Ostrich ejaculate characteristics and male libido around equinox and solstice dates

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Abstract

The study evaluated the effect of time of the year in which changes in photoperiod occurs on ostrich semen characteristics and male libido. Semen was collected for five days before, on and five days after winter solstice (21st June 2016), spring equinox (22nd September 2016), summer solstice (21st December 2016) and autumn equinox (20th March 2017) in the southern hemisphere. Semen was collected from 10 South African Black ostrich males (average age ± standard deviation: 4.5 ± 2.27 years) using the dummy female. Semen volume, sperm concentration, total sperm per ejaculate, sperm motility traits, percentage of normal sperm, head and tail abnormalities and percentage of affected sperm in the hypo-osmotic swelling test (HOS) were evaluated. Male libido defined as the willingness of males to mount the dummy female was also recorded. Semen samples collected around summer solstice, spring and autumn equinox were higher in sperm concentration and sperm output compared to winter solstice ($P < 0.05$). Study periods did not influence semen volume, sperm motility traits, the percentage of normal sperm, head abnormalities and HOS. Tail abnormalities were higher around winter solstice than around spring equinox ($P < 0.05$). Male libido and the success of semen collection were significantly higher around spring equinox ($P < 0.05$). Changes in photoperiod in the southern hemisphere do not affect semen production in ostriches. However, high sperm output and male libido around spring equinox

and summer solstice dates suggest that these periods may be preferred for semen collection for artificial insemination and storage purposes.

Keywords: Artificial insemination, Photoperiod, Seasonal variation, Semen quality, Semen production, *Struthio camelus*

Introduction

Commercial ostrich farming has been practiced in South Africa for more than 150 years and has gained interest worldwide (Cloete et al. 2012). Although great strides have been made in the development of ostrich farming, reproductive performance is still challenged by low fertility (Lambrechts et al. 2004). Assisted reproductive technologies such as artificial insemination (AI) have been suggested as a potential breeding tool to alleviate fertility challenges (Malecki et al. 2008). This technology has been used successfully in the livestock (dairy and pig) and poultry industries as well as for the conservation of endangered wildlife species (Foote 2001; Blanco et al. 2009). Studies on commercially farmed ostriches have indicated that selection of birds with desirable behaviour (i.e. a lack of fear and/or aggression; Bonato et al. 2013), allows the training of ostrich females for routine artificial insemination using voluntary crouching behaviour (Rybnik-Trzaskowska 2009; Bonato and Cloete 2013). It is also possible to train males for routine, stress-free semen collection using the dummy female method (Rybnik et al. 2007; Bonato et al. 2012). Another prerequisite for a successful AI programme is a flock of females that would lay in the absence of males, while showing desirable behavioural attributes. Bonato et al. (2017) provided evidence that this objective has also largely been met. Hence, the development of these techniques has demonstrated the need to characterise the ostrich ejaculates and established a protocol for optimum semen preservation (Rybnik et al. 2007; Malecki et al. 2008; Bonato et al. 2010; Smith et al. 2018). However, substantial research on male and female ostrich reproductive performance is still required for this technology to be successfully implemented in the ostrich industry.

Ostriches have been described as seasonal breeders that increase their reproductive activity along with increasing photoperiod, but also as opportunistic breeders, relying on food availability (Degen et al. 1994; Bronneberg et al. 2007). In birds, seasonal variation in photoperiod plays a significant role in reproductive activities as photoperiod changes can stimulate the secretion of gonadotropin releasing hormone responsible for development of the gonads and sexual behaviour (Johnson 2015; Vizcarra et al. 2015). Some bird species may cease gamete production during a certain time of the year due to the lack of synthesis of the gonadotropin

stimulating hormone because they do not respond to photoperiod due to them becoming photorefractory (Dawson and Sharp 2007). Within a commercial farming setting this may influence the quality and quantity of semen produced and potentially impact on fertility. For example, emus (*Dromaius novaehollandiae*), another commercially exploited ratite displayed a strong seasonal breeding activity in winter and a complete cessation of sperm production in spring and summer (Malecki and Martin 2000).

Previous studies on ostriches have highlighted that traits such as sperm output, sperm viability, morphology and motility as well as male libido appear to be affected by factors such as season, male age and the frequency of collections (Rybnik et al. 2012; Bonato et al. 2011, 2014). These studies have however used a small number of individual animals or a subjective method to measure sperm motility, which may have produced less accurate results than using more recent objective computer assisted methods (Mocé and Graham 2008; Smith et al. 2016; Farooq et al. 2017, 2018). Smith et al. (2016) recently reported sperm motility characteristics evaluated by computer-aided sperm analysis as well as sperm membrane integrity during the conventional ostrich breeding season (May to December). No information on the above parameters is however available beyond confines of the standard breeding season (January-April). Therefore, the aim of this study was to evaluate the potential effect of time of year during which changes in photoperiod occur on ostrich ejaculate characteristics and male libido. It was hypothesised that semen production in ostriches will vary between times when seasonal changes do occur (solstice and equinox) and that ejaculate characteristics and male libido will be affected.

Materials and methods

Study area and animals sampled

The study was conducted at the Oudtshoorn Research Farm of the Western Cape Department of Agriculture, situated outside Oudtshoorn, South Africa (33°63′ S, 22°25′ E). A total of 10 South African Black (SAB) male ostriches aged between 3 and 10 years, kept in individual paddocks and trained for semen collection using the dummy female method, were used (Rybnik et al. 2007). The males were selected based on their desirable behavioural responses such as willingness to approach and associate with human, no fear of human and an absence of aggression towards humans (Bonato et al. 2013) and their cooperation during semen collection. Briefly, the dummy female method required male ostriches to direct their sexual behaviour towards humans. While the male was at display, the dummy female was pushed gently between its legs for it to mount, and ejaculate into a fitted artificial cloaca (Rybnik et al. 2007). Throughout the entire experiment, the birds were fed

an ostrich breeder diet (10.99 MJ/kg and 180.9 g/kg protein on as-fed basis) formulated at the research farm and fresh water was available *ad libitum.*

Semen collection and measurements

Semen samples were collected once a day for five days around solstice and equinox dates (five days before a season ended and five days after a new season commenced and on the day the change occurred: N = 11 days). The study periods under which semen was collected were as follow: 21st June 2016 (winter solstice), 22nd September 2016 (spring equinox), 21st December 2016 (summer solstice) and 20th March 2017 (autumn equinox). These dates corresponded to the transition of shorter to longer days in winter and from longer days to shorter days in summer and the period of maximum change in sunshine hours during autumn and spring in the southern hemisphere.

Semen volume was measured using an automatic pipette, while sperm concentration was determined by means of a spectrophotometer (Spectrawave, WPA, S800, Biochrom), using a 20 µL aliquot of semen diluted 1:400 (v/v) with a phosphate buffered saline solution containing 10% formalin. The total number of sperm per semen sample was then calculated by multiplying semen volume and sperm concentration.

Evaluation of sperm motility

Sperm motility traits were measured using the Sperm Class Analyzer® (SCA) version 5.3 (Microptic S.L., Barcelona, Spain) with a Basler A312fc digital camera (Basler AG, Ahrensburg, Germany) mounted on an Olympus BX41 microscope (Olympus Optical Co., Tokyo, Japan) equipped with phase contrast optics. An aliquot of neat semen at a concentration of 20 × 10^6 sperm cells/mL was diluted in 245 µL of standard motility buffer containing sodium chloride (150 mM) and TES (20 mM) with 2% male specific seminal plasma (Smith et al. 2016). The tube containing the diluted semen was placed in an incubator at 38°C for 1 min, before loading 2 µl of the diluent onto a pre-warmed slide (at 38°C), covered gently with a cover slip (22 × 22 mm) and allowed to settle for 20 s prior to recording. At least 300 clearly presented motile sperm tracks per semen sample were recorded from random fields. Sperm motility traits evaluated were progressive motility (PMOT, %), total motility (MOT, %), curve-linear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), straightness (STR, %), amplitude of lateral head displacement (ALH, µm), wobble (WOB, %) and beat cross frequency (BCF, Hz).

Sperm morphology

The procedure described by Du Plessis et al. (2014) was used to evaluate sperm morphology. Briefly, an aliquot from each ejaculate was fixed in 2.5% glutaraldehyde in 0.13 Millonig's phosphate buffer, pH 7.4 until used. Semen smears were prepared from the fixed samples, air-dried for 24 h and stained with Wrights' stain (Rapidiff®, Clinical Sciences Diagnostics, Johannesburg, South Africa). The number of normal sperm (filiform in appearance) and sperm with defects (defects classified into head or tail defects) were determined by counting 300 sperm cells per slide from each semen sample with an Olympus BX63 light microscope (Olympus Corporation, Tokyo, Japan) using a 100x oil immersion objective (phase-contrast illumination). The specific defects associated with the head and tail has previously been described and illustrated (Du Plessis et al. 2014).

Evaluation of the sperm membrane integrity

The hypo-osmotic swelling test (HOS) was used to evaluate sperm membrane integrity (Jeyendran et al. 1992). An aliquot of neat semen at a concentration of 20×10^6 sperm cells/mL was diluted in 250 µL of a standard saline solution ($NaCl/H_2O$) adjusted to 25 mOsm (Smith et al. 2016). After dilution, 2 µl of the semen diluent was loaded onto a pre-warmed slide (at 38°C), covered gently with a glass cover slip (22×22 mm) and allowed to settle for 20 s prior to recording. At least 300 clearly presented sperm per semen sample were recorded in random fields and the proportion of swollen sperm (Fig. 1) indicating HOS was calculated. The recording was performed using the software and hardware described for sperm motility assessment.

Evaluation of male libido and success of semen collections

Male libido was defined as the willingness to mount the dummy female as described by Bonato et al. (2011) on a scale from 0 to 3 (0 = no reaction; 1 = approach with interest but no willingness to mount; 2 = no courtship but willingness to mount the dummy; 3 = courtship and willingness to mount the dummy). The number of attempts to successfully collect semen was recorded ranging from 1 to 3 attempts. The success of semen collection was determined binomially (where: 1 = did collect and 0 = did not collect) and the reaction time (defined as the time spent by males during copulation) was recorded in seconds.

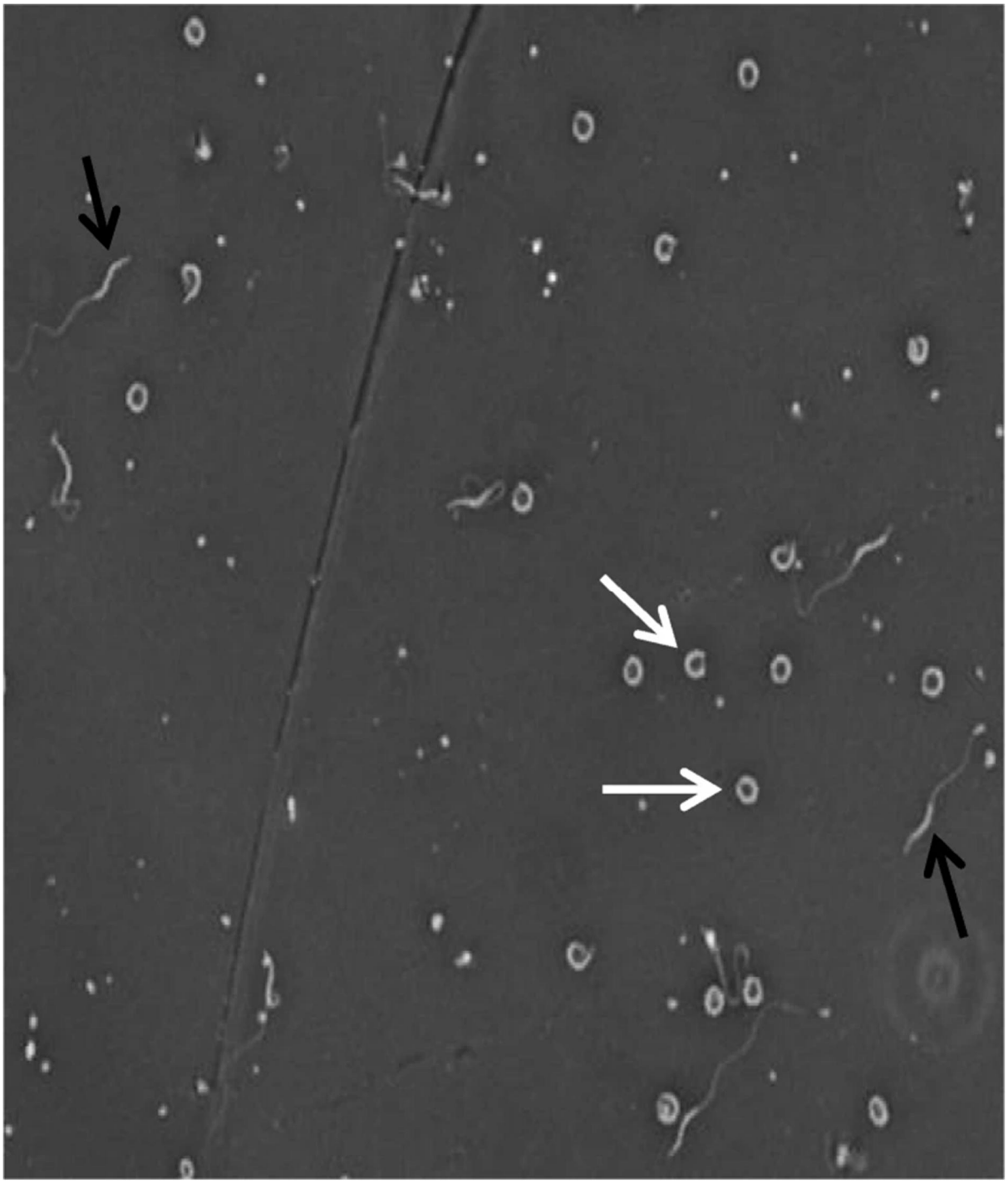

Figure 1. Ostrich sperm under hypo-osmotic stress. 'Round' shapes are the swollen sperm (pointed by the white arrows) and indicate live sperm with an intact membrane, responsive to the hypo-osmotic solution, while straight sperm (pointed by the black arrows) indicate sperm with damaged (compromised) membrane that ruptured when in the hypo-osmotic solution

Statistical analysis

The data were analysed using SAS, version 9.3 (SAS 2012). The Generalized Linear Mixed Models (GLMM) procedure was performed on the data with the study periods and male age as independent variables. Male age was divided in two categories, young and old, to facilitate the analysis. Males of 3 and 4 years old (7 males) were classified as young males while males older than 4 years (3 males) were classified as old males. This categorization allowed the analysis of the interaction between male age and semen collection periods. The number of days semen was collected was entered as a linear covariate. Dependent variables were semen volume, sperm concentration, number of sperm per ejaculate, sperm motility traits, sperm morphology (normal, overall sperm abnormalities, head and tail abnormalities) traits, HOS and reaction time to collect semen. Sperm concentration was entered as a linear covariate in the analysis when ejaculate characteristics were analyzed. Relationships among sperm traits were depicted by Pearson's correlation coefficients. A GLMM with an ordered multinomial distribution model with a cumulative logit link function was performed on male libido (0-3) and the number of attempts to collect ejaculates (1-3). A GLMM with a binary distribution and a logit link function was performed for ejaculate collection success (1: collected; 0: did not collect). Male identity was entered as a random variable in all the models to account for repeated measurements on the same male. In a different analysis, male identity was entered as an independent variable to evaluate the differences among males. Variables were considered significantly different at $P < 0.05$. Tukey's pairwise comparisons were used to test for significant different.

Results

Descriptive statistics for semen characteristics and male responses to semen collection

The results for the variables across the four studied periods were reported as means and standard errors (mean ± SE). The mean ejaculate volume was 1.16 ± 0.04 mL, with a concentration of 2.08 ± 0.05 × 10^9 sperm/mL and a mean sperm count of 2.53 ± 0.12 × 10^9 sperm per ejaculate. The mean values for the sperm motility traits were: PMOT = 40.01 ± 0.93%, MOT = 87.85 ± 1.07%, VCL = 66.79 ± 0.89μm/s; VSL = 32.36 ± 0.53μm/s; VAP = 52.14 ± 0.87μm/s, ALH = 2.54 ± 0.03μm, LIN = 48.07 ± 0.46%, STR = 62.38 ± 0.52%, WOB = 76.73 ± 0.48%, BCF = 7.12 ± 0.07Hz. The mean percentages of normal sperm, abnormal sperm, head and tail abnormalities and HOS affected sperm were 90.00 ± 0.46%, 9.43 ± 0.54%, 3.81 ± 0.35% and 5.32 ± 0.26% and 72.10 ± 1.21%, respectively. The means for male libido score, number of attempts to collect semen, and reaction time amounted

to 2.64 ± 0.03, 1.73 ± 0.04 and 52.98 ± 0.94s. The rate of successful collection of semen amounted to 75.68%. The incidence of aspermia (failure to produce semen after completing the copulation process) represented 8% of all successful collections.

Effect of study collection periods on ejaculate characteristics

Ejaculate volume was not influenced by the study period (Table 1). A higher sperm concentration was recorded in ejaculates collected around autumn and spring equinox, and summer solstice, while the winter solstice reflected the lowest sperm concentration ($P < 0.001$; Table 1). The number of sperm per ejaculate was higher around the autumn and spring equinox, and summer solstice, while samples collected in winter had the lowest number of sperm per ejaculate ($P = 0.004$; Table 1). Study period did not have an effect on any sperm motility trait (Table 2). Male age did not have an effect on semen characteristics; however, a significant interaction between study period and male age was recorded for BCF ($P < 0.05$). Ejaculates collected from younger males (< 4 years old) around the autumn equinox had a lower BCF (6.61 ± 0.23 Hz) than ejaculates of males of the same age collected around spring equinox (7.51 ± 0.18 Hz). The percentages of normal sperm, overall abnormal sperm, head abnormalities and HOS sperm were not affected by study periods (Table 3), but tail abnormalities were higher around the winter solstice when compared to data collected around the spring equinox ($P = 0.011$; Table 3). No significant interactions were recorded in these traits between study periods and male age.

Table 1 Least square means and standard errors (± SE) of ejaculate volume, sperm concentration and number of sperm per ejaculate during the study periods. Semen was collected from 10 South African Black ostrich males around solstice and equinox dates

Variables	Autumn equinox	Winter solstice	Spring equinox	Summer solstice	P-value
Ejaculate volume (mL)	1.20 ± 0.24	1.20 ± 0.22	1.16 ± 0.22	1.06 ± 0.23	0.575
Sperm concentration ($\times 10^9$/mL)	2.51 ± 0.18^b	1.24 ± 0.15^a	2.28 ± 0.15^b	2.42 ± 0.16^b	<0.001
Number of sperm ($\times 10^9$)	2.60 ± 0.51^b	1.59 ± 0.44^a	2.79 ± 0.43^b	2.78 ± 0.46^b	0.004

^{a,b} Least square means with different superscript within a row differ significantly ($P < 0.05$)

Table 2 Least square means (±SE) for sperm motility traits during the study periods. Semen was collected from 10 South African Black ostrich males around solstice and equinox dates

Variables	Autumn equinox	Winter solstice	Spring equinox	Summer solstice	P-value
ALH (μm)	2.63 ± 0.10	2.74 ± 0.10	2.54 ± 0.06	2.37 ± 0.08	0.341
BCF (Hz)	6.74 ± 0.27	7.69 ± 0.27	7.12 ± 0.16	6.82 ± 0.21	0.275
LIN (%)	46.51 ± 1.74	50.09 ± 1.77	48.81 ± 1.09	45.38 ± 1.38	0.542
MOT (%)	88.43 ± 4.03	85.13 ± 4.07	88.43 ± 2.57	86.12 ± 3.21	0.547
PMOT (%)	45.53 ± 3.62	37.16 ± 3.63	39.58 ± 2.40	36.90 ± 2.94	0.087
STR (%)	60.13 ± 1.99	65.47 ± 2.00	61.63 ± 1.30	59.45 ± 1.60	0.813
VAP (μm/s)	56.54 ± 3.22	48.96 ± 3.25	52.84 ± 2.06	50.80 ± 2.58	0.584
VCL (μm/s)	71.37 ± 3.28	63.58 ± 3.33	67.26 ± 2.05	65.12 ± 2.59	0.396
VSL (μm/s)	33.91 ± 2.02	32.15 ± 2.03	32.22 ± 1.30	30.61 ± 1.62	0.377
WOB (%)	77.09 ± 1.86	75.97 ± 1.86	77.64 ± 1.27	75.51 ± 1.46	0.466

ALH- amplitude of lateral head displacement; BCF- beat cross frequency; LIN- linearity; MOT- total motility; PMOT- progressive motility; STR- straightness; VAP- average path velocity; VCL- curve-linear velocity; VSL- straight-line velocity; WOB- wobble

Table 3 Least square means (±SE) for the percentage of normal sperm, overall abnormal sperm, head and tail abnormalities, hypo-osmotic swelling test (HOS) affected sperm, as well as male libido, number of attempts to collect semen and reaction time to ejaculate around the study periods. Male libido was measured as the willingness to mount the dummy female and scored on a scale from 0 to 3 (0: no reaction; 1: approach with interest but no willingness to mount; 2: no courtship but willingness to mount the dummy; 3: courtship and willingness to mount the dummy). Number of attempts to successfully collect semen was measured on a range from 1 to 3 attempts. Reaction time represents the time taken by males during the process of copulation. Semen was collected from 10 South African Black ostrich males around solstice and equinox dates

Variables	Autumn equinox	Winter solstice	Spring equinox	Summer solstice	P-value
Normal sperm (%)	89.38 ± 3.03	89.94 ± 3.03	90.27 ± 2.92	88.83 ± 2.97	0.082
Abnormal sperm (%)	10.47 ± 3.17	9.83 ± 3.18	9.57 ± 2.92	11.24 ± 3.02	0.211
Head abnormality (%)	4.45 ± 2.62	2.50 ± 2.64	4.19 ± 2.38	4.61 ± 2.48	0.653
Tail abnormality (%)	5.95 ± 1.20^{ab}	7.31 ± 1.20^{b}	5.38 ± 1.05^{a}	6.63 ± 1.05^{ab}	0.011
HOS (%)	74.78 ± 5.05	68.12 ± 4.49	64.78 ± 4.32	67.06 ± 4.62	0.277
Male libido	1.97 ± 0.13^{a}	2.65 ± 0.13^{b}	2.92 ± 0.13^{c}	2.67 ± 0.13^{b}	<0.001
Number of attempts to collect	2.24 ± 0.19^{c}	1.63 ± 0.19^{b}	1.35 ± 0.19^{a}	1.83 ± 0.19^{b}	<0.001
Reaction time (s)	56.09 ± 5.34	47.45 ± 5.05	50.22 ± 5.00	56.73 ± 5.10	0.373

[a,b,c] Least square means with different superscript within a row differ significantly ($P < 0.05$)

Effect of study periods on male libido, number of attempts to collect semen, success of semen collection and reaction time to ejaculate

Male libido scores were highest around the spring equinox compared to the autumn equinox, as well as winter and summer solstice, while libido scores around the summer and winter solstices were higher than scores around autumn equinox ($P < 0.001$; Table 3). However, the libido scores around the summer and winter solstices did not differ (Table 3). Consequently, the number of attempts to collect semen was high around the autumn equinox compared to the other study periods, while attempts to collect semen around the summer and winter solstice were higher than attempts around the spring equinox ($P < 0.001$; Table 3). The number of attempts to collect semen around summer solstice and winter solstice did not differ. Semen collection success rate was high ($P = 0.005$) around spring equinox (86.36%) compared to the success rate around summer solstice (73.63%) and autumn equinox (56.36%), but did not differ from the success rate around the winter solstice (86.36%). Moreover, the success rate for collecting semen was higher around the summer and winter solstices compared to around the autumn equinox. Success rate around the winter solstice was also higher than the summer solstice ($P < 0.001$). Male age did not have an effect on libido scores, reaction time to collect semen and number of attempts to successfully collect semen. Study period or the interaction between study period and male age did not influence the reaction time to ejaculate. However, a significant interaction between study period and male age was recorded for male libido and the number of attempts to collect semen ($P < 0.05$). Around the autumn solstice, younger males had higher libido scores (2.62 ± 0.15) than older males (1.33 ± 0.22). Likewise, more attempts to collect semen were performed on older males (2.67 ± 0.32) than on younger males (1.81 ± 0.21) for that specific study period.

Relationship among sperm motility traits

There was a significant and positive correlation between PMOT and all other sperm motility traits ($P < 0.0001$; Table 4), except for STR. All sperm velocity traits recorded significant positive correlations among each other ($P < 0.001$), except for VCL vs STR and VAP vs STR where significant negative correlations were recorded ($P < 0.001$), and STR vs WOB were not correlated (Table 4).

Table 4 Pearson's correlation coefficients among sperm motility traits collected from 10 South African Black ostrich males around solstice and equinox dates. Sperm motility traits measured were: progressive motility (PMOT), motility (MOT), curve linear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN), amplitude of lateral head displacement (ALH), straightness (STR), wobble (WOB) and beat cross frequency (BCF)

Sperm traits	PMOT (%)	MOT (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	ALH (μm)	LIN (%)	STR (%)	WOB (%)	BCF (Hz)
PMOT (%)										
MOT (%)	0.78***									
VCL (μm/s)	0.91***	0.75***								
VSL (μm/s)	0.89***	0.65***	0.85***							
VAP (μm/s)	0.89***	0.70***	0.97***	0.88***						
ALH (μm)	0.42***	0.49***	0.42***	0.33***	0.31***					
LIN (%)	0.37***	0.23***	0.21***	0.65***	0.30***	0.14*				
STR (%)	-0.00	-0.07	-0.21***	0.22***	-0.20***	0.15***	0.76***			
WOB (%)	0.64***	0.54***	0.69***	0.78***	0.81***	0.15***	0.61***	0.06		
BCF (Hz)	0.19***	0.25***	0.12*	0.36***	0.13*	0.30***	0.63***	0.57***	0.34***	

*** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$

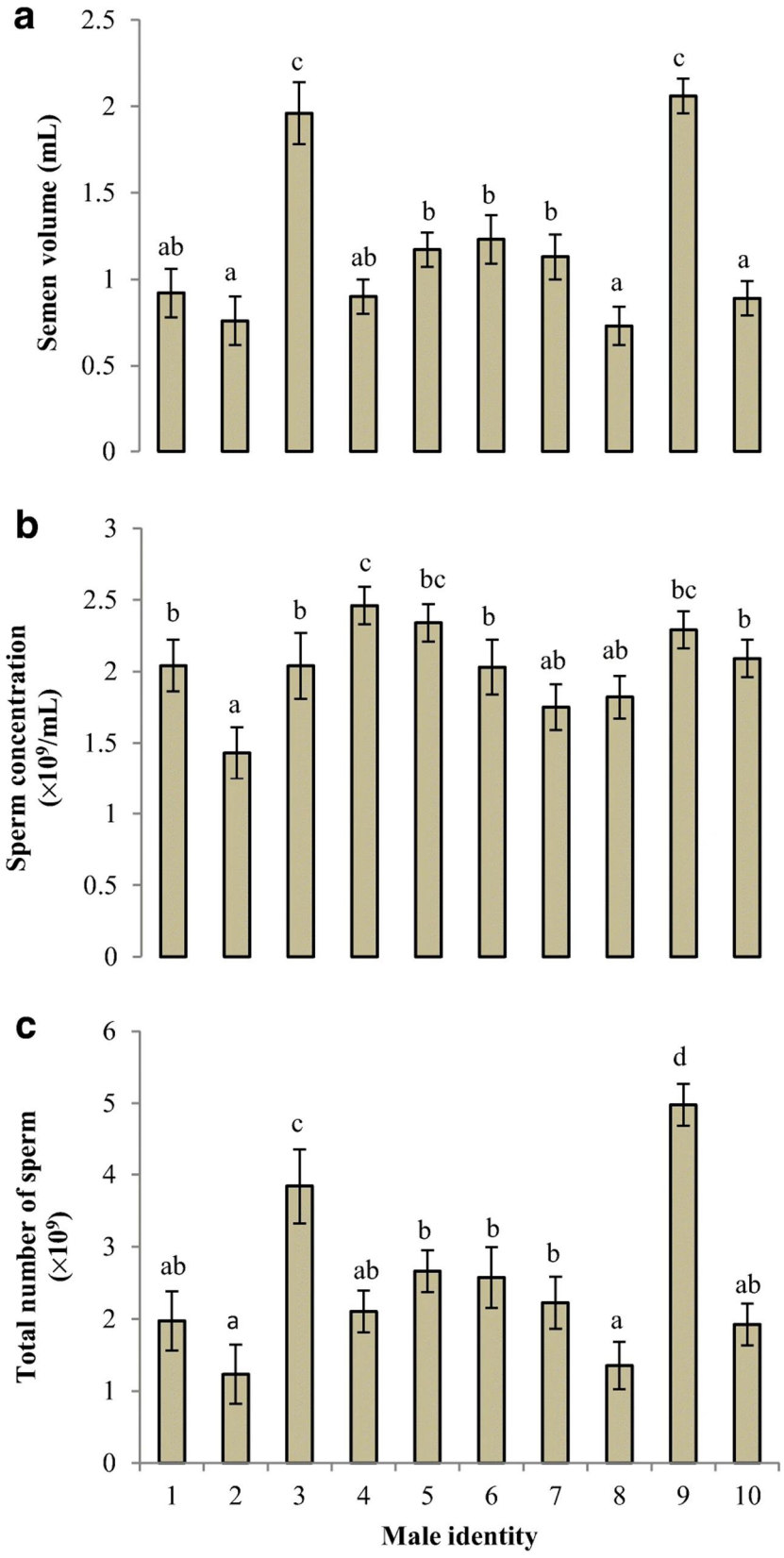

Figure 2. Means (± SE) depicting the effect of male identity on a) semen volume, b) sperm concentration and c) total number of sperm per ejaculate, as derived from 10 South African Black ostrich males around winter and summer solstice as well as autumn and spring equinox dates in the southern hemisphere

Table 5 Least square means (±SE) for sperm motility traits for 10 individual South African Black ostrich males around solstice and equinox dates. Sperm motility traits measured were: progressive motility (PMOT), motility (MOT), curve linear velocity (VCL), straight line velocity (VSL), average path, velocity (VAP), linearity (LIN), amplitude of lateral head displacement (ALH), straightness (STR), wobble (WOB) and beat cross frequency (BCF)

Male identity	PMOT (%)	MOT (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	ALH (μm)	STR (%)	WOB (%)	BCF (Hz)
1	35.68 ± 3.38^{ab}	88.33 ± 3.9^{ab}	62.94 ± 3.27^{ab}	31.46 ± 1.92^{abc}	49.97 ± 3.14^{ab}	49.74 ± 1.72^{ab}	2.45 ± 0.1	60.81 ± 1.94^{ab}	78.46 ± 1.72^{cb}	7.00 ± 0.28
2	26.60 ± 3.38^{a}	71.05 ± 3.90^{a}	53.15 ± 3.27^{ab}	26.16 ± 1.92^{a}	39.61 ± 3.14^{a}	45.47 ± 1.72^{ab}	2.35 ± 0.10	63.15 ± 1.94^{ab}	70.04 ± 1.72^{c}	6.55 ± 0.28
3	46.77 ± 4.29^{b}	94.18 ± 4.96^{b}	74.40 ± 4.15^{b}	36.46 ± 2.44^{abc}	60.74 ± 4.00^{b}	49.35 ± 2.18^{ab}	2.57 ± 0.12	60.87 ± 2.46^{ab}	81.38 ± 2.19^{c}	6.91 ± 0.35
4	40.79 ± 2.42^{b}	89.98 ± 2.79^{b}	69.46 ± 2.34^{b}	32.70 ± 1.37^{abc}	55.44 ± 2.25^{b}	47.07 ± 1.23^{ab}	2.55 ± 0.07	59.83 ± 1.39^{a}	78.97 ± 1.23^{cb}	7.04 ± 0.20
5	41.45 ± 2.39^{b}	94.23 ± 2.76^{b}	68.15 ± 2.31^{b}	32.03 ± 1.36^{abc}	51.87 ± 2.22^{ab}	46.78 ± 1.21^{ab}	2.62 ± 0.07	62.08 ± 1.37^{ab}	75.43 ± 1.22^{abc}	7.34 ± 0.20
6	41.76 ± 3.55^{ab}	86.14 ± 4.10^{ab}	65.75 ± 3.44^{ab}	33.24 ± 2.02^{abc}	48.12 ± 3.30^{ab}	49.66 ± 1.80^{ab}	2.67 ± 0.10	68.19 ± 2.04^{d}	72.05 ± 1.81^{bc}	7.71 ± 0.29
7	45.46 ± 3.03^{b}	89.11 ± 3.51^{b}	68.67 ± 2.94^{b}	33.43 ± 1.72^{abc}	55.28 ± 2.82^{b}	48.35 ± 1.54^{ab}	2.37 ± 0.09	61.11 ± 1.74^{ab}	78.67 ± 1.55^{ab}	7.07 ± 0.25
8	38.39 ± 2.78^{ab}	88.13 ± 3.21^{b}	66.45 ± 2.69^{ab}	31.42 ± 1.58^{abc}	49.61 ± 2.59^{ab}	47.08 ± 1.42^{ab}	2.71 ± 0.08	63.81 ± 1.42^{ab}	73.80 ± 1.42^{bc}	7.35 ± 0.23
9	45.01 ± 2.39^{b}	88.09 ± 2.76^{b}	70.29 ± 2.31^{b}	36.68 ± 1.36^{c}	56.95 ± 2.22^{b}	52.12 ± 1.21^{b}	2.53 ± 0.07	65.40 ± 1.37^{ab}	79.82 ± 1.22^{c}	7.31 ± 0.20
10	36.62 ± 2.39^{ab}	85.18 ± 2.76^{ab}	65.27 ± 2.31^{ab}	29.97 ± 1.36^{bc}	50.81 ± 2.22^{ab}	46.16 ± 1.21^{a}	2.49 ± 0.07	60.11 ± 1.37^{a}	76.91 ± 1.22^{ab}	6.84 ± 0.20
P-value	0.006	0.002	0.002	0.002	0.003	0.019	0.05	0.012	<0.001	0.118

^{a,b,c} Least square means with different superscript within a column differ significantly ($P < 0.05$)

Table 6 Least square means and standard errors (±SE) of percentage normal sperm, overall abnormal sperm, head and tail abnormality, hypo-osmotic swelling test (HOS), male libido, number of attempts to collect semen and reaction time to ejaculate around the study periods. Male libido was measured as the willingness to mount the dummy female and scored on a scale from 0 to 3 (0: no reaction; 1: approach with interest but no willingness to mount; 2: no courtship but willingness to mount the dummy; 3: courtship and willingness to mount the dummy). Number of attempts to successfully collect semen was measured on a range from 1 to 3 attempts. Reaction time represents the time taken by males during copulation. Semen was collected from 10 South African Black ostrich males around solstice and equinox dates

Male identity	Normal sperm (%)	Abnormal sperm (%)	Head abnormality (%)	Tail abnormality (%)	HOS (%)	Male libido	Attempts to collect	Reaction time to ejaculate (s)
1	94.32 ± 0.95^{de}	5.66 ± 1.48^{ab}	1.90 ± 0.59^{ab}	3.82 ± 0.74^{abc}	72.79 ± 4.41^{ab}	2.45 ± 0.08^{b}	2.18 ± 0.11^{b}	24.95 ± 2.70^{b}
2	90.70 ± 0.95^{cd}	8.34 ± 1.84^{abc}	2.73 ± 0.59^{ab}	6.18 ± 0.74^{cd}	62.22 ± 4.41^{a}	2.59 ± 0.08^{bc}	2.20 ± 0.11^{b}	81.76 ± 2.53^{c}
3	86.50 ± 1.21^{bc}	12.74 ± 1.89^{bc}	2.55 ± 0.75^{ab}	10.42 ± 0.94^{ef}	85.36 ± 5.61^{b}	1.73 ± 0.08^{a}	2.45 ± 0.11^{b}	43.23 ± 3.52^{a}
4	93.05 ± 0.68^{d}	7.19 ± 1.14^{ab}	3.74 ± 0.42^{b}	3.25 ± 0.53^{ab}	76.87 ± 3.15^{ab}	2.91 ± 0.08^{cd}	1.43 ± 0.11^{a}	55.14 ± 1.96^{ab}
5	96.35 ± 0.67^{e}	3.55 ± 1.06^{c}	1.75 ± 0.42^{a}	1.73 ± 0.52^{a}	73.94 ± 3.15^{ab}	2.98 ± 0.08^{d}	1.14 ± 0.11^{a}	56.88 ± 1.93^{b}
6	68.77 ± 0.97^{a}	31.15 ± 1.52^{d}	23.50 ± 0.61^{c}	7.74 ± 0.76^{de}	63.57 ± 4.52^{ab}	2.45 ± 0.08^{b}	2.30 ± 0.11^{b}	47.86 ± 2.87^{ab}
7	93.92 ± 0.87^{de}	6.57 ± 1.48^{ab}	2.59 ± 0.54^{ab}	3.53 ± 0.68^{abc}	80.03 ± 4.12^{ab}	2.93 ± 0.08^{cd}	1.30 ± 0.11^{a}	48.51 ± 1.98^{ab}
8	86.38 ± 0.8^{b}	13.55 ± 1.31^{c}	2.04 ± 0.49^{ab}	11.58 ± 0.62^{f}	67.93 ± 3.69^{ab}	2.41 ± 0.08^{b}	1.60 ± 0.11^{a}	55.67 ± 2.21^{ab}
9	91.93 ± 0.68^{d}	$9.92 \pm 1.08b^{c}$	2.15 ± 0.42^{ab}	5.92 ± 0.53^{cd}	71.21 ± 3.12^{ab}	2.95 ± 0.08^{cd}	1.27 ± 0.11^{a}	53.17 ± 1.96^{ab}
10	93.36 ± 0.67^{de}	6.63 ± 1.05^{ab}	2.09 ± 0.42^{ab}	4.55 ± 0.52^{bc}	69.43 ± 3.20^{ab}	2.98 ± 0.08^{d}	1.48 ± 0.11^{a}	52.04 ± 1.93^{ab}
P-value	<0.001	<0.001	<0.001	<0.001	0.009	<0.001	<0.001	<0.001

^{a,b,c,d,e,f} Least square means with different superscript within a column differ significantly ($P < 0.05$)

Effect of male on ejaculate characteristics, libido, number of attempts to collect semen and reaction time to ejaculate

Variation between males was recorded for semen volume ($P < 0.001$; Fig. 2a), sperm concentration ($P < 0.05$; Fig. 2b) total number of sperm per ejaculate ($P < 0.001$; Fig. 2c) and the sperm motility traits ($P < 0.05$; Table 5) except for ALH and BCF (Table 5). The percentages of normal or abnormal sperm, head and tail abnormalities and HOS sperm also differed between males ($P < 0.001$; Table 6). Variation between males was also recorded for libido, number of attempts to collect semen and reaction time to ejaculate ($P < 0.001$; Table 6). The incidence of aspermia was distributed evenly across study periods. One male had a high occurrence of aspermia of 36% while other males had no incidence recorded ($P < 0.05$).

Discussion

This study demonstrated that no variation in semen production around the time of the year when daylength changes occurs, suggesting that semen production in ostriches is not a seasonal trait. However, ejaculate characteristics and male libido were affected by the study periods. These findings corroborate the results of a previous study which revealed that male ostriches produce semen throughout the year (Bonato et al. 2014). Ecological studies on ostriches also indicate that ostriches are opportunistic breeders whose reproduction relies on forage quality and quantity (Cooper et al. 2010). Ejaculates collected around the summer solstice as well as spring and autumn equinoxes were highly concentrated and contained higher number of spermatozoa than those collected around the winter solstice. This finding is consistent with previous studies that reported that semen samples collected during the spring, summer and autumn months contain a higher sperm concentration than samples collected in winter (Bonato et al. 2014, Smith et al. 2016; 2018). This suggests that sperm output in ostriches may be influenced by a range of environmental variables such as daylength, rainfall and temperature (Ball and Ketterson 2008). Furthermore, an increase in the sperm output during this study periods may result from gonadal increase in size as stimulated by increasing photoperiod as reported in other bird species (Dixit and Singh 2011; 2020). Although the overall sperm concentration recorded in this study appear low compared to literature (Bonato et al. 2011; 2012; 2014, Smith et al. 2016), the sperm concentration value in this study was within the range recorded for ostriches (1.73-4.73 $\times 10^9$sperm cells/mL) as reported by Smith et al. (2016). Furthermore, a yearly variation in ostrich sperm concentration has also been observed (Bonato et al. 2014).

This study revealed that sperm motility traits did not vary across the study periods. This lack of differences between study periods is inconsistent with a previous study by Smith et al. (2016) on ostrich semen which reported higher PMOT, MOT and VAP in summer compared to spring and winter. The inconsistency between studies on sperm motility traits may be due to the fact that Smith et al. (2016) investigated the effect of an ostrich specific diluent on sperm traits, while the present study evaluated the quality of raw semen soon after collection, a critical variable for successful semen processing in AI protocols. Positive significant correlations were recorded between several sperm motility traits such as PMOT, MOT, sperm velocity traits (VAP, VCL and VSL) and LIN, WOB and BCF. These correlations are similar to what has been reported previously for ostriches and Japanese quail (Smith et al. 2016; Farooq et al. 2018). Such positive correlations are important since they disclose linked sperm motility traits. The lack of a significant correlation between PMOT and STR reported in this study confirms that sperm considered as progressive does not need to be swimming in a linear path. In that sense, sperm swimming in a large circular motion have been considered as progressive sperm in cockatiels (*Nymphicus hollandicus*; Fischer et al. 2014).

The present study could not report any effect of study periods on the percentage of normal sperm, overall sperm abnormalities, and head abnormalities. However, a higher incidence of tail abnormalities were recorded around winter solstice compared to other study periods. Even though sperm tail abnormalities varied with study periods, BCF, a measure of the frequency at which the sperm track crosses the mean path and the flagellar beat cycle around its longitudinal axis, was not affected (Hong et al. 1993). This might indicate that BCF was measured for the whole sperm specimen and not only for the flagellum section of the sperm (Hong et al. 1993; Chen et al. 1998). The HOS test, which evaluates the functional integrity of the sperm membrane under hypo-osmotic conditions (Jeyendran et al. 1992; Santiago-Moreno et al. 2009), revealed no variation in sperm membrane integrity during the study periods. This suggests that male ostriches produce good quality sperm around solstice and equinox times, which may still be effective in achieving fertilization. It is however noteworthy to mention that the study periods were composed of only five days around solstice and equinox when seasonal changes take place. Thus the seasonal variation in some variables reported in previous studies (Bonato et al. 2014; Smith et al. 2016) might indicate that the differences/changes in sperm characteristics may only become apparent as the season progressed.

Male libido was high around spring equinox, winter and summer solstice but decreased around autumn equinox. This result is consistent with previous studies on ostriches (Bonato et al. 2011, 2014). In addition, the number of

attempts to collect ejaculates was lower during the spring equinox, and the winter and summer solstice compared to the autumn equinox. This resulted in a higher rate of success in collecting semen during these three study periods. A lower libido around the autumn equinox may be attributed to photorefractoriness which is known to set in the long day breeding birds after the summer solstice (Dixit and Singh 2011). During photorefractoriness, a decrease in the synthesis of the hypothalamic gonadotropin-releasing hormone production is commonly observed, which results in lower testosterone production and consequently a lower male libido (Garamszegi et al. 2005; Dawson and Sharp 2007; Vizcarra et al. 2015). When the photorefractory birds are exposed to shorter daylength (around winter solstice in the southern hemisphere), photorefractoriness may dissipate rending the birds sensitive again to stimulatory effects increasing daylength (Dawson and Sharp 2007; Dixit and Singh 2011; 2020). The dissipation of photorefractoriness then stimulates the synthesis of the hypothalamic gonadotropin-releasing hormone which in turn leads to increase in testosterone production and therefore high male libido around winter solstice than the autumn equinox (Dawson and Sharp 2007; Dixit and Singh 2011; 2020). Lower libido indicated in the current study did however not lead to a longer reaction time prior to ejaculate into the artificial cloaca, which is in contrast to what was reported for emus (Malecki et al. 1997). However, the lower libido scores around the autumn equinox increased the number of attempts to collect semen at a lower success rate of 43%. This lower rate of collection success outside of the conventional breeding season (around the autumn equinox) reported in this study is consistent with experience on the red-winged tinamou (*Rhynchotus rufescens*) where semen collection success outside the breeding season was compromised (Paranzini et al. 2018). This highlight greater difficulty in obtaining ejaculates during autumn and implies that semen collection in ostriches should be restricted to periods when higher chances of collection success are maximized.

Contrary to previous studies (Rybnik et al. 2012; Bonato et al. 2011; 2014), no effect of male age on ejaculates characteristics or male libido was detected. However, these three studies included two-year-old males, which could have potentially contributed to these differences in sperm output and libido with regards to age. Two-year-old male ostriches may still be in a transition to full sexual maturity and thus have not reached their optimum reproductive potential. This corresponds well with the findings that female ostriches mated to younger males (two year olds) produced a higher proportion of infertile eggs than when mated to males older than two years (Bunter 2002). The lack of differences of male age on semen characteristics in the present study might suggest that males of 3 years and older have reached their optimum sexual maturity and may be reliable for breeding purposes (Bunter 2002). Significant variations between males were recorded for the majority of ejaculate

characteristics and male libido. Similar observations were reported for ostriches (Bonato et al. 2010, 2011, 2014; Smith et al. 2016) as well as in broiler chickens (Floyd and Tyler 2011). The differences between males in these traits may stem from genetic variation, variation in body weight and hormonal regulation between individual birds (Malik et al. 2013). The variation between males indicates the potential to identify and select for superior males for future breeding purposes, an observation supported by repeatable male performance in a previous study (Cloete et al. 2015). It is therefore a priority to establish indicator traits that reflect superiority in sperm quality and output. Incidents of aspermia were recorded several times in this study and were distributed evenly across the study periods. Although it was frequently recorded in one male, compared to the others, aspermia has not yet been reported in ostriches. However, this condition has been observed in other bird species such as the Northern pintail duck (*Anas acuta*) and broilers (Penfold et al. 2000; Tyler et al. 2011). The description of aspermia in ostriches is particularly important as it may partly explain the high proportion of infertility in naturally mated flocks (Lambrechts et al. 2004). This also highlights the need to screen individual males before selection for AI programs in this species.

Conclusions

Semen production did not vary across study periods indicating that changes in daylight in the southern hemisphere do not have any effect on semen production in ostriches. However, sperm output and male libido were affected by study periods. It can be recommended to collect semen during spring and summer when sperm output, male libido and the success of semen collection are optimum. The variation between males in most ejaculate characteristics as well as male libido in this study suggests that some males produce quality semen and cooperate better than others during semen collection. It thus might be possible to identify and select for superior males of high sperm production and libido for breeding purposes.

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Compliance with ethical standards

Ethical approval The study was conducted following the recommendations of the Western Cape Department of Agriculture's Departmental Ethical Committee for Research on Animals (Ref No.: R9/24).

Competing interests

The authors declare that they have no competing interests.

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