

Original Research Paper

Seasonal Variations in Semen Parameters of Zulu Rams Preserved at 10°C for 72 H During Breeding and Non-Breeding Season

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Abstract: The study was conducted to evaluate the seasonal variations in semen parameters of Zulu rams preserved at 10°C for 72 h. The study site was the Agricultural Research Council (ARC) Irene, during the breeding season and the non-breeding season. Zulu rams (n = 6, age = 3 years and average weight = 35 kg ± 2.29) were used in this study. Semen was collected with the aid of artificial vagina. Macroscopic (volume and pH), microscopic (sperm concentration, progressions, velocities and velocity ratios and morphology) semen parameters were evaluated immediately after semen collection. Data were analyzed using General Linear Model (GLM) in Minitab 17®. Semen volume was higher during breeding season (0.97±0.25 mL) than non-breeding season (0.72±0.5 mL). Total sperm motility was higher (92.01±1.40%) at 24h during breeding season than non-breeding season (88.69±1.40%), thereafter declined drastically in both seasons. However, non-breeding season yielded a high progressive motility (50.86±1.63%, 33.77±1.63% and 27.56±1.63%) after 24, 48 and 72 h than breeding season (21.32±1.63%, 11.89±1.63% and 10.29±1.63%). It was concluded that, semen parameters from Zulu rams does vary with seasons. Nevertheless, despite seasonal variations observed, Zulu rams' semen quality is acceptable in both breeding and non-breeding seasons hence can breed throughout the year. Notwithstanding, studies involving fertility are recommended and the nutrition effect of lambing during dry period should be considered.

Keywords: Conservation, Zulu Sheep Seasonal Variations, Semen Quality

Introduction

Zulu sheep is a fat-tailed South African indigenous sheep along with BaPedi and Swazi breed, hardy and well adapting to harsh environmental conditions (Ramsay *et al.*, 2000; Kunene *et al.*, 2011). Zulu people brought this breed to the east coast of South Africa 200-400 AD ago hence named after Zulu tribe (Mavule *et al.*, 2013; Du Toit, 2018). Despite their adaptability, Zulu sheep are endangered, with the breeding rams' numbers left not known with only 48 semen straws cryopreserved (FAO-DAD-IS, 2020) hence Cryo-gene bank is required urgently. Other studies on Zulu sheep revealed that they suffer from inbreeding depression (Kunene *et al.*, 2009;

2011; Chella *et al.*, 2017; Selepe *et al.*, 2018) due to uncontrolled extensive rearing (Mavule *et al.*, 2016).

Genetic improvement of endangered species and the control of diseases affecting their conservation programmes and production are vital (Bailey *et al.*, 2000) and can be accomplished through Advanced Reproductive biotechnologies (ART) (Palacín *et al.*, 2012). For example, using Artificial Insemination (AI), one ejaculate from a superior ram can impregnate multiple females depending on the dilution ratio, semen quality and sperm concentration (Paulenz *et al.*, 2002), limiting physical contacts, spread of contagious diseases and introducing superior genotypes (Kershaw *et al.*, 2005). Besides that, other ART such as semen cryopreservation facilitates long-

term semen storage for proper transportation over distance locations thus enabling the use of superior sires even after death (Albiaty *et al.*, 2016).

Nevertheless, AI success depends on the spermatozoa ability to survive frozen-thawed stress (Peruma *et al.*, 2013) and the male fertility produced semen (Susilawati *et al.*, 2020). On other hand, semen cryopreservation has many challenges in ovine compare to bovine (Watson, 2000). To mention few, its leads to irreversible loss of motility (40-50%) and DNA integrity even with the optimised freezing protocols (D'alessandro and Martemuci, 2003) resulting to low pregnant rate (25-35%) with cervical AI (Anel *et al.*, 2005) or even 5-15% with vaginal AI (Cseh *et al.*, 2012).

Alternatively, ram semen can and has been stored in a liquid form at different storage temperatures ranging from 4°C (Kasimanickam *et al.*, 2007; 2011), 5°C (Purdy, 2006; Purdy *et al.*, 2010) and 15°C (Yániz *et al.*, 2005; 2011). However, when stored at 4°C and 5°C permanent Cryo-damage has been observed due to the detrimental effects associated with cold shock (Gheller *et al.*, 2018) targeting the plasma membrane (Bailey *et al.*, 2000; Mortimer and Maxwell, 2004; Aitken and Nixon, 2013) hence failing to retain motility and lose fertility especially after 24 h (O'Hara *et al.*, 2010). On other hand, (Yániz *et al.*, 2010) revealed high metabolic activities when spermatozoa stored at 15°C resulting to the short lifespan not exceeding 24 h (Purdy *et al.*, 2010).

No extensive studies on Zulu rams' semen parameters stored at 10°C and assessed by Sperm Class Analyser® (SCA) until this study so far. Likewise, no published study available addressing the preservation of Zulu sheep genetic resources using ART and preserved up to 72 h in both breeding and non-breeding season. Research published by (Chella *et al.*, 2017) on Zulu sheep, covers the semen variation based on age, season in the fresh semen, with motility assessed subjectively. Therefore, it is vital to identify the seasonal variations in semen parameters from Zulu rams using objective semen evaluation methods such as Computer-Aided Sperm Analysis (CASA). This would favour the use of superior ram for successful cervical AI in Zulu sheep which will in turn improve *in-vivo* conservation program and yield comparable results to the sophisticated laparoscopic AI.

Materials and Methods

Study Ethics Approval

The Agricultural Research Council (ARC) and Tshwane University of Technology animal research ethics committee approved all study procedures with reference number: APIEC16/034 and AREC2018/03/004, respectively. The South African National Department of Agriculture, Land Reform and Rural Development (DALRRD) granted the section 20 of the Animal Disease Act (Act no. 35 of 1984).

Study Area

The study site was the Agricultural Research Council (ARC) Irene, small stock unit and Germplasm, Conservation and Reproduction Biotechnologies (GCRB) laboratory. The Agricultural Research Council area extends between 25°53'59.6'' South latitude and 28°12'51.6'' East longitudes. The ARC, Irene area situated in the Highveld at an altitude of 1525 m above sea level. According to (Grobbelaar *et al.*, 2010), weather condition at ARC Irene ranges between hot days and cool nights in summer (17.5°C to 32°C) with moderate winter days and cold nights (1°C to 17°C).

Study Animals

Zulu rams ($n = 6$, age = 3 years, weight = 35 kg ± 2.29) were used in this study. These rams were part of the Zulu sheep conservation program formed between the South African National Department of Agriculture, Land Reform and Rural Development (DALRRD) and Agricultural Research Council (ARC), Irene. All rams were raised at the ARC Irene. Initially (preliminary study), eighteen rams (average weight = ± 35 kg 2.29 and age = 3 years) were selected from the base population, divided into the groups of 6 and kept at the camps with ± 4 m² shelter to provide shades. Rams were taken cared for and fed balanced diet for maintenance as described by (NRC, 1985). Same diet was provided in both breeding and non-breeding season, consisting of free access to *Eragrostis curvula* hay and 400g of pellets per ram/day. Pellets were purchased from Meadow feeds with the nutrient composition of protein 15, moisture 12, fat 2.5, fiber 12, calcium 1.08, phosphorus 0.5, potassium 0.8 magnesium 0.08, sodium 1.1, chloride 1.7, sulphur 0.35%.

Famacha was conducted to detect internal parasite infections as described by (Ferreira *et al.*, 2019), prior the commencement of semen collection. In case when internal parasites were suspected, Lintex-1 [Bayer South Africa, Registration number G447 (Act 36/1947)] was dosed orally, subjected to the Famacha score.

Semen Collection

Semen was collected with the aid of Artificial Vagina (AV) using estrogenized ewes as stimulus. These ewes ($n = 4$, aged = 3 years, average weight = ± 35 kg) were estrous synchronized using Controlled Intravaginal Drug Released (CIDR) as described by (Ungerfeld and Rubianes, 2012) with few amendments. In brief, controlled intravaginal drug released was inserted into the vagina for seven days. On the 6th day, injection site was allocated and disinfected with the spirit to eliminate bacteria and 0.5 mL estrumate was injected subcutaneously.

During semen collection, rams were free from hunger and thirst, pain, injury and diseases, fear, distress and discomfort. Consequently, all expressed normal behaviour throughout the trial. However, in case where animal showing mild discomfort signs, was placed in the handling pen and habituated. Only experienced personnel were allowed to handle the animals.

Rams were given an adaptation period of ± 10 days to human handling, using an ear massage every morning, precisely at a similar time every day (9 h00). After 5 days of habituation, all rams showed signs of comfort and calmness by allowing hand feeding and masticating during ear massage. Thereafter, a training to serve AV begun. All rams responded to AV training within three days and were advanced to the semen collection step. Three semen samples were collected during the preliminary study with the aid of artificial vagina and 2 days of resting period between collections was maintained. Semen was thereafter analyzed using Sperm Class Analysis (CASA). Only those produced semen with $>90\%$ total motility were considered for the experiment to limit individual variation. Those failed preliminary study tests were taken back to the base population. Hence, the final experimental sample size was six rams.

Semen Storage

Unless stated, all chemical products were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Steinem, Germany). The diluents were prepared using Sabax purified water (Adcock Ingram, South Africa PTY LTD). Commercial extender (Tris-based) was used for semen dilution and preservation. This was prepared on weekly basis and stored at 5°C (Hafez and Hafez, 2000; Salamon and Maxwell, 2000). Before use in the semen collection day, it was warmed up using warm plate adjusted to 37°C . The Tris-based extender was composed of Tris 1.211 g, citric acid 0.68 g, monohydrate glucose 0.5 g, sterile water 40 mL, egg yolk 10 mL and gentamycin sulphate 0.05 g and dissolved in 50 mL Falcon tube as described by (Salamon and Maxwell, 2000). For sperm washing solution, (dilution) egg yolk was not included. Tris-based extender was added to the semen at a ratio of 1:2 before storing at 10°C incubator [MCO-20 AIC Sanyo® CO₂ incubator (Sanyo, Japan)]. Semen quality analysis was performed at fresh 0h and after 24, 48 and 72 h.

Semen Evaluation

Two hundred and eighty-eight ejaculates were collected during breeding season and another two hundred and eighty-eight ejaculates during non-breeding season. These gave the total of five hundred and seventy-six ejaculates for the whole study. Late summer to autumn (May to July) was considered as a breeding season while

late winter to spring (September to October) taken as a non-breeding season. Semen was evaluated as macroscopic (semen pH, volume), microscopic (sperm concentration, morphology and motility).

Macroscopic Semen Evaluation

Semen pH was measured using Oakton pH meter (Eutech Instruments Pte Ltd/Oakton Instrument, CyberScan pH 11/110). Semen volume was measured in milliliters (mL) using the graduation marks in the AV lining glass tube before dilution.

Microscopic Semen Evaluation

Spermatozoa concentration was evaluated with the use of grey Beckam spectrophotometer (Jenway 6310, Spectrophotometer, Bibby Scientific, England) as described by (Brito *et al.*, 2016). Spermatozoa morphology and viability was examined using eosin-nigrosin stain (Onderstepoort Faculty of Veterinary Science' Pharmacy, South Africa). Stain was pre-warmed in the warm plate to 37°C for about 5 min before mixed with semen. For smear, 20 μL of stain was pipetted to Eppendorf tube and mixed with 5 μL of semen. Five μL of mixed semen and stain was pipetted and placed near the labelled end of the microscope slide (76 \times 26 \times 1 mm, Germany). The second microscope slide was held at 45° angle to the slide with the mixture of stain and semen. Thereafter was pulled gently without destroying spermatozoa as described by (Dolatpanah *et al.*, 2008) across the slide. Smear microscope slides were air dried for one day before examination. In the following day, 30 μm of oil immersion was pitted to the dried smeared slide and place under fluorescent microscope (Olympus BX 51TF, Japan), magnification (UPlanFLN 100 \times /1.30) for morphology analysis. The spermatozoa morphology was recorded as abnormal mid-piece, loose head and tail, cytoplasmic droplets, bent and coiled tail (Bester *et al.*, 2004). In each smeared slide, at least 200 cells were counted and percentage of mentioned defects was calculated and recorded on the Microsoft® Excel sheet.

Sperm motility was analyzed using Computer-Aided Sperm Analysis (CASA), Sperm Class Analyzer® system (SCA) 5.0 version (Microptic, Barcelona, Spain) at a magnification of 10 \times (Nikon, Japan). Sperm motility parameters evaluated were sperm progressions (progressive, non-progressive, total, slow, medium, rapid and static motility), sperm velocities (Curvilinear Velocity (VCL), Straight-Line Velocity (VSL) and average path Velocity (VSL)) and sperm velocity ratios (Linearity (LIN), Straightness (STR) and Wobble (WOB)). Careful precautions were taken to eliminate debris from egg yolk particles, known to affect CASA precisions (Holt *et al.*, 2007). Five microliters of semen were diluted with one hundred

microliters of extender (SWS) using Eppendorf tube. Thereafter, five microliters of the mixture were pipette and place in the warmed microscope slide (76×26×1 mm, Germany) and gently enclosed with cover slip (22×22 mm, Germany). Enclosed slide was placed over a warm plate (Omron) adjusted to 37°C, under the CASA camera at the magnification of 10× Ph1 BM. For each sample, three fields were randomly selected containing 100 to 200 spermatozoa and examined. Generated data was saved in the Microsoft Excel® sheet.

Statistical Analysis

Data was analyzed using General Linear Model (GLM) in Minitab 17® wherein the season (breeding and non-breeding), storage period and their interactions were fitted as independent variables. Means were compared using Fisher's LSD method and considered significantly when $p < 0.05$.

Results

Data on the macroscopic semen analysis results are set out in Table 1. Semen volume (mL) during breeding season was higher (0.97 ± 0.25 mL) than the non-breeding season (0.72 ± 0.5 mL). Non-breeding season recorded high (6.83 ± 0.54) semen pH than breeding season (6.54 ± 0.98). Spermatozoa concentration did not differ ($p > 0.05$) among seasons.

Semen was further evaluated as fresh in both breeding and non-breeding season hence the effect of season on semen quality of Zulu rams are shown in Table 2. The breeding season in this study showed high total motility and rapid motility (91.30 ± 0.70 and $55.38 \pm 1.05\%$) than non-breeding season (81.06 ± 0.81 and $34.34 \pm 1.05\%$), respectively. Moreover, breeding season recorded high non-progressive motility ($69.12 \pm 0.81\%$) than non-breeding season ($38.24 \pm 0.81\%$). Whereas high progressive motility was attained during non-breeding ($42.82 \pm 0.82\%$) than breeding season ($22.19 \pm 0.82\%$). Nevertheless, breeding season and non-breeding did not differ ($P > 0.05$) for slow motility however, breeding season had high curvilinear velocity (VCL) (137.78 ± 1.57 ms⁻¹) and average path velocity (VAP) (79.57 ± 1.21 ms⁻¹) than non-breeding season (111.52 ± 1.57 ms⁻¹ and 71.53 ± 1.21 ms⁻¹) respectively. Notwithstanding, straight-line velocity (VSP) could not differ significantly ($p > 0.05$) between the seasons.

Spermatozoa viability and abnormalities parameters results are shown in Table 3. Live spermatozoa were higher ($79.44 \pm 1.66\%$) during breeding season than non-breeding season ($72.33 \pm 0.99\%$), with dead spermatozoa showing the opposite direction. Loose head and coiled tail were significant greater ($21.23 \pm 1.64\%$; $19.56 \pm 1.60\%$) during breeding season than non-breeding season ($15.81 \pm 1.33\%$; $10.70 \pm 0.75\%$). Nevertheless,

breeding season did not differ ($p > 0.05$) to non-breeding season for mid-piece abnormality, distal droplet, bent tail and reacted acrosome.

The mean (\pm SE) GLM summary on the influence of storage temperature (10°C) on spermatozoa progression motility, velocities and velocity ratios kept for 72 h are set out in Table 4. Progressive motility was higher ($52.18 \pm 1.15\%$) at 0h than 24, 48 and 72 h ($36.09 \pm 1.15\%$, $22.83 \pm 1.15\%$ and $18.92 \pm 1.15\%$), respectively. Non-progressive motility increased drastically from 0, 24 and 48 h (42.92 ± 1.14 , 54.74 ± 1.14 and $57.92 \pm 1.14\%$), respectively, though, non-significant different ($p > 0.05$) was observed between 48 and 72 h for non-progressive motility. Total motility was higher at 0 h ($95.10 \pm 0.99\%$) but significantly declined at 24 h ($90.83 \pm 0.99\%$) even more at 48h ($80.75 \pm 0.99\%$) however, 48 h ($80.75 \pm 0.99\%$) did not differ significantly ($p > 0.05$) with 72 h ($78.04 \pm 0.99\%$).

No differences ($p > 0.05$) observed for VCL between 0h (135.66 ± 2.22 ms⁻¹) and 24h (130.91 ± 2.22 ms⁻¹) and between 48 h (117.37 ± 2.22 ms⁻¹) and 72 h (114.65 ± 2.22 ms⁻¹) but 24 h and 48 h differed significantly ($p < 0.05$), losing $\pm 10\%$ between 24 h (130.91 ± 2.22 ms⁻¹) and 48 h (117.37 ± 2.22 ms⁻¹). Straight-line velocity (82.13 ± 1.51 , 55.36 ± 1.51 , 36.46 ± 1.51 ms⁻¹ and 32.10 ± 1.51 ms⁻¹) and average path velocity (103.93 ± 1.71 ms⁻¹, 79.38 ± 1.71 ms⁻¹, 61.42 ± 1.71 ms⁻¹ and 57.47 ± 1.71 ms⁻¹) shrank gradually from 0, 24, 48 and 72 h, respectively. However, VAP could not differ ($p < 0.05$) between 48 and 72 h. Velocity ratios such as Linearity (LIN), Straightness (STR) and Wobble (WOB) followed analogous trend, where all considerably dropped from 0, 24, 48 and 72 h (Table 4). However, Wobble (WOB) could not differ significantly between 48 h ($52.62 \pm 0.72\%$) and 72 h ($50.87 \pm 0.72\%$) on the semen kept at 10°C.

Data on the interaction between seasons (Breeding and non-breeding season) and storage period (0 h, 24 h, 48h and 72 h) are found in Table 5. Total motility during breeding season was higher (92.97 ± 1.40 , 86.68 ± 1.40 and $88.56 \pm 1.40\%$) at 24, 48 and 72 h than non-breeding season (88.69 ± 1.40 , 74.83 ± 1.40 and $67.52 \pm 1.40\%$), whereas could not differ significantly ($p > 0.05$) at 0 h.

Progressive motility was greater during non-breeding season (59.09 ± 1.63 ; 50.86 ± 1.63 ; 33.77 ± 1.63 ; $27.56 \pm 1.63\%$) than the breeding season (45.26 ± 1.63 ; 21.32 ± 1.63 ; 11.89 ± 1.63 ; $10.29 \pm 1.63\%$) at all storage periods (0, 24, 48 and 72 h), respectively. Despite lesser spermatozoa motility during breeding season, spermatozoa during breeding season was moving at a rapid motility or speed (64.22 ± 2.10 ; 59.85 ± 2.10 ; 48.81 ± 2.10 ; $48.63 \pm 2.10\%$) than non-breeding season (48.12 ± 2.10 ; 39.57 ± 2.10 ; 26.59 ± 2.10 ; $23.08 \pm 2.10\%$) at all storage periods (0, 24, 48 and 72 h), respectively.

Curvilinear velocity was greater during breeding season (147.14 ± 3.14 ms⁻¹; 147.55 ± 3.14 ms⁻¹;

129±3.14 ms⁻¹ and 127.40±3.14 ms⁻¹) at 0, 24, 48 and 72h than non-breeding season (124.19±3.14 ms⁻¹; 114.27±3.14 ms⁻¹; 105.70±3.14 ms⁻¹ and 101.90±3.14 ms⁻¹), respectively. Straight-line velocity during breeding season (92.46±2.14 ms⁻¹) was higher at 0h than non-breeding (71.79±2.14 ms⁻¹).

Nevertheless, VSL was lesser at 24h during breeding season (51.41±2.14 ms⁻¹) than non-breeding season (59.32±2.14 ms⁻¹). Nevertheless, VSL could not differ (p>0.05) at 48 h and 72 h between seasons (breeding and non-breeding). Average path velocity was higher at 0h during breeding season (114.82±2.42 ms⁻¹) than non-breeding season (93.04±2.42 ms⁻¹), however did not

differ (p> 0.05) at 24 and 48h among seasons. At 72 h, VAP was greater during breeding season (61.28±2.42 ms⁻¹) than non-breeding season (53.65±2.42 ms⁻¹). Velocity ratios (LIN, STR and WOB) followed alike trend where breeding season performed better (62.81±1.15; 80.02±0.99 and 77.46±1.02) at 0 h than non-breeding season (53.63±1.15; 70.52±0.99 and 70.09±1.02%), respectively.

Thereafter, at 24, 48 and 72 h, LIN during non-breeding was higher (48.00±1.15; 37.25±1.15 and 33.21±1.15%) than the breeding season (35.80±1.15; 26.26±1.15 and 25.58±1.15%), respectively.

Table 1: Mean (±SE) Fresh Semen Evaluation During Breeding Season and Non-Breeding Season

Seasons	n of rams	Volume (mL)	Sperm Conc. ×10 ⁹ /mL	pH
Breeding season	6	0.97±0.25 ^a	5.29±0.36 ^a	6.54±0.98 ^b
Non-breeding season	6	0.72±0.5 ^b	5.00±0.37 ^a	6.83±0.54 ^a

^{ab} Column means with different superscripts differ significantly at p<0.05

Table 2: Mean (±SE) Effect of Seasons (Breeding and Non-Breeding Season) on Spermatozoa Motility Parameters from Fresh Zulu Rams' Semen

Parameters	Seasons	
	Breeding season	Non-Breeding season
Progressions (%)		
Progressive motility	22.19±0.82 ^b	42.82±0.82 ^a
Non-progressive motility	69.12±0.81 ^a	38.24±0.81 ^b
Total motility	91.30±0.70 ^a	81.06±0.81 ^b
Static	8.70±0.70 ^b	18.96±0.81 ^a
Rapid motility	55.38±1.05 ^a	34.34±1.05 ^b
Medium motility	9.15±0.65 ^b	20.38±0.65 ^a
Slow motility	26.46±0.69 ^a	26.34±0.69 ^a
Velocities (ms ⁻¹)		
VCL	137.78±1.57 ^a	111.52±1.57 ^b
VSP	52.50±1.07 ^a	50.53±1.07 ^a
VAP	79.57±1.21 ^a	71.53±1.21 ^b
Velocity ratios (%)		
LIN	37.43±0.57 ^b	43.02±0.57 ^a
STR	62.53±0.49 ^b	64.06±0.49 ^a
WOB	57.14±0.51 ^b	61.44±0.51 ^a

^{ab}Row means with different superscripts differ significantly at p<0.05

Table 3: Spermatozoa morphology parameters (mean ±S. E) from fresh Zulu rams' semen

Spermatozoa morphology	Seasons	
	Breeding season	Non-breeding season
Viability (%)		
Live spermatozoa	79.44±1.66 ^a	72.33±0.99 ^b
Dead spermatozoa	20.56±1.66 ^b	27.67±0.99 ^a
Abnormalities (%)		
Loose head	21.23±1.64 ^a	15.81±1.33 ^b
Loose tail	11.65±1.43 ^a	11.56±0.75 ^a
Mid-piece abnormality	10.06±1.02 ^a	11.98±0.89 ^a
Acrosome damage	7.69±0.73 ^a	6.15±0.45 ^a
Distal droplets	10.02±0.80 ^a	8.48±0.60 ^a
Bent tail	14.25±1.33 ^b	30.23±3.14 ^a
Coiled tail	19.56±1.60 ^a	10.70±0.75 ^b
Reacted acrosome	5.54±0.42 ^a	5.15±0.37 ^a

^{ab}Means that do not share a letter within a row differ significantly (p<0.05)

Table 4: Mean (\pm SE) influence of storage temperature (10°C) on spermatozoa progressions, velocities and velocity ratios from the semen kept until 72h

Parameters	Storage h at 10°C			
	0 H	24 H	48 H	72 H
Progressions (%)				
Progressive motility	52.18 \pm 1.15 ^a	36.09 \pm 1.15 ^b	22.83 \pm 1.15 ^c	18.92 \pm 1.15 ^d
Non-progressive motility	42.92 \pm 1.14 ^c	54.74 \pm 1.14 ^b	57.92 \pm 1.14 ^a	59.12 \pm 1.14 ^a
Total motility	95.10 \pm 0.99 ^a	90.83 \pm 0.99 ^b	80.75 \pm 0.99 ^c	78.04 \pm 0.99 ^c
Static	4.92 \pm 0.99 ^c	9.17 \pm 0.99 ^b	19.26 \pm 0.99 ^a	21.97 \pm 0.99 ^a
Rapid motility	56.17 \pm 1.48 ^a	49.71 \pm 1.48 ^b	37.70 \pm 1.48 ^c	35.85 \pm 1.48 ^c
Medium motility	15.17 \pm 0.92 ^{ab}	16.54 \pm 0.92 ^a	14.96 \pm 0.92 ^{ab}	13.12 \pm 0.92 ^b
Slow motility	23.76 \pm 0.97 ^b	24.67 \pm 0.97 ^b	28.10 \pm 0.97 ^a	29.08 \pm 0.97 ^a
Velocities (ms ⁻¹)				
VCL	135.66 \pm 2.22 ^a	130.91 \pm 2.22 ^a	117.37 \pm 2.22 ^b	114.65 \pm 2.22 ^b
VSP	82.13 \pm 1.51 ^a	55.36 \pm 1.51 ^b	36.46 \pm 1.51 ^c	32.10 \pm 1.51 ^d
VAP	103.93 \pm 1.71 ^a	79.38 \pm 1.71 ^b	61.42 \pm 1.71 ^c	57.47 \pm 1.71 ^c
Velocity ratios (%)				
LIN	58.22 \pm 0.81 ^a	41.54 \pm 0.81 ^b	31.56 \pm 0.81 ^c	29.40 \pm 0.81 ^d
STR	75.67 \pm 0.70 ^a	65.97 \pm 0.70 ^b	57.21 \pm 0.70 ^c	54.73 \pm 0.70 ^d
WOB	73.78 \pm 0.72 ^a	59.89 \pm 0.72 ^b	52.62 \pm 0.72 ^c	50.87 \pm 0.72 ^c

^{abcd}Row means with different superscripts differ significantly at p<0.05

Table 5: Mean (\pm SE) seasons (Breeding and non-breeding season) and storage period (0 h, 24 h, 48 h and 72 h) effect on spermatozoa motility kept at 10°C

Parameters	Seasons							
	Breeding Season				Non-Breeding Season			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Progressions (%)								
Progressive Motility	45.26 \pm 1.63 ^c	21.32 \pm 1.63 ^f	11.89 \pm 1.63 ^g	10.29 \pm 1.63 ^g	59.09 \pm 1.63 ^a	50.86 \pm 1.63 ^b	33.77 \pm 1.63 ^d	27.56 \pm 1.63 ^e
Non-progressive motility	51.75 \pm 1.61 ^c	71.65 \pm 1.61 ^b	74.79 \pm 1.61 ^{ab}	78.28 \pm 1.61 ^a	34.10 \pm 1.61 ^e	37.83 \pm 1.61 ^{de}	41.05 \pm 1.61 ^d	39.9 \pm 1.61 ^d
Total motility	97.01 \pm 1.40 ^a	92.97 \pm 1.40 ^b	86.68 \pm 1.40 ^c	88.56 \pm 1.40 ^c	93.13 \pm 1.40 ^{ab}	88.69 \pm 1.40 ^c	74.83 \pm 1.40 ^d	67.52 \pm 1.40 ^e
Static	3.01 \pm 1.40 ^e	7.03 \pm 1.40 ^d	13.32 \pm 1.40 ^c	11.44 \pm 1.40 ^c	6.83 \pm 1.40 ^e	11.31 \pm 1.40 ^c	24.20 \pm 1.40 ^b	32.50 \pm 1.40 ^a
Rapid motility	64.22 \pm 2.10 ^a	59.85 \pm 2.10 ^a	48.81 \pm 2.10 ^b	48.63 \pm 2.10 ^b	48.12 \pm 2.10 ^b	39.57 \pm 2.10 ^c	26.59 \pm 2.10 ^d	23.08 \pm 2.10 ^d
Medium motility	10.63 \pm 1.29 ^d	8.82 \pm 1.29 ^d	8.72 \pm 1.29 ^d	9.87 \pm 1.29 ^d	19.70 \pm 1.29 ^{bc}	24.27 \pm 1.29 ^a	21.20 \pm 1.29 ^{ab}	16.36 \pm 1.29 ^c
Slow motility	22.16 \pm 1.38 ^d	24.47 \pm 1.38 ^d	29.16 \pm 1.38 ^{ab}	30.07 \pm 1.38 ^a	25.35 \pm 1.38 ^{bcd}	24.88 \pm 1.38 ^{cd}	27.03 \pm 1.38 ^{abc}	28.08 \pm 1.38 ^{abc}
Velocities (ms ⁻¹)								
VCL	147.14 \pm 3.14 ^a	147.55 \pm 3.14 ^a	129.04 \pm 3.14 ^b	127.40 \pm 3.14 ^b	124.19 \pm 3.14 ^b	114.27 \pm 3.14 ^c	105.70 \pm 3.14 ^{cd}	101.90 \pm 3.14 ^d
VSL	92.46 \pm 2.14 ^a	51.41 \pm 2.14 ^d	33.73 \pm 2.14 ^{ef}	32.40 \pm 2.14 ^f	71.79 \pm 2.14 ^b	59.32 \pm 2.14 ^c	39.19 \pm 2.14 ^e	31.80 \pm 2.14 ^f
VAP	114.82 \pm 2.42 ^a	79.75 \pm 2.42 ^c	62.44 \pm 2.42 ^d	61.28 \pm 2.42 ^d	93.04 \pm 2.42 ^b	79.01 \pm 2.42 ^c	60.40 \pm 2.42 ^d	53.65 \pm 2.42 ^e
Velocity ratios (%)								
LIN	62.81 \pm 1.15 ^a	35.80 \pm 1.15 ^e	26.26 \pm 1.15 ^f	25.58 \pm 1.15 ^f	53.63 \pm 1.15 ^b	48.00 \pm 1.15 ^c	37.25 \pm 1.15 ^d	33.21 \pm 1.15 ^e
STR	80.02 \pm 0.99 ^a	63.66 \pm 0.99 ^c	53.79 \pm 0.99 ^f	52.64 \pm 0.99 ^f	70.52 \pm 0.99 ^b	68.27 \pm 0.99 ^b	60.63 \pm 0.99 ^d	56.82 \pm 0.99 ^e
WOB	77.46 \pm 1.02 ^a	54.28 \pm 1.02 ^{de}	48.41 \pm 1.02 ^f	48.39 \pm 1.02 ^f	70.09 \pm 1.02 ^b	65.49 \pm 1.02 ^b	56.83 \pm 1.02 ^d	53.36 \pm 1.02 ^e

^{abcdefg} Row means with different superscripts differ significantly at P < 0.05

Discussion

Semen volume obtained in the current study fell within a normal range in both seasons. Ram semen volume normally ranges from 0.5 to 2.0 mL (Karagiannidis *et al.*, 2000a; Hafez and Hafez, 2000) but highly affected by the collection frequency, season (Karagiannidis *et al.*, 2000b), collection technique (Bopape *et al.*, 2015), nutrition (Guan *et al.*, 2014) and age (Chella *et al.*, 2017). In temperate zones, photoperiod is the main factor defining the onset of breeding season (Pourseif and Moghaddam, 2012). Consequently, the evaluation of the fertilizing potential of a semen sample has been the utmost objective of

semen analysis to estimate the fertility results of a future artificial insemination (Del Olmo *et al.*, 2013). High semen volume obtained in the present study during breeding season was expected and in line with numerous studies (Barkawi *et al.*, 2006; Talebi *et al.*, 2009; Wang *et al.*, 2015; Chella *et al.*, 2017; Kulaksiz *et al.*, 2019). This is because, testicle volume shrink during non-breeding season and are highly affected by the heat stress (Hedia *et al.*, 2019). Seminiferous tubules during breeding season occupies the majority of the testicular tissues (76.6%) and the spermatic layers (Barkawi *et al.*, 2006; Kadili *et al.*, 2019). Therefore, low semen volume obtained during non-breeding season is an indication of the positive correlation between semen volume and

testicles size (Sarlós *et al.*, 2013). Despite that, semen volume obtained in this study during non-breeding season fell within the normal range as reported previously (Karagiannidis *et al.*, 2000a; Hafez and Hafez, 2000; Chella *et al.*, 2017). Moreover, there was high differences among rams (data not shown) that was comparable to that reported by (Benia *et al.*, 2018).

Semen pH is an exogenous factor influencing spermatozoa motility and its imbalances reduce spermatozoa motility, viability and capacitation (Hafez and Hafez, 2000). In this study, semen pH was higher ($p < 0.05$) during non-breeding season than breeding season. According to (Chella *et al.*, 2017) semen pH in Zulu rams' semen ideally ranges from 5.9 to 7.3. Moreover, (Al-Anazi *et al.*, 2017), reported that semen pH in Naimi and Najdi rams was somewhat alkaline and lower during autumn than spring. Nevertheless, contradicted to (Talebi *et al.*, 2009; Wang *et al.*, 2015), where they found semen pH to not differ ($p > 0.05$) throughout the year. However, this might due to the delays in semen processing after collection leading to acidic semen pH and the degradation of fructose by the sperm cells (Zamiri *et al.*, 2010).

Ram sperm concentration ideally ranges from 3, 5×10^9 to 6, 0×10^9 sperm/ml (Hafez and Hafez, 2000). In the current study, spermatozoa concentration could not differ ($p > 0.05$) among seasons (breeding and non-breeding season). Benia *et al.* (2018) obtained similar results in Ouled-Djellal rams. However, other authors (Barkawi *et al.*, 2006; Gündoğan, 2007; Azawi and Ismael, 2012; Golher *et al.*, 2018; Hedia *et al.*, 2019) reported huge seasonal variation on spermatozoa concentration, with breeding season recording higher concentration than non-breeding season with winter yielding lesser sperm concentration (Wang *et al.*, 2015; Kadili *et al.*, 2019). Noteworthy, the sperm concentration from the current study was higher than that reported by (Chella *et al.*, 2017) in Zulu rams. Nevertheless, the effect pose by the environmental condition as well as the ability of each ram to respond to a particular environmental condition might explain these variations (Carvajal-Serna *et al.*, 2019).

Semen was further evaluated for fresh spermatozoa motility in both breeding and non-breeding season. High total motility and rapid motility during breeding season than non-breeding season in the current study was expected in line to numerous studies (Gündoğan, 2007; Badi *et al.*, 2018; Kadili *et al.*, 2019). This is because semen quality is highly affected by testicular volume (Gündoğan, 2007) as mentioned above and scrotal circumference (Badi *et al.*, 2018). Regardless of that, the effect of season in semen quality has been reported even in stallions (Janett *et al.*, 2003), goats (Kumar *et al.*, 2014) and Bulls (Perumal *et al.*, 2017). Furthermore, although testosterone is not part of the current study, its high concentration during summer can explain these seasonal variations (Mallick *et al.*,

2016). Ntemka *et al.* (2019) reported contradicting results where no significant different for sperm motility was declared between breeding and non-breeding season (Kulaksiz *et al.*, 2019). Nevertheless, (Kulaksiz and Sen, 2019) reported high sperm motility and freezing-thawed survival during non-breeding season.

Breeding season recorded high non-progressive motility than non-breeding season. Whereas high progressive motility was attained during non-breeding than breeding season which were in line to (Kulaksiz and Sen, 2019). Previous studies reported seasonal variations of sperm quality numerous breeds ranging from United Arab Emirates crossbred rams (Ibrahim, 1997), Suffolk and Ile-de-France rams (Mandiki *et al.*, 1998), indigenous breeds of Greece (Karagiannidis *et al.*, 2000b) and Naimi and Najdi rams of Saudi Arabia (Al-Anazi *et al.*, 2017). Therefore, this high progressive motility obtained during non-breeding indicate that Zulu rams produce acceptable semen quality in both breeding and non-breeding season. Furthermore, although seminal plasma composition was not a part of the current study, its composition during non-breeding may explain these differences (Domínguez *et al.*, 2008) and has been reported to improve sperm frozen-thawed rate (Rickard *et al.*, 2014; Kumar *et al.*, 2014). Breeding season and non-breeding did not differ ($p > 0.05$) for slow motility though breeding season had high VCL and VAP than non-breeding season. Temperature-humidity index has significant effect on physiological, biochemical, hormonal and enzymatic indices of indigenous sheep (Rathwa *et al.*, 2017). Wobble spermatozoa was higher during non-breeding season than breeding season. Wobble is a comparison between average path and curvilinear (Mortimer, 1997). Therefore, spermatozoa collected during non-breeding season was moving unsteadily side to side.

Spermatozoa morphology and viability evaluation are ordered as normal, abnormal and live spermatozoa based on the set standards for characteristics evaluated on the constant scales: Loose head, loose tail, mid-piece, acrosome damage, distal droplets, bent tail and reacted acrosome (Björndahl, 2010). Live spermatozoa are vital to bypass cervix folds through crypt folds and to survive the selection and bio-fluid from the ewe reproductive tract (Fair *et al.*, 2019; Rickard and de Graaf, 2020). It was not surprising to find high live spermatozoa during breeding season than non-breeding season, with the dead spermatozoa in the opposite direction in the current study. However, (Kadili *et al.*, 2019) found high spermatozoa viability during non-breeding season than breeding season. Normal spermatozoa have a significant role in different ways. For example, sperm head is covered by the membranous sac contain nucleus and involved in transporting the DNA to the egg (Barbas and Mascarenhas, 2009). The membranous sac delivers the

hydrolytic enzymes such as acrosin and hyaluronidase are released by exocytosis during fertilization to aid the spermatozoon to bind with zona pellucida during the so-called process of acrosome reaction. Conversely, loose head and coiled tails in this study were higher during breeding season than non-breeding season. These results were controversial to (Karagiannidis *et al.*, 2000b; Barkawi *et al.*, 2006; Francis *et al.*, 2019).

No significant different ($p>0.05$) observed for mid-piece abnormality, distal droplet, bent tail and reacted acrosome in this study. This implies that, irrespective of season, spermatozoa produced by Zulu rams are normal and the rams producing superior semen quality throughout the season. Ram that produce good quality semen throughout the year can be utilized for AI throughout the year (Al-Anazi *et al.*, 2017) without hormone supplementation, to reduce costs of breeding. The sperm mid-piece is an energy power station containing mitochondria which is responsible for energy generation through a highly specialized Adenosine Triphosphate (ATP) (Barbas and Mascarenhas, 2009). As a result, it was remarkable to find non-significant different ($p>0.05$) for distal droplets in both seasons because retaining residual cytoplasm droplet correlate with Reactive Oxygen Species (ROS) that are normally produced by the glucose-6-phosphate dehydrogenase enzyme (Agarwal *et al.*, 2003). There is an association between the membrane fatty acid profile and the acrosome reaction as well sperm-oocyte fusion (De Vriese and Christophe, 2003). Reacted acrosome could not differ among seasons. Therefore, non-significant different observed in the current study could mean that the sperm-oocyte fusion may not be affected by the season in Zulu sheep.

On the other hand, there was a noticeable gradual decrease of spermatozoa quality when the storage h' increases. For instance, after 24 h progressive motility numerically decreased by 16.09, 29.35% after 48 h and 33.26% at 72 h. These results were comparable to (Mata-Campuzano *et al.*, 2015) and implies that, after 24 h spermatozoa are barely retaining motility hence, it is not recommended to inseminate with semen kept for more than 24 h (O'Hara *et al.*, 2010) especial using cervical AI (Anel *et al.*, 2005). D'alessandro and Martemuci (2003), evaluated the seasonal effects on semen freezability and found that the highest post-thawed survival was during summer and autumn (breeding season) due to seminal plasma protective effect (Domínguez *et al.*, 2008). However, liquid stored semen represents an inexpensive alternative way to preserve endangered breeds through semen transfer from research stations to different farms for artificial insemination (Benmoula *et al.*, 2017) than frozen state (Maxwell *et al.*, 2007). In this regard, spermatozoa motility is a vital parameter extensively used to predict fertility

(Hirano *et al.*, 2001; Broekhuijse *et al.*, 2012) after freezing and thawing (D'Alessandro and Martemuci, 2003). However, it is highly susceptible to the environmental changing condition such as excessive heat and or cold shock (Hafez and Hafez, 2000). Furthermore, have high metabolic activities after semen collection at the body temperature (37°C) hence, have less lifespan when not diluted (Salamon and Maxwell, 2000) and samples analysis should take place immediately after collection (Amann and Weberski, 2014). Therefore, in the current study it was expected that the progressive motility would be higher at 0 h than 24, 48 and 72 h. However, following semen-chilling, spermatozoa metabolic activities are suspended to prolong lifespan and activated after thawing (Salamon and Maxwell, 2000).

Non-progressive motility is inversely proportional to progressive motility (Holt *et al.*, 2007). Therefore, when progressive motility decrease gradually as reported in the current study, non-progressive motility increase automatically at a similar pace. Hence, the increase of non-progressive motility as the storage period increases in the current study was determined by the increase of progressive motility and was expected.

The sum of progressive motility and non-progressive contributes to the total motility (Holt *et al.*, 2007). In this study, total motility was greater at 0 h but steadily decreased as the storage period increases. These results were comparable to (Yániz *et al.*, 2005; Sinha *et al.*, 2019) where sperm motility and live sperm declined as preservation period increases. Nonetheless, contradicted to (Purdy, 2006; Purdy *et al.*, 2010) on ram semen held at 5°C for 24 and 48 h prior cryopreservation, respectively. Numerically, Zulu ram semen in this study loosed 5% of total motility after 24 h of storage which is acceptable for cervical AI. Nevertheless, the ability of the spermatozoa to move through the cervical passage and mucus after natural mating or AI depends on rapid motility (Björndahl, 2010). Spermatozoa at 0 h was moving at relatively rapid motility (56.17 ± 1.48) but prominently diminished as storage period increases to 24, 48 and 72 h though did not differ ($p>0.05$) between 48 and 72 h. These results were comparable to those reported by (Wang *et al.*, 2015). Menchaca *et al.* (2005) obtained 43 and 35% motility when semen was stored on a tris based extender for 12 and 24 h respectively, at 5°C. In ram semen preserved for 48 h in MOPS titrated buffer, total motility did not differ from 0 h until 48 h. However, in tris based extender, drastically decline was observed (Yániz *et al.*, 2011). Therefore, it might happen that, the decrease of motility (rapid and total motility) observed in the current study after 24 h was due to the less protecting effect from tris based extender (Albiaty *et al.*, 2016). However, the effect extender might have in semen stored at 10°C still need more attention.

Curvilinear velocity in the current study was always higher than VSL and VAP though did not differ ($p>0.05$) between 0 h and 24 h as well between 48 h and 72 h. However, VCL at 24 h was higher than 48 h. These results were in line to (Yániz *et al.*, 2011), where VCL in semen extended with tris based extender did not differ from 0 h until 24 h. The distance spermatozoa transit along its curvilinear path is called curvilinear velocity (Mortimer, 1997). Curvilinear velocity is a product from the sum of the distances along trajectory and corrected time. When male fertility is not compromised, curvilinear should be high followed by the average path velocity and the lowest being a straight-line velocity (Verstegen *et al.*, 2002). Conversely, when male fertility is compromised, curvilinear tend to be a lowest and straight-line velocity value becomes the highest (Mortimer, 1997). The development of three velocity ratios allows advance explanations of the sperm trajectory (Mortimer, 1997; Holt *et al.*, 2007). Spermatozoa velocity motions determines the velocity ratios and always expressed in the percentage form.

There is a close relationship between the Computer-Aided Sperm Analysis (CASA) motility (total and rapid motility) and in vitro fertilization rate in human (Hirano *et al.*, 2001; Verstegen *et al.*, 2002) and pigs (Hirai *et al.*, 2001). Moreover, spermatozoa motility in sheep is associated with in vivo fertility and influence Artificial Insemination (AI) results (David *et al.*, 2015). In the current study, total and rapid motility were higher during breeding season than non-breeding season. These results contradicted to (Benia *et al.*, 2018) nonetheless, the method use might cause the differences since they used subjective method but were in line to (Kadili *et al.*, 2019) who observed high total motility during autumn than winter in Beni Arous bucks. Other authors reported a seasonal effect on sheep reproduction efficiency where semen quality decreased (Zaragaza *et al.*, 2009), depending on the latitude of origin (Carvajal-Serna *et al.*, 2019). However, humidity and ambient temperature might cause negative effects (Ntemka *et al.*, 2019).

Progressive motility determines the fertilizing ability of the sperm cell even after cryopreservation (Del Olmo *et al.*, 2013). Progressive motility during non-breeding season differed ($p<0.05$) from the one during breeding season and so does with static and medium motility. These results were comparable to (Benmoula *et al.*, 2017) in INRA180 rams, (Nitharwal *et al.*, 2017), in Murrah buffalo, (Kadili *et al.*, 2019) in Beni Arous bucks. Although fertility evaluation was out of the scope, these results might imply that, high spermatozoa population from non-breeding season will be able to pass cervix better than those from breeding season, due to the selection pressure cervix apply to the spermatozoa (Rickard *et al.*, 2019; Rickard and de Graaf, 2020),

selecting only viable spermatozoa with high progressive motility (Sakkas *et al.*, 2015).

Only CASA parameters determines the velocity spermatozoa (VSL, VCL and VAP) (Hirai *et al.*, 2001) and provide valuable physiological status of the spermatozoa (Broekhuijse *et al.*, 2011). Spermatozoa velocities (VCL and VAP) are higher during breeding season (Kadili *et al.*, 2019; Wang *et al.*, 2015). Moreover, Sperm Velocities (VCL and VSL) can be used to determine fertile and infertile males (Hirai *et al.*, 2001). In the current study, VCL and VAP was higher during breeding season than non-breeding season. Therefore, semen collected from breeding season can be regarded as more superior to the one during non-breeding season. Although STR does indicator fertilization outcome in human being (Hirano *et al.*, 2001), it did not differ ($p>0.05$) in the current study. The effect of season on semen parameters from several ram breeds have been previous reported (Chella *et al.*, 2017; Benmoula *et al.*, 2017; Badi *et al.*, 2018). However, the present study is the first study reporting the seasonal variation in semen parameters from Zulu rams, assessed objectively and preserved at 10°C for up to 72 h.

Conclusion

These findings showed that semen parameters from Zulu rams does vary with season. Breeding season have better semen quality than non-breeding season. However, the differences observed cannot prevent Zulu rams from breeding during non-breeding season. Therefore, based on these results Zulu rams' can be classified as all year around breeders but studies involving fertility are still needed to verify these findings. On other hand, these results disclosed that preserving semen at 10°C result in a measly reduction of quality in both breeding and non-breeding seasons especially, when preservation period exceed 24 h until 48 h after that remain stable until 72 h. Therefore, Zulu ram semen stored at 10°C can retain quality until 72 h however, based on the spermatozoa quality obtained it is advisable to use the semen before storage period exceed 24 h in both seasons. Nevertheless, more studies are needed to test fertility and to look at the effect female may have when bred during non-breeding season and consider the feed effect when the lambing season take place during dry periods.

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Authors' Contribution

Jabulani Nkululeko Ngcobo: Designed the study, collected and analyzed the data, as well wrote the manuscript.

Tshimangadzo Lucky Nedambale: Designed the study and revised the manuscript.

Khathutshelo Agree Nephawe: Designed the study and revised the manuscript.

Ayanda Maqhashu: Designed the study and revised the manuscript.

Ethics

This article is the original work containing unpublished material. The corresponding would like to confirm that all authors read and approved the final manuscript.

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