a heated stage (4).

3.4 Sperm motility

Various sperm motility parameters were assessed, including percentage total motility, percentage progressive motility, percentages rapid, medium and slow swimming sperm and eight kinematic parameters (see Table 3.4.1). These parameters were assessed by placing 3 µl of semen into a pre-heated 4 chamber Leja slide, where after the semen was flushed with phosphate buffered saline (PBS) (Sigma-Aldrich, Cape Town, South Africa) and using the Motility module of SCA® version 6.1 (Microptic S.L., Barcelona, Spain). The SCA® selection criteria for the measurement of the various motility and kinematic parameters are indicated in Table 3.4.2. The same microscopy equipment was used as mentioned above in

section 3.3.3. A total number of at least 200 motile spermatozoa were evaluated for each semen sample.

Table 3.4.1. Motility and kinematic parameters of spermatozoa assessed by CASA

Parameter	Definition
VCL ($\mu\text{m/s}$)	Time-averaged velocity of a sperm head along its actual curvilinear path
VSL ($\mu\text{m/s}$)	Time-averaged velocity of a sperm head along the straight line between its first detected position and its last
VAP ($\mu\text{m/s}$)	Time-averaged velocity of a sperm head along its average path
LIN (%)	Linearity of the curvilinear path = VSL/VCL
STR (%)	Linearity of the average path = VSL/VAP
WOB (%)	Measure of oscillation of the actual path about the average path = VAP/VCL
BCF (Hz)	Average rate at which the curvilinear path crosses the average path
ALH (μm)	Magnitude of lateral displacement of a sperm head about its average path

*VCL = curvilinear velocity, VSL= straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness , WOB= wobble, BCF= beat cross frequency, ALH = amplitude of later head displacement

Table 3.4.2. SCA® motility analysis properties used for the assessment of Tankwa goat spermatozoa

Parameter	Setting
Particle area (μm^2)	3<PA<70
VCL* Slow ($\mu\text{m/s}$)	10<S<20
VCL Medium ($\mu\text{m/s}$)	20<M<80
VCL Rapid ($\mu\text{m/s}$)	R>180
Progressivity (% of STR)	>80
Circular (% of LIN*)	<50
Connectivity	14
VAP* points 50f/s	7
Number of images	30
Images per second	50
Optic	Ph- (negative phase contrast)
Chamber	Leja 20

*VCL- Curvilinear velocity STR- Straightness (%), LIN- Linearity (%), VAP- Average path velocity.

3.5 Sperm morphology and morphometry

Sperm morphology and morphometry were assessed and quantified using SpermBlue, a universal stain for automated sperm morphology analysis (ASMA) (van der Horst & Maree 2009). The CASMA system automatically detects the acrosome, head and mid-piece of spermatozoa.

Morphology smears were made using 10 μl semen diluted with phosphate buffered saline (PBS) and left to air dry. Dried smears were submerged in a Wheaton Coplin jar containing SpermBlue stain-fixative mixture for 45 seconds, followed by a 6 second washing step in

distilled water. Stained slides were left to air dry at an angle to allow any excess stain to run off. Once dried, slides were mounted with a coverslip using DPX mounting medium. Sperm morphometry were assessed using a 60x objective, bright field optics and a blue filter on a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa). Images of at least 100 spermatozoa were captured with a Basler A312fc digital camera (Microptic S.L., Barcelona, Spain) attached to the microscope, and further analysed with the Morphology Module of SCA®, version 6.0, (Microptic S.L., Barcelona, Spain).

In total, 11 sperm morphometry parameters were measured by SCA, including head surface area, head width, head perimeter, head length, head ellipticity, head elongation, head regularity, head roughness and the percentage acrosome coverage over the head. Two mid-piece dimensions were also measured, namely width and area.

Since no data was available on sperm morphology and morphometry of Tankwa goats prior to this study, there was no reference values available for assessing the percentage spermatozoa with “normal” morphology present in each semen sample. Cut-off points for normal morphology were determined as described by van der Horst *et al.* (2018) by grouping different morphometric measures for each percentile (0-100%, 2.5-95% etc.). Minimum and maximum values for each parameter were derived from different percentile intervals as analysed using routine statistical methods. Morphometric values for Tankwa goat (season 1 samples) from 95% confidence interval (CI) were used in the Tankwa goat configuration function for normal morphometry. Subsequently, the following sperm morphology parameters were analyzed: percentage normal morphology. SCA® capturing properties for Tankwa goat sperm analysis using the Morphology Module of SCA® were set as follows: detection = automatic, criteria = Customized, stain = SpermBlue and particle area = 1 - 100.

3.6 Sperm vitality

Sperm vitality was quantified using a dye-exclusion assay to assess sperm plasma membrane integrity. Nigrosin-eosin stain (Evans and Maxwell, 1987) was aliquoted (20 µl) into an amber eppendorf tube and preheated (37°C) together with microscope slides, prior to use. A volume of 5 µl semen was mixed with 15 µl preheated nigrosineosin stain, and incubated for 30 seconds. Smears were made using 20 µl of the semen and nigrosin-eosin mixture, and left to air dry in a dark room. Once dried, slides were mounted with coverslips using DPX mounting medium. All slides were viewed using a 40x objective and

brightfield optics on a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa), with an attached digital camera (same cameras for different seasons, as indicated in section 3.3.3). Vitality of at least 100 spermatozoa from each semen sample was assessed with the Vitality module of SCA®, version 6.0 (Microptic S.L., Barcelona, Spain). All captured fields were verified visually to ensure that viable and dead spermatozoa were correctly analysed (pink= dead; white= alive). Sperm vitality was expressed as percentage live spermatozoa.

3.7 Hyperactivation

Sperm hyperactivation was induced by exposing spermatozoa to five different media namely, neat PBS, 5 mM procaine hydrochloride in PBS, 4% lignocaine in PBS, and BO wash as proposed by Brackett and Oliphant (1975) (consisted of 1.4845g NaCl, 0.0875g KCl, 0.024g MgSO₄, 0.0125g KH₂PO₄, 0.525g NaHCO₃, 0.009g Na pyruvate, 0.12525g Glucose, 0.075g Phenol red, 0.784g Na Lactate and 1% Bovine Serum Albumin (BSA) with an addition of 0.105g NaHCO₃, 1.1915g HEPES and 0.6ml NaOH) (Sigma Aldrich, Cape Town, South Africa). Neat PBS was used as a control throughout the study. Of the mentioned chemicals above, only the procaine hydrochloride solution was assessed for hyperactivation for all the seasons, whereas only the BO and Lignocaine were assessed in season two. All of the solutions were aliquoted into their own eppendorf tubes and pre-warmed to 37°C. Using a positive displacement pipette, 1 µl of semen was placed into each chamber of a pre-heated 4 chamber Leja slide (Leja Products B.V., Nieuw-Venep, Netherlands). Following the flush technique which disperses spermatozoa and allows for capturing of sperm motility tracks (van der Horst *et al.*, 2010, Boshoff *et al.* 2018), 2 µl of each solution was used to flush the semen into each chamber, whilst keeping the slide on the heated stage. A minimum of 200 motile spermatozoa in at least two frames were evaluated for each semen sample within 10 minutes.

Sperm kinematic parameter cut-offs for identifying spermatozoa displaying hyperactive motility had to be determined for Tankwa goat spermatozoa. Firstly, kinematics of individual spermatozoon from each semen sample (Season 2 samples) after exposure to PBS and procaine hydrochloride, respectively, were constructed to an Excel file and imported to MedCalc software (version 12.3.0; Medcalc Software, Mariakerke, Belgium). Receiver Operating Characteristics (ROC) curves were constructed (de Long *et al.*, 1988) for each of the eight kinematic parameters.

Only those parameters that displayed both high sensitivity and specificity when comparing the control and procaine treated samples were used to determine cut-off values for hyperactivation. After creating a sort function for Tankwa goat sperm hyperactivation on the SCA Motility module using the cut-offs, the number of hyperactivated spermatozoa was calculated as a percentage of motile spermatozoa using the same equipment as for sperm motility evaluation mentioned above.

3.8 Acrosome integrity

The fluorescein isothiocyanate-labelled Peanut Agglutinin (FITC-PNA) assay (SigmaAldrich, Cape Town, South Africa) was used to assess the acrosome intactness of spermatozoa. Semen was diluted with PBS (Sigma Aldrich, Cape Town, South Africa), thereafter two 5 µl drops were placed 2 cm apart on the slides and allowed to air dry. The slides were then fixed with pre-cooled 95% ethanol at 4°C for 30 minutes in the dark. Controls were made and some slides were then left overnight at 4°C to induce the acrosome reaction. An equal volume of Hoechst (H33258) (Sigma Aldrich, Cape Town, South Africa) was placed on the different semen dots and placed at room temperature for 7 minutes in the dark. Thereafter, the slides were dipped in distilled water and excess water was shaken off. A 40 µl volume of FITC-PNA was placed on each semen dot and was allowed to stain at room temperature for 60 minutes, after which the slides were dipped once in distilled water in the dark and allowed to air dry. Dako fluorescent mounting medium (Diagnostech, Gauteng, South Africa) was used to mount a coverslip onto the slides. The slides were then viewed under a Nikon Eclipse 50i fluorescence microscope (IMP, Cape Town, South Africa) using the 40X objective with either the FITC filter (B-2A Nikon: ex450-490; DM 505; BA 520) or broadband filter (MXV 44005 C127214). A minimum of 100 spermatozoa were evaluated for each semen sample.

3.9 Statistical analysis

All the data was collected and saved as specific CASA files, and thereafter transferred onto MedCalc software (version 12.3.0; Medcalc Software, Mariakerke, Belgium). 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 among groups were further analysed

using the Student-Newman-Keuls test for pairwise comparisons. 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 applied. Data are represented as mean \pm standard deviation (SD) in the tables while $P \leq 0.05$ was considered significant.

Multivariate statistical visualization analysis was also performed using the StatGraphics Centurion data analysis software system, version XVII (Dittrich & Partner Consulting GmbH, Solingen, Germany). The dataset analysed consisted of several semen and sperm parameters (including macro-movement, total motility, progressive motility, rapid progressive, VCL, ALH, R-VCL, R-VSL, and R-VAP) from ejaculates with no missing data. Two visualization techniques were applied to this dataset, namely an Andrews plot, and Star and sunray plots. The graphical displays of each of these visualizations assisted to distinguish small seasonal differences of above mentioned parameters as well as association among these parameters.



Chapter 4: Results

4.1 Standard semen analysis

A total number of 108 ejaculates were obtained over four seasons, with 26 ejaculates collected in season 1, 30 ejaculates in season 2, 25 ejaculates in season 3 and 27 ejaculates in season 4.

The overall results were as follow; the mean Tankwa goat sperm volume was 1.2 ml and ranged between 0.9 ml and 1.6 ml. The mean pH was 6.7 and ranged between 6.0 and 7.0. Macro-movement of spermatozoa had a mean of 4 and ranged between 3.6 and 4.3. The sperm concentration had a mean of $2436.3 \times 10^6/\text{ml}$ and ranged between $1971.5 \times 10^6/\text{ml}$ and $2971.4 \times 10^6/\text{ml}$. Total motility had a mean of 78.2% and ranged between 70.8% and 83.6%.

Statistically significant differences were observed for most of the semen parameters among the four collection seasons compared (Table 4.1.1), with the exception of the sperm concentration where there was no significant difference observed. A higher mean semen volume was observed in February and August 2016 compared to August 2015 and February 2017. A higher average was observed in August 2016 and February 2017 for pH while the lowest average was in February 2016. The mean macro-movement in February 2016 was significantly higher than the mean observed in August 2016. Total motility in February 2016 was significantly higher than August 2016. When comparing the summer (February 2016 and 2017) and winter (August 2015 and 2016) period (Table 4.1.2), statistically significant differences were observed for macro-movement and percentage total motility. Both macro-movement and total motility had higher averages in the summer period. Statistically different characteristics are further illustrated in Fig 4.1.1-4.1.3.

Table 4.1.1. Standard Tankwa goat semen parameters over a two year period (mean \pm SD)

Parameter	Aug-15 n=26	Feb-16 n=30	Aug-16 n=25	Feb-17 n=27
Volume (ml)	1,0 \pm 0,6 ^a	1,3 \pm 0,5 ^b	1,6 \pm 0,5 ^b	0,9 \pm 0,4 ^a
pH*	6,3 \pm 0,7 ^a	6,0 \pm 0,8 ^a	7,0 \pm 0,7 ^b	7,0 \pm 0,6 ^b
Macromovement [#]	-	4,3 \pm 0,9 ^a	3,6 \pm 1,2 ^b	4,0 \pm 0,7 ^{ab}
Sperm Concentration ($\times 10^6$ /ml)	1971,5 \pm 1388,0	2335,5 \pm 1840,8	2520,9 \pm 1402,9	2917,4 \pm 1573,9
Total Motility (%)	74,8 \pm 16,8 ^{ab}	83,6 \pm 10,3 ^a	70,8 \pm 15,6 ^b	80,9 \pm 15,9 ^{ab}

Superscript a-b in the same row indicates significant difference where $p < 0.05$ *pH, n=24 for

Aug 2015, n= 6 for Feb 2016.

#macro-movement, n=28 for Feb 2016.

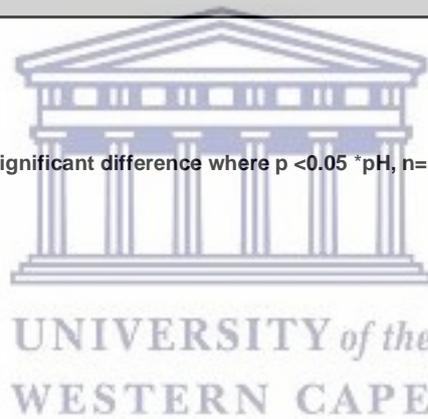


Table 4.1.2. Standard Tankwa goat semen parameters (mean \pm SD) for summer and winter seasons and all samples combined.

Characteristic	Summer n=57	Winter n=51	Combined n=108
Volume (ml)	1,1 \pm 0,5	1,3 \pm 0,6	1,2 \pm 0,5
pH*	6,3 \pm 0,7	5,9 \pm 0,8	6,7 \pm 0,8
Macromovement[#]	4,1 \pm 0,8 ^a	3,6 \pm 1,2 ^b	4,0 \pm 1,0
Sperm Concentration ($\times 10^6$/ml)	2611,2 \pm 1729,4	2240,8 \pm 1480,8	2436,3 \pm 1589,7
Total Motility (%)	82,4 \pm 13,1 ^a	73,1 \pm 16,3 ^b	78,2 \pm 15,3

Superscript a-b indicates significant difference where $p < 0.05$

*pH, n =82 for combined data

[#]macro-movement, n =80 for combined data , summer, n = 55 and winter, n = 25.



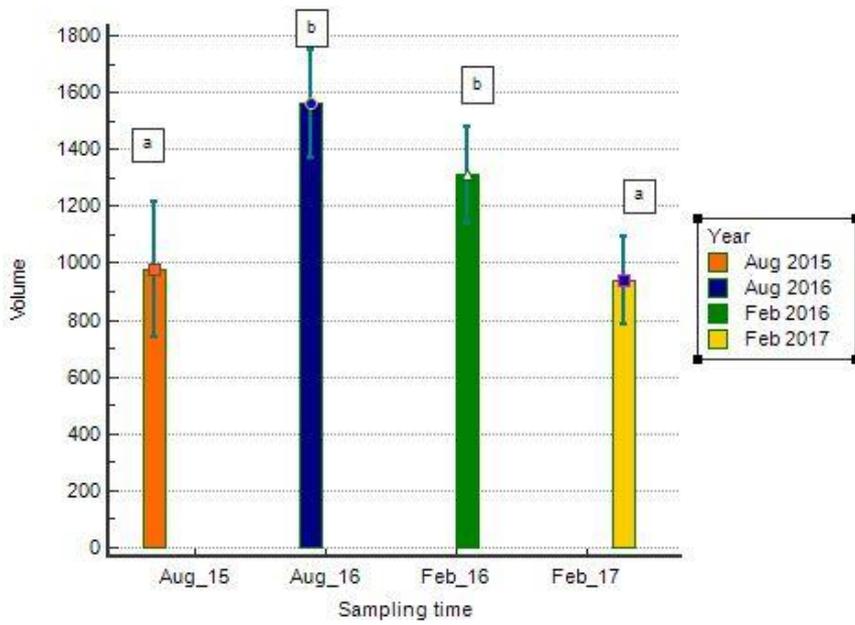


Fig 4.1.1. Clustered comparison graph (mean \pm SD) of Tankwa goat semen volume (μ l) over a two year period.

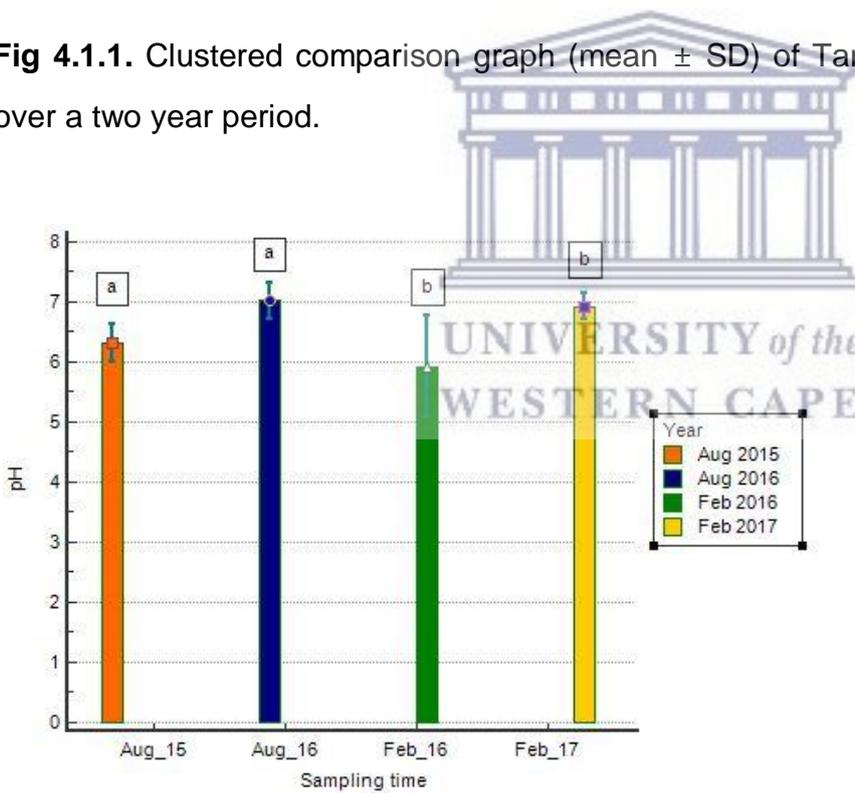


Fig 4.1.2. Clustered comparison graph (mean \pm SD) of Tankwa goat semen pH over a two year period.

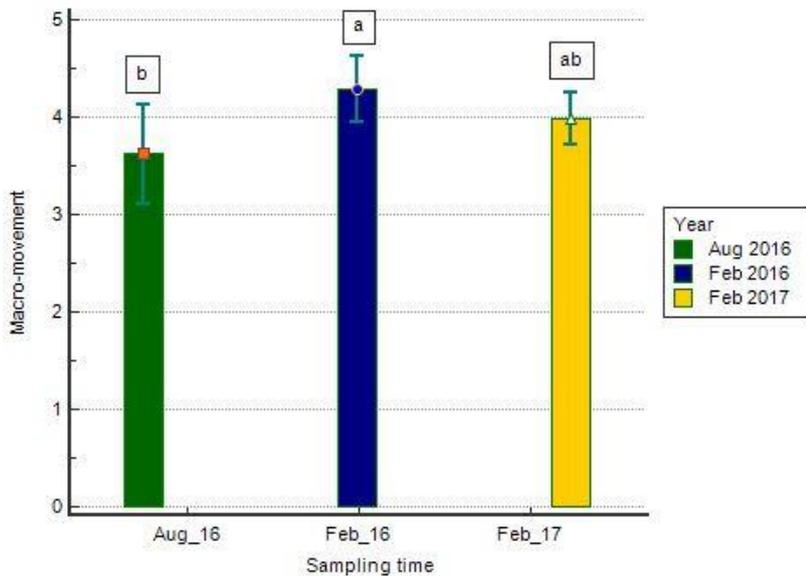


Fig 4.1.3. Clustered comparison graph (mean \pm SD) of Tankwa goat semen macromovement over a two year period.

4.2 Sperm motility and kinematics

Statistically significant differences were observed for most of the sperm motility and kinematic parameters among the four collection seasons, with the exception of percentage progressive motility. The mean total motility for Tankwa goat sperm was 78.2% and ranged from 70.8% to 83.6%, progressive motility had a mean value of 54.1% and ranged from 44.9% to 65.9%. Rapid swimming sperm had a mean of 16.5%, and ranged between 4.3% and 33.6%. Medium swimming sperm had a mean of 47.2%, and ranged between 35.1% and 57.5%. Slow swimming sperm had a mean of 16.1% and ranged between 11.8% and 24%. Typical Tankwa goat sperm tracks are illustrated in Fig 4.2.1.

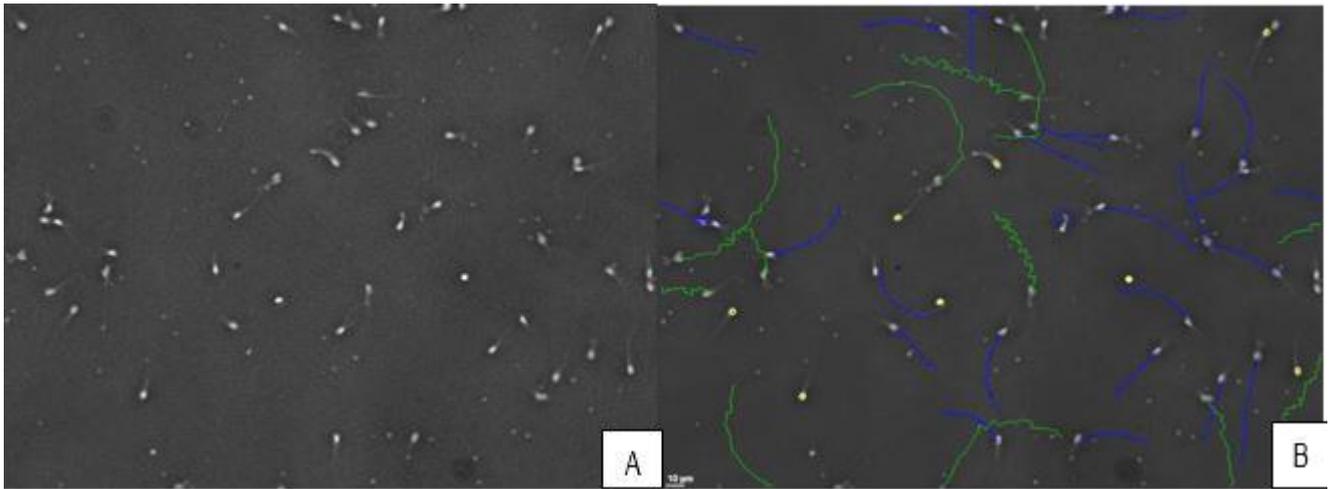


Fig 4.2.1. A typical Tankwa goat sperm motility analysis in phosphate buffered saline (PBS) medium without (A) and with sperm tracks (B) (Green line; Medium progressive sperm, Blue; non-progressive and Yellow circles; Immotile sperm).

August 2016 had the lowest average for total motility and was significantly lower than February 2016. Rapid progressive in February 2017 was significantly higher than all other seasons. Medium progressive significantly differed from each season and recorded the highest average in February 2016. August 2015 was significantly higher than all three seasons for non-progressive (slow). Furthermore, August 2015 was significantly lower than February and August 2016 for VCL. August 2015 and February 2017 were significantly lower than February 2016 and August 2016 for VSL, VAP and STR. February 2016 was significantly higher than August 2015 and August 2016 for LIN. February 2017 had the highest mean for ALH while August 2016 had the highest mean for BCF. The WOB parameter (Table 4.2.1) indicated that each season differed from one another. The results are further illustrated in Fig 4.2.2-4.2.4.

Table 4.2.1. Sperm motility and kinematic parameters of Tankwa goats (mean \pm SD) over a two year period.

Parameter	Aug-2015 n=23	Feb-2016 n=30	Aug-2016 n=20	Feb-2017 n=26
Total motility (%)*	74,8 \pm 16,8 ^{ab}	83,6 \pm 10,3 ^a	70,8 \pm 15,6 ^b	81,0 \pm 15,9 ^{ab}
Progressive motility (%)*	44,9 \pm 16,7	65,9 \pm 13,0	50,9 \pm 16,5	52,1 \pm 26,4
Rapid (%)	4,3 \pm 4,5 ^a	11,9 \pm 10,7 ^a	15,9 \pm 18,2 ^a	33,6 \pm 25,1 ^b
Medium (%)	49,4 \pm 23,0 ^a	57,5 \pm 17,9 ^b	44,3 \pm 16,6 ^c	35,1 \pm 16,9 ^d
Slow (%)	24,0 \pm 13,2 ^a	15,6 \pm 13,8 ^b	11,8 \pm 6,4 ^b	12,5 \pm 10,4 ^b
VCL (μm/s)	111,4 \pm 20,1 ^a	137,2 \pm 20,4 ^b	140,6 \pm 24,0 ^b	157,8 \pm 37,3 ^{ab}
VSL (μm/s)	63,6 \pm 17,9 ^a	87,7 \pm 15,2 ^b	80,6 \pm 13,4 ^b	63,7 \pm 27,1 ^a
VAP (μm/s)	79,0 \pm 18,7 ^a	101,3 \pm 14,7 ^b	92,3 \pm 13,6 ^b	82,1 \pm 27,5 ^a
STR (%)	75,5 \pm 9,6 ^a	83,3 \pm 7,1 ^b	82,6 \pm 6,8 ^b	70,4 \pm 11,2 ^a
LIN (%)	55,7 \pm 11,8 ^a	64,2 \pm 10,3 ^b	55,9 \pm 6,9 ^a	37,7 \pm 10,4 ^{ab}
ALH (μm)	2,9 \pm 0,5 ^a	3,1 \pm 0,7 ^a	2,7 \pm 0,6 ^a	3,8 \pm 0,8 ^b
BCF (Hz)	22,6 \pm 5,2 ^a	25,7 \pm 5,3 ^a	30,0 \pm 4,5 ^b	23,0 \pm 4,9 ^a
WOB (%)	70,1 \pm 9,4 ^a	74,6 \pm 8,3 ^b	65,0 \pm 5,0 ^c	50,5 \pm 7,4 ^d

Superscript a-d in the same row indicates significant difference where $p < 0.05$

*VCL = curvilinear velocity, VSL= straight-line velocity, VAP = average path velocity, STR = straightness , LIN = linearity,

ALH = amplitude of lateral head displacement, BCF = beat cross frequency,, WOB = wobble

*Total motility/Progressive motility, n=26 for Aug 2015

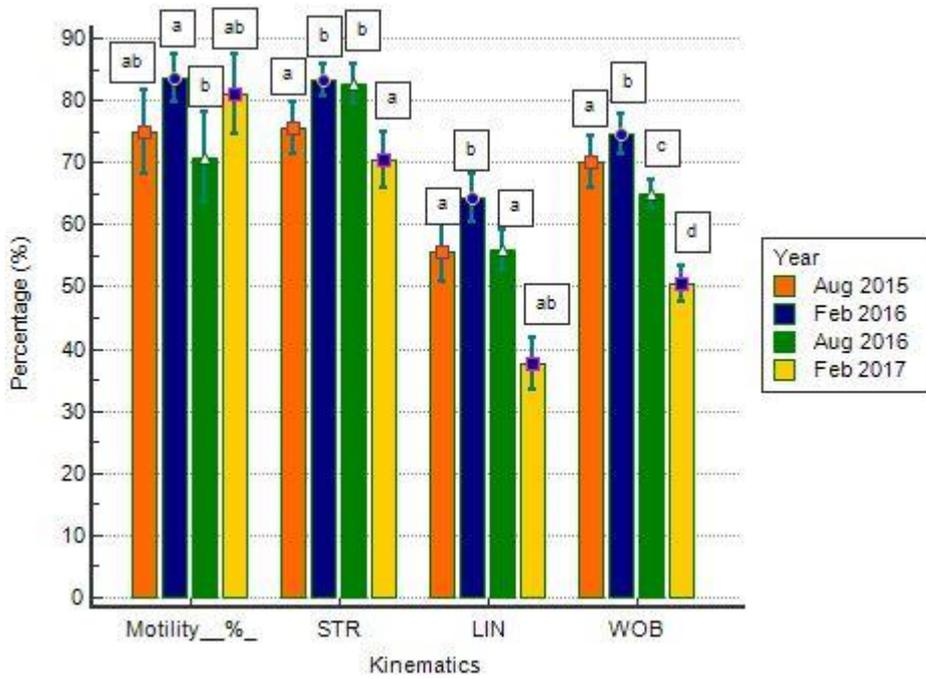


Fig 4.2.2. Clustered comparison graph (mean \pm SD) of Tankwa goat sperm motility and kinematics (%) over a two year period. Motility (%) = Total motility (%)

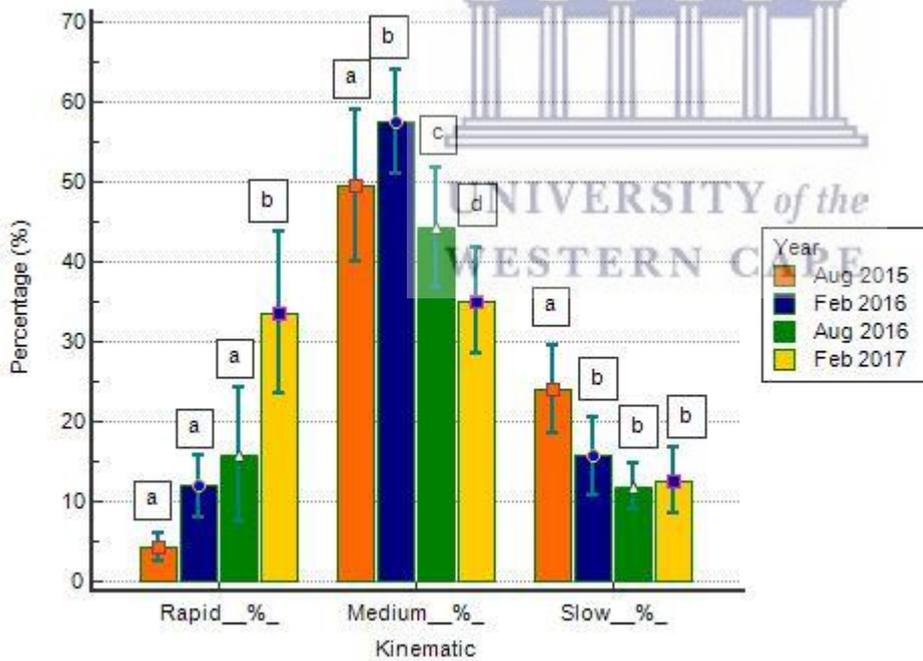


Fig 4.2.3. Clustered comparison graph (mean \pm SD) of rapid, medium and slow swimming Tankwa goat sperm (%) over a two year period.

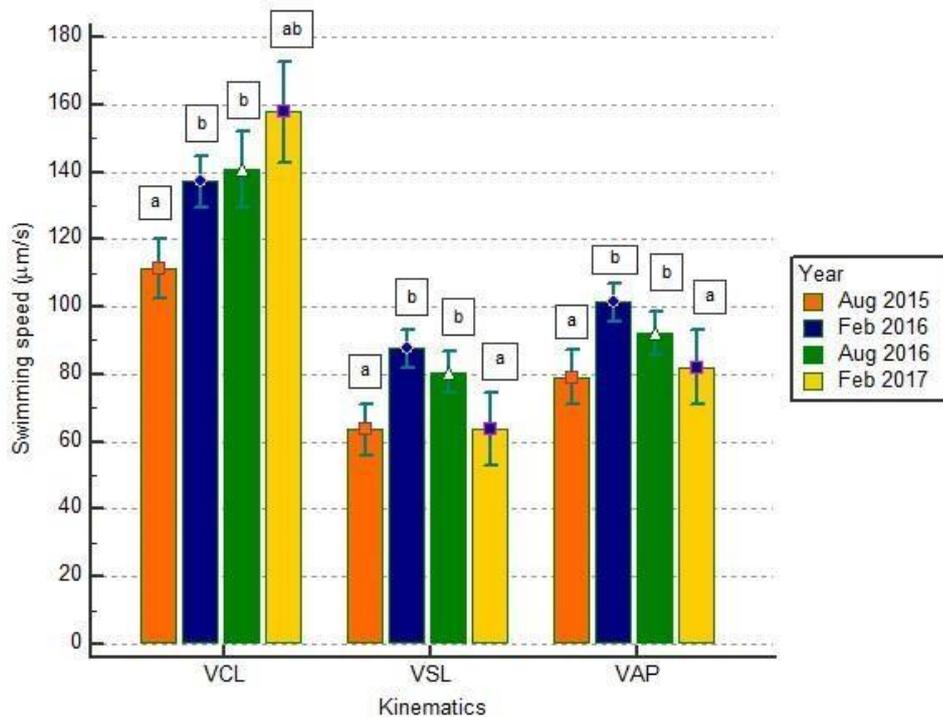


Fig 4.2.4. Clustered comparison graph (mean \pm SD) of Tankwa goat sperm swimming speed ($\mu\text{m/s}$) over a two year period. (VCL = curvilinear velocity, VSL = straightline velocity, VAP = average path velocity)

Significant differences were observed for percentage progressive motility, rapid, slow, VCL and ALH between summer and winter period (Table 4.2.2). The summer period revealed higher averages than winter for percentage progressive motility, rapid progressive, VCL and ALH. (Fig 4.2.3-4.2.4).

Table 4.2.2. Sperm motility and kinematic parameters of Tankwa goats (mean \pm SD) for summer and winter seasons and all samples combined.

Parameter	Summer n=56	Winter n=43	Combined n=99
Total motility (%)	82,4 \pm 13,1	73,1 \pm 16,3	78,2 \pm 15,3
Progressive motility (%)	59,5 \pm 21,3 ^a	47,9 \pm 16,7 ^b	54,1 \pm 20,2
Rapid (%)	21,8 \pm 21,7 ^a	9,6 \pm 13,9 ^b	16,5 \pm 19,6
Medium (%)	47,2 \pm 20,6	47,1 \pm 20,3	47,2 \pm 20,4
Slow (%)	14,5 \pm 12,4 ^a	18,5 \pm 12,2 ^b	16,1 \pm 12,4
VCL (μm/s)	146,7 \pm 31,0 ^a	125,0 \pm 26,3 ^b	137,3 \pm 30,9
VSL (μm/s)	76,6 \pm 24,5	71,5 \pm 18,0	74,3 \pm 21,9
VAP (μm/s)	92,4 \pm 23,5	85,2 \pm 17,7	89,3 \pm 21,4
STR (%)	77,3 \pm 11,2	78,8 \pm 9,1	78,0 \pm 10,3
LIN (%)	51,9 \pm 16,8	55,8 \pm 9,7	53,6 \pm 14,3
ALH (μm)	3,4 \pm 0,8 ^a	2,8 \pm 0,5 ^b	3,1 \pm 0,8
BCF (Hz)	24,4 \pm 5,2	26,0 \pm 6,1	25,1 \pm 5,6
WOB (%)	63,4 \pm 14,4	67,7 \pm 8,0	65,3 \pm 12,2

Superscript a-d in the same row indicates significant difference where $p < 0.05$

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*VCL = curvilinear velocity, VSL= straight-line velocity , VAP = average path velocity, STR = straightness , LIN = linearity,

ALH = amplitude of lateral head displacement BCF= beat cross frequency, , WOB= wobble,

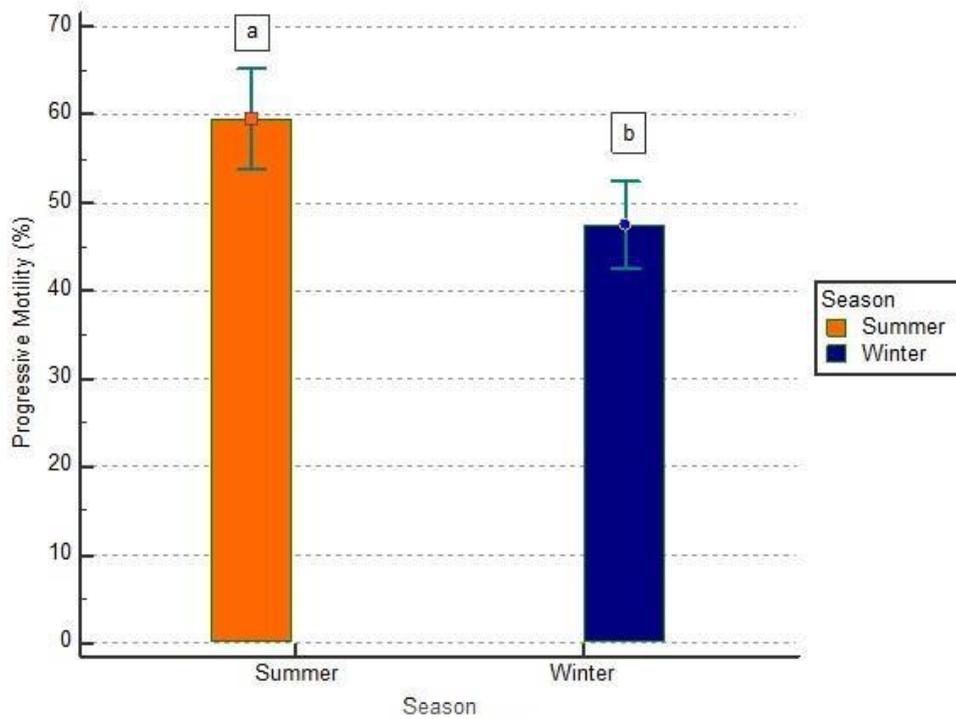


Fig 4.2.3. Clustered comparison graph (mean \pm SD) of Tankwa goat progressive sperm motility (%) over a two year period.

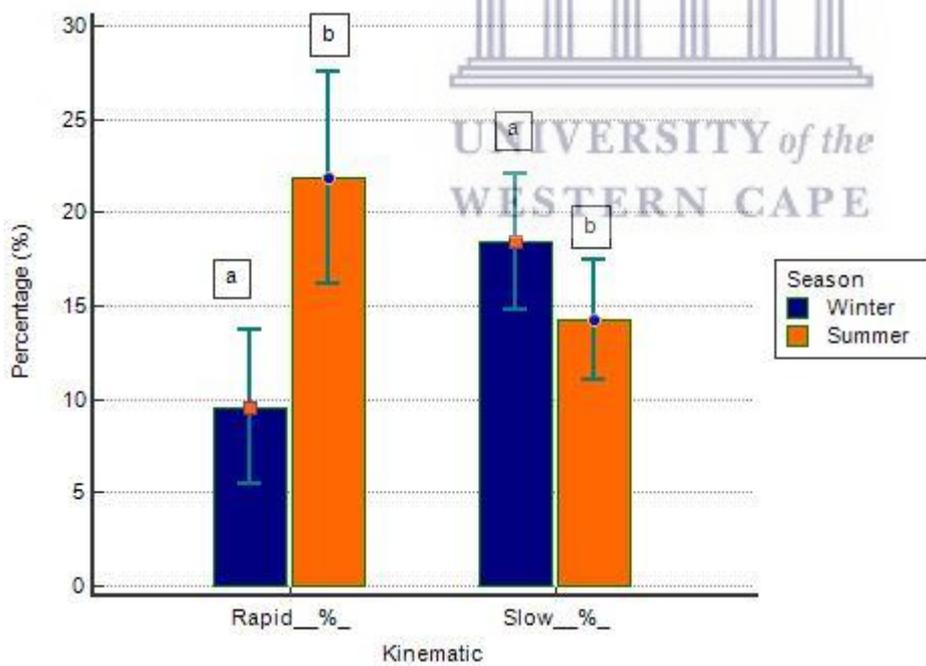


Fig 4.2.4. Clustered comparison graph (mean \pm SD) of rapid and slow swimming Tankwa goat sperm (%) over a two year period.

4.3 Sperm morphology and morphometry

4.3.1 Sperm morphometry

Significant differences were observed among the four collection seasons for some head morphometric parameters namely head elongation, head regularity, head width and head area. Significant differences were also observed for the midpiece and acrosome components (Table 4.3.1.1).

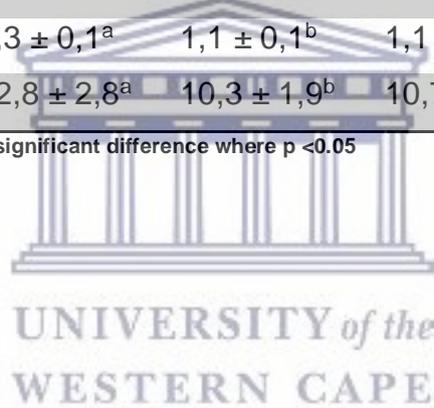
The head length had a mean of 8.7 μm and ranged from 8.5 to 8.9 μm , head width had a mean of 4.1 μm and ranged from 4.0 to 4.2 μm . The head area recorded a mean of 35.9 μm^2 and ranged from 34.0 to 37.1 μm^2 . The head perimeter had a mean of 18.8 μm , and ranged from 18.5 to 18.9 μm . Head ellipticity was constant at 2.1. Head elongation, roughness and regularity had a mean of 0.4, 1.3 and 0.8, respectively and ranged from 0.3 to 0.4, constant at 1.3 and 0.8 respectively. The acrosome coverage had a mean of 55.4% and ranged from 55.1 to 55.7%. The midpiece width and area had a mean of 1.1 μm^2 and 11.4 μm^2 , and ranged from 1.1 to 1.3 μm^2 and 10.3 to 12.8 μm^2 .

August 2015 and February 2017 had the largest head width while February 2016 had the lowest (Fig 4.3.1.2). August 2015 recorded the largest head area (37.1 μm^2) while February 2016 had the smallest (34 μm^2), as illustrated in Fig 4.3.1.1. For head elongation, August 2015 was lower than in other months and for head regularity, the mean was the same (0.8) over the two year period. For midpiece area, August 2015 had the largest midpiece area while February 2016 had the smallest. For midpiece width, August 2015 had a larger midpiece width as compared to the rest. The acrosome coverage was lower in August 2015 compared to the other three collection seasons (Fig 4.3.1.3).

Table 4.3.1.1. Sperm morphometry parameter measurements (Mean \pm SD) of Tankwa goats over a two-year period.

Parameter	Aug-15 n=18	Feb -16 n=8	Aug-16 n=21	Feb-17 n=22
Head length (μm)	8,7 \pm 0,2	8,5 \pm 0,1	8,6 \pm 0,3	8,9 \pm 0,3
Head width (μm)	4,2 \pm 0,1 ^a	4,0 \pm 0,1 ^b	4,1 \pm 0,1 ^{ab}	4,2 \pm 0,2 ^a
Head area (μm^2)	37,1 \pm 2,0 ^a	34,0 \pm 1,1 ^b	35,2 \pm 2,1 ^c	36,1 \pm 2,0 ^d
Head perimeter (μm)	18,9 \pm 0,3	18,5 \pm 0,4	18,7 \pm 0,6	18,9 \pm 0,6
Head ellipticity	2,1 \pm 0,1	2,1 \pm 0,1	2,1 \pm 0,1	2,1 \pm 0,1
Head elongation	0,3 \pm 0,01 ^a	0,4 \pm 0,01 ^{ab}	0,4 \pm 0,01 ^b	0,4 \pm 0,01 ^b
Head roughness	1,3 \pm 0,04	1,3 \pm 0,05	1,3 \pm 0,07	1,3 \pm 0,05
Head regularity	0,8 \pm 0,03 ^a	0,8 \pm 0,02 ^{ab}	0,8 \pm 0,03 ^b	0,8 \pm 0,03 ^b
Acrosome coverage (%)	55,1 \pm 0,6 ^a	55,4 \pm 1,1 ^{ab}	55,3 \pm 0,6 ^{ab}	55,7 \pm 0,7 ^b
Midpiece width (μm)	1,3 \pm 0,1 ^a	1,1 \pm 0,1 ^b	1,1 \pm 0,1 ^c	1,1 \pm 0,1 ^c
Midpiece area (μm^2)	12,8 \pm 2,8 ^a	10,3 \pm 1,9 ^b	10,7 \pm 2,0 ^b	11,1 \pm 1,5 ^b

Superscript a-d in the same row indicates significant difference where $p < 0.05$



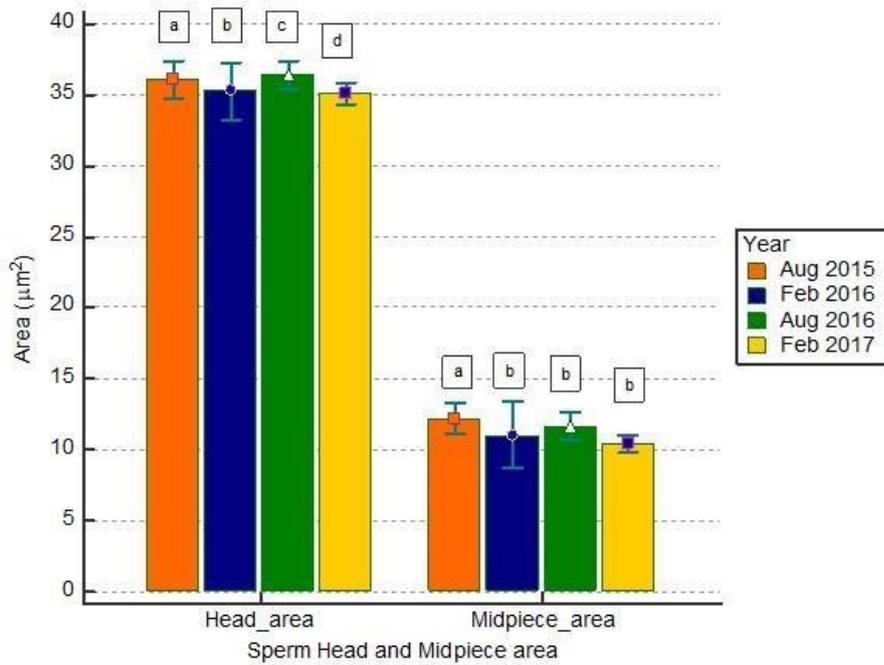


Fig 4.3.1.1. Multiple comparison graph of Tankwa goat sperm head and midpiece area (μm^2) over a two-year period.

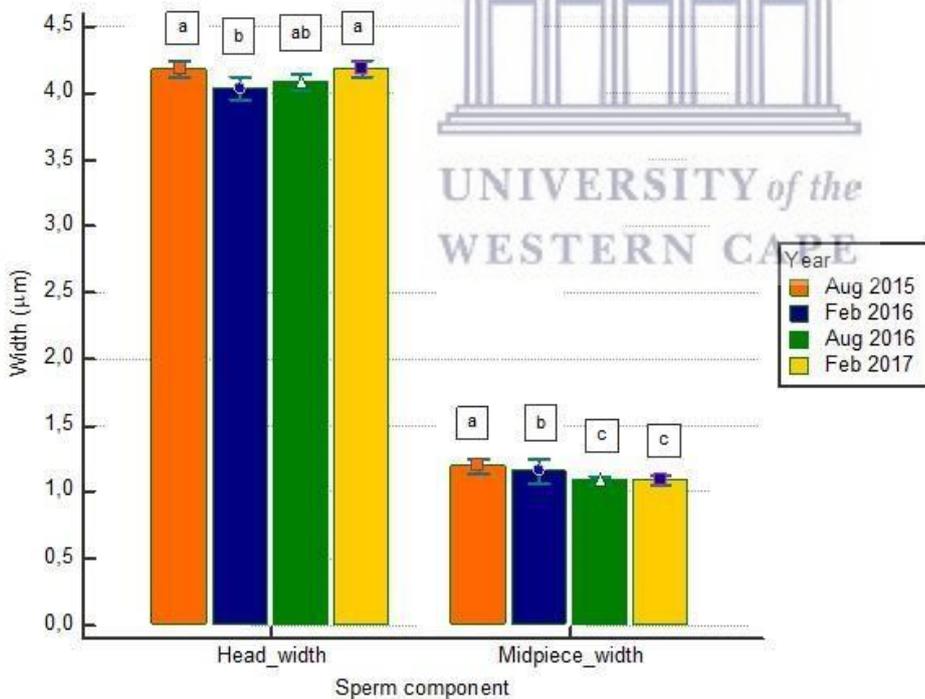


Fig 4.3.1.2. Multiple comparison graph of Tankwa goat sperm head and midpiece width (μm) over a two-year period.

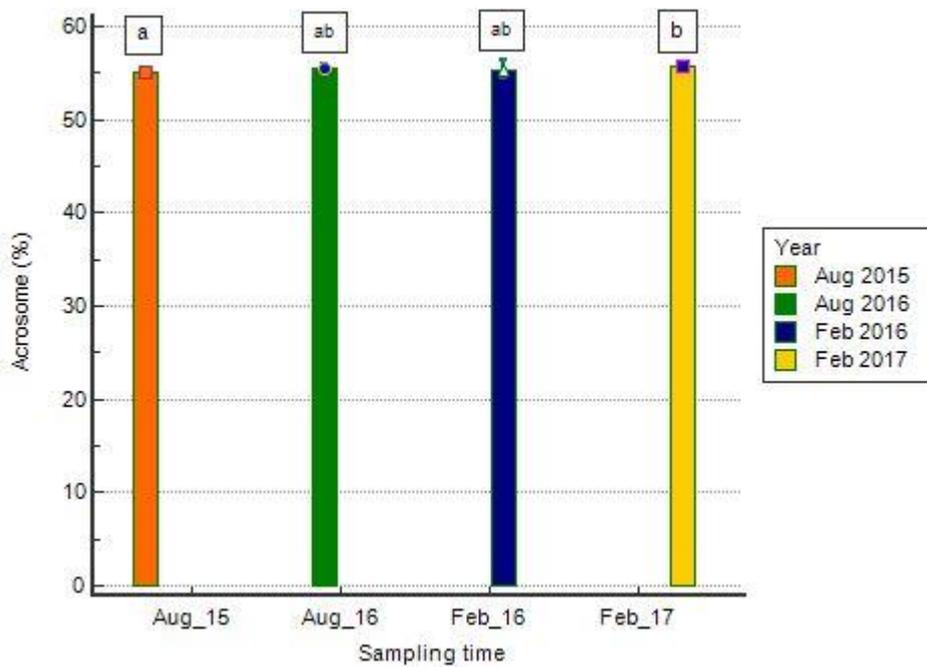


Fig 4.3.1.3. Clustered comparison of acrosome coverage (%) of Tankwa goat sperm over a two year period.

There was a significant difference in head regularity, roughness and width observed between winter and summer (Table 4.3.1.2 and Fig 4.3.1.4). However, there was no difference in the average for head regularity and roughness whereas for head width, summer had a larger average than winter. The midpiece width and area were larger in winter (1.2 μm and 11.8 μm^2 respectively) as compared to summer (1.1 μm) and 10.9 μm^2 (Fig4.3.1.5).

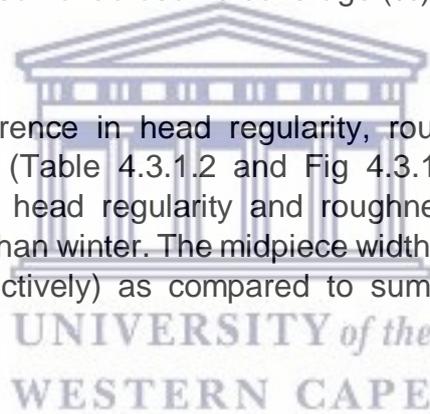


Table 4.3.1.2. Sperm morphometric parameter measurements (mean \pm SD) Tankwa goats for summer and winter seasons and all samples combined

Parameter	Summer n=30	Winter n=39	Combined n=69
Head length (μm)	8,7 \pm 0,3	8,7 \pm 0,2	8,7 \pm 0,2
Head width (μm)	4,2 \pm 0,2	4,1 \pm 0,1	4,1 \pm 0,1
Head area (μm^2)	35,6 \pm 2,0	36,2 \pm 2,2	35,9 \pm 2,2
Head perimeter (μm)	18,9 \pm 0,6	18,8 \pm 0,5	18,8 \pm 0,5
Head ellipticity	2,1 \pm 0,1	2,1 \pm 0,1	2,1 \pm 0,1
Head elongation	0,4 \pm 0,01	0,4 \pm 0,01	0,4 \pm 0,01
Head roughness	1,3 \pm 0,05 ^a	1,3 \pm 0,1 ^b	1,3 \pm 0,05
Head regularity	0,8 \pm 0,02 ^a	0,8 \pm 0,03 ^b	0,8 \pm 0,03
Acrosome (%)	55,1 \pm 0,6	55,4 \pm 1,1	55,4 \pm 0,7
Midpiece width (μm)	1,1 \pm 0,1 ^a	1,2 \pm 0,1 ^b	1,1 \pm 0,1
Midpiece area (μm^2)	10,9 \pm 1,6 ^a	11,8 \pm 2,2 ^b	11,4 \pm 2,0

Superscript a-d in the same row indicates significant difference where $p < 0.05$

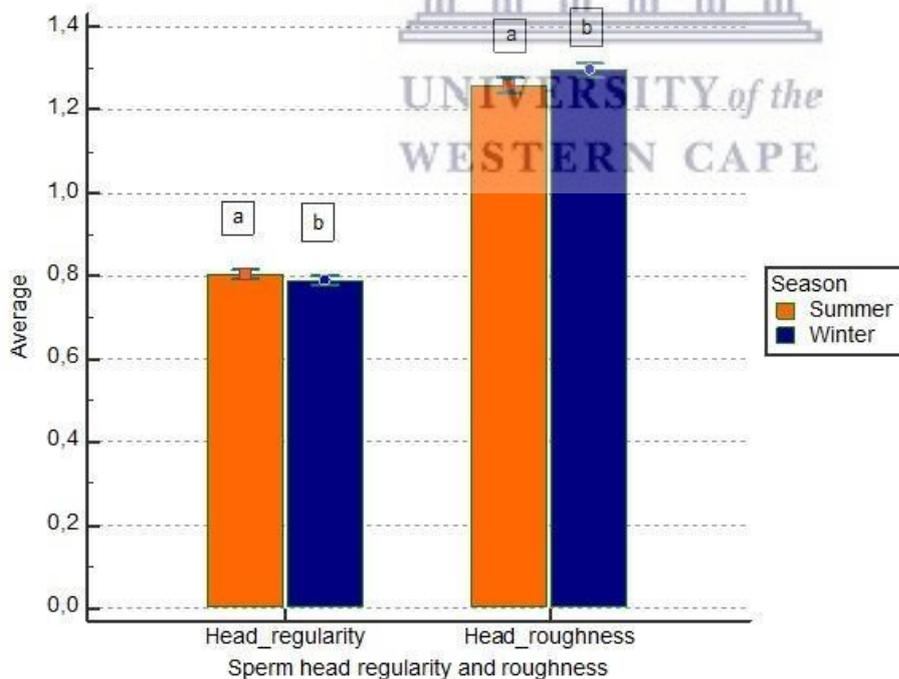


Fig 4.3.1.4. Multiple comparison of head regularity and roughness of Tankwa goat sperm between summer and winter.

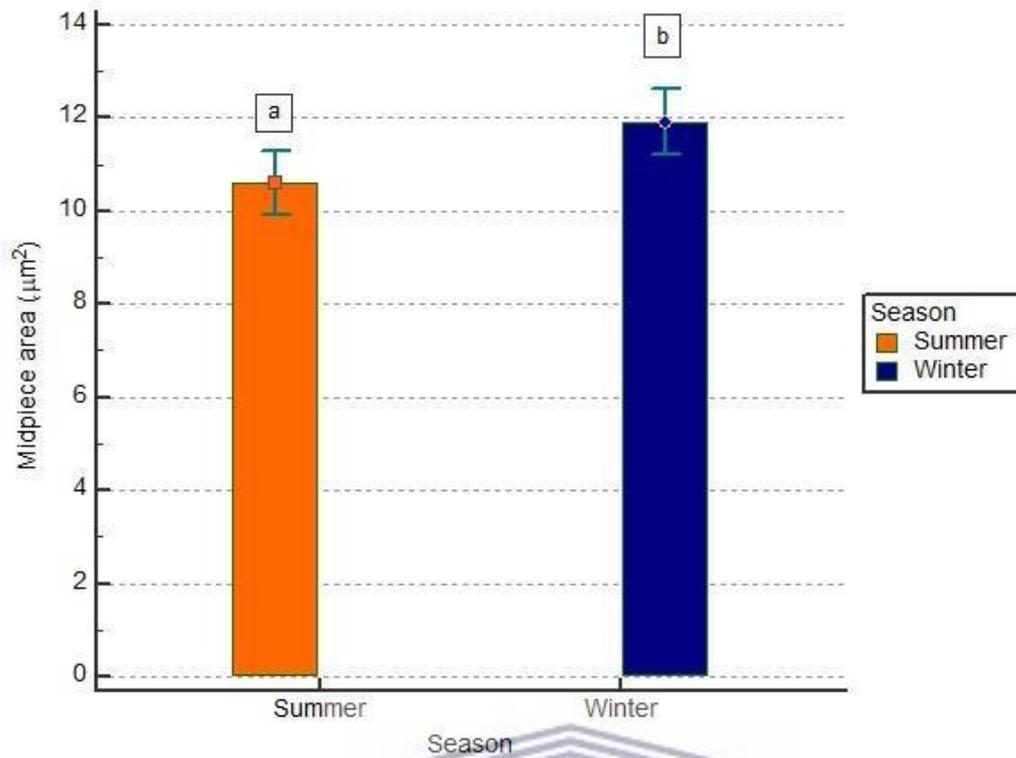


Fig 4.3.1.5. Clustered comparison of midpiece area (μm^2) of Tankwa goat sperm between summer and winter.



4.3.2 Sperm morphology

The sperm morphometry properties determined and set for Tankwa goat sperm morphology analysis using the Confidence Intervals and the Morphology Module of SCA® are shown in Table 4.3.2.1. Fig 4.3.2.1. shows an analysed morphology field of the Tankwa goat spermatozoa.

Table 4.3.2.1. Morphometry properties used to determine percentage normal morphology for Tankwa goat spermatozoa.

Sperm Component	Parameter	Formula	Minimum	Maximum
Head	Length (μm)	L	7.45	10.87
	Width (μm)	W	3.50	5.10
	Area (μm^2)	A	26.30	51.52
	Perimeter (μm)	P	16.67	23.59
	Ellipticity	L/W	1.78	2.53
	Elongation	$(L-W)/(L+W)$	0.28	0.43
	Roughness	$4\pi (A/P^2)$	0.79	1.75
	Regularity	$\pi (L*W/4*A)$	0.61	0.97
	Acrosome		47	100
	Vacuoles (%)		0	20
Midpiece	Width (μm)		0	1.99
	Area (μm^2)		0	27.50

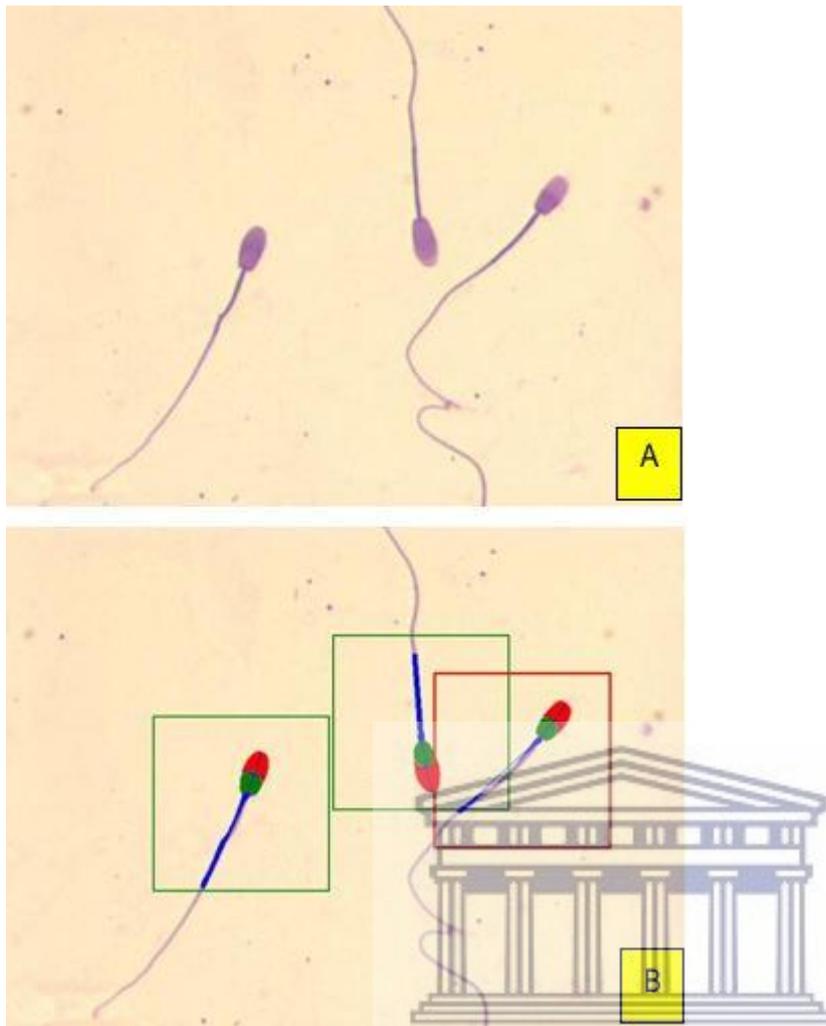


Fig4.3.2.1. An analysed morphology field of Tankwa goat spermatozoa without (A) and with masks (B), stained with SpermBlue. Boxes: Red box=Abnormal sperm, Green box=Normal sperm; Sperm component: Acrosome=Red, Head=Green and Midpiece=Blue.

Significant differences were observed in percentage normal sperm morphology, August 2015 (80.3 ± 14.0) was different from February 2016 (61.0 ± 24.5) but August 2016 (73.1 ± 16.3) and February 2017 (71.7 ± 15.2) did not show any significant difference. The highest percentage for normal sperm was observed in August 2015 (80.3%), the lowest was observed in February 2016 (61%). A very high standard deviation was also observed in February 2016 (Fig 4.3.2.2). Between the summer (68.9 ± 18.2) and winter (76.7 ± 15.8), there was no significant difference observed for the two seasons. February 2018 data ($n = 19$) had relatively high mean for normal sperm morphology (90 ± 5.9) but the results were not included as it was not the part of the study.

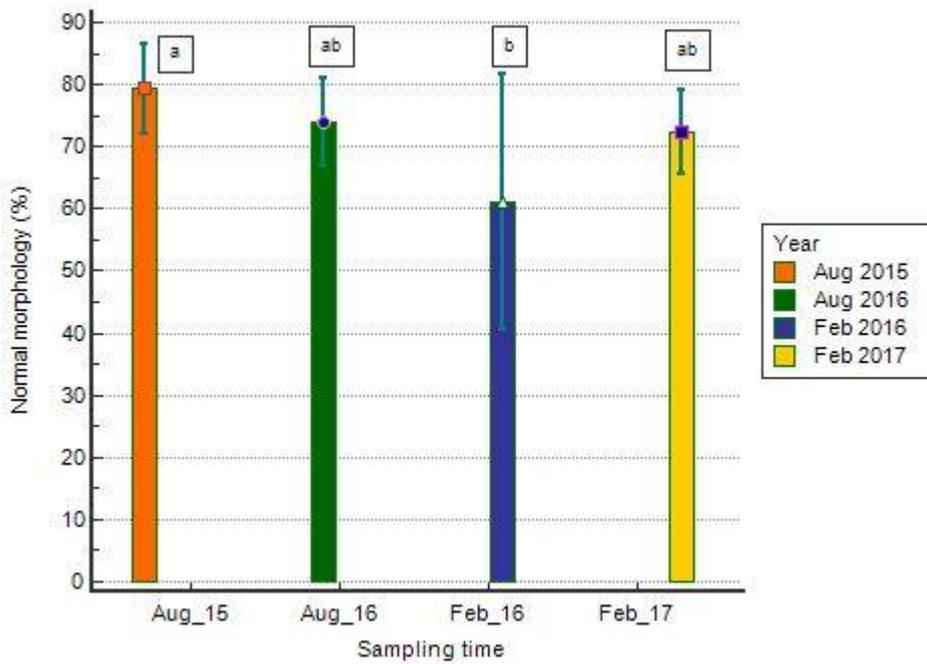


Fig 4.3.2.2. Multiple comparison graph of Tankwa goat normal sperm morphology (%) over a two year period.



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4.4 Sperm vitality

Sperm vitality was $66.3 \pm 6.9\%$ in August 2015, $67.9 \pm 18.2\%$ in February 2016, $69.6 \pm 13.7\%$ in August 2016 and $81.0 \pm 8.6\%$ in February 2017. Typical Tankwa goat sperm stained and analysed are illustrated in Fig 4.4.1. There was a statistically significant difference observed in percentage vitality over the two-year period, with August 2015, February 2016 and August 2016 revealing a significantly lower percentage sperm vitality compared to February 2017 (Fig 4.4.2). In August 2015 (Fig 4.4.3) and August 2016 (Fig 4.4.5), a small sample size was included (readable NE slides) while a relatively large variation (Fig 4.4.4) was observed in February 2016, which ranged from 25% to 93%. February 2017 had a relatively large sample size (Fig 4.4.6)



Fig 4.4.1. SCA analysis of Tankwa goat sperm stained with Nigrosin-Eosin to assess vitality (white = live spermatozoa, pink = dead spermatozoa; Red box = dead, green box = live)

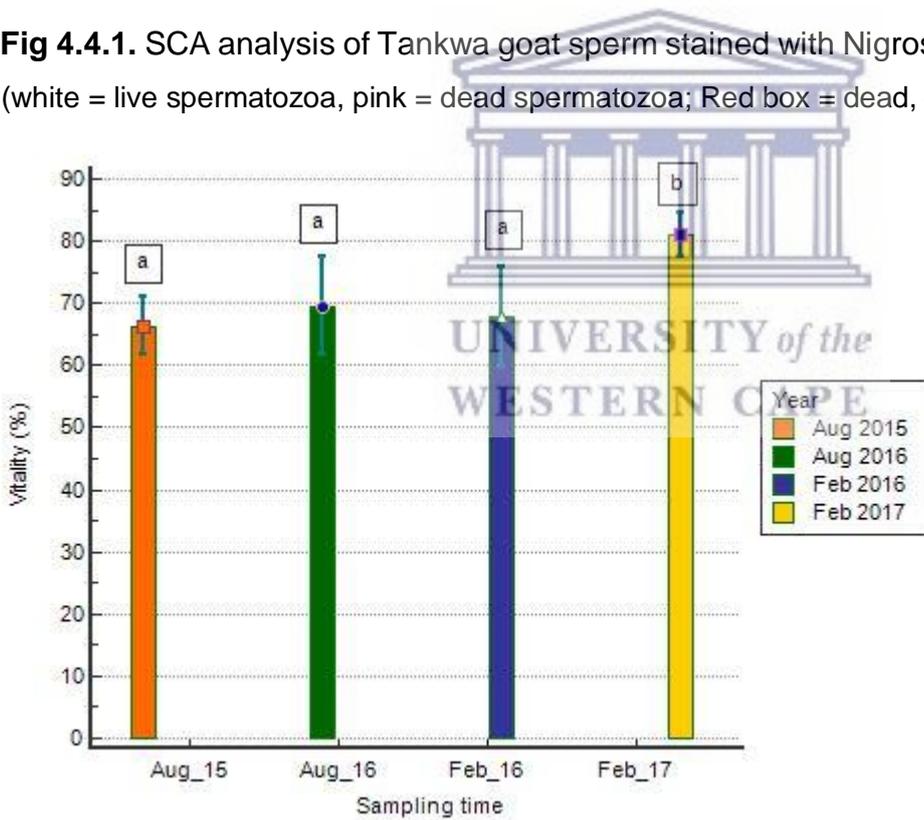


Fig 4.4.2. Multiple comparison graph of Tankwa goat sperm vitality (%) over a two year period.

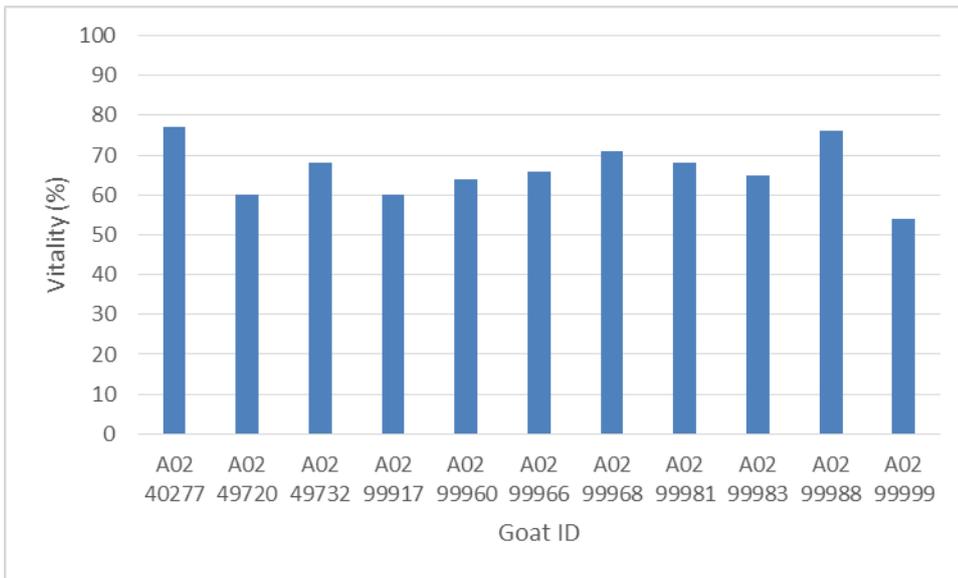


Fig 4.4.3 Sperm vitality (%) of individual Tankwa goats in August 2015 (n = 11).

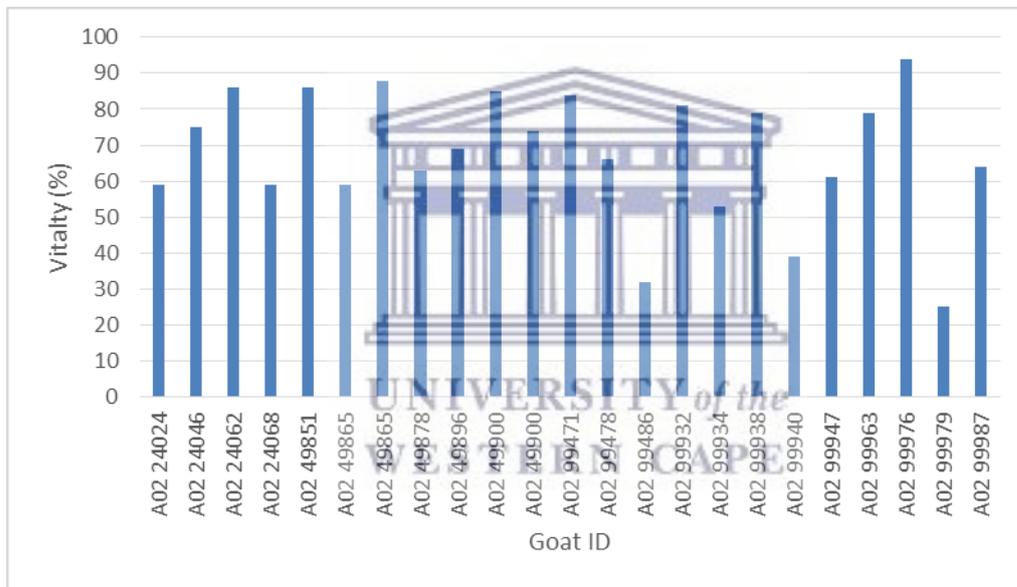


Fig 4.4.4. Sperm vitality (%) of individual Tankwa goats in February 2016 (n = 23).

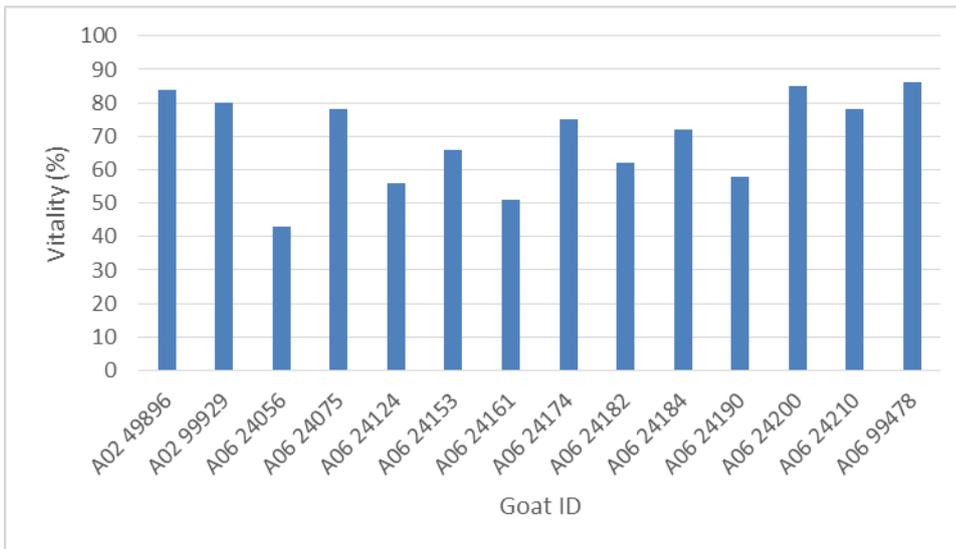


Fig 4.4.5. Sperm vitality (%) of individual Tankwa goats in August 2016 (n = 14).

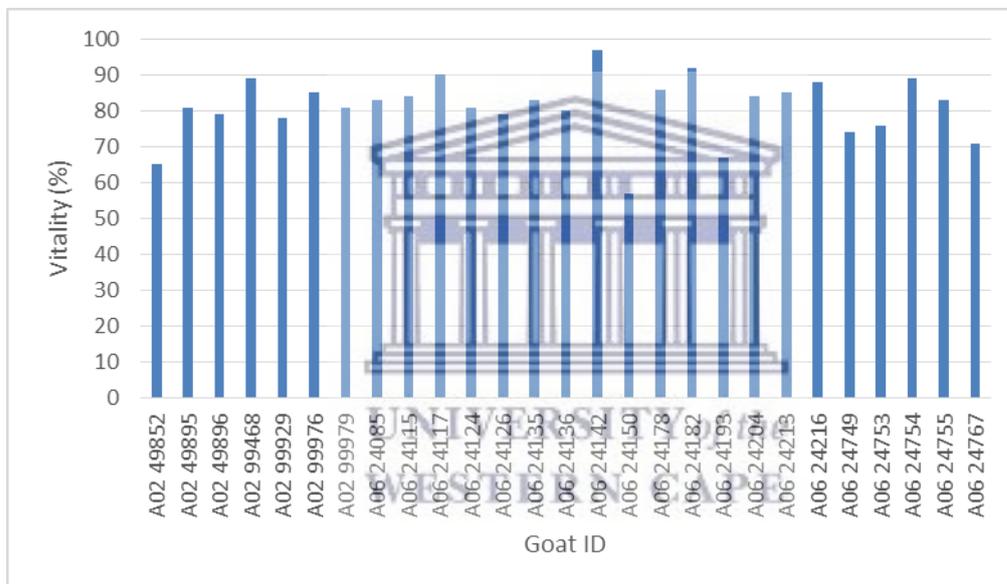


Fig 4.4.6. Sperm vitality (%) of individual Tankwa goats in February 2017 (n = 27).

The summer season had a significantly higher percentage sperm vitality of 74.9% compared to 68.1% in the winter season (Fig 4.4.7).

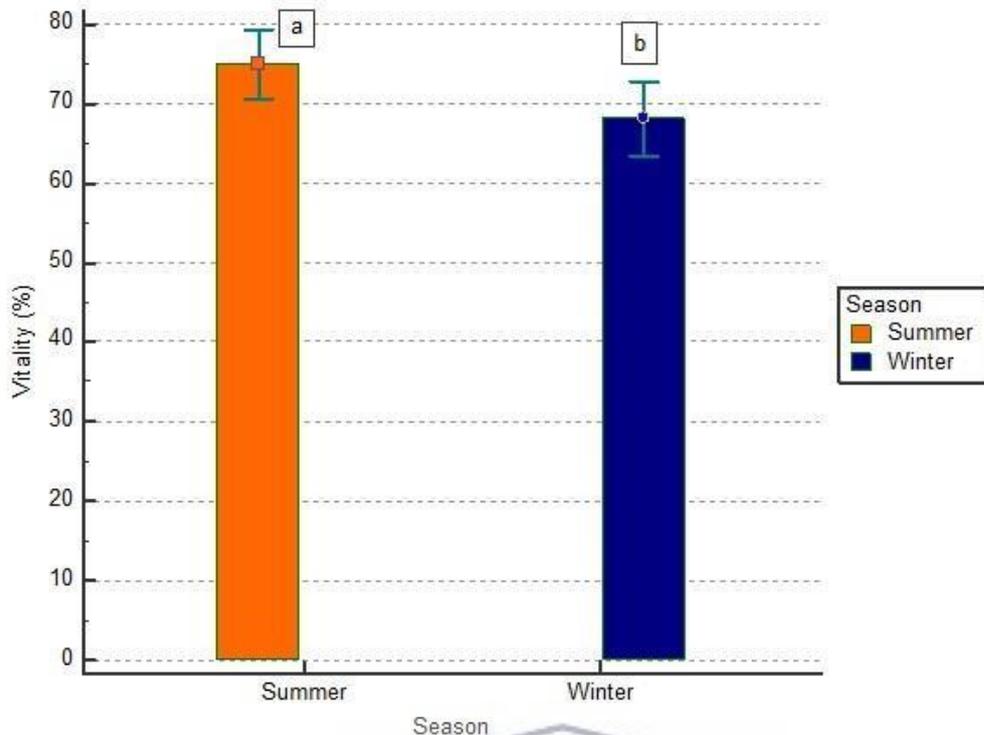


Fig4.4.7. Multiple comparison of sperm vitality (%) of Tankwa goats between summer (n = 50) and winter (n = 25) periods.

4.5 Sperm hyperactivation

4.5.1 Determination of cut-offs for Tankwa goat sperm hyperactivation

Kinematics of individual spermatozoa from each February 2016 semen samples after exposure to PBS and procaine hydrochloride, respectively, were extracted to an Excel file and imported to MedCalc software (version 12.3.0; Medcalc Software, Mariakerke, Belgium). ROC curve analysis graphs were constructed (Fig 4.5.1.1-4.5.1.8) and used to determine cut-off values for Tankwa goat sperm hyperactivation (Table 4.5.1). The VAP parameter was not used as a cut-off point as it had sensitivity and specificity values below 80% and was thus not considered as a restriction.

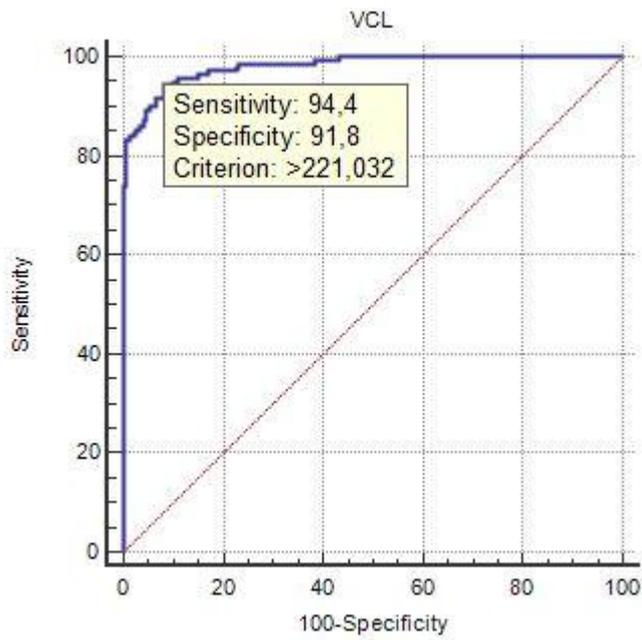


Fig4.5.1.1. ROC curve analysis for curvilinear velocity (VCL) ($\mu\text{m/s}$)

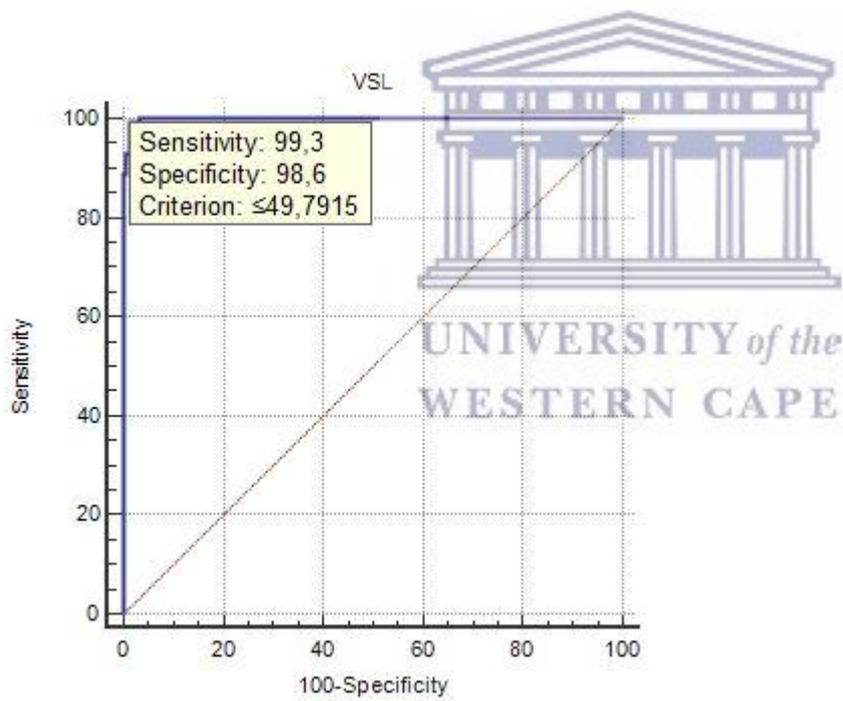


Fig 4.5.1.2. ROC curve analysis for straight-line velocity (VSL) ($\mu\text{m/s}$)

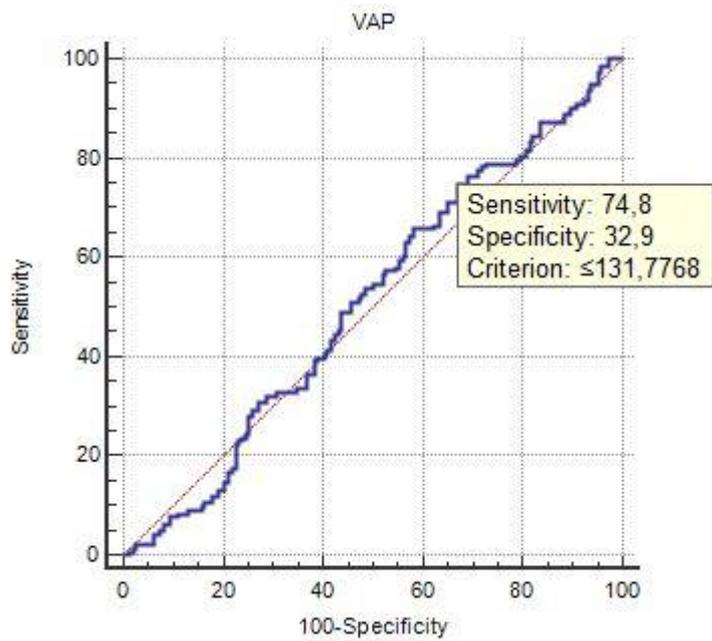


Fig4.5.1.3. ROC curve analysis for average path velocity (VAP) ($\mu\text{m/s}$)

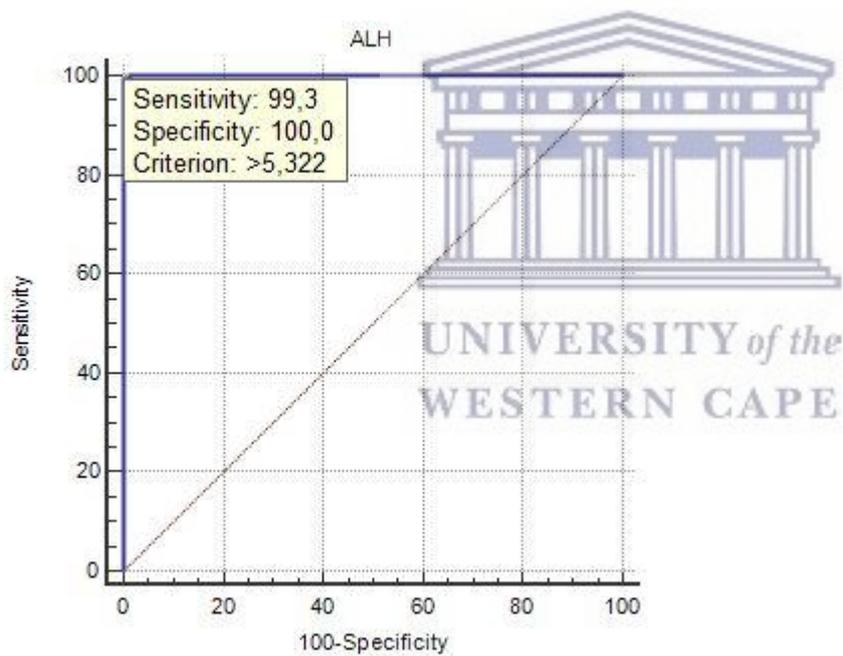


Fig4.5.1.4. ROC curve analysis for amplitude of lateral head displacement (ALH) (μm)

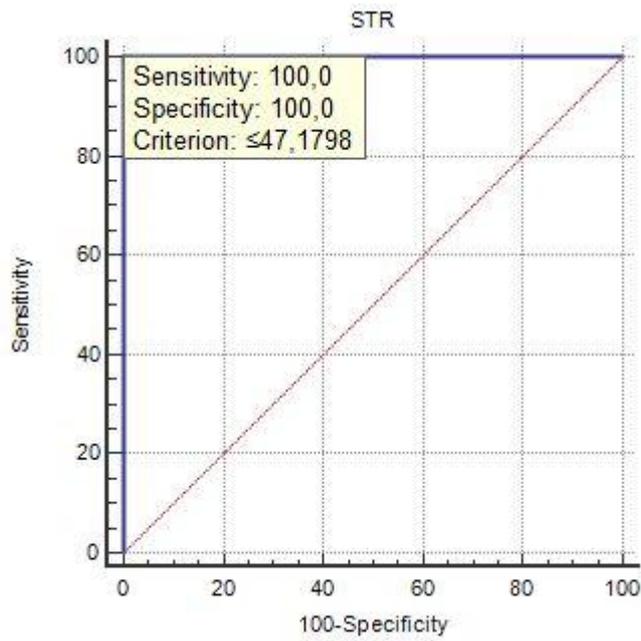


Fig4.5.1.5. ROC curve analysis for straightness (STR) (%)

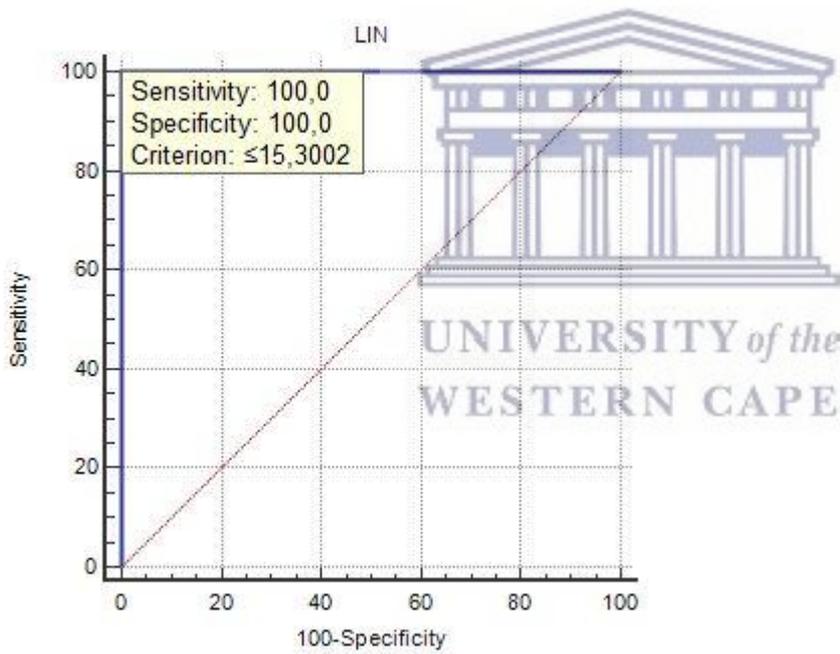


Fig4.5.1.6. ROC curve analysis for linearity (LIN) (%)

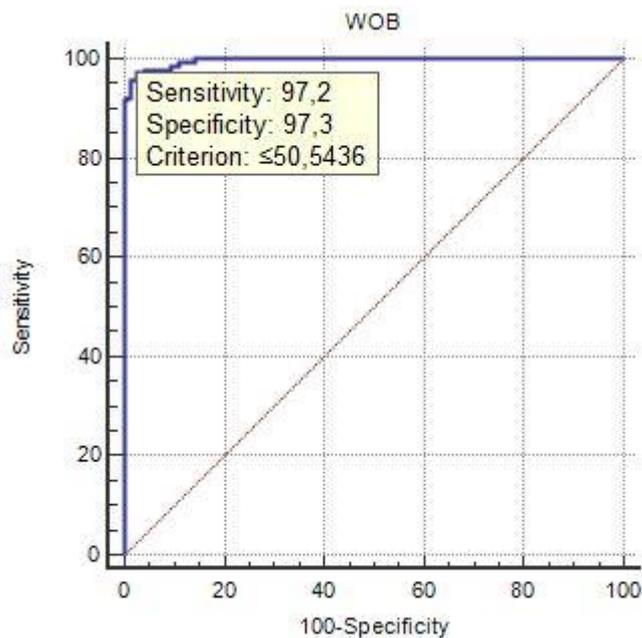


Fig4.5.1.7. ROC curve analysis for wobble (WOB) (%)

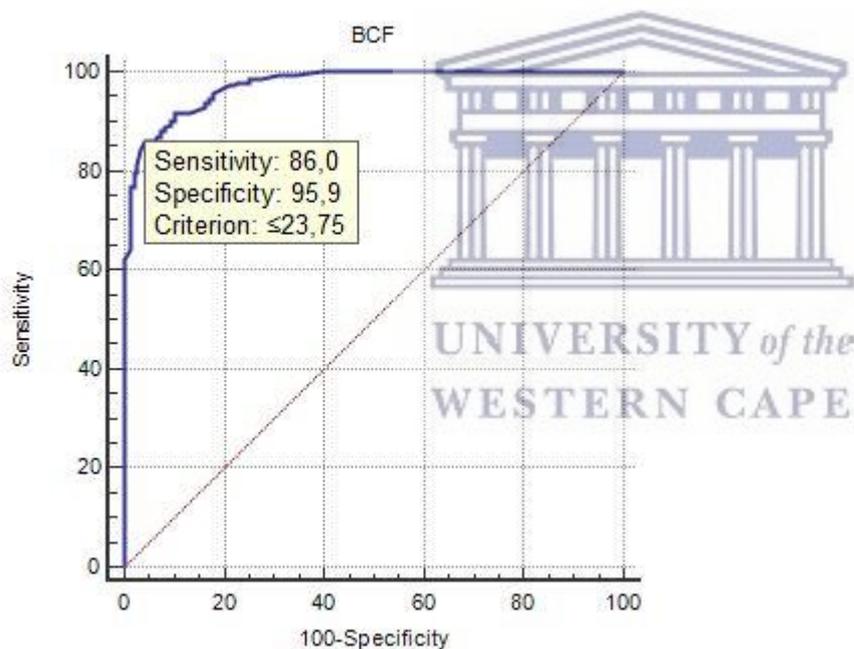


Fig4.5.1.8. ROC curve analysis for beat cross frequency (BCF) (Hz)

After creating a sort function for Tankwa goat sperm hyperactivation on the SCA® Motility module using the cut-off points in Table 4.5.1. The number of hyperactivated spermatozoa was automatically calculated as a percentage of motile spermatozoa.

Table 4.5.1.1. SCA® motility analysis restrictions for Tankwa goat hyperactivated spermatozoa.

Parameter	Restriction Setting
VCL (µm/s)	221>VCL<500
VSL (µm/s)	<49.8
STR (%)	<47.2
LIN (%)	<15.3
ALH (µm)	5.3>ALH<15.3
BCF (Hz)	<23.8
WOB (%)	<50.5

* VCL = curvilinear velocity, VSL= straight-line velocity, VAP = average path velocity, STR = straightness , LIN = linearity,

ALH = amplitude of lateral head displacement, BCF = beat cross frequency, WOB = wobble

4.5.2 Comparison of media for sperm hyperactivation in Tankwa goats

Lignocaine had significant lower percentage progressive motility than all other media. Procaine had the highest average of rapid swimming sperm while BO wash had the lowest average. Additionally, procaine had a significant higher average than all three media for medium swimming sperm. Procaine had the lowest average of slow swimming sperm while BO had the highest average, the procaine medium further recorded the highest average for VCL than all three media. Procaine and lignocaine were significantly lower than PBS and BO for VSL. PBS and procaine were significantly higher than BO and lignocaine for VAP. PBS and BO wash were significantly higher than procaine and lignocaine for STR, LIN and WOB. Furthermore PBS and BO were significantly higher than procaine and lignocaine for BCF. The highest average recorded for percentage sperm hyperactivation was in procaine medium (Table 4.5.2.1 and Fig 4.5.2.1.)

Table 4.5.2.1. Sperm kinematic parameter measurements and percentage hyperactivation (mean \pm SD) with the use of phosphate buffered saline (PBS), BO wash, 5 mM procaine hydrochloride and 4% lignocaine in February 2016.

Parameter	PBS n=29	BO n=29	Procaine n=28	Lignocaine n=27
Total motility (%)	86,6 \pm 13,1	84,3 \pm 13,5	83,9 \pm 13,5	83,4 \pm 17,7
Progressive motility (%)	48,9 \pm 22,1 ^a	40,0 \pm 15,2 ^a	47,9 \pm 20,4 ^a	25,4 \pm 14,1 ^b
Rapid (%)	13,0 \pm 11,6 ^a	6,2 \pm 6,1 ^b	44,4 \pm 20,8 ^c	17,7 \pm 13,4 ^d
Medium (%)	51,6 \pm 20,2 ^a	48,2 \pm 19,8 ^a	24,5 \pm 11,1 ^b	46,0 \pm 16,6 ^a
Slow (%)	22,0 \pm 14,7 ^a	29,8 \pm 10,8 ^b	15,0 \pm 7,6 ^c	19,8 \pm 13,1 ^d
VCL (μ m/s)	106,4 \pm 32,6 ^a	88,8 \pm 22,4 ^b	149,0 \pm 41,0 ^c	107,0 \pm 31,1 ^d
VSL (μ m/s)	55,5 \pm 21,0 ^a	45,7 \pm 14,6 ^b	33,7 \pm 9,3 ^c	29,8 \pm 9,1 ^c
VAP (μ m/s)	67,4 \pm 23,1 ^a	56,1 \pm 16,4 ^b	67,8 \pm 18,0 ^a	53,3 \pm 15,6 ^b
STR (%)	71,1 \pm 12,2 ^a	71,1 \pm 9,6 ^a	46,2 \pm 6,9 ^b	50,4 \pm 8,2 ^b
LIN (%)	46,6 \pm 12,5 ^a	45,8 \pm 11,4 ^a	26,5 \pm 6,5 ^b	27,7 \pm 6,7 ^b
ALH (μ m)	2,2 \pm 0,6 ^a	1,9 \pm 0,4 ^a	4,0 \pm 1,0 ^b	3,0 \pm 0,8 ^c
BCF (Hz)	21,9 \pm 6,0 ^a	21,5 \pm 5,2 ^a	12,3 \pm 2,7 ^b	15,2 \pm 3,8 ^c
WOB (%)	58,9 \pm 10,2 ^a	58,5 \pm 10,1 ^a	47,0 \pm 5,9 ^b	48,1 \pm 5,9 ^b
HA (%)	0,1 \pm 0,2 ^a	0,04 \pm 0,2 ^b	25,3 \pm 13,6 ^b	3,4 \pm 3,0 ^a

Superscript a-d in the same row indicates significant difference where $p < 0.05$

VCL = curvilinear velocity, VSL= straight-line velocity, VAP = average path velocity, STR = straightness, LIN = linearity,

ALH = amplitude of lateral head displacement, BCF = beat cross frequency, WOB= wobble, HA = Hyperactivation

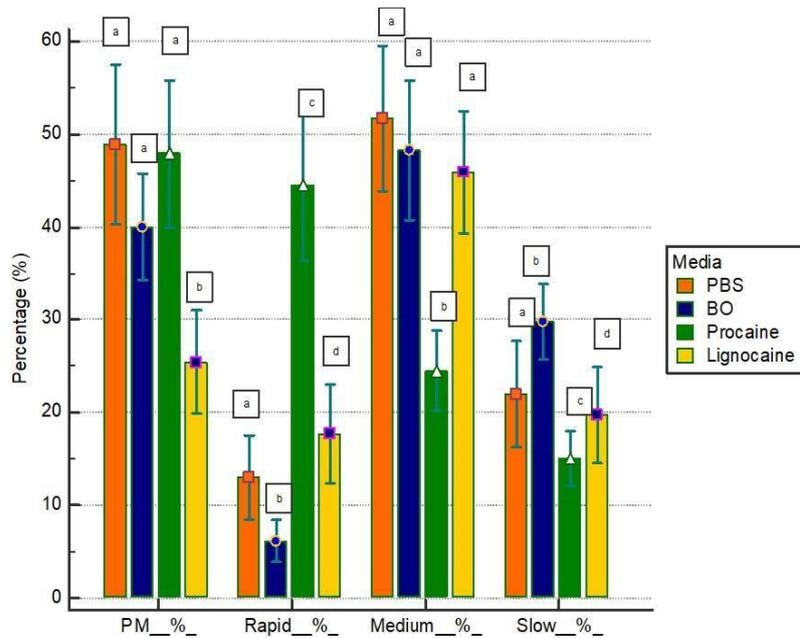


Fig 4.5.2.1. Multiple comparison graph of sperm swimming percentages (%) with the use of phosphate buffered saline (PBS), BO wash, 5mM procaine hydrochloride and 4% lignocaine in February 2016. *PM (%) = progressive motility.

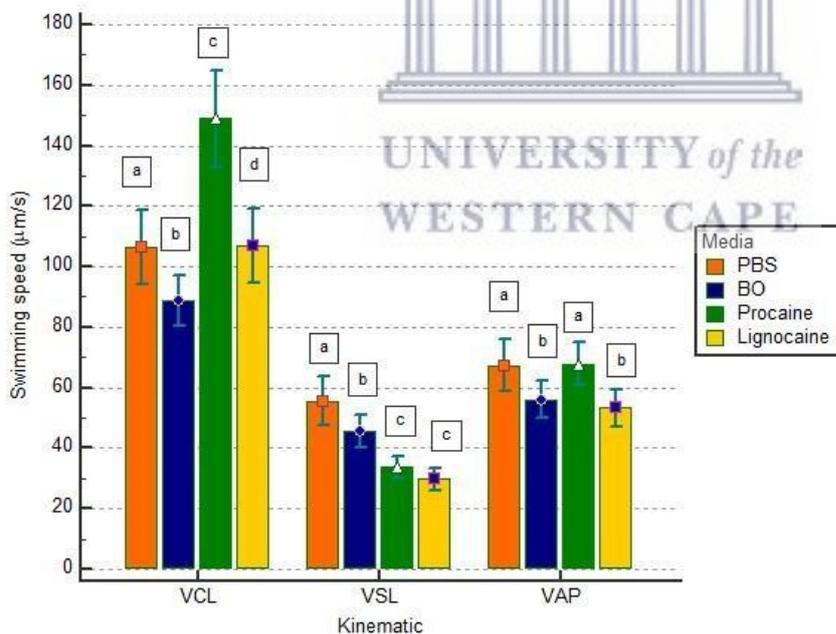


Figure 4.5.2.2. Multiple comparison graph of sperm swimming speed parameters with the use of phosphate buffered saline (PBS), BO wash, 5mM procaine hydrochloride and 4% lignocaine in February 2016. *VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity

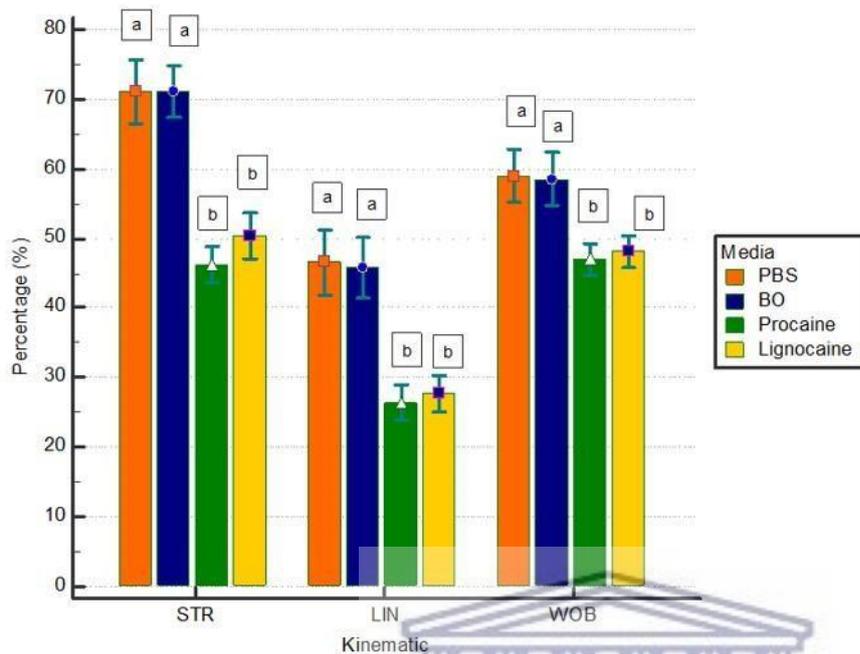


Fig 4.5.2.3. Multiple comparison graph of straightness (STR), linearity (LIN) and wobble (WOB) with the use of phosphate buffered saline (PBS), BO wash, 5mM procaine hydrochloride and 4% lignocaine in February 2016.

Procaine was significantly higher for rapid VCL than all the media (Fig 4.5.2.4). BO and lignocaine were significantly higher than PBS and procaine for medium VCL while BO was significantly lower than lignocaine for slow VCL. The parameters; rapid and medium VSL, rapid and medium VAP, rapid, medium and slow STR, rapid and medium LIN, rapid, medium and slow BCF, and rapid and medium WOB were significantly higher in PBS and BO. Furthermore lignocaine had a significant lower average than all media for slow VSL, slow LIN and a higher average than all media for slow ALH (Fig 4.5.2.5-4.5.2.10).

Table 4.5.2.2. Induced hyperactivation (%) and kinematic parameters (rapid, medium and slow) (mean \pm SD) with the use of phosphate buffered saline (PBS), BO wash, 5mM procaine hydrochloride and 4% lignocaine in February 2016.

Media	PBS	BO	Procaine	Lignocaine
R-VCL ($\mu\text{m/s}$)	228,1 \pm 41,0 ^a	232,9 \pm 43,4 ^a	259,5 \pm 22,2 ^b	239,6 \pm 14,3 ^a
M-VCL ($\mu\text{m/s}$)	134,9 \pm 15,0 ^{ab}	129,5 \pm 8,1 ^a	140,2 \pm 8,2 ^b	132,4 \pm 9,2 ^a
S-VCL ($\mu\text{m/s}$)	63,3 \pm 6,7 ^{ab}	60,5 \pm 5,1 ^a	62,9 \pm 3,7 ^{ab}	64,8 \pm 5,0 ^b
R-VSL ($\mu\text{m/s}$)	118,3 \pm 64,2 ^a	122,6 \pm 41,6 ^a	45,8 \pm 14,5 ^b	70,6 \pm 42,5 ^c
M-VSL ($\mu\text{m/s}$)	72,7 \pm 17,3 ^a	70,7 \pm 11,5 ^a	42,2 \pm 10,0 ^b	38,6 \pm 8,5 ^b
S-VSL ($\mu\text{m/s}$)	32,7 \pm 11,5 ^a	29,9 \pm 7,5 ^a	28,6 \pm 7,5 ^a	23,6 \pm 7,3 ^b
R-VAP ($\mu\text{m/s}$)	139,4 \pm 59,7 ^a	144,8 \pm 37,4 ^a	109,5 \pm 15,3 ^b	121,0 \pm 30,2 ^{ab}
M-VAP ($\mu\text{m/s}$)	87,3 \pm 16,0 ^a	85,2 \pm 11,0 ^a	73,0 \pm 7,8 ^b	67,5 \pm 6,9 ^b
S-VAP ($\mu\text{m/s}$)	40,3 \pm 10,0	37,4 \pm 6,9	39,0 \pm 6,6	36,0 \pm 6,8
R-STR (%)	80,8 \pm 10,7 ^a	81,6 \pm 8,9 ^a	38,2 \pm 7,9 ^b	52,4 \pm 14,7 ^c
M-STR (%)	80,3 \pm 8,8 ^a	81,3 \pm 6,0 ^a	52,4 \pm 7,6 ^b	54,1 \pm 8,9 ^b
S-STR (%)	69,5 \pm 14,4 ^a	72,0 \pm 8,8 ^a	63,5 \pm 10,1 ^b	58,5 \pm 10,6 ^b
R-LIN (%)	50,6 \pm 17,1 ^a	52,5 \pm 14,9 ^a	17,9 \pm 5,0 ^b	29,4 \pm 17,1 ^c
M-LIN (%)	54,2 \pm 12,8 ^a	54,8 \pm 7,8 ^a	31,2 \pm 7,6 ^b	29,5 \pm 7,0 ^b
S-LIN (%)	48,6 \pm 15,0 ^a	47,8 \pm 11,2 ^a	46,0 \pm 12,5 ^a	37,5 \pm 11,0 ^b
R-ALH (μm)	4,1 \pm 1,1 ^a	4,0 \pm 1,1 ^a	7,3 \pm 0,6 ^b	5,8 \pm 1,2 ^c
M-ALH (μm)	2,8 \pm 0,6 ^a	2,6 \pm 0,3 ^a	3,6 \pm 0,7 ^b	3,8 \pm 0,5 ^b
S-ALH (μm)	1,4 \pm 0,3 ^a	1,5 \pm 0,3 ^a	1,4 \pm 0,3 ^a	1,8 \pm 0,4 ^b
R-BCF (Hz)	23,2 \pm 8,0 ^a	23,5 \pm 8,6 ^a	14,6 \pm 3,3 ^b	18,0 \pm 4,6 ^b
M-BCF (Hz)	27,7 \pm 4,9 ^a	28,7 \pm 3,7 ^a	14,9 \pm 3,7 ^b	18,9 \pm 4,1 ^c
S-BCF (Hz)	19,1 \pm 6,0 ^a	20,8 \pm 3,8 ^a	12,8 \pm 3,8 ^b	15,3 \pm 3,5 ^c
R-WOB (%)	59,8 \pm 14,2 ^a	62,5 \pm 12,5 ^a	42,5 \pm 4,3 ^b	50,6 \pm 11,7 ^c
M-WOB (%)	65,2 \pm 11,1 ^a	66,0 \pm 6,8 ^a	52,9 \pm 5,4 ^b	52,1 \pm 4,7 ^b
S-WOB (%)	61,6 \pm 11,7	60,7 \pm 9,4	62,0 \pm 10,4	55,9 \pm 9,5

Superscript a-c in the same row indicates significant difference where $p < 0.05$

VCL = curvilinear velocity, VSL= straight-line velocity , VAP = average path velocity, STR = straightness , LIN = linearity, BCF= beat cross frequency, ALH = amplitude of lateral head displacement , WOB= wobble, , HA = Hyperactivation (%) # R-Rapid, M-Medium and S-Slow.

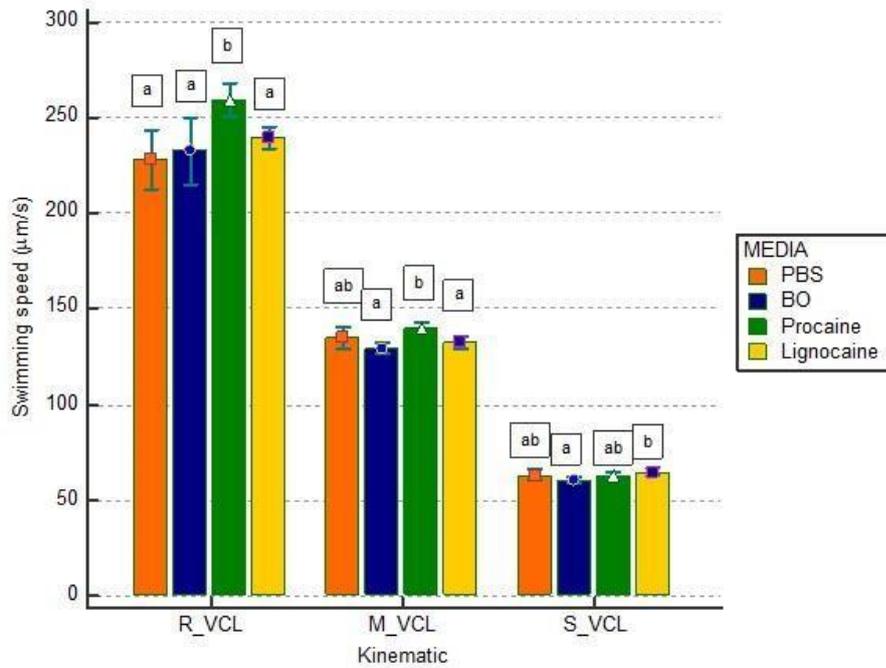


Fig 4.5.2.4. Multiple comparison of rapid, medium and slow curvilinear velocity (VCL) with the use of phosphate buffered saline (PBS), BO wash, 5mM procaine hydrochloride and 4% lignocaine in February 2016.

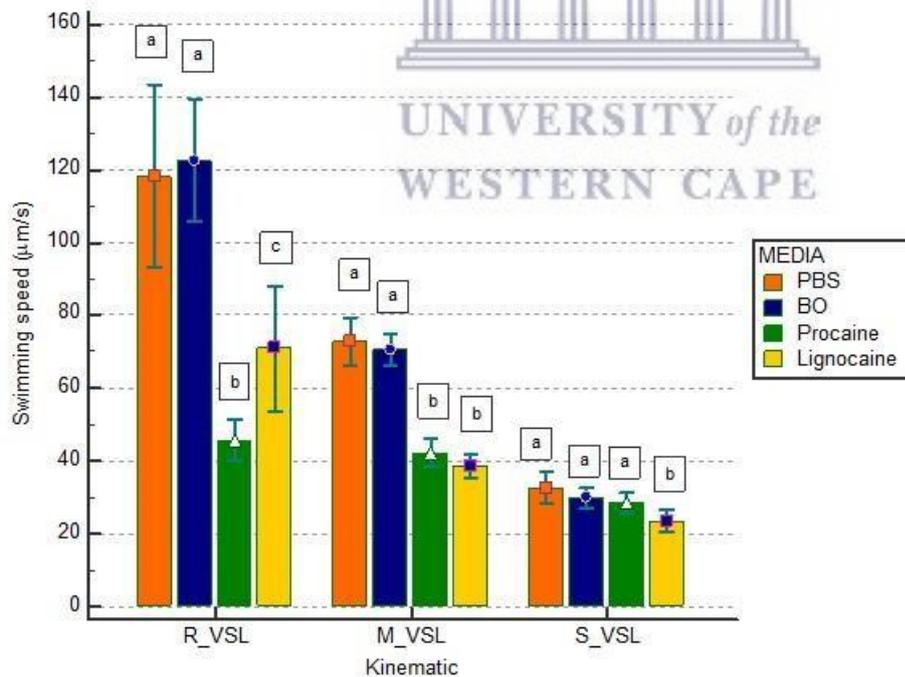


Fig 4.5.2.5. Multiple comparison of rapid, medium and slow straightline velocity (VSL) with the use of phosphate buffered saline (PBS), BO wash, 5mM procaine hydrochloride and 4% lignocaine in February 2016.

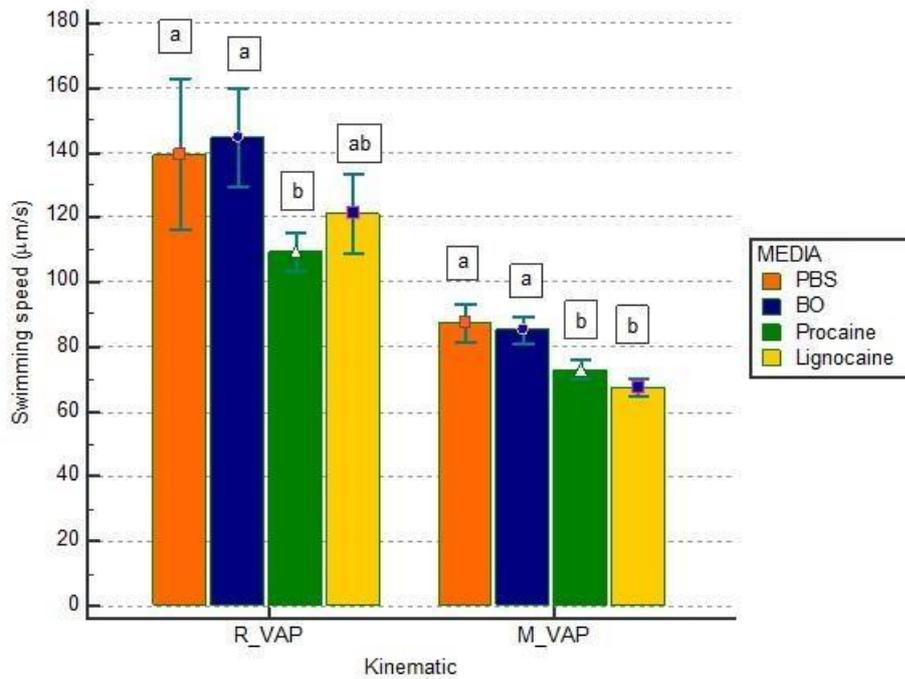


Fig 4.5.2.6. Multiple comparison of rapid and medium average path velocity (VAP) with the use of phosphate buffered saline (PBS), BO wash, 5mM procaine hydrochloride and 4% lignocaine in February 2016.

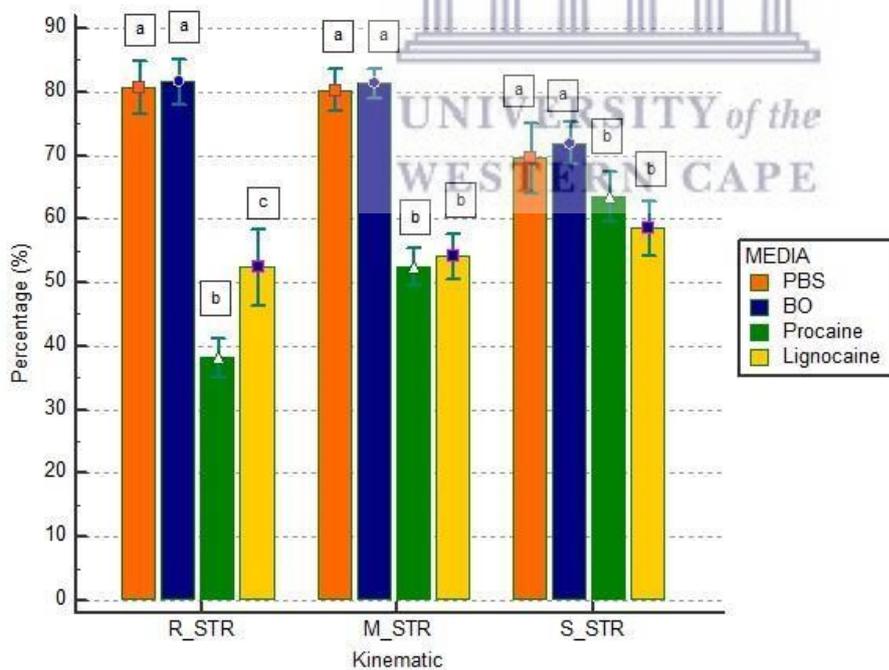


Fig 4.5.2.7. Multiple comparison of rapid, medium and slow straightness index (STR) with the use of phosphate buffered saline (PBS), BO wash, 5mM procaine hydrochloride and 4% lignocaine in February 2016.

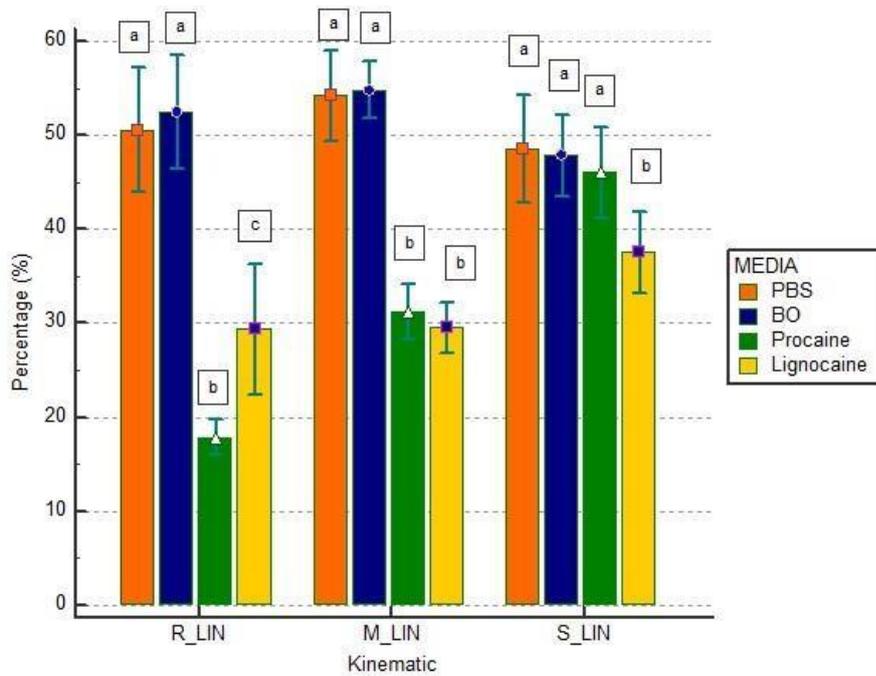


Fig 4.5.2.8. Multiple comparison of rapid, medium and slow linearity (LIN) with the use of phosphate buffered saline (PBS), BO wash, 5mM procaine hydrochloride and 4% lignocaine in February 2016.

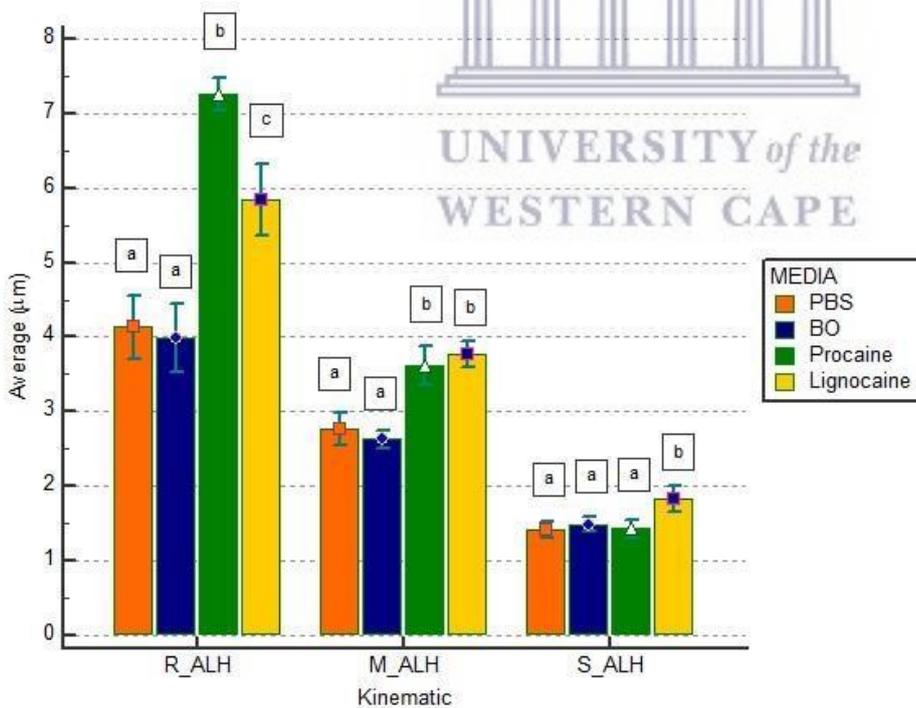
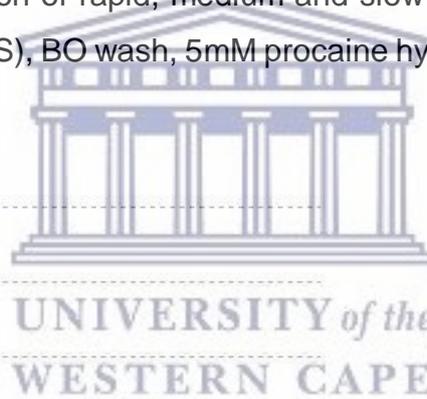


Fig 4.5.2.9. Multiple comparison of rapid, medium and slow amplitude of lateral head displacement (ALH) with the use of phosphate buffered saline (PBS), BO wash, 5mM procaine hydrochloride and 4% lignocaine in February 2016.

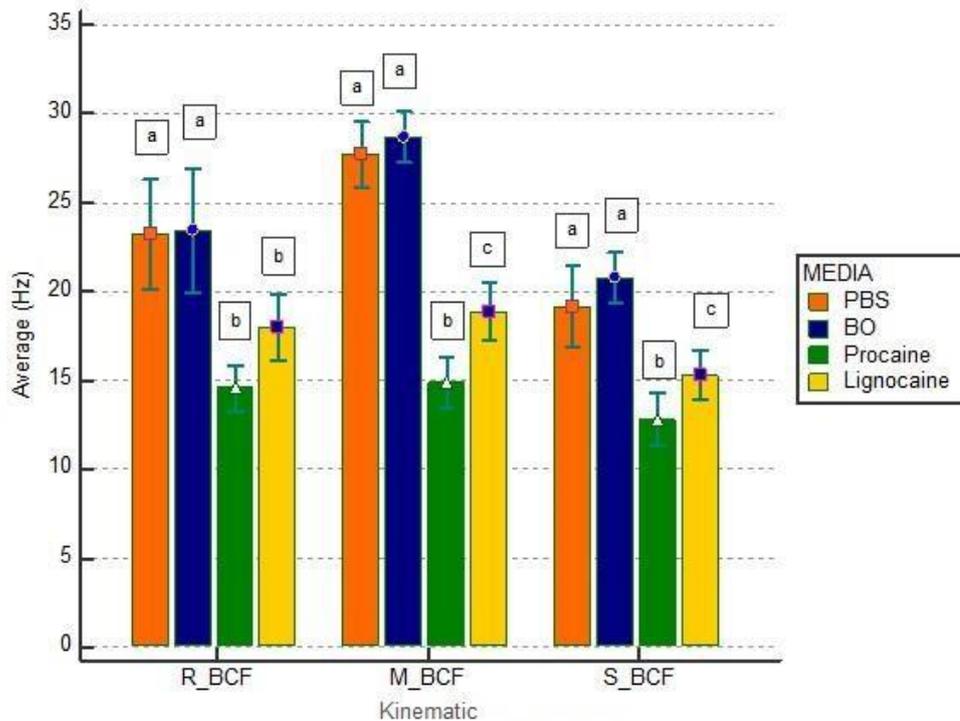


Fig 4.5.2.10. Multiple comparison of rapid, medium and slow beat cross frequency (BCF) with the use of phosphate buffered saline (PBS), BO wash, 5mM procaine hydrochloride and 4% lignocaine in February 2016.

Compared to the control (PBS), it was observed that procaine hydrochloride caused a significant amount of hyperactivation. An example of the differences in swimming tracks for diluted semen and spermatozoa displaying typical hyperactivation patterns in different media are displayed in Fig 4.5.2.11 to Fig 4.5.2.14.

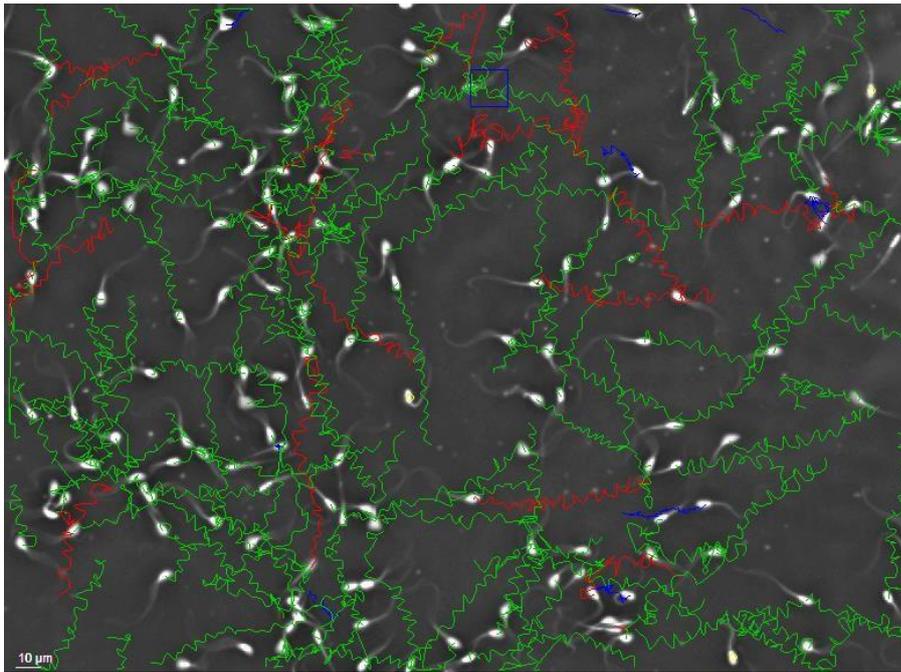


Fig 4.5.2.11. An analysed Tankwa goat motility field after exposure to PBS using 100x magnification. (Red tracks = Rapid progressive sperm, Green = Medium progressive sperm, Blue = Non-progressive sperm and Yellow = immotile sperm. Blue box = hyperactivated spermatozoa)

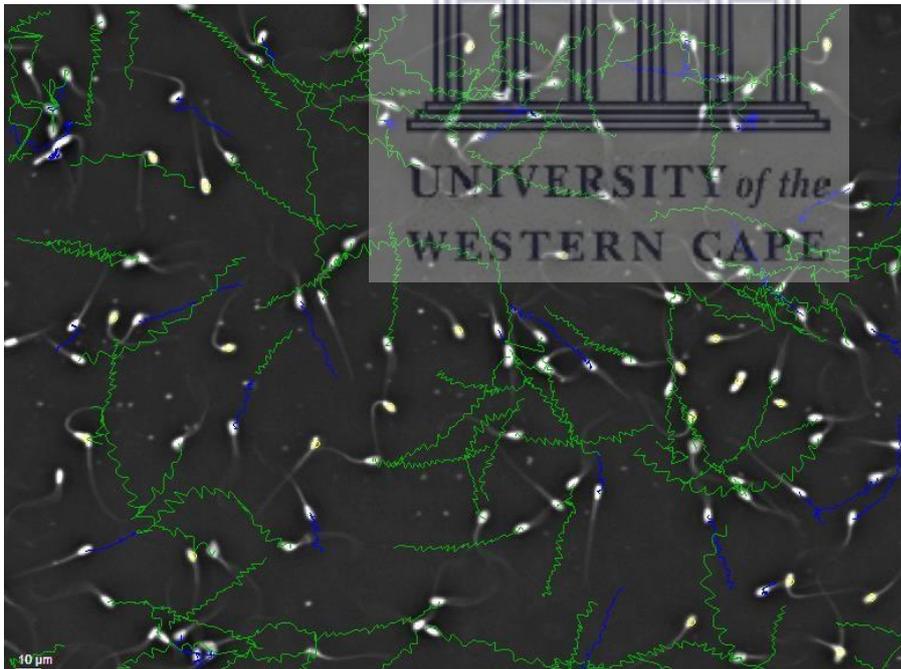


Fig 4.5.2.12. An analysed Tankwa goat motility field after exposure to BO wash using 100x magnification. (Red tracks = Rapid progressive sperm, Green = Medium progressive sperm, Blue = Non-progressive sperm and Yellow = immotile sperm)

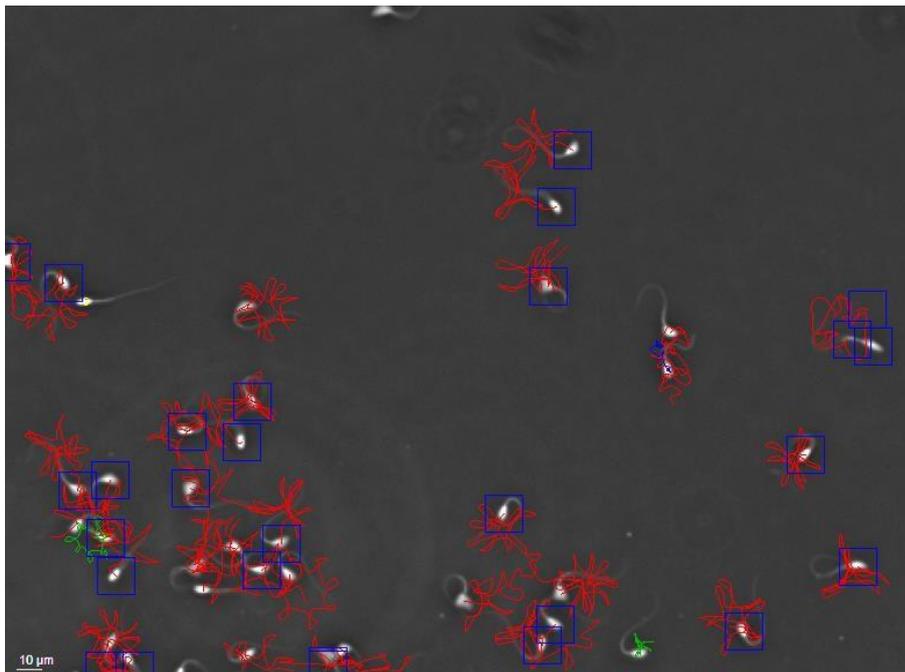


Fig 4.5.2.13. An analysed Tankwa goat motility field after exposure to 5 mM procaine hydrochloride using 100x magnification. (Red tracks = Rapid progressive sperm, Green = Medium progressive sperm, Blue = Non-progressive sperm and Yellow = immotile sperm. Blue box = hyperactivated spermatozoa)

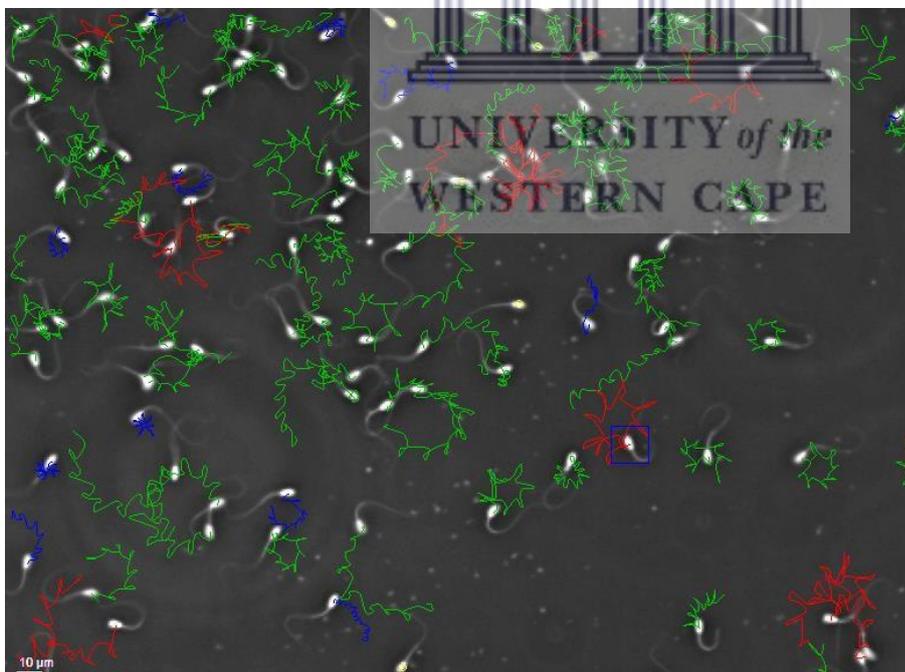


Fig 4.5.2.14. An analysed Tankwa goat motility field after exposure to 4% lignocaine using 100x magnification. (Red tracks = Rapid swimming sperm, Green = Medium swimming sperm, Blue = Slow swimming sperm and Yellow = immotile sperm. Blue box = hyperactivated spermatozoa)

4.5.3 Comparison of sperm hyperactivation over three seasons

Percentage sperm hyperactivation for individual goats were recorded over a year period (February 2016 – February 2017) and are illustrated in Fig 4.5.3.1-4.5. 3.3. February 2016 had a mean of 26.8% sperm that were hyperactivated and ranged from 3.1-54.5%. August 2016 had a mean of 16.1% and ranged from 1.6-48.2%, February 2017 had a mean of 25.4% and ranged from 0-54.4%. The number of goats showing hyperactivated sperm was 16, 9 and 17 in February 2016, August 2016 and February 2017 respectively.



Fig 4.5.3.1. Induced hyperactivation (%) of individual Tankwa goats with the use of phosphate buffered saline (PBS) and 5 mM procaine hydrochloride in February 2016 (n = 28). (note: not all individual goats are labelled)

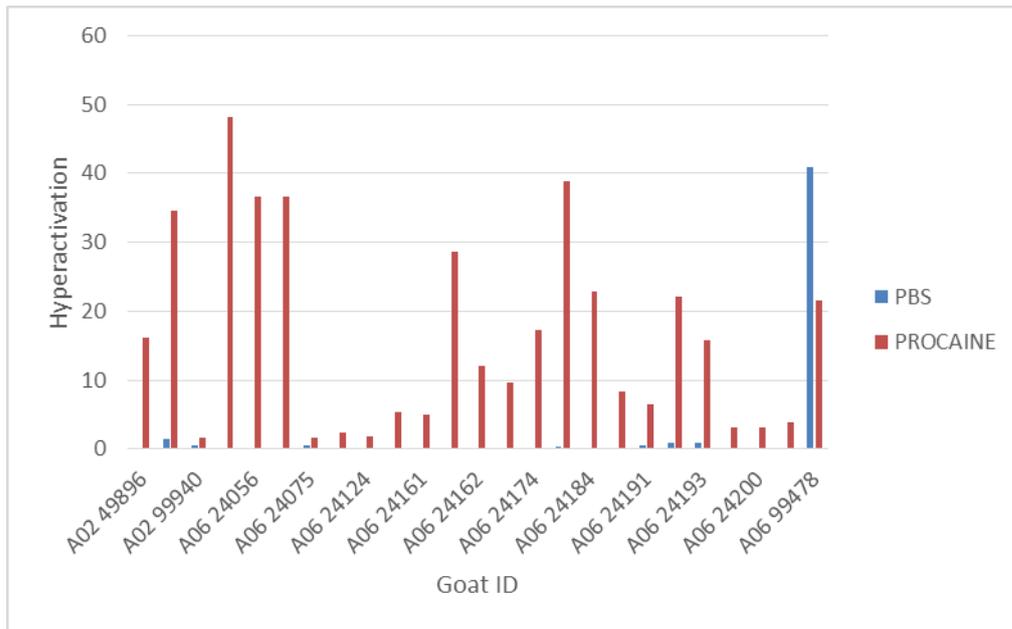


Fig 4.5.3.2. Induced hyperactivation (%) of individual Tankwa goats with the use of phosphate buffered saline (PBS) and 5 mM procaine hydrochloride in August 2016 (n = 23). (note: not all individual goats are labelled)

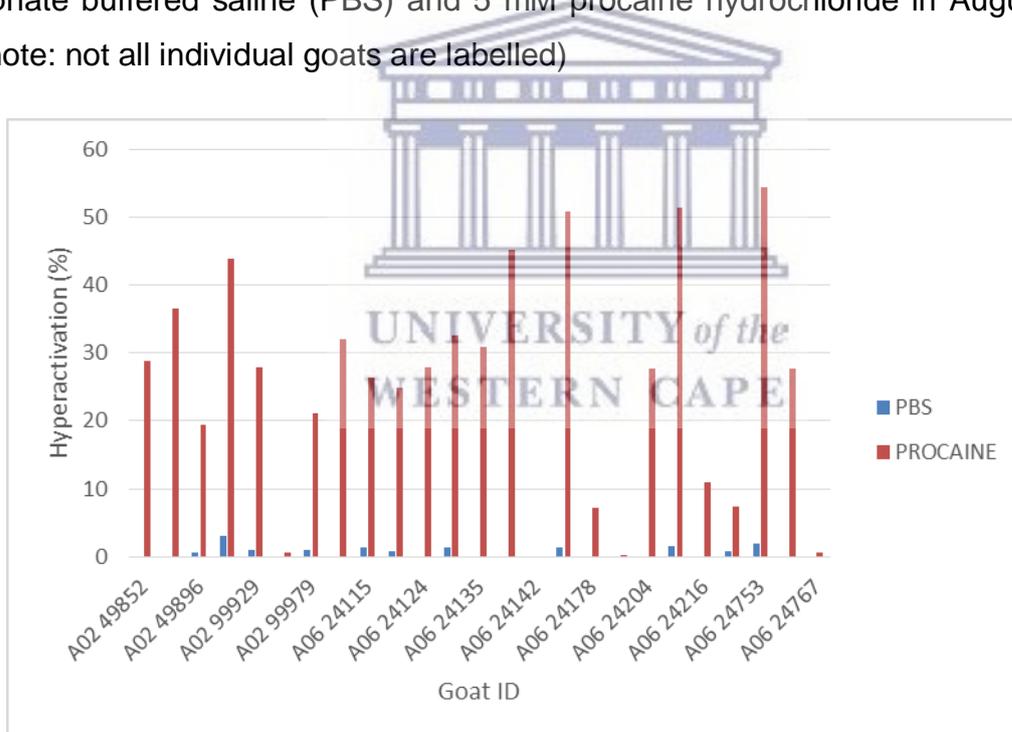


Fig 4.5.3.3. Induced hyperactivation (%) of individual Tankwa goats with the use of phosphate buffered saline (PBS) and 5 mM procaine hydrochloride in February 2017 (n = 23). (note: not all individual goats are labelled)

With data from all three mentioned seasons combined, a significant difference was observed in all the kinematic parameters except for VAP between PBS and procaine (Table 4.5.3.1). A higher average VCL for procaine was observed (Fig 4.5.3.6) and a lower average of VSL,

STR, LIN, BCF and WOB as compared to PBS (Fig 4.5.3.6 – 4.5.3.9). Procaine also had a significantly higher averages for ALH (Fig 4.5.3.8) and hyperactivation. Procaine induced an average of 22.9% hyperactivation as compared to only 0.8% in PBS (Table 4.5.3.1).

Table 4.5.3.1. Induced hyperactivation (%) and kinematic parameters (mean \pm SD) with the use of phosphate buffered saline and 5mM procaine hydrochloride over a year period.

Parameter	PBS n=75	Procaine n=76
VCL ($\mu\text{m/s}$)	103,7 \pm 36,7 ^a	149,2 \pm 53,5 ^b
VSL ($\mu\text{m/s}$)	52,3 \pm 22,0 ^a	36,4 \pm 11,7 ^b
VAP ($\mu\text{m/s}$)	63,1 \pm 24,3	67,8 \pm 21,0
STR (%)	67,7 \pm 11,6 ^a	48,4 \pm 7,9 ^b
LIN (%)	42,4 \pm 11,6 ^a	27,3 \pm 6,8 ^b
ALH (μm)	2,2 \pm 0,7 ^a	3,9 \pm 1,4 ^b
BCF (Hz)	19,2 \pm 6,3 ^a	12,9 \pm 3,9 ^b
WOB (%)	54,6 \pm 9,6 ^a	46,4 \pm 5,9 ^b
HA (%)	0,8 \pm 4,6 ^a	22,9 \pm 15,5 ^b

Superscript a-d in the same row indicates significant difference where $p < 0.05$

VCL = curvilinear velocity, VSL= straight-line velocity, VAP = average path velocity, STR = straightness, LIN = linearity, ALH = amplitude of lateral head displacement BCF= beat cross frequency, WOB= wobble, HA = Hyperactivation (%)

Compared to the control (PBS) it was observed that procaine hydrochloride caused a significantly higher percentage of hyperactivation (Fig 4.5.3.4). An example of the differences in swimming tracks for diluted semen and spermatozoa displaying typical hyperactivation patterns are displayed in Fig 4.5.3.5.

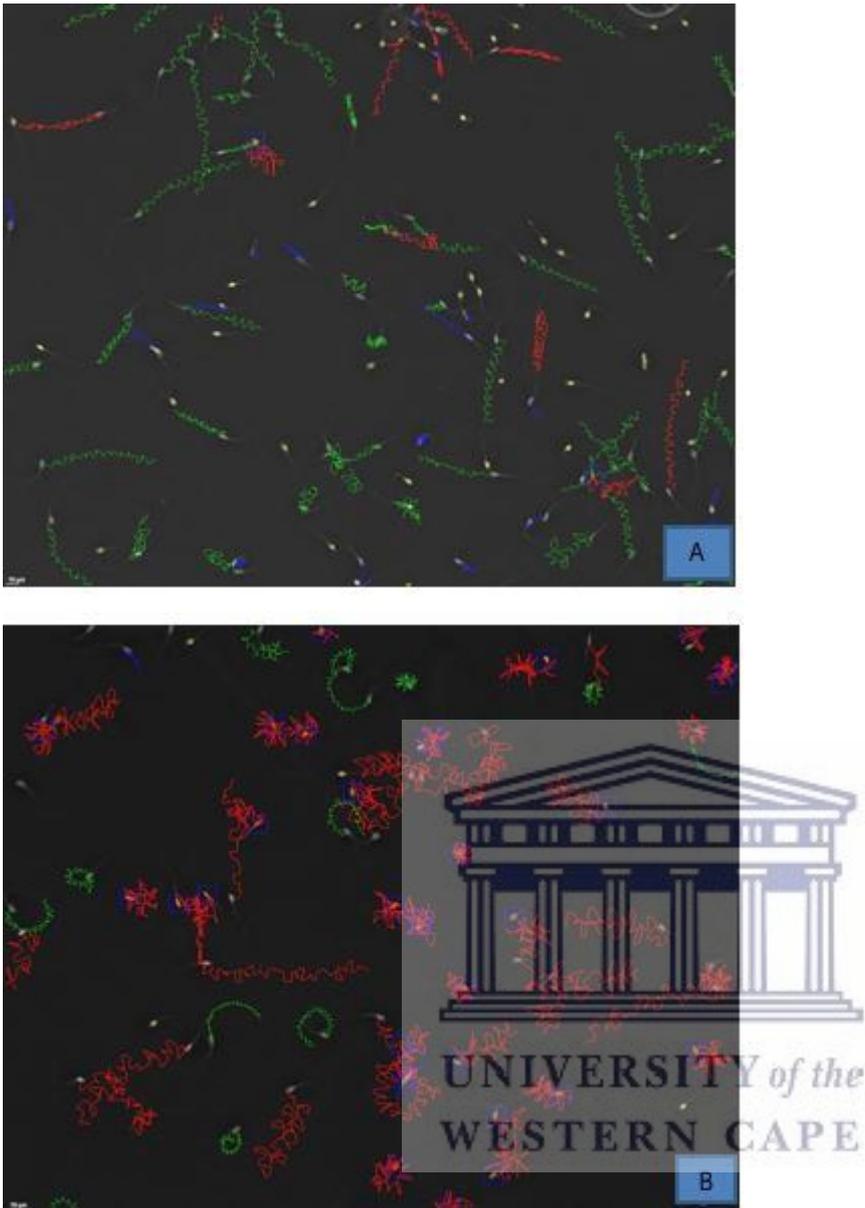


Figure 4.5.3.4. An analysed motility field (with tracks) after PBS incubation (A) and Procaine-HCl-exposure (B) for the same Tankwa goat semen sample using 100x magnification. (Red tracks = Rapid progressive sperm, Green = Medium progressive sperm, Blue = Non-progressive sperm and Yellow = immotile sperm. Blue box = hyperactivated spermatozoa)

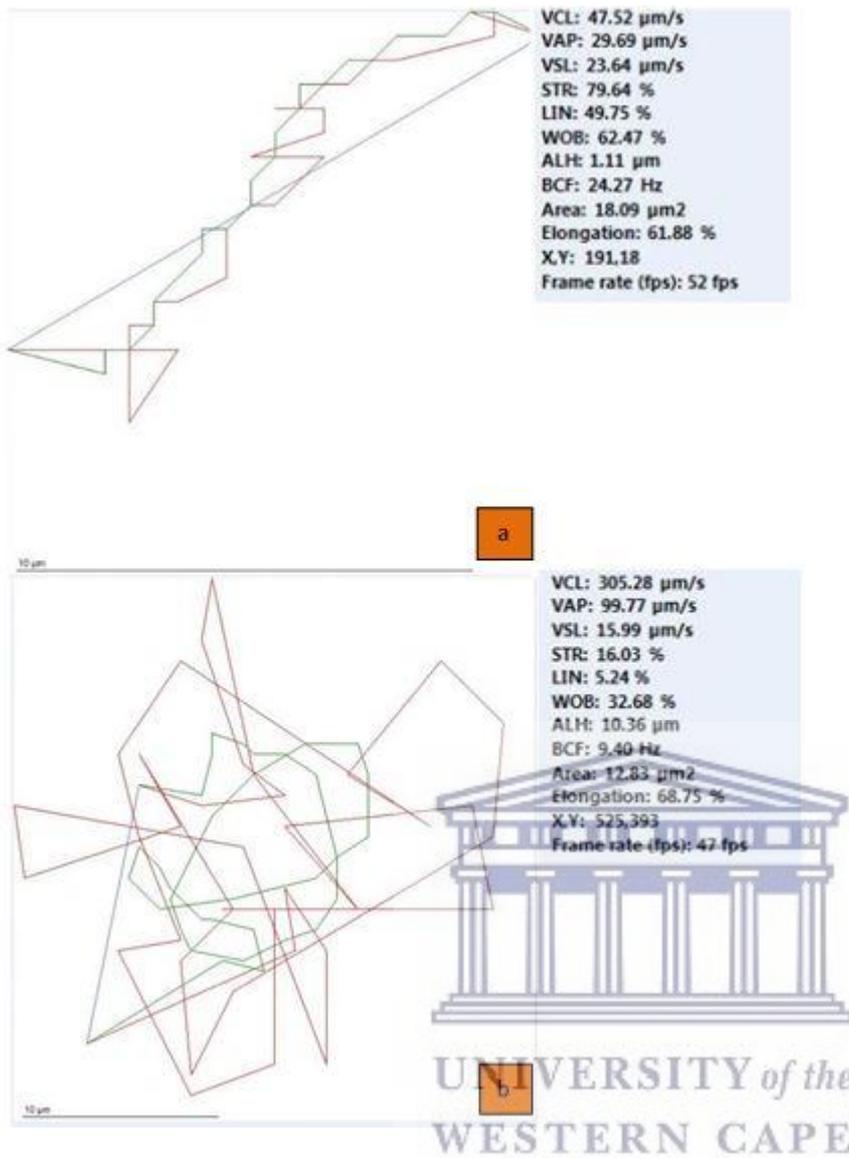


Fig 4.5.3.5. Typical movement patterns of individual spermatozoa after PBS incubation (A), and starspin pattern after procaine HCl exposure (B).

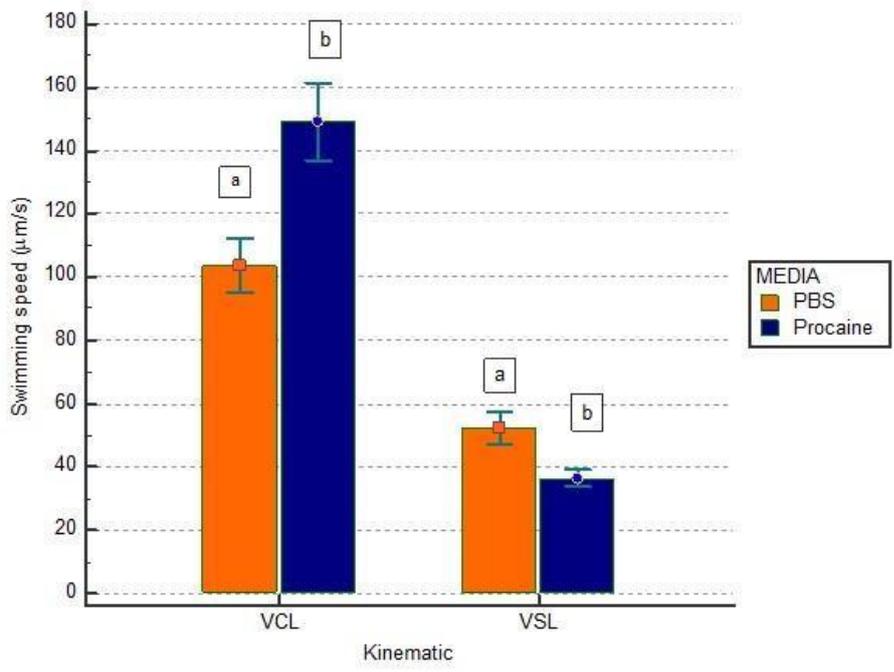


Fig 4.5.3.6. Multiple comparison of swimming speed ($\mu\text{m/s}$) with the use of phosphate buffered saline (PBS) and 5mM procaine hydrochloride over a year period.

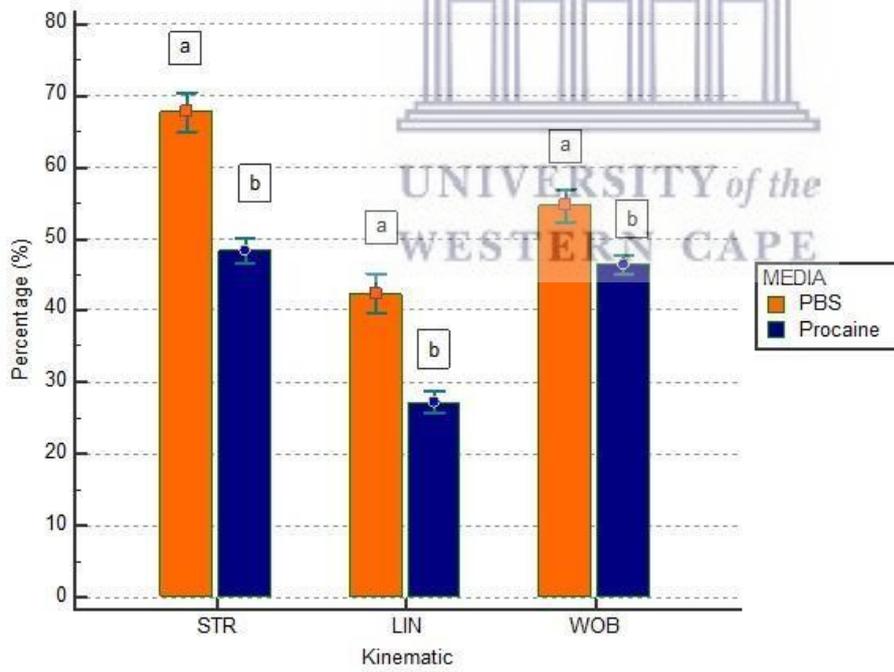


Fig 4.5.3.7. Multiple comparison of STR, LIN and WOB (%) with the use of phosphate buffered saline (PBS) and 5mM procaine hydrochloride over a year period.

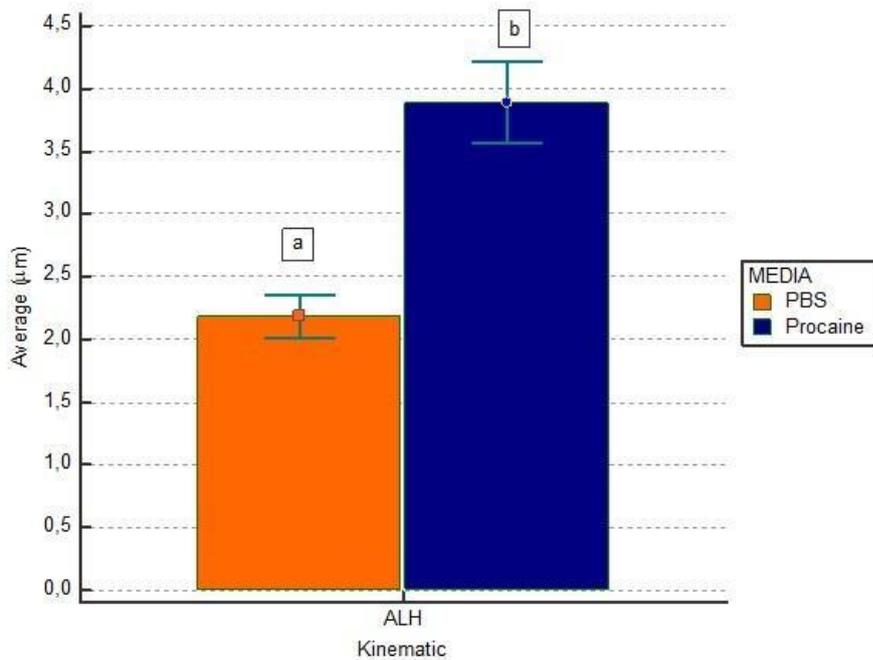


Fig 4.5.3.8. Multiple comparison of amplitude of the lateral head displacement (ALH) with the use of phosphate buffered saline (PBS) and 5mM procaine hydrochloride over a year period.

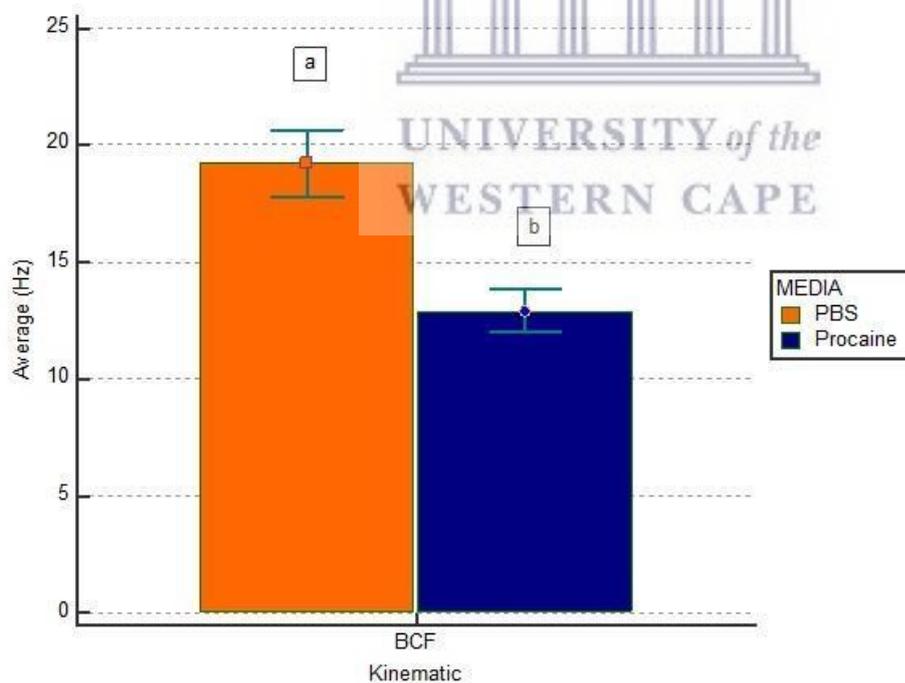


Fig 4.5.3.9. Multiple comparison of beat cross frequency (BCF) with the use of phosphate buffered saline (PBS) and 5mM procaine hydrochloride over a year period.

Statistically significant differences were observed in the kinematics for procaine among the three collection seasons, except for STR, LIN and WOB (Table 4.5.3.2). In VCL, VSL, VAP, STR, ALH and BCF, February 2017 had the highest average values while VCL, VAP and ALH were also significantly lower in August 2016 compared to February 2016 (Fig 4.5.3.10-4.5.3.12). While there was no significant difference in the average percentage hyperactivation induced in February 2016 and 2017, both were significantly higher than the average percentage hyperactivation induced in August 2016 (see Fig 4.5.3.13).

Table 4.5.3.2. Induced hyperactivation (%) and kinematic parameters (mean \pm SD) with the use of 5 mM procaine hydrochloride over a year period.

Parameter	Feb-16 n=28	Aug-16 n=23	Feb-17 n=25
VCL ($\mu\text{m/s}$)	149,0 \pm 41,0 ^a	116,2 \pm 48,2 ^b	179,6 \pm 54,1 ^c
VSL ($\mu\text{m/s}$)	33,7 \pm 9,3 ^a	31,6 \pm 9,7 ^a	43,7 \pm 12,4 ^b
VAP ($\mu\text{m/s}$)	67,8 \pm 18,0 ^a	55,4 \pm 19,1 ^b	79,1 \pm 20,2 ^c
STR (%)	46,2 \pm 6,9	49,5 \pm 8,0	49,9 \pm 8,5
LIN (%)	26,5 \pm 6,5	29,2 \pm 7,7	26,5 \pm 6,1
ALH (μm)	4,0 \pm 1,0 ^a	3,0 \pm 1,3 ^b	4,6 \pm 1,5 ^a
BCF (Hz)	12,3 \pm 2,7 ^a	11,2 \pm 3,8 ^a	15,1 \pm 4,3 ^b
WOB (%)	47,0 \pm 5,9	47,1 \pm 6,6	45,0 \pm 5,2
HA (%)	26,8 \pm 14,1 ^a	16,1 \pm 14,1 ^b	25,4 \pm 16,6 ^a

Superscript a-d in the same row indicates significant difference where $p < 0.05$

VCL = curvilinear velocity, VSL= straight-line velocity , VAP = average path velocity, STR = straightness , LIN = linearity, ALH = amplitude of lateral head displacement BCF= beat cross frequency, , WOB= wobble, , HA = Hyperactivation (%)

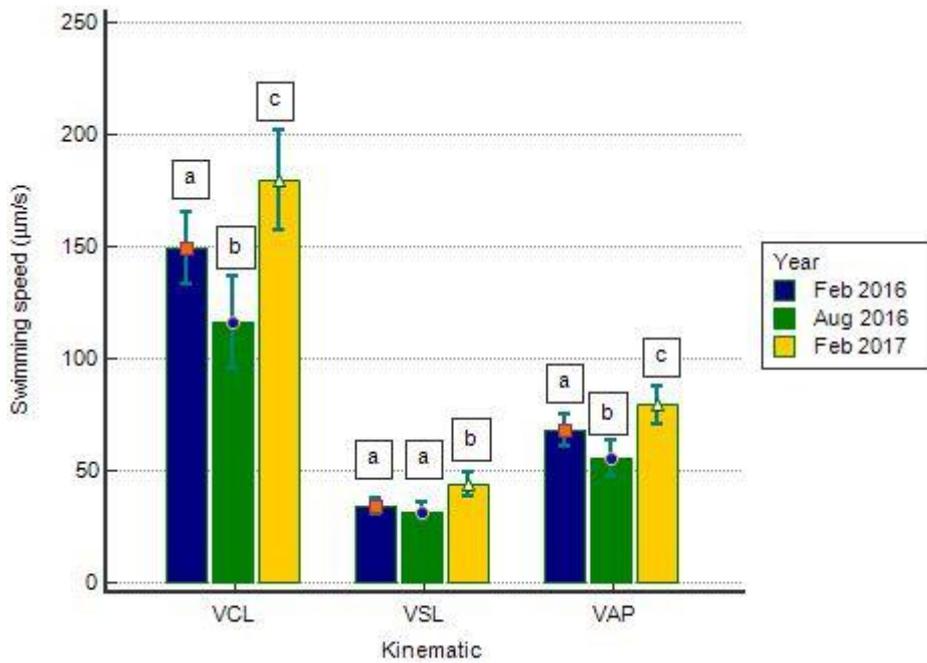


Fig 4.5.3.10. Multiple comparison of swimming speed of Tankwa goat sperm with the use of 5 mM procaine hydrochloride over a year period. *VCL = curvilinear velocity, VSL = straightline velocity, VAP = average path velocity.

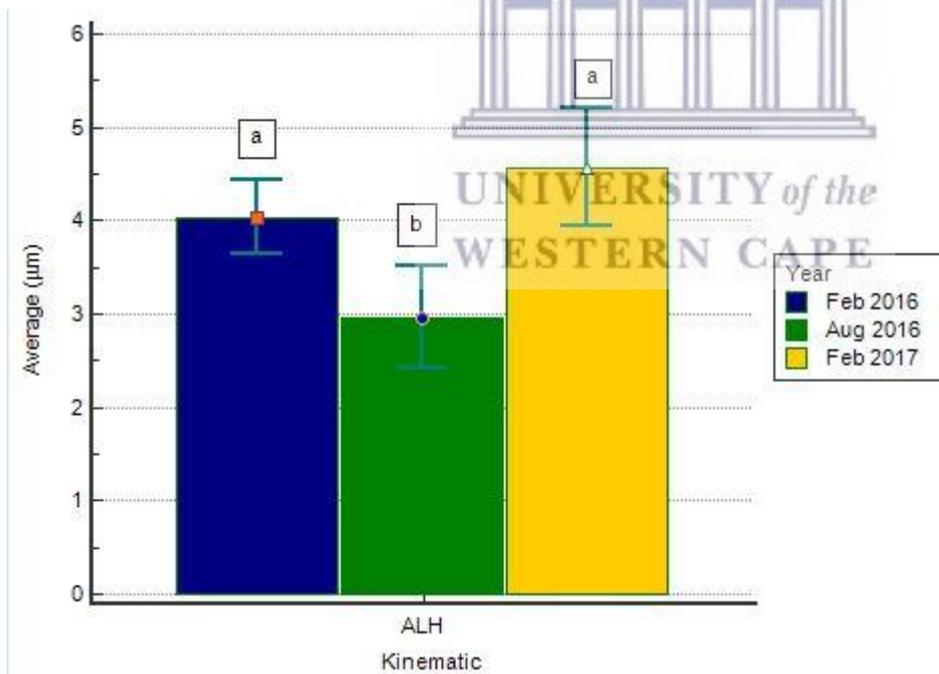


Fig 4.5.3.11. Multiple comparison of amplitude lateral head displacement (ALH) of Tankwa goat sperm with the use of 5 mM procaine hydrochloride over a year period.

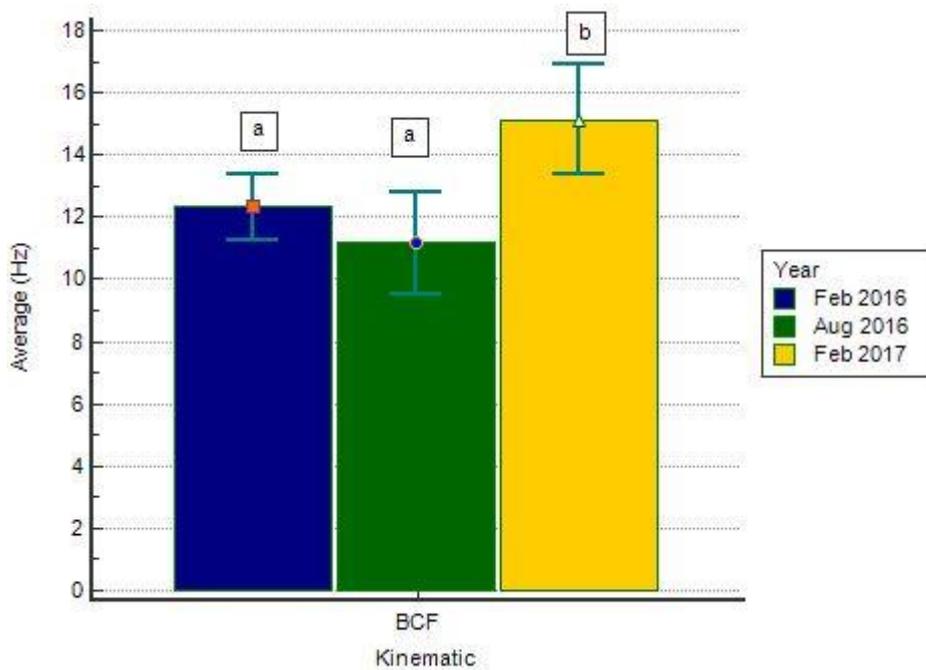


Figure 4.5.3.12. Multiple comparison of beat cross frequency (BCF) of Tankwa goat sperm with the use of 5mM procaine hydrochloride over a year period.

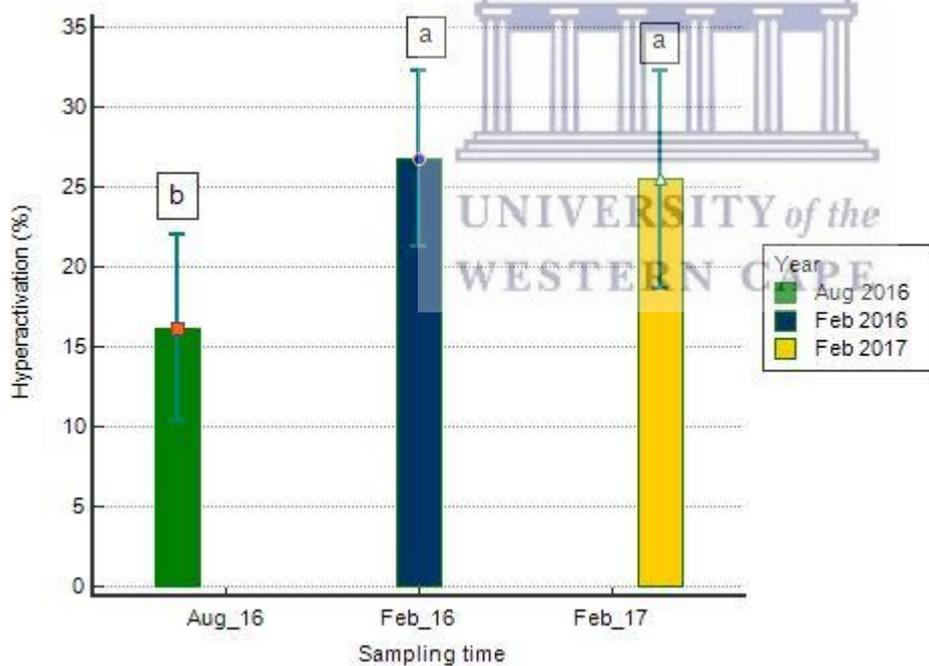


Fig 4.5.3.13. Multiple comparison of hyperactivation (%) of Tankwa goat sperm with the use of 5 mM procaine hydrochloride over a year period.

Between summer and winter, significant differences were observed in VCL, VAP, ALH and HA (%) (Table 4.5.3.3). The summer had a higher average VCL, VAP (Fig 4.5.3.14) and

ALH than winter. A significantly higher average percentage hyperactivation was also observed in summer (26.8%) compared to winter (16.1%) (Fig 4.5.3.15).

Table 4.5.3.3. Induced hyperactivation (%) and kinematic parameters (mean \pm SD) with the use of 5mM procaine hydrochloride between summer and winter.

Parameter	Summer n=53	Winter n=23	Combined n=76
VCL ($\mu\text{m/s}$)	163,4 \pm 49,6 ^a	116,2 \pm 48,2 ^b	149,2 \pm 53,5
VSL ($\mu\text{m/s}$)	38,4 \pm 11,9	31,6 \pm 9,7	36,4 \pm 11,7
VAP ($\mu\text{m/s}$)	73,1 \pm 19,7 ^a	55,4 \pm 19,1 ^b	67,8 \pm 21,0
STR (%)	48,0 \pm 7,8	49,5 \pm 8,0	48,4 \pm 7,9
LIN (%)	26,5 \pm 6,3	29,2 \pm 7,7	27,3 \pm 6,8
ALH (μm)	4,3 \pm 1,3 ^a	3,0 \pm 1,3 ^b	3,9 \pm 1,4
BCF (Hz)	13,7 \pm 3,8	11,2 \pm 3,8	12,9 \pm 3,9
WOB (%)	46,0 \pm 5,6	47,1 \pm 6,6	46,4 \pm 5,9
HA (%)	26,8 \pm 14,1 ^a	16,1 \pm 14,1 ^b	22,9 \pm 15,5

Superscript a-d in the same row indicates significant difference where $p < 0.05$

VCL = curvilinear velocity, VSL= straight-line velocity, VAP = average path velocity, STR = straightness , LIN = linearity,

ALH = amplitude of lateral head displacement BCF= beat cross frequency , WOB= wobble , HA = Hyperactivation (%)

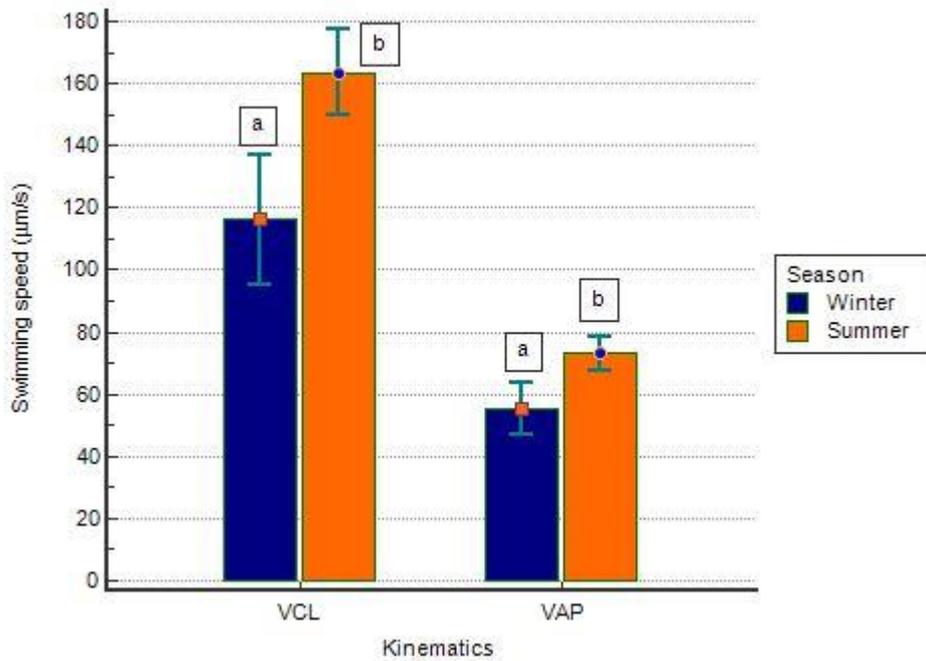


Fig 4.5.3.14. Multiple comparison of swimming speed of Tankwa goat sperm with the use of 5 mM procaine hydrochloride between summer and winter.

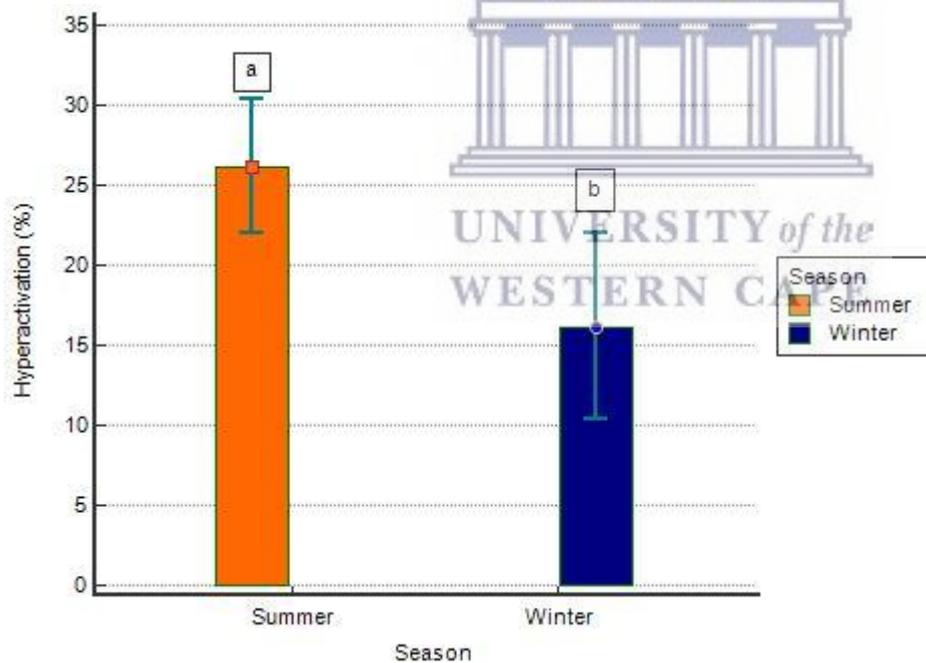


Fig 4.5.3.15. Multiple comparison of hyperactivation (%) of Tankwa goat sperm with the use of 5 mM procaine hydrochloride between summer and winter.

4.6 Acrosome integrity

Most Tankwa goat sperm from selected individuals (n = 6) had intact acrosomes (Fig3.6.1) and values varied from 80% to 95% intact acrosomes in individual goat samples. Acrosome reacted goat sperm was illustrated in Fig 3.6.2 and 3.6.3.

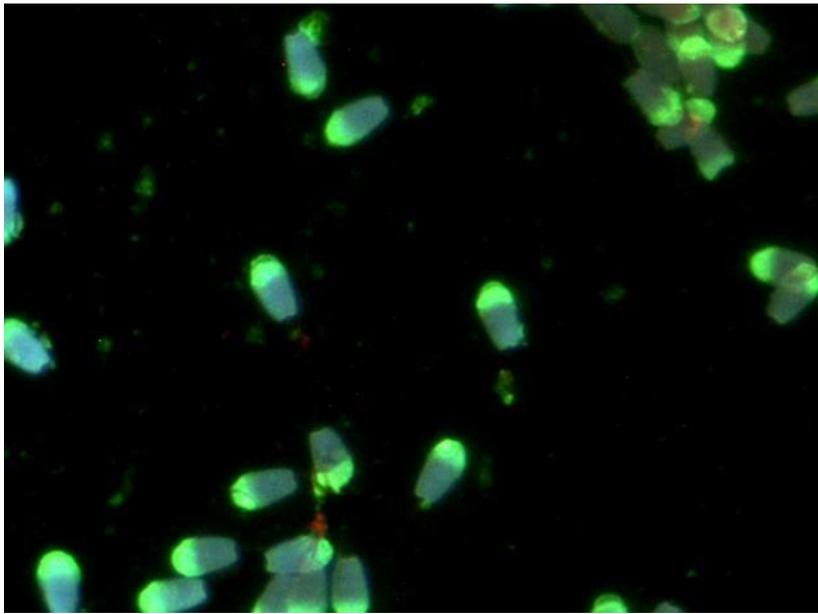


Fig 3.6.1. Examples of FITC-PNA stained spermatozoa with acrosome intact (green fluorescence) viewed with a fluorescence microscope using 400X magnification

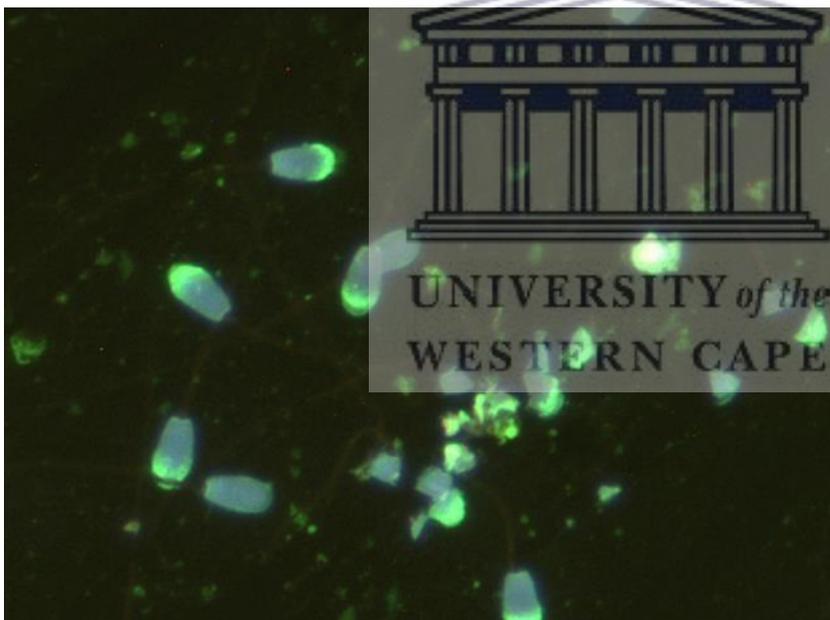


Fig 3.6.2. Examples of FITC-PNA stained spermatozoa with acrosome intact (green fluorescence) and reacted (white arrow) viewed with a fluorescence microscope using 400X magnification

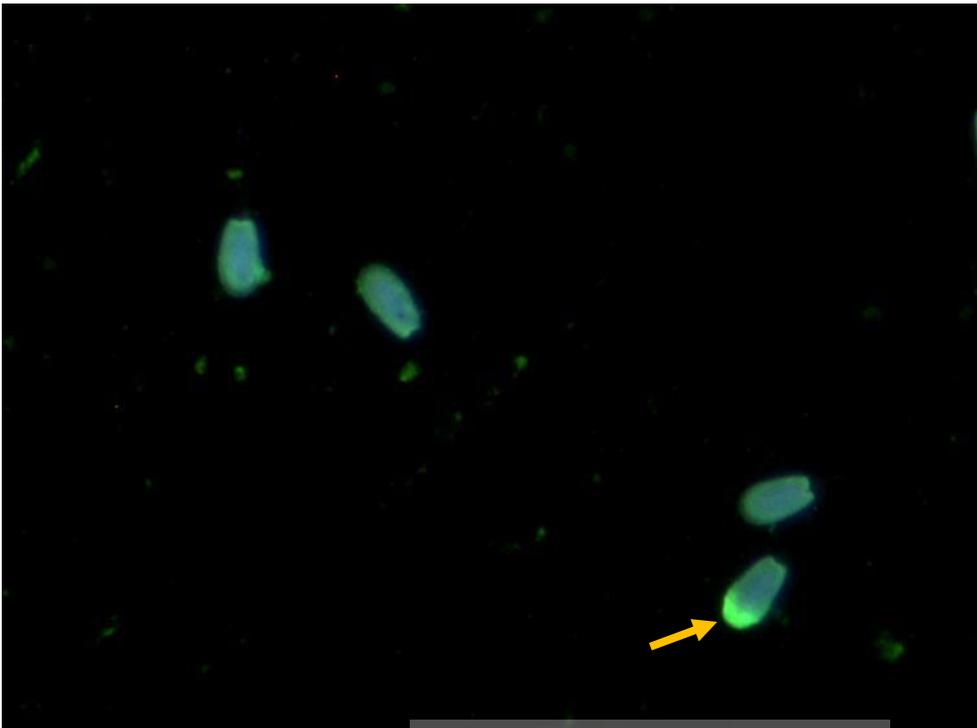


Fig 3.6.3. Examples of FITC-PNA stained spermatozoa with intact (green fluorescence) and reacted acrosome viewed with a fluorescence microscope using 400X magnification (Orange arrow = intact acrosome)

4.7 Multivariate analysis

In the two multivariate analyses, it was possible to visualize separation of the two seasons (Fig 3.7.1) and distinctly different seasonal patterns (Fig 3.7.2) using macromovement, percentage total motility, percentage progressive motility, percentage rapid progressive, VCL, ALH, rapid VCL, rapid VSL and rapid VAP data (Table 3.7.1)

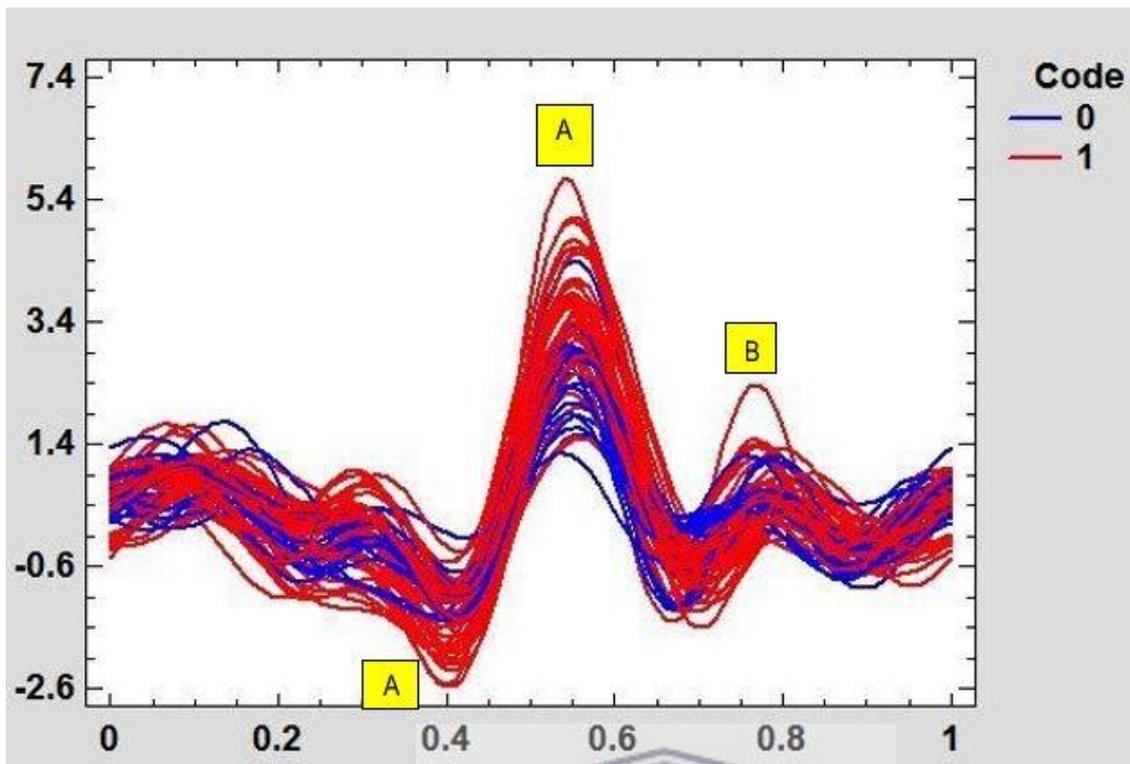


Fig 3.7.1. Andrews plot for 108 ejaculates to distinguish cases that have similar values for 10 input variables. Peaks and valleys on the plot do not represent individual variables. **A)** Clear separation is seen for season 0 (blue; winter) and season 1 (red; summer) ejaculates at various peaks and valleys. **B)** while there is an overlap between the two seasons ejaculates, season 1 ejaculates (red) seem to have a larger variation among individuals. Input variables: macro-movement, percentage total motility, percentage progressive motility, percentage rapid progressive, VCL, ALH, rapid VCL, rapid VSL and rapid VAP

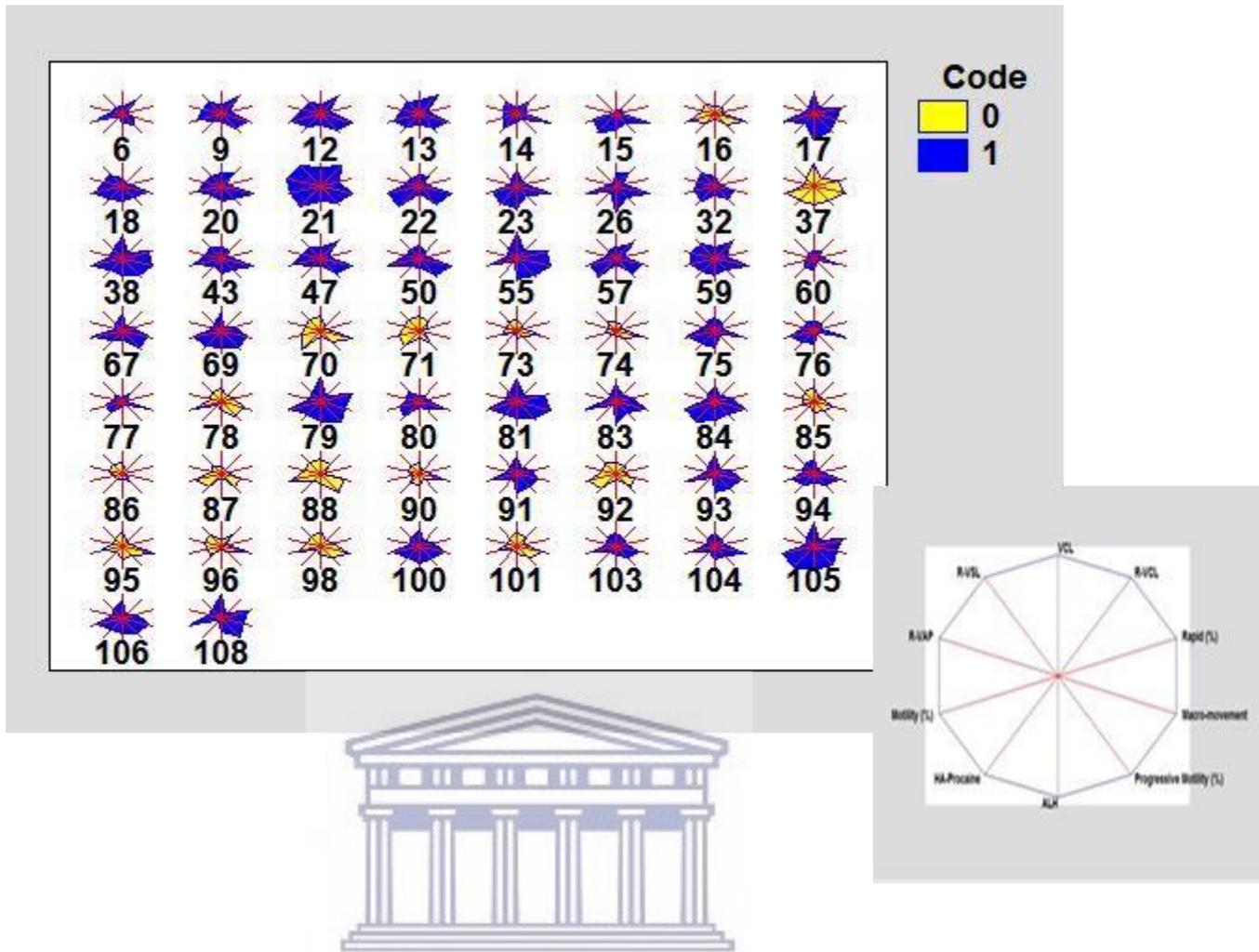


Fig 3.7.2. Sunray plots for 108 ejaculates to distinguish cases that have similar values for 10 input variables. Season 1 ejaculates (blue; summer) had proportionately higher values for most of the selected variables when compared to season 0 ejaculates (yellow; winter). Ejaculates from season 1 revealed mostly similar patterns (association among input variables) when compared to one another, while season 0 ejaculates had more heterogeneous patterns.

Table 3.7.1. Input variable data for Andrews plot and Sunray plot.

Variable	Sample mean	Standard deviation	Minimum	Maximum
VCL	144.67	30.7679	85.13	213.61
R-VCL	237.632	37.0265	196.04	341.04
Rapid (%)	20.4141	22.1977	1.63934	85.1685
Macro-movement	4.06897	0.905385	1.0	5.0
Progressive Motility (%)	55.9153	20.3575	12.62	94.16
ALH	3.26759	0.857465	1.94	5.19
HA-Procaïne	22.9172	15.9245	0.0	54.5
Motility (%)	79.86	14.2355	37.2	99.58
R-VAP	142.366	43.9524	76.01	341.04
R-VSL	118.004	50.3437	0.0	341.04

*VCL = curvilinear velocity, R-VCL= rapid curvilinear velocity, ALH = lateral amplitude of head, HA-Procaïne = hyperactivation-procaïne, R-VAP = rapid average path velocity, R-VSL = rapid straightline velocity.

Chapter 5: Discussion

The overall body condition and health of the Tankwa goat population assessed at Carnarvon Research Station is generally sound, thus their reproductive performance is not compromised. This goat population breeds throughout the year and the majority of females evaluated during the semen sampling events were either pregnant or had young offspring. Females as young as nine months of age have been reported to have healthy offspring and twin kids were often observed during the four sampling events (T Jonker, personal communication, 8 October 2018). It is thus expected that males also have a healthy reproductive status with most males having high quality semen samples, as reported by Ramukhithi (2016).

The aim of the current study was to assess and compare seasonal differences of the semen and sperm characteristics of Tankwa goats.

Standard semen parameters, sperm motility and kinematics, sperm morphometry and morphology, percentage sperm vitality, sperm hyperactivation as well as acrosome intactness were evaluated. The mentioned parameters were significantly higher ($p < 0.05$) in summer as compared to winter with the exception of percentage normal sperm morphology.

In the comparison of four media for sperm hyperactivation, procaine induced a higher percentage hyperactivation in spermatozoa than PBS, BO and lignocaine. The highest number of hyperactivated spermatozoa was observed in both summer periods (February 2016 and February 2017) compared to winter (August 2016). Preliminary studies were done on the acrosome intactness test and was generally high (80-95%) although the sample size was small ($n=7$), acrosome intactness was not included in seasonal comparisons.

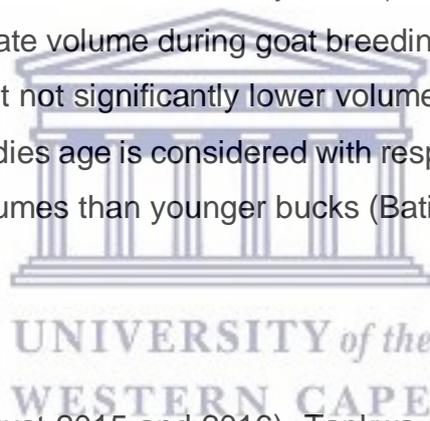
5.1 Standard semen analysis

5.1.1 Volume

The average Tankwa goat ejaculate volume (1.2 ± 0.5 ml) was considerably higher compared to 0.7 ± 0.08 ml obtained from South African indigenous goats (Ramukhithi *et al.*, 2016) but lower when compared to Korean native bucks (2.1 ± 0.4 ml) as reported by Choe *et al.* (2006). Although the method of collection used for these three goat breeds was the same, i.e. electroejaculation, some other factors such as breed, age of buck and breeding season may play a role in the differences in semen volume reported for the Tankwa goat

and other goat breeds. Furthermore, semen volume was also suggested to be affected by physiological status such as age of the bucks (Webb *et al.*, 2004; David *et al.*, 2007).

It has been reported in bucks and rams that changes in day light length may have an impact on testicular activity by altering the release of GnRH and in turn gonadotropins. With reference to seasonal breeders, it has been observed that ejaculate volume is higher in the breeding season than in the non-breeding season. The changes in the ejaculate volume are influenced by the accessory glands and epididymis secretions. During the breeding season, ejaculate volume from crossbred goats is estimated to range between 0.8 ml to 2.0 ml (Huat, 1976). Huat (1976) also observed that the quality of semen (sperm concentration) may decrease as the total volume of the ejaculate increases, but generally a larger volume means more spermatozoa. Over the two-year period during which samples were collected, the highest mean recorded for Tankwa goat ejaculate volume was in August 2016 (1.6 ± 0.5 ml) and the lowest mean recorded was in February 2017 (0.9 ± 0.4 ml), which all fall within the range suggested for ejaculate volume during goat breeding season. Summer ejaculates (1.1 ± 0.5 ml) had a slightly but not significantly lower volume than winter ejaculates (1.3 ± 0.6 ml). However, in some studies age is considered with respect to this parameter as older buck generally have larger volumes than younger bucks (Batista *et al.*, 2011).



5.1.2 pH

During the winter months (August 2015 and 2016), Tankwa goats had slightly more acidic semen (5.9 ± 0.7) than the summer months (6.3 ± 0.7), but for both seasonal periods the pH was below 7.4. The average Tankwa goat semen pH was 6.7 ± 0.8 which is comparable with the results obtained by Bopape *et al.* (2015) in South African indigenous goats (6.9 ± 0.3) and by Ngoula *et al.* (2012) in West African Dwarf goat breeds (6.7 ± 0.3) using the electroejaculation collection method. The acidic semen could be an indication of excessive seminal vesicle secretion due to electrical stimulation in the rectum of the buck (Ortiz-de-Montellano *et al.*, 2007; Sundararaman *et al.*, 2007; Ramukhithi *et al.*, 2011). The alkaline secretions from the bulbourethral (Cowper's) glands are responsible for the buffering capacity of semen (Ramukhithi *et al.*, 2011). Latif *et al.* (2005) reported that in an acidic pH environment, the motility of sperm is negatively affected, probably due to a change in the metabolic activity and a disturbance in the cellular respiration of spermatozoa. The acidity of semen could be one possible explanation for the variation in semen quality that was

observed in Tankwa goats as semen samples from the winter season with more acidic pH (5.9) had the lower percentage total sperm motility (73.1%) as compared to summer semen samples where pH was 6.3 but had a higher percentage total sperm motility (82.4%).

However, it should be noted that the method of semen collection used could have had an effect on the physiological status of the semen. It can be assumed that the electroejaculator used in semen collection had an effect on the pH of goat semen. In previous studies, this method of semen collection was found to stimulate bucks in releasing acidic urine which could contaminate semen and reduce the semen pH (Jimenez *et al.*, 2008; Moreno *et al.*, 2009). Goats normally produce alkaline urine but diet can have an effect on the urine's pH.

5.1.3 Macro-movement

Gross motility, or the amount of swirling (or wave motion) present in an undiluted semen sample, is a function of both sperm concentration and individual motility. Semen samples with good motility will show wavy motion when it is observed and semen with poor quality will not show wave motion (Chenoweth, 2002).

The summer season had a significantly higher average macro-movement (4.1 ± 0.9) than the winter season (3.6 ± 1.2). However, it should be noted that the macromovement was only recorded once in the winter period as there were no records for this semen parameter in August 2015.

The Tankwa goat semen mass-movement average for both summer (4.1) and winter (3.6) were higher compared to that of the British goat breeds (4.03 and 3.4 respectively) as recorded by Ahmad and Noakes (1996) but lower as compared to the Iranian Markhoz (Angora) breed (4.3 and 4.0 respectively) as recorded by Talebi *et al.* (2009).

The average Tankwa goat semen macro-movement was 4.0 ± 1.0 , and it was observed that when macro-movement is high then percentage total motility is also high. However, there were instances where low macro-movement was scored but the sample had a high percentage total motility. This could have been due to a human error during the observation and recording process.

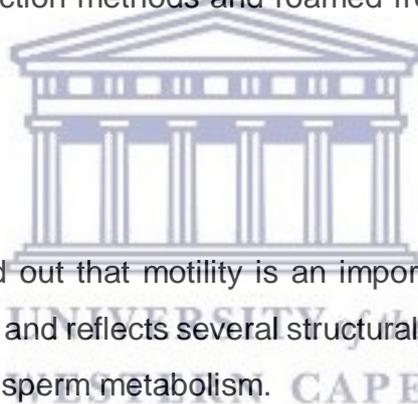
5.1.4 Sperm concentration

The sperm concentration of an ejaculate is a function of several factors which could affect the production and release of spermatozoa, including degree of sexual preparation of the buck, age of the buck, time of year the collection is made, amount of sexual rest before

collection, health of the buck, his nutritional state, inherent sperm storage, and production capacity of the buck (Nutti, 2002).

Corteel (1975) reported that the satisfactory sperm concentration for a high fertilization rate for bucks is $40 \times 10^6/\text{ml}$ in a French breed. Paulenz *et al.* (2005) in Norwegian goats and Hildago *et al.* (2008) in Florida goats reported that a good fertile buck must produce a standard sperm concentration of $\geq 2000 \times 10^6/\text{ml}$. The average sperm concentration ($2463.3 \pm 1589.7 \times 10^6/\text{ml}$) of the Tankwa goats thus falls within the high fertility range as reported by these previous studies.

Although there was no significant difference observed between the two seasons for sperm concentration, summer had a higher average when compared to winter. Memon *et al.* (1986) reported that electro-ejaculation yield ejaculates of greater volumes but lower sperm concentration than the artificial vagina. However, it should be noted that these goats were all subjected to the same collection methods and roamed freely in the same geographical area.



5.1.5 Total motility

Penaz-Martinez (2004) pointed out that motility is an important attribute of spermatozoa, because it is readily identifiable and reflects several structural, and functional competencies, as well as essential aspects of metabolism.

The average percentage total sperm motility for Tankwa goats was $78.2 \pm 15.3\%$, which is in the same range as previously reported for Saanen goats (62.5%, Nur *et al.*, 2005), South African indigenous goats (83.1%, Ramukhithi *et al.*, 2011; 86.3%, Bopape *et al.*, 2015), the South African Boer breed (96%, Ajao, 2015) and the Tankwa goats from the same population as the present study (70.2%, Ramukhithi, 2016). The summer season ($82.4 \pm 13.1\%$) had a significantly higher average percentage total sperm motility in semen than the winter season ($73.1 \pm 16.3\%$). This was higher when compared to the British goat breeds for summer (65.58%) and winter (76.33%) as reported by Ahmad and Noakes (1996).

5.2 Sperm motility and kinematics

In general, sperm motility and especially characteristics of sperm motion could be some of the indicators of the quality of spermatozoa. The progressively forward motion of mammalian spermatozoa by means of beating movement of its tail is essential to enable spermatozoa to cover the distance through the cervix, uterus and oviductal ampulla in order to penetrate the ovum (Katz and Overstreet, 1981).

5.2.1 Percentage total and progressive motility

For percentage total motility, February 2016 was significantly higher than August 2016 over the two-year period. In addition, for percentage progressive motility, the summer months had significantly higher values than the winter months. Both these findings are in agreement with what was hypothesised; summer months have a better semen quality. Progressive forward motility is a crucial feature, because this is the only way spermatozoa can cover the distance to reach the ovum which is to be fertilized (Ludwick and Frick, 1990). A study by Ramukhithi (2016) on the same Tankwa goat population yielded a lower average for percentage progressive motility (35.1%) when compared to the present study (54.1%). The difference could be attributed to the CASA analysis settings and sample size could also have had an influence as Ramukhithi's study had a smaller sample size (n=41).

The summer Tankwa goat percentage progressive motility (59.5%) was lower as compared to the Zaraibi goats (81.2%) during their breeding period as reported by Barkawi *et al.* (2005). Environmental conditions, age, inbreeding and body weight could have an impact on the semen quality (Abagair *et al.*, 1999; Abagair *et al.*, 2001). It should be noted that the Tankwa goats were from the same population and no inbreeding was observed.

5.2.2 Sperm subpopulations for swimming speed (Rapid, Medium and Slow)

Various sperm characteristics such as biochemical parameters (Meggiolaro *et al.*, 2003), functional tests (Holt *et al.*, 1997; Petrunkina and Topfer-Petersen, 2000; Gualtieri, and Talevi, 2003; Pena *et al.*, 2003 and Perez-Llano *et al.*, 2003) or sperm morphology (Gravance *et al.*, 1996; Thurston *et al.*, 1999; Thurston *et al.*, 2001; Estes *et al.*, 2003; Pena *et al.*, 2005; Rubio-Guillen *et al.*, 2007; Hildago *et al.*, 2008; Ferraz *et al.*, 2014; Barbas *et al.*, 2018) have been used to identify sperm subpopulations within an ejaculate. Different subpopulations have also been identified in mammalian species' ejaculates on the basis of motility characteristics displayed by individual spermatozoa (Hildago *et al.*, 2008; Dorado *et al.*, 2010 and Vázquez *et al.*, 2015). Maree and van der Horst (2013) described a method of

using species-specific swimming speed (VCL) cut-off values for six species to identify three motility subpopulations (rapid-, medium- and slow-swimming spermatozoa) within the total motile sperm population. Motility characteristics have also been successfully used to classify sperm into subpopulations by Quintero-Moreno *et al.* (2003) in stallion ejaculates and Martinez-Pastor *et al.* (2005) in Iberian red deer ejaculates.

Different subpopulations of spermatozoa coexist within any typical mammalian ejaculate (Chang and Hunter, 1975; Bedford, 1983). These are thought to owe their origins to variations in the assembly of individual spermatozoa during spermatogenesis as well as to the differential maturational status and age through mixing in the epididymis. Davis *et al.* (1995), using a complex interactive multiple regression technique to analyze human sperm motion parameters prior to freezing, were able to demonstrate the existence of subpopulations this way, reflecting the physiological status of individual cells. The notion that motility is an indicator for fertilization to occur (Fraczek *et al.*, 2014) is important, however, the degree of motility as indicated by detailed kinematic parameters is of vital importance. The value of using such sperm swimming characteristics have been indicated by Mortimer and Mortimer (2013), who reported on hyperactivation (indicator for fertilization) cut-off values for VCL, ALH and LIN.

While Tankwa goat semen in February 2017 had a significantly higher percentage rapid swimming spermatozoa (33.6%) than the other three collection periods, there was also more rapid swimming spermatozoa in the summer months ($21.8 \pm 21.7\%$) compared to the winter months ($9.6 \pm 13.9\%$). The average Tankwa goat percentage rapid swimming sperm (16.5%) was very low, but it was due to the low percentage rapid swimming spermatozoa in the winter months (ranged from 0% to 60.9%).

5.2.3 Kinematics

Sperm movement characteristics are important for spermatozoa to be able to fertilize oocytes. In earlier studies, Holt *et al.* (1994) reported that the swimming speed (VCL) of ejaculated spermatozoa measured with a semi-automatic image analysis system was strongly correlated with *in vitro* fertilization (IVF) rates. Jeulin *et al.* (1996) showed that the ALH of motile spermatozoa selected by the swim-up technique correlated positively with fertilization rates, which was also confirmed by Liu and Baker (1992) and Sukcharoen *et al.* (1995) in humans. The significance of VCL and VAP as predictors of fertilization may imply the importance of capacitation in the process of fertilization, because both of these are

enhanced after capacitation. The ALH is one of the parameters affecting the outcome of IVF (Barlow *et al.*, 1991 and Jeulin *et al.*, 1996) and the mammalian spermatozoa's ability to penetrate cervical mucus and fuse with the oocytes (Aitken *et al.*, 1982: Feneux *et al.*, 1985: Aitken *et al.*, 1986: Aitken *et al.*, 1992: Aitken and Fisher, 1994: Mortimer *et al.*, 1995). This parameter is of importance as it indicates the vigour of flagellar beating together with the frequency of cell rotation (David *et al.*, 1981: Serres *et al.*, 1984), which are probably important for the progression of spermatozoa into the cervical mucus and the peri-oocyte envelopes.

The summer Tankwa goat sperm velocity for VCL, VSL and VAP were 146.7 $\mu\text{m/s}$, 76.6 $\mu\text{m/s}$ and 92.4 $\mu\text{m/s}$ respectively, which were lower to the values recorded in Florida goats except for VCL (146.4 $\mu\text{m/s}$, 104.06 $\mu\text{m/s}$ and 117 $\mu\text{m/s}$). The average summer Tankwa goat sperm STR, LIN and WOB were 77.3%, 51.9%, 63.4% respectively, they were also lower than those of Florida goats except for STR (71.1%, 88.3% and 79%) as reported by (Dorado *et al.*, 2010). The average Tankwa goat sperm ALH parameter (3.4 μm) was in agreement to that observed by Dorado *et al.* (2010) (3.95 μm). Dorado *et al.* (2010) used Dulbecco phosphate buffered saline to dilute the semen and like the present study, used SCA® to analyse the kinematics.

The summer sperm kinematics for VCL (146.7 \pm 31.0 $\mu\text{m/s}$) and ALH (3.4 \pm 0.8 μm) were higher than winter (125.0 \pm 26.3 $\mu\text{m/s}$ and 2.8 \pm 0.5 μm respectively). High VCL and ALH are characteristics of spermatozoa that can penetrate cervical mucus, therefore summer season indicate a superior fertilisation potential.

5.3 Sperm morphology and morphometry

The fertility potential of a male is often analysed through classifying spermatozoa according to the normality of their different components (head, midpiece and tail) and various defects such as nuclear vacuoles and cytoplasmic droplets. Morphology has become a vital structural sperm test, due to the correlation it has with other functional and structural parameters such as percentage motility and vitality (Maree, 2011). Poor semen morphology is an important indicator of decreased fertility in humans (Kruger *et al.*, 1988), stallions (Jasko *et al.*, 1990), bulls (Sekoni and Gustafsson, 1987), goats, and rams (Oshinowo *et al.*, 1988; De Jarnette *et al.*, 1992; Gravance *et al.*, 1995).

The Tankwa goat sperm morphometric values did not show any significant differences for seasonal variation with the exception of head roughness and head regularity, midpiece width

and midpiece area. The average Tankwa goat sperm head length, width, elongation and regularity ($8.7 \pm 0.2\mu\text{m}$, $4.1 \pm 0.1\mu\text{m}$, 0.4 ± 0.01 and 0.8 ± 0.02 respectively) were similar to that of the Florida goats ($8.47 \pm 0.27\mu\text{m}$, $4.16 \pm 0.18\mu\text{m}$, 0.34 ± 0.02 and 0.95 ± 0.03 respectively) (Vazquez *et al.*,2015).

Although significant differences were obtained for head roughness and regularity between summer and winter, the values were the same for these two sperm components and a small variation was observed. The summer season morphometric values ($1.1 \pm 0.1 \mu\text{m}$) was slightly smaller than winter ($1.2 \pm 0.1 \mu\text{m}$) for midpiece width, and the summer ($10.9 \pm 1.6 \mu\text{m}^2$) was smaller than winter ($11.8 \pm 2.2 \mu\text{m}^2$) for midpiece area.

The average Tankwa goat percentage normal morphology was lower (73.3%) when compared to the Tankwa goats from the same population (84.7%) (Ramukhithi ,2016). The difference could be due to analysis equipment used. This study used Morphology module of the SCA® while Ramukhithi employed fluorescence microscopy. The staining technique could also have influenced the results, Ramukhithi used nigrosin-eosin while the Sperm Blue was used in this study.

The Tankwa goat percentage normal sperm morphology was considerably lower in both summer ($68.9 \pm 18.2\%$) and winter ($76.7 \pm 15.8\%$) as compared to Zaraibi goats (88.6% and 81.7% respectively) as observed by Barkawi *et al.* (2005) and to Markhoz (Angora) goats (90.8% and 88.8% respectively) as observed by Talebi *et al.* (2009). Both summer and winter season were lower compared to unspecified Brazilian indigenous goats as observed by Aguiar *et al.* (2013) where normal sperm morphology was $82.8 \pm 2.5\%$ and $86.9 \pm 1.8\%$ for autumn and spring, respectively. However, it should be noted that these mentioned studies had employed subjective methods in determining sperm morphology as compared to the present study which used objective method namely CASA. Van der Horst *et al.* (2018) demonstrated the use of CASA as a superior method compared to manual techniques as CASA offers the advantage of reduced bias.

Due to human error in the morphology staining technique, unreadable Tankwa goat semen samples were encountered in February 2016, with spermatozoa either being over-stained or under-stained. This resulted in only a few ($n = 8$) of the stained samples ($n = 22$) to be usable and the majority not being correctly analysed by the SCA® CASA system and were therefore not included in the final results.

Despite the apparent higher percentage normal morphology found in the winter, no significant difference was observed between the two seasons. This is probably a result of the large variation and accordingly large standard deviation that was observed within the two seasons. As mentioned above, sample size differences could have contributed to this observation, since summer had a smaller sample size ($n = 30$) compared to winter ($n = 39$).

It should be noted that August 2015 had the highest percentage normal sperm as compared to other periods. Another factor that could have played a role in this observation are the restrictions for morphology classification, as the morphometric data for August 2015 was used to set the restriction for normal Tankwa sperm morphology. There is a possibility that the restriction settings might have been too strict for other periods.

5.4 Sperm vitality

The average percentage Tankwa goat sperm vitality of this study ($72.7 \pm 14.3\%$) was similar to the one observed by Ramukhithi (2016) in Tankwa goats from the same population ($70.6 \pm 2.1\%$). The average percentage Tankwa goat sperm vitality was higher for both summer ($74.9 \pm 15.3\%$) and winter ($68.1 \pm 11.1\%$) when compared to other indigenous goats as observed by Webb *et al.* (2012) where percentage vitality was between 37.64% and 42.5% in winter and improved to 68% in summer. Furthermore, Tankwa goat sperm vitality was lower compared to that of the British goat breeds for summer (82%) and winter (87.65%) as recorded by Ahmad and Noakes (1996), as well as to that of Zaraibi goat (87.2% and 77.2% for summer and winter respectively) as recorded by Barkawi *et al.* (2005), and to that of Markhoz (Angora) goats for summer (88.2%) and winter (84.9%) as observed by Talebi *et al.* (2009). However, the sample size in the above studies were lower ($n=20$ for Webb *et al.* (2012) and $n=10$ for the subsequent studies) as compared to that of this present study ($n=75$). This could have influenced the difference between these studies. Moreover, the current study had a small sample size for the winter ($n=25$) as compared to the summer ($n=50$) period. This could have influenced the results in favour of summer period in our study.

Percentage sperm vitality was lower than the percentage sperm total motility for August 2015, February 2016 and August 2016. Large variations in percentage vitality were also observed during 2016. A possible factor that played a role in the variation and anomaly mentioned above was that the vitality staining technique used during 2015-2016 period was

not ideal for the Tankwa goat spermatozoa. Difficulties were experienced whilst analysing nigrosin-eosin stained spermatozoa with the Sperm Class Analyser® CASA system. Analyses might have been inaccurate, as it was difficult to deduce whether a spermatozoon was alive or dead, possibly because of the over-staining with eosin. In addition to this, time delay and temperature control could have had a significant effect on the vitality of spermatozoa, and therefore it is possible that these factors could also have contributed to the inconsistent results.

5.5 Hyperactivation

Hyperactivation is thought to provide strong thrusting power to the spermatozoa while passing through the cells and layers surrounding the oocyte, particularly the zona pellucida (Yanagimachi, 1984). Hyperactivation is a flagellar phenomenon, even though it is often measured by changes in the movement of the sperm head. The difference between the flagellar beat patterns of hyperactivated and nonhyperactivated spermatozoa is caused by changes in the degree of bending of the axoneme, as well as changes in the propagation of the flagellar beats (Katz *et al.*, 1978; Katz *et al.*, 1986; Mortimer *et al.*, 1997). In previous studies, it has been shown that if spermatozoa are unable to become hyperactivated, they are unable to fertilize the oocytes *in vitro* (Fleming and Yanagimachi, 1982; Fleming and Kuehl, 1985). It was observed in human spermatozoa co-cultured with epithelial cells from fallopian tubes (Pacey *et al.*, 1995) and mouse spermatozoa in oviducts of naturally mated mice (Demott and Suarez, 1992) that hyperactivation helps spermatozoa to break free and move along the oviduct. The objective measure of this physiological process can serve as a biological marker to evaluate the functional capabilities of spermatozoa.

5.5.1 Determination of cut-off points for Tankwa goat sperm hyperactivation

Receiver operating curves (ROC) graphs were constructed from the kinematics of individual spermatozoa from the February 2016 data, to determine the cut-off values for Tankwa goat sperm hyperactivation. The VAP was the only parameter that did not have high sensitivity (74.8) and high specificity (32.8) and thus was not used to set the hyperactivation restriction. It should be noted that the kinematics used were the ones that were treated with procaine in February 2016 and could have influenced the results in favour of procaine.

This was a novel approach as most studies have not reported on the sperm hyperactivation in goats. Determining sperm hyperactivation is a useful aspect in the improvement of sperm functional characteristics.

5.5.2 Comparison of media for sperm hyperactivation in Tankwa goats

Individual Tankwa goat semen samples were exposed to four different media namely phosphate buffered saline (PBS), BO medium, 5 mM procaine hydrochloride and 4% lignocaine to evaluate its capability to induce sperm hyperactivation.

Procaine has been widely reported to induce hyperactivation in numerous species such as guinea pig and bovine (Ho and Suarez, 2001) and stallions (McPartlin *et al.*, 2009). According to Meyers and Baumber (2006) and Mortimer and Mortimer (1990) hyperactivation induced by procaine resembles non-progressive hyperactivation or star-spin pattern. Lignocaine has been observed to cause sperm hyperactivation in human sperm (Bennett *et al.*, 1992) and it was mainly used in this study for comparison with procaine in terms of sperm trajectories.

In the present study, procaine displayed lower mean and rapid VSL, LIN, and STR, and high mean, rapid and medium VCL than the other three media. The significant values in these kinematic parameters suggest that sperm tracks induced by procaine display a typical asymmetrical hyperactivation pattern as described by Mortimer and Mortimer (1990). Spermatozoa displaying low VSL, LIN and STR are an indication of non-progressive hyperactivation tracks (Baumber and Meyers, 2006).

Spermatozoa exposed to PBS and BO media showed more linear and progressive sperm tracks as compared to the asymmetrical and non-progressive tracks induced by procaine hydrochloride. Spermatozoa exposed to lignocaine also showed asymmetrical sperm tracks but they were not as pronounced as compared to those induced by procaine.

5.5.3 Comparison of sperm hyperactivation over three seasons

Procaine had significant low VSL, STR, LIN, WOB, and BCF averages than PBS. It also displayed higher averages for VCL, ALH and HA (%). The average for procaine was 22.9% which is higher than the cut-off point (20%) for good fertilizing potential for a spermatozoan. However, there was a large variation observed among the individual goats as is evident from the high standard deviation ($22.9 \pm 15.5\%$) and ranged from 0% to 54.5%.

The goats with the most hyperactivated spermatozoa, i.e number of spermatozoa that had a HA (%) of greater than 20%, were recorded in February 2017 (n=17), while August 2016 had the least amount of males with a HA (%) of greater than 20%. This could mean the Tankwa goat spermatozoa are more likely to be hyperactivated in the summer month, showing potentially a higher chance for fertilization in this period.

5.6 Acrosome integrity

The acrosome reaction is an essential event for mammalian fertilization. The acrosome may contain enzymes that aid in penetration of the extracellular matrix surrounding the oocyte, part of which is a secretion known as the zona pellucida (ZP) (Florman, 1994; Breitbart 2003). Further, the release of this vesicle may enable the spermatozoa to attach to the ZP via either the now exposed inner acrosomal membrane or plasma membrane (Yanagimachi and Phillips, 1984)

O'Toole *et al.* (2000), Harper *et al.* (2006), and Florman *et al.* (2008) have proposed two primary physiologically relevant triggers for this reaction *in vivo*, the zona pellucida glycoprotein (ZP3 in humans) and progesterone. Both of these agonists can induce the acrosome reaction in laboratory settings. Until recently, zona pellucida glycoproteins were thought to be the more biologically relevant agonist, as it was thought that the AR happens when the sperm binds to the egg where the ZP glycoproteins are located in successful fertilization events. It has been observed that the ZP can trigger or accelerate the acrosome reaction (Florman and Storey 1982; Bleil and Wassarman 1983; Crozet and Dumont 1984; Cherr *et al.* 1986; Uto *et al.* 1988; Tollner *et al.* 2003; Abou-haila and Tulsiani 2009; Buffone *et al.* 2009) However, recent *in vivo* mouse studies have suggested that the acrosome reaction is often initiated before reaching the ZP (Jin *et al.* 2011; Inoue *et al.* 2011; Hino *et al.* 2016; La Spina *et al.* 2016), indicating that ZP-induced acrosome reactions may not be the primary mechanism *in vivo*.

Although only a small number of samples were evaluated for acrosome integrity (n=6), 80%-95% acrosome intactness was observed in these samples, implying high quality spermatozoa for Tankwa goats. It should be noted that these are preliminary results and this functional test needs to be investigated further on the Tankwa goat sperm. At least this study has confirmed that the routine protocol suggested for the staining of the sperm acrosome with FITC-PNA seems to give accurate results with Tankwa goat spermatozoa.

A future experiment where the acrosome reaction is induced is needed to confirm the results and accuracy of this functional test in Tankwa goats. The assessment of the acrosomal status remains a research interest, as acrosomal status represents one of the most important approaches to evaluating the sperm's fertilizing ability (Esteves *et al.*, 2007).



Chapter 6: Conclusion

6.1 General conclusion

The CASA system has proved to be a valuable tool in assessing semen of the Tankwa goat. The number of motile and morphologically normal spermatozoa was determined. The novel approach of the study was determination of cut-off points for hyperactivation, which was successful as a number of hyperactivated sperm were quantified. Employing the SCA® has proved to be useful in this aspect.

There was distinct seasonal difference between summer and winter for various semen and sperm characteristics, although large variations were observed among individual goats. Semen samples collected in the summer season seem to have a higher percentage of functionally intact and potentially fertile spermatozoa as compared to winter. Seasonal differences and individual variations were also observed with the multivariate analysis. After exposing Tankwa goat spermatozoa to different hyperactivation-inducing media, procaine hydrochloride is believed to be the best stimulant for evaluating sperm hyperactivation. The results from this study can be used as baseline recommendation for future studies on the potential fertilising ability of the Tankwa goats. Determining sperm hyperactivation is a useful aspect in the improvement of sperm functional characteristics. Furthermore, this functional test can be optimised and include more hyperactivation-inducing media.

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Limitations within the study included:

- Human error. Unreadable slides were encountered and thus, could have led to inconsistent results as evident in percentage normal sperm parameter.
- Staining techniques. In the vitality test, it was difficult to deduce whether a sperm cell was alive or dead. It is therefore recommended to optimise the staining technique in future studies.
- Sample size. In acrosome intactness tests, there was low sample size and the technique needs to be evaluated further.

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