Classification of ostrich sperm characteristics

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ABSTRACT

The success of assisted reproduction techniques is dependent on a sound foundation of understanding sperm characteristics to evaluate so as to improve semen processing. This study offers a descriptive basis for ostrich semen quality in terms of sperm function characteristics (SFC) that include motility, measured by computer assisted sperm analysis CASA (SCA®), viability (SYBR14/PI) and membrane integrity (hypo-osmotic swelling test). Relationships among these SFC's were explored and described by correlations and regressions. Certain fixed effects including the dilution of semen, season, year and male associated with semen collection were interpreted for future applications. The seasonal effect on sperm samples collected throughout the year suggested that it is prudent to restrict collections to spring and summer when SFC's and sperm concentration are maximized, compared to winter when these aspects of sperm quality are suppressed. Dilution of ejaculates helped to maintain important SFC's associated with fertilization success. The SFC's and sperm concentration varied among males, with specific males, having greater values for the percentage of motile (MOT) and progressively motile (PMOT) sperm, as well as sperm velocity (VCL, VSL, VAP) and linearity (LIN) variables. Males may thus be screened on these variables for inclusion in an artificial insemination (AI) programme to optimize fertility success rates.

Keywords: Sperm function characteristics, Sperm variables, Seasonality, Semen dilution, Male variation; Artificial insemination; *Struthio camelus*

58 1. Introduction

59 Variation in semen quality in terms of functionality within and between species, males as
60 well as ejaculates, has been well documented (Songsasen and Leibo, 1997; Blanco et al., 2000;
61 King et al., 2000; Yu et al., 2002; Blesbois et al., 2005; Roca et al., 2006; Chaveiro et al., 2006;
62 Leahy and Gadella, 2011). Because of inter- and intra-male variation in ejaculate quality, semen
63 samples should be evaluated before processing for storage and AI. The initial ejaculate quality is
64 of utmost importance for successful semen processing because sperm cells are irreparable
65 (Blesbois et al., 2005; Graham and Moce 2005). Damage that is likely to occur during processing
66 will lead to a decrease in sperm function after storage, manifested more explicitly in cryopreserved
67 semen than in chilled or neat semen. A 40% to 70% reduction in different sperm functions have
68 been reported in the literature for cryopreserved sperm of both domestic and non-domestic avian
69 species, emphasizing the importance of an ejaculate with good initial quality (Park, 1992;
70 Donoghue and Wishart, 2000; Watson, 2000; Gee et al., 2004; Malecki et al., 2008; Moce et al.,
71 2010). In the ostrich, semen cryopreservation has been attempted by Malecki and Kadokawa,
72 (2011) and liquid storage has also been assessed by Ya-jie et al. (2001), but with limited success.
73 Malecki and Kadokawa (2011) reported a mean of $11 \pm 1\%$ and Ya-jie et al. (2001) a mean of 26.1
74 $\pm 10.1\%$ overall live sperm.

75 Semen processing technology can be technical, costly and time consuming and should
76 thus not be wasted on a poor quality semen sample. Assessing semen throughout the processing
77 protocol can also give an indication of the type and amount of damage exerted on the cell during
78 the different stages and can be used as a basis for protocol optimizations.

79 Poor sperm production and supply has been noted as one of the primary reasons for poor
80 fertility in the ostrich industry and has stressed the importance of effective male fertility evaluation
81 (Bertshinger et al., 1992; Hemberger et al., 2001; Malecki and Martin, 2003; Malecki et al., 2008).
82 The evaluation and selection of males for semen quality and potential fertility is a very important
83 factor to consider before including a male in a breeding scheme (natural or artificial reproduction,
84 stored or non-stored). Knowledge of the capacity of an ostrich male to contribute to an artificial
85 insemination (AI) programme would allow the timely exclusion of males with inferior sperm quality.
86 The maintenance of a resource population for AI is a costly and hazardous practice that includes

many challenges. The ratio of males to females kept in a natural reproduction scheme, where a colony breeding system is most prevalent, can also be reduced with greater knowledge of the male's sperm quality (Lambrechts et al., 2004). The latter will potentially increase overall profitability by increasing chick numbers while maintaining fewer males with greater sperm functional quality.

Recent advances in ostrich semen collection by means of the “dummy-female” method developed by Rybnik et al. (2007) facilitated obtaining representative biological ejaculates, suitable for evaluation. Ejaculate quality was not compromised at a collection frequency of up to two times per day (Bonato et al., 2011). Ejaculate quality can, therefore, be assessed according to different sperm functional tests developed as adapted specifically for ostrich by Smith (2016). Sperm functional tests have been well correlated with sperm survivability after storage and acceptable fertility after AI in most other species, including men (Mahmoud et al., 1998), bulls (Ericsson et al., 1993; Farrell et al., 1998; Kasimanickam et al., 2006), roosters (Wishart and Palmer, 1986) and turkey toms (King et al., 2000). Subjective visual measures of conventional semen variables (commonly used to evaluate sperm variables in various livestock industries) are not highly repeatable or reliable when predicting fertility and are thus not recommended (Linford et al., 1976; Neuwinger et al., 1990; Hoflack et al., 2005; Moce and Graham, 2008). Sperm function variation can, therefore, be used to develop an objective, cost effective, time efficient and reliable classification system for objective evaluation of ostrich ejaculates and male screening. The aim of the present study was, thus, to describe the variation of functional sperm variables within and among ostrich ejaculates.

2. Material and methods

2.1. Animal population

Ten South African Black (SAB) ostrich males (*Struthio camelus var. domesticus*), aged between 3 and 7 years, were allocated to the study over a period of 5 years (2011 to 2015),

although ejaculates collected in 2013 and 2014 were primarily used. Ejaculates ($n = 326$) were collected from these males using the “dummy” female method as described by Rybnik et al. (2007). Briefly, the dummy was made of hemp sack that inside had a steel frame structure cushioned with dense foam, providing firm support for the male chest and leg, and the PVC tube to which the artificial cloaca was inserted. Ejaculates were collected during winter (June to August), spring (September to November), and summer (December to February). Males in the resource population were screened from the commercial ostrich breeding flock, maintained at the Oudtshoorn Research Farm situated in the Klein Karoo, South Africa region (33°63' S, 22°25' E), on the basis of behavioural attributes rendering them suitable for AI (referred as desirable behaviour as described by Bonato et al., 2013). The origin of the ostrich flock and the general management procedures implemented therein were described previously (Van Schalkwyk et al., 1996; Bunter & Cloete, 2004).

2.2. Semen preparation

Ejaculates were diluted 1:1 (Malecki and Kadokawa, 2011; Sood et al., 2011) after collection with the ostrich specific diluent (OS1) developed by Smith (2016). The OS1 diluent content was based on the macro mineral composition of ostrich seminal plasma. Sperm concentrations were obtained by use of a spectrophotometer (Spectrawave, WPA, S800, Biochrom) in 20 μ L semen diluted 1:400 (v/v) with a phosphate buffered saline solution containing 10% formalin. The transmittance values of the spectrophotometer were used to calculate sperm concentration using a regression equation pre-experimentally developed using the actual sperm counts from a haemocytometer for the ostrich. Neat and diluted samples were evaluated for sperm specific functions that included sperm cell motility, viability and membrane integrity.

2.3. Sperm function evaluation

2.3.1. Sperm cell motility

Sperm images were captured 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. All sperm cell motility recordings were made after re-suspension of neat sperm as well as treated sperm in a standard motility buffer using sodium chloride (150 mM) and TES (20 mM) with male specific seminal plasma (2%) to a final sperm concentration of 20×10^6 sperm cells/ml. After re-suspension, the tube was placed in a 38 °C water bath for 1 minute. For sperm cell motility recording, 2 µl of diluted semen was placed onto a pre-warmed slide covered gently with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds prior to recording. Images of seven to nine different fields were captured until at least 500 motile sperm images were obtained. The fields were captured randomly to eliminate bias towards a greater sperm cell concentration or motility. Sperm motility variables included motility (MOT, %), progressive motility (PMOT, %), curve-linear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), and beat cross frequency (BCF, Hz).

2.3.2. Sperm cell viability evaluation

Sperm cell viability was measured using the LIVE/DEAD® Sperm Viability Kit from Life technologies, that contained the SYBR® 14 and Propidium Iodide (PI) fluorescent stains. All sperm cell viability recordings were made after re-suspension of neat sperm, as well as treated sperm in the standard ostrich diluent pH7 to a final sperm concentration of 20×10^6 sperm cells/ml. The SYBR® 14 working solution was prepared in a HEPES/NaCl medium to a 1:49 concentration (v/v) of SYBR® 14 to HEPES/NaCl solution. Sperm suspension aliquots of 250 µl were re-suspended with 1.5 µl membrane-permeant SYBR® 14 working solution and incubated for

10 minutes in a temperature controlled environment of 38 °C. After incubation, 2 µl of the next fluorescent stain, propidium iodide (PI), was added and incubated for 10 minutes where after the cells were evaluated. For evaluation of viable (green) and non-viable (red/or green with red) sperm a 2 µl droplet was placed on a glass slide and covered with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds prior to recording. The fluorescent sperm was observed and photographed under 10x microscopy with an Olympus BX41 -epifluorescent microscope (Olympus Optical Co., Tokyo, Japan), equipped with a filter, camera (ColorView Illu Soft Imaging System) and software package (analysis FIVE, Olympus Soft Imaging Solutions GmbH, Münster) to count viable and non-viable sperm. Nine to ten different fields were randomly captured until at least 500 sperm were recorded. Distorted fields as well as fields that included drift or debris or clumps of sperm were excluded. The SYBR® 14 nucleic acid stain labels live sperm with green fluorescence, and membrane-impermeant PI labels the nucleic acids of membrane-compromised sperm with red fluorescence.

2.3.3. Sperm cell membrane integrity evaluation

Sperm cell membrane integrity was measured using the hypo-osmotic swelling test (Jeyendran *et al.*, 1984), adapted specifically for the ostrich by means of preliminary experimental exploration. The neat sperm samples for the hypo-osmotic swelling test (HOS, %) were prepared at the same time as that of sperm motility evaluation. All sperm membrane integrity recordings were made after re-suspension of neat sperm and treated sperm in a standard salt (NaCl/H₂O) solution adapted to 25 mOsm to a final sperm concentration of 20×10^6 sperm cells/ml. For HOS recording, 2 µl of diluted semen was placed onto a pre-warmed slide, using a heated stage set at 38 °C, covered gently with a cover glass (22 x 22 mm) and allowed to settle for 20 seconds prior to recording. Sperm images were captured 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. Seven to nine different fields of sperm images were captured

randomly until accurate representations (500 sperm) were attained and to eliminate biasness towards greater sperm concentration. Distorted fields as well as fields that included drift or debris or clumps of sperm were excluded.

2.4. Statistical analyses

Sperm variables as percentages, and with skewed distribution (as determined by the Shapiro-Wilk test: $P < 0.05$) were transformed using the arc sine of the percentage mean square-root ($\text{degree} \cdot \arcsin \sqrt{\%}$), while the sperm concentration was transformed to natural logarithms. Analyses included a distribution analysis and summary statistics to obtain variance parameters and graphs, describing the sperm variables. The total number of records, means, standard deviations, minima, maxima and coefficients of variation (CV) were determined for each sperm variable. The contribution of each FE to a particular SFC was evaluated by expressing the sum of squares for such an effect as a percentage of the total corrected sum of squares (TCSS; Leighton et al., 1982; Smith, 2010).

General Linearized Mixed Models (GLMM) were performed to evaluate the influence of factors such as dilution rate (D), season (S), year (A) and sperm concentration (C; as a linear covariate) affecting the different sperm variables with the inclusion of male (M) as random effect to account for the repeated sampling of the same males. General Linear Models (GLM) were used to evaluate the specific effect of variation between males and its interactions with other fixed effects (D, S, A, C). Sperm concentrations (C) as the response variable in analyses that included the fixed effects of M, S, D and A were also evaluated using GLM. Sperm quality function characteristics or variables (SFC) included motility derived from CASA (SCA®), viability (LIVE/DEAD®) and membrane integrity (Hypo-osmotic swelling test) that were fitted individually to each model, as dependent variables. Least squares means, standard errors (S.E.) and variation coefficients (CV) were calculated and subjected to Tukey's multiple range tests to investigate differences between least squares means. Correlations (Pearson) and regressions (linear and non-linear) were applied

to describe significant ($P < 0.05$) relationships among variables. Statistical Analysis System (SAS, version 9.3) was used for analyses performed.

An example of the GLMM fitted with Y being the dependent sperm characteristic are:

$$Y_{ijkl} = \mu + M_i + D_j + S_k + A_l + b_0(C)_{ijkl} + e_{ijkl}$$

Where: Y_{ij} = Sperm variable under assessment

μ = population mean

M_i = random effect of the i^{th} male ($i = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10$)

D_j = fixed effect of the j^{th} dilution rate ($j = 1, 2$)

S_k = fixed effect of the k^{th} season ($k = 1, 2, 3$)

A_l = fixed effect of the l^{th} year ($l = 1, 2, 3, 4, 5$)

C_{ijkl} = sperm concentration fitted as a linear covariate

b_0 = regression coefficients of Y_{ijkl} on sperm concentration (C)

e_{ijkl} = random error

3. Results

3.1. Descriptive statistics

Across the whole analyses, sperm concentration (mean $\pm$ SE) was $3.26 \times 10^9 \pm 3.27 \times 10^7$ sperm cells/mL, with a minimum of 1.73×10^9 and a maximum of 4.73×10^9 sperm cells/mL. The values for variables were: LIVE = 83.69 ± 0.70 , HOS = 79.53 ± 0.86 , PMOT = 48.03 ± 1.03 , MOT = 82.55 ± 0.67 , VCL = 67.68 ± 0.78 , VSL = 40.72 ± 0.79 , VAP = 54.54 ± 0.88 , ALH = 2.44 ± 0.02 , LIN = 59.27 ± 0.64 , STR = 73.77 ± 0.60 , WOB = 79.45 ± 0.52 and BCF = 8.77 ± 0.08 . Fewer records, 102 and 97 respectively, were obtained for LIVE and HOS, compared to sperm cell motility variables due to the 2014 year limitation, with relatively small CV values (8.75% and 10.66 % respectively). The CV's for sperm cell motility and kinematic sperm variables ranged from 11.10% to 36.52%.

The R^2 values indicate variable response for the different SFC's ranging from 7% (LIVE) to 80% (BCF) of the variation explained by the fixed effects fitted. The lesser R^2 values associated with LIVE and HOS can partially be explained by not being able to fit year as a fixed effect when compared to analyses on sperm cell motility and kinematic variables for which there were data available for both years that data were analysed. The least squares means for the different SFC's are presented in Table 1 and Table 2, indicating the variation for each fixed effect on the SFC's. Pearson's correlation coefficients between all sperm function variables and sperm concentrations are presented in Table 3 while the categorization of ostrich males according to sperm quality variables are reported in Table 4.

3.2. Effect of season on sperm variables

All SFC's, namely LIVE, HOS, motility and kinematic characteristics were influenced ($P < 0.05$) by season of collection. The contribution of seasonal variation to the overall variation ranged from 0.23% to 24.82% across SFC's with sperm cell motility and kinematic variables being more dependent ($P < 0.001$) on season compared with LIVE and HOS. It is evident from information included in Table 1 that there was no LIVE and HOS sperm data obtained during the summer season and this may be partially responsible for the lack of marked seasonal effects on the variation in LIVE and HOS variables. Data from the spring semen collection, resulted in the greatest ($P < 0.05$) LIVE (mean $\pm$ SE = $90.72 \pm 3.67\%$) and HOS (mean $\pm$ SE = $89.54 \pm 7.85\%$) compared with data from the winter collection. Data from the summer collection indicated there was greater sperm cell motility (e.g., PMOT, mean $\pm$ SE = $59.60 \pm 3.08\%$ and MOT, mean $\pm$ SE = $85.63 \pm 2.11\%$) and more desirable kinematic variables for sperm cells as indicated VCL (mean $\pm$ SE = $72.95 \pm 2.07 \mu\text{m/s}$), VSL (mean $\pm$ SE = $49.29 \pm 1.99 \mu\text{m/s}$), VAP (mean $\pm$ SE = $61.89 \pm 2.18 \mu\text{m/s}$, LIN (mean $\pm$ SE = $66.92 \pm 1.50 \%$), and WOB (mean $\pm$ SE = $83.84 \pm 1.40 \%$). The STR was greatest in both the summer (mean $\pm$ SE = $78.76 \pm 2.02\%$) and spring (mean $\pm$ SE = $76.42 \pm 2.19\%$) semen collections with there being no difference ($P > 0.05$) between the two seasons for

this variable. These means, however, were greater than the STR at the winter (mean $\pm$ SE = 68.55 $\pm$ 1.90%) collection. The BCF was greatest with the summer (mean $\pm$ SE = 9.26 $\pm$ 0.22 Hz) semen collection and differed ($P < 0.05$) from values at the spring (mean $\pm$ SE = 8.51 $\pm$ 0.26 Hz) collection in which the VCF was least, but the VCF for the spring semen collection was not different from that at the winter (mean $\pm$ SE = 8.86 $\pm$ 0.19 Hz) collection. The ALH was greatest at the winter (mean $\pm$ SE = 2.51 $\pm$ 0.05 μ m) semen collection and did not differ ($P > 0.05$) from the spring (mean $\pm$ SE = 2.36 $\pm$ 0.07 μ m) collection, but differed significantly from values at the summer collection where the ALH was least (mean $\pm$ SE = 2.29 $\pm$ 0.06 μ m). Medium positive Pearson's correlations ($P < 0.001$) were recorded between season and PMOT ($r = 0.55$), VCL ($r = 0.44$), VSL ($r = 0.61$), VAP ($r = 0.53$), LIN ($r = 0.59$), STR ($r = 0.42$) and WOB ($r = 0.53$), suggesting linear relationships. These relationships of season with each of the SFC's reported were validated by linear regressions. Figure 1 depicts the linear regressions and R^2 values ($P < 0.001$) obtained for PMOT, VCL, VSL, VAP, LIN, STR and WOB. The R^2 values indicate that the linear regressions that were fitted accounted for 18% to 35% of the variation for the different SFC's. The difference between winter and summer collections for important variables such as the PMOT was as great as 22% taking into consideration that the PMOT could increase by 10.88% for each season from winter to summer.

3.3. Effect of dilution rate on sperm variables

Diluting the ejaculate after collection affected ($P < 0.05$) most SFC's except ($P > 0.05$) MOT, LIN, STR and BCF. Dilution contributed to a lesser extent to the variation explained by the FE% ranging from 2.04 to 6.71. Furthermore, dilution had a marked effect ($P < 0.0001$) on some of the more important sperm velocity variables associated with fertilizing capacity, namely VCL, VSL and VAP. Dilution at 1:1 decreased ($P < 0.05$) LIVE and HOS by ~3% whereas PMOT, VCL, VSL, VAP, ALH, WOB improved ($P < 0.05$). Dilution enhanced PMOT (mean $\pm$ SE = 49.60 $\pm$ 2.86%), VCL (mean $\pm$ SE = 69.80 $\pm$ 1.85 μ m/s), VSL (mean $\pm$ SE = 43.16 $\pm$ 1.81 μ m/s), VAP (mean $\pm$ SE =

57.02 $\pm$ 1.94 μ m/s), ALH (mean $\pm$ SE = 2.45 $\pm$ 0.05 μ m) and WOB (mean $\pm$ SE = 80.45 $\pm$ 1.26%). The effects of dilution on PMOT, VCL, VAP and VSL are depicted in Figures 2, 3, 4 and 5, respectively, with data for undiluted and diluted samples for these and other SFC's being included in Table 1 and Table 2.

3.4. Effect of sperm concentration and year on sperm variables

Sperm concentration as a linear regression ($P < 0.01$) only affected HOS with a FE contribution of 5.86%. There was a negative correlation ($P < 0.05$) between HOS ($r = -0.27$) and sperm concentration (Table 3). A non-linear quadratic equation ($Y = \alpha + \beta_1 X + \beta_2 X^2$; $P < 0.01$) was best fit (Figure 6) for describing the relationship ($Y = -527 \times 10^{-20} X^2 + 2.94 \times 10^{-8} X + 40.20$) between HOS and concentration. The R^2 value indicated 10% of the variation in HOS was accounted for by the quadratic regression. The point ($X = -(\beta_1/2\beta_2)$) where HOS would be maximized (81.20%) amounted to $X = 2.79 \times 10^9$ whereafter the HOS decreased with further increases in sperm concentration.

The BCF was the only SFC that was influenced ($P < 0.001$) by year, with year contributing 3.60% to the observed variation in this variable. The BCF increased ($P < 0.05$) from 2013 (mean $\pm$ SE = 8.51 $\pm$ 0.21 Hz) to 2014 (mean $\pm$ SE = 9.24 $\pm$ 0.21 Hz).

3.5. Relationships among sperm variables

Data for relationships among sperm variables as estimated using Pearson correlation coefficients are included in Table 3. The PMOT had the greatest positive correlations ($P < 0.001$) with sperm motility (MOT) and kinematic variables (VCL, VSL, VAP, LIN, STR, WOB) that ranged from 0.52 to 0.83, but there was no correlation ($P > 0.05$) with LIVE, HOS, ALH and BCF. The LIVE variable was positively correlated ($P < 0.05$) with MOT, VSL, VAP, LIN and WOB with correlation values ranging from 0.22 to 0.41 and negatively correlated with ALH ($r = -0.18$; $P <$

0.01) and BCF ($r = -0.26$; $P < 0.05$). The HOS variable was only correlated ($P < 0.05$) with ALH ($r = 0.28$). The correlations ($P < 0.001$) among variables reflecting sperm motility, namely MOT and PMOT, VCL, VSL, VAP and WOB were all moderately to highly positive. The VCL, VSL and VAP variables were positively correlated with each other, as well as with the PMOT, MOT, LIN, STR and WOB variables. The ALH variable was negatively correlated ($P < 0.05$) with the VSL, LIN, STR, WOB and BCF variables and was slightly and positively ($P < 0.01$) correlated with the VCL variable.

3.6. Effect of male on sperm variables

The R^2 values associated with each of the models fitted for the different SFC's, that considered all other fixed effects of concentration, season and dilution, indicated there was a male effect as the largest single contributor to variation in the SFC's. The male effect contributed 4.175 to 19.3% of the variation associated with all SFC's with the largest contribution to the percentage MOT and smallest to HOS variables. Males differed ($P < 0.01$) from one another for most of the SFC's except for BCF ($P > 0.05$). Variation between males for the MOT, STR, VCL, LIN, VAP, PMOT and VSL variables are depicted in Figure 7.

Male semen was categorized as good, average and poor as summarised in Table 4. Categorization depended on the distribution of closely related sperm variables (PMOT, MOT, VCL, VSL, VAP, LIN), based on Pearson correlation coefficients among males. Although the sample sizes of males were very small in this study ($n = 10$), males could be subjectively categorized on average values for the different SFC's according to the variation between males.

3.7. Effect of season, dilution, male and year on sperm concentration

Sperm concentration means were differed ($P < 0.001$) when fitted as the response variable in a GLM model with the fixed effect of season, dilution, male and year. The latter FE contributed

28% to the total variation associated with sperm concentration. Sperm concentration was influenced by the fixed effects of season ($P < 0.001$) and male ($P < 0.001$), but not by dilution rate ($P > 0.05$) or year ($P > 0.05$). Season contributed 6.17% towards sperm concentration. Sperm concentration was different ($P < 0.001$) between seasons, with the greatest ($P < 0.001$) concentration in the summer (mean $\pm$ SE = $3.42 \times 10^9 \pm 7.33 \times 10^7$ / mL) and the least in winter (mean $\pm$ SE = $3.17 \times 10^9 \pm 6.30 \times 10^7$ /mL) and spring (mean $\pm$ SE = $2.97 \times 10^9 \pm 8.13 \times 10^7$ /mL) with no difference between the latter seasons. The relationship between seasonality and sperm concentration was confirmed by a highly significant positive Pearson's correlation ($r = 0.3$) and a linear relationship of $y = 1.8 \times 10^8 X + 2.9 \times 10^9$ ($R^2 = 0.08$; $P < 0.001$).

The individual male variable had the greatest contribution to the variation associated with sperm concentration (FE = 16.04%), compared with season, dilution rate and year. However, only some males differed ($P < 0.05$) from one another for sperm concentration with variation depicted in Figure 8.

4. Discussion

4.1. Effects of season, dilution and year on sperm variables

Results indicated that season is the most influential effect on sperm variables, compared with semen dilution, year and sperm concentration if male is considered as a random effect. Seasonality effects on sperm quality are a common phenomenon in most species, including the ostrich and emu (a close relative of the ostrich). Seasonality may limit reproduction to specific times of the year for the greater likelihood of offspring survival (Jarvis et al., 1985; Malecki et al., 1997; Williams et al., 1995; Blache et al., 2001). Extensive husbandry systems, such as those employed in ostrich, emu or free-range chicken management are more prone to the effects of seasonality compared to management in indoor housing systems. An important variable, such as seasonality, may impact survivability through natural selection but may also be detrimental for production efficiency in terms of consistent production of offspring throughout the year.

Seasonality effects are induced by seasonal changes in photoperiod, temperature, rainfall, social interactions and resource availability (Blache et al., 2001; Hemberger et al., 2001; Lambrechts et al., 2004). Different seasons may affect fresh semen variables, resulting in impacts on fertilization success of the male (natural or AI systems) as well as the percentage of sperm surviving processing for short- and long-term storage. The first account of ostrich seasonality on ejaculate quality in terms of sperm cell volume, concentration, motility score, morphology as well as libido was reported by Bonato et al. (2014). However, a detailed functional assessment of sperm had not been considered for the ostrich until the present study was conducted.

Sperm cell viability and membrane integrity (in terms of LIVE and HOS), respectively, were less dependent on season compared with sperm cell motility and kinematic variables although the same seasonal trend existed between all variables. The LIVE and HOS variable means in the present study were different between seasons being greatest in the spring for LIVE and HOS, compared to the winter. These results are consistent with those of Bonato et al. (2014) where it was reported that percentage of live-normal sperm as determined from Nigrosin/Eosin stained slides was greater for ostrich males in the spring to early summer compared to the winter season. The greater sperm cell viability and membrane integrity is possibly be associated with the ostrich's greater reproductive activity during the warmer spring months compared with the colder winter months (Degen et al., 1994; Soley and Groenewald, 1999; Rybnik et al., 2012). Results from the present study, however, were inconsistent with previous results with other avian species where it was reported that there was a lesser HOS due to greater temperatures caused by heat stress and testicular function disturbances (Saeid and AL-Soudi, 1975; Datta et al., 1980; Santiago-Moreno et al., 2009). The difference between the ostrich and other domestic avian species such as chickens can possibly be explained by the intra-abdominal location of the ostrich testes, as well as other physiological adaptations that make them more resistant to heat stress (Maclean, 1996; Soley and Groenewald, 1999; Hemberger et al., 2001).

The greatest sperm motility in terms of the PMOT and MOT variables were obtained in the summer, while the lesser values for these variables were observed during winter, with distinct differences between each of the seasons. Results for kinematic sperm variables, specifically sperm velocity (VCL, VSL and VAP) and sperm cell swim quality (LIN and WOB) indicated there was a similar trend as that for the PMOT and MOT variables. The swim straightness (STR) of sperm cells appeared to be less sensitive towards seasonal change, as there was no difference between summer and spring seasons in STR although STR was still greater in these seasons compared with the winter season. The latter results are inconsistent with previous results obtained for the ostrich where sperm motility was relatively consistent over different seasons of the year. The latter result can possibly be explained by the method of motility evaluation (Bonato et al., 2014). Results from the present study, however, were consistent with previous results in studies with free-range chickens where sperm motility decreased with decreased photoperiod and temperature particularly during the winter season (Santiago-Moreno et al., 2009).

Variations in sperm concentration due to seasonal changes in the present study were observed with there being a greater sperm concentration in the summer compared with the spring and winter seasons with no difference between the latter two seasons. The effect of seasonal changes in sperm variables for the ostrich has also been reported by Rybnik et al. (2012) and Bonato et al. (2014). Furthermore, Degen et al. (1994) provided results that indicated an increase of day light length in the spring and early summer months was associated with elevated androgen concentrations, specifically testosterone, a hormone that impacts sperm cell production and maturation, which could potentially increase sperm concentration (Degen et al., 1994).

Results of the present study indicate sperm cell function variables follow the same pattern as that of the ostrich breeding season with the greatest reproduction activity and sperm cell function traits occurring in spring and early summer months. Knowledge regarding ejaculate quality and quantity in the different seasons will allow managerial manipulation through assisted reproduction techniques to increase reproduction efficiency. For example, greater quality

ejaculates cryopreserved during spring and early summer may be used for insemination during the winter when females are in production, but when ejaculates are of a poorer quality.

Dilution of neat ejaculates in ostriches caused an initial loss (~3%) of LIVE and HOS, but is an important attribute to maintain sperm cell function for further processing. Dilution is important to prolong sperm cell viability for evaluation and storage purposes, specifically for ejaculates of lesser volumes and greater sperm cell concentration to avoid substrate depletion, toxin accumulation, pH change and increased metabolic activity (Clarke et al., 1982; Bilgili et al., 1987). A significant increase in PMOT was observed upon semen dilution and there was also an increase in PMOT that was related to important sperm cell velocity variables. The improvement of VCL, VSL and VAP associated with semen dilution can possibly be explained by the capacity of diluted semen samples to maintain sperm cell function, compared to undiluted samples. Sperm cells in undiluted semen samples deteriorate quickly after collection due to cell agglutination and hence are difficult to evaluate compared with sperm cells in diluted samples (Malecki et al., 2008). Ciereszko et al. (2010) similarly observed these effects in a study conducted with ostrich semen that was evaluated in undiluted and diluted (1:3) conditions with a non-specific ostrich extender.

The BCF was the only sperm variable influenced ($P < 0.05$) by year and varied between 2013 and 2014. The BCF is an indication of sperm cell oscillation and is based on specific sperm cell movement paths expressed in Hz (number of video frames per second). Because BCF was the only SFC influenced by year, it is suggested that the upgrade in the CASA system during this time frame could explain the variation in BCF values because Boryshpolets et al., (2013) reported variation in BCF values associated with a change in the CASA system, although the same system settings were applied.

4.2. Effect of male and sperm concentration on sperm variables and relationship between the two variables

The effect of male contributed substantially to the variation observed in all the SFC evaluated in the present study. The effect of male was greater as compared with other variables assessed in the present study on the PMOT, MOT, VCL, VSL, VAP and LIN. These sperm cell motility and kinematic traits could be used to categorize the male and quality of ejaculates. Sperm function characteristics and reproductive performance variation between males has been reported in other avian reproductive studies specifically for the ostrich (Kamar and Badreldin, 1959; Bonato et al., 2010, 2011, 2014). Genetic differences between males may possibly explain the effect of male observed in the present study for a variable such as MOT, where variation was the greatest between males. Documented genetic differences in terms of seminal plasma protein concentration, amidase activity and fatty acid composition could contribute to the variation associated with sperm variables as reported by Surai et al. (1998) and Ciereszko et al. (2010). For example, plasma proteins, unique to a specific male, have been found to affect sperm motility (Yoshida et al., 2008; Rodrigues et al., 2012) either negatively (Schoneck et al., 1996) or positively (Somlev et al., 1996). The percentage motile sperm has been found to be highly heritable in both mammals (cattle: 0.79; Pepper-Yowell, 2011), and avian species (Beijing-You: 0.85; Hu et al., 2013). It is thus possible that ostrich males may have the same genetic variation in the MOT variable, which would allow for genetic selection and the improvement of sperm motility and associated variables. This would be very convenient because the MOT variable is also associated with sperm cell structural and functional integrity, thus, may be used to identify males with greater reproductive capacity because of the high correlation with fertilization potential (Wishart and Palmer, 1986; Froman, 1999; Blesbois et al., 2008; Pepper-Yowell, 2011). Variation in sperm concentration between ostrich males was observed in the present study. Previous studies on ostrich sperm concentration are inconsistent with some reports indicating variations between males in sperm concentration (Rybnik et al., 2012) while in other studies there were not inter-male

variations in sperm concentrations (Bonato et al., 2010). Results of studies where sperm concentrations in ostriches have been evaluated may differ because of number of males included in the studies (Bonato et al., 2010).

The variation in sperm concentration between males can involve several factors such as feed intake, body size, androgen concentrations, age, semen collection frequency and the individual's genetic make-up (Malik et al., 2013). For example, large cockerels with greater body weights are associated with increased testicular size and produce more sperm cells during spermatogenesis resulting in a greater sperm concentration (Adeyemo et al., 2007, Mosenene, 2009). Furthermore, in the present study sperm concentration influenced sperm function in terms of HOS resistant sperm: very low and very high sperm concentrations were detrimental to sperm cell membrane integrity as associated with a lesser percentage HOS indicating fewer cells with functional membrane integrity. Greater and lesser sperm concentrations are associated with greater sperm cell oxidative stress and are often associated with lesser fertility (Murphy et al., 2013; Agarwal et al., 2014a,b). Oxidative stress causes peroxidative damage to the sperm cell membrane that primarily contains unsaturated fatty acids which lack the necessary cytoplasmic components containing antioxidants (Lenzi et al., 2002; Murphy et al., 2013). The loss of fatty acids, up to 60% in severe oxidative stress conditions, compromises sperm cell membrane function by decreasing its fluidity, increasing non-specific permeability to ions, and inactivating membrane bound receptors and enzymes. This ultimately contributes to poor sperm cell membrane integrity, which may result in the lesser numbers of HOS resistant sperm cells observed.

Most of the correlations obtained for ostrich sperm variables in the present study are in agreement with those reported in other avian and mammalian studies that offer the opportunity for indirect selection based on ϕ associated variables. The sperm motility variables, PMOT and MOT, are strongly correlated, while the kinematic sperm variables VCL, VSL, VAP, LIN and WOB. VCL, VSL and VAP are highly and positively correlated with each other and with STR, LIN and WOB.

Although there was no correlation between PMOT and sperm viability (LIVE) in the present study, LIVE was positively correlated with MOT, VSL, VAP, LIN and WOB. The latter results are consistent with other avian studies and suggest that values for any of the two variables will be representative of values for the other variables (Kramar and Badreldin, 1959). In the present study, there was no correlation between the HOS and LIVE variables, which is inconsistent with results of Santiago-Moreno et al. (2009) where it was reported that there was a very high correlation of $r = 0.86$ ($P < 0.001$) between the HOS and LIVE variables for free-range chickens.

The close association between most sperm cell motility and kinematic variables enabled male identification on the basis of greater values for some of the most important sperm motility and kinematic variables. Greater sperm cell velocity variables and a decreased deviation from linearity were important determinants of fertilization success because these sperm cell variables influence the capacity of the sperm to traverse the female reproductive tract to reach the site of sperm storage and fertilization (Froman, et al., 1999; King et al., 2000). King et al. (2000) reported the categorization of turkey males according to a sperm mobility index and found VSL, VCL, VAP, LIN and BCF to be significantly greater in the group with greater sperm mobility with a strong positive correlation between sperm mobility and certain sperm cell kinematic variables. Sperm velocity variables have also been used as indirect indicators of mitochondrial function of sperm cells (Graham et al. 1984). The latter can be used for a rapid evaluation to determine sample quality for AI or suitability for further processing that may include short or long term storage protocols.

5. Conclusion

The variation between and within ostrich males for ejaculate sperm functional variables, including sperm cell motility, kinematic variables, viability and membrane integrity, indicate the importance of evaluation prior to breeding of birds or semen processing for storage. A variable such as sperm concentration which has been used as an indicator of semen quality in most

commercial ejaculate evaluation systems together with ejaculate volume and sperm cell motility evaluations are inadequate for ostrich sperm cell quality assessments because some SFC's decrease when the sperm concentration increases above an upper threshold value. Moreover, variation between ostrich males is difficult to identify with a subjective sperm cell motility scoring system. The identification of ostrich males with SFC values *in vitro* may potentially improve fertilization ability *in vivo* because these SFC's are highly correlated with fertilization success in other species. Favourable relationships among sperm function variables simplify evaluation of males for semen storage because it is only necessary to consider two or three of these SFC's for accurate assessments of semen quality in ostriches. The effects of season and dilution should be considered when using AI for ostrich breeding, or when semen processing for storage or evaluation occurs because both factors significantly affect the SFC's. Late spring and early summer ejaculate evaluations should be sufficient to give a good representation of the ostrich male's SFC status and ejaculate suitability for further storage processing. Winter collections would be suitable when sexually aggressive males are considered for evaluation because testosterone concentrations are associated with photoperiod length and are less during the winter season (Degen et al., 1994). The SFC values, however, should be corrected for the losses associated with seasonality. Dilution of ejaculates is necessary to maintain sperm function for further evaluation and processing purposes to maintain progressive sperm cell motility and velocity in ostriches, variables that are directly correlated with fertilizing capacity when compared to neat ejaculates. It is, however, important that an optimal dilution rate in the most appropriate medium at a suitable temperature be established, specifically for the ostrich, to guarantee maximal sperm function maintenance for evaluation purposes and further processing. The latter would allow maximum utilization of desirable males for semen collection for artificial insemination purposes because several inseminations of multiple females would be possible.

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Fig. 1. Influence of season on ostrich sperm function variables, namely progressive motility (PMOT, %), curve linear velocity (VCL, $\mu\text{m/s}$), straight line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), linearity (LIN, %), straightness (STR, %) and wobble (WOB, %); Standard errors are indicated by vertical bars about means

Fig. 2. Effect of dilution on Ostrich sperm progressive motility (PMOT, %); Standard errors indicated by vertical bars about means; Means with different letters differ ($P < 0.05$);

Fig. 3. Effect of dilution on ostrich sperm curve linear velocity (VCL, $\mu\text{m/s}$); Standard errors indicated by vertical bars about means; Means with different letters differ ($P < 0.05$)

Fig. 4. Effect of dilution on ostrich sperm average path velocity (VAP, $\mu\text{m/s}$); Standard errors indicated by vertical bars about means; Means with different letters differ ($P < 0.05$)

Fig. 5. Effect of dilution on ostrich sperm straight line velocity (VSL, $\mu\text{m/s}$); Standard errors indicated by vertical bars about means; Means with different letters differ ($P < 0.05$)

Fig. 6. Quadratic relationship between membrane integrity (HOS, %) and sperm concentration; Standard errors indicated by vertical bars about means

Fig. 7. Ostrich male variation for sperm variables, namely motility (MOT, %), straightness (STR, %), curve-linear velocity (VCL, $\mu\text{m/s}$), linearity (LIN, %), average path velocity (VAP, $\mu\text{m/s}$), progressive motility (PMOT, %), straight line velocity (VSL, $\mu\text{m/s}$); Standard errors indicated by vertical bars about means

Fig. 8. Between-male variation in sperm concentration; Standard errors indicated by vertical bars about the means

Table 1

Least square mean ($\pm$ S.E.) of sperm concentration, season, dilution and year on sperm viability (LIVE, %), membrane integrity (HOS, %), progressive motility (PMOT, %) and motility (MOT, %)

Variation source	LIVE	HOS	PMOT	MOT
Concentration	$P > 0.05$	**	$P > 0.05$	$P > 0.05$
Season	*	**	***	***
Winter	83.36 ± 1.11^a	78.25 ± 3.33^a	38.91 ± 2.93^a	81.91 ± 1.96^a
Spring	90.72 ± 3.67^b	89.54 ± 7.85^b	41.61 ± 3.36^a	78.81 ± 2.35^a
Summer	na	na	59.60 ± 3.08^b	85.63 ± 2.11^b
Processing stage	*	**	***	$P > 0.05$
Non-diluted_0	88.31 ± 2.09^a	85.53 ± 3.72^a	43.82 ± 2.99^a	82.81 ± 2.04^a
Diluted_1:1	85.78 ± 2.09^b	82.27 ± 3.72^b	49.60 ± 2.86^b	81.43 ± 1.91^a
Year	na	na	$P > 0.05$	$P > 0.05$
2013	na	na	46.06 ± 2.98^a	81.56 ± 2.04^a
2014	na	na	47.36 ± 2.99^a	82.68 ± 2.03^a

na: not applicable because these sperm variables were not recorded in 2014; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$;

^{a,b} Means with different superscripts within the column and factor differ $P < 0.05$

Table 2

Least square means (S.E.) of sperm concentration, season, dilution and year on the sperm kinematic traits, curve-linear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average-path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat cross frequency (BCF, Hz)

Variation source	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF
Concentration	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
Season	***	***	***	***	***	***	***	**
Winter	61.54 $\pm$ 1.91 ^a	31.92 $\pm$ 1.86 ^a	46.18 $\pm$ 2.01 ^a	2.51 $\pm$ 0.05 ^a	52.32 $\pm$ 1.36 ^a	68.55 $\pm$ 1.90 ^a	74.57 $\pm$ 1.30 ^a	8.86 $\pm$ 0.19 ^a
Spring	63.29 $\pm$ 2.33 ^b	39.37 $\pm$ 2.22 ^b	51.53 $\pm$ 2.48 ^b	2.36 $\pm$ 0.07 ^{ba}	61.76 $\pm$ 1.75 ^b	76.42 $\pm$ 2.19 ^b	80.16 $\pm$ 1.56 ^b	8.51 $\pm$ 0.26 ^{ba}
Summer	72.95 $\pm$ 2.07 ^c	49.29 $\pm$ 1.99 ^c	61.89 $\pm$ 2.18 ^c	2.29 $\pm$ 0.06 ^b	66.92 $\pm$ 1.50 ^c	78.76 $\pm$ 2.02 ^b	83.84 $\pm$ 1.40 ^c	9.26 $\pm$ 0.22 ^a
Processing stage	***	***	***	**	$P > 0.05$	$P > 0.05$	*	$P > 0.05$
Non-diluted_0	62.05 $\pm$ 1.99 ^a	37.22 $\pm$ 1.93 ^a	49.27 $\pm$ 2.10 ^a	2.32 $\pm$ 0.06 ^a	74.74 $\pm$ 2.00 ^a	82.81 $\pm$ 2.04 ^a	78.60 $\pm$ 1.35 ^a	9.01 $\pm$ 0.21 ^a
Diluted_1:1	69.80 $\pm$ 1.85 ^b	43.16 $\pm$ 1.81 ^b	57.02 $\pm$ 1.94 ^b	2.45 $\pm$ 0.05 ^b	74.41 $\pm$ 1.86 ^a	81.43 $\pm$ 1.91 ^a	80.45 $\pm$ 1.26 ^b	8.75 $\pm$ 0.19 ^a
Year	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$	***
2013	64.56 $\pm$ 1.99 ^a	39.94 $\pm$ 1.92 ^a	51.53 $\pm$ 22.0 ^a	2.42 $\pm$ 0.06 ^a	61.11 $\pm$ 1.45 ^a	75.82 $\pm$ 2.00 ^a	78.85 $\pm$ 1.34 ^a	8.51 $\pm$ 0.21 ^a
2014	67.29 $\pm$ 1.98 ^a	40.44 $\pm$ 1.92 ^a	54.77 $\pm$ 2.08 ^a	2.35 $\pm$ 0.05 ^a	59.56 $\pm$ 1.42 ^a	73.33 $\pm$ 2.00 ^a	80.20 $\pm$ 1.34 ^a	9.24 $\pm$ 0.21 ^b

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ^{a,b,c} Means with different superscripts within the column and factor differ $P < 0.05$

Table 3

Pearson's correlation coefficients among ostrich sperm variables, progressive motility (PMOT, %), sperm viability (LIVE, %), membrane integrity (HOS, %), motility (MOT, %), curve linear velocity (VCL, $\mu\text{m/s}$), straight line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), linearity (LIN, %), straightness (STR, %), wobble (WOB, %) and beat cross frequency (BCF, Hz)

Sperm variable	PMOT	LIVE	HOS	MOT	VCL	VSL	VAP	ALH	LIN	STR	WOB	BCF	CONC
PMOT	1	0.14	-0.02	0.60***	0.76***	0.83***	0.77***	-0.04	0.66***	0.52***	0.61***	0.07	0.10
LIVE	0.14	1	-0.05	0.22*	0.05	0.26*	0.22*	-0.34	0.35**	0.08	0.41***	-0.26*	- 0.07
HOS	-0.02	-0.05	1	0.15	0.18	0.10	0.09	0.28*	-0.15	0.00	-0.12	0.16	- 0.23**
MOT	0.60***	0.22*	0.15	1	0.48***	0.38***	0.49***	0.00	0.19	0.02	0.41***	-0.04	- 0.03
VCL	0.76***	0.05	0.18	0.48***	1	0.86***	0.96***	0.17**	0.47***	0.19**	0.66***	-0.07	0.09
VSL	0.83***	0.26*	0.10	0.38***	0.86***	1	0.91***	-0.14*	0.84***	0.61***	0.80***	0.17**	0.15
VAP	0.77***	0.22*	0.09	0.49***	0.96***	0.91***	1	-0.07	0.60***	0.27***	0.84***	-0.04	0.13
ALH	-0.04	-0.34**	0.28*	0.00	0.17**	-0.14*	-0.07	1	-0.39***	-0.28***	-0.46***	-0.18**	- 0.09
LIN	0.66***	0.35**	-0.15	0.19	0.47***	0.84***	0.60***	-0.39***	1	0.82***	0.74***	0.36***	0.19
STR	0.52***	0.08	0.00	0.02	0.19**	0.61***	0.27***	-0.28***	0.82***	1	0.35***	0.52***	0.10
WOB	0.61***	0.41***	-0.12	0.41***	0.66***	0.80***	0.84***	-0.46***	0.74***	0.35***	1	0.03	0.16
BCF	0.07	-0.26*	0.16	-0.04	-0.07	0.17**	-0.04	-0.18**	0.36***	0.52***	0.03	1	0.07
CONC	0.10	- 0.07	- 0.23**	- 0.03	0.09	0.15	0.13	- 0.09	0.19	0.10	0.16	0.07	1

CONC: Sperm concentration; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 4

Categorization of ostrich males in relation to the quality of sperm variables, progressive motility (PMOT, %), motility (MOT, %), curve linear velocity (VCL, $\mu\text{m/s}$), straight line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$) and linearity (LIN, %)

Sperm variable	Poor	Average	Good
PMOT	< 40%	40 – 50%	> 50%
MOT	< 70%	70 – 80%	> 80%
VCL	< 60 $\mu\text{m/s}$	60 – 70 $\mu\text{m/s}$	> 70 $\mu\text{m/s}$
VSL	< 30 $\mu\text{m/s}$	30 – 40 $\mu\text{m/s}$	> 40 $\mu\text{m/s}$
VAP	< 40 $\mu\text{m/s}$	40 – 50 $\mu\text{m/s}$	> 50 $\mu\text{m/s}$
LIN	< 50 $\mu\text{m/s}$	50 – 60 $\mu\text{m/s}$	> 60 $\mu\text{m/s}$

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8