Sperm macrocephaly syndrome in the ostrich *Struthio camelus*: morphological characteristics and implications for motility

L. du Plessis[A,F], *M. Bonato*[B], *C. Durandt*[C], *S. W. P. Cloete*[B,D] *and J. T. Soley*[E]

[A]Electron Microscope Unit, Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa.

[B]Department of Animal Sciences, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa.

[C]Department of Immunology, Institute for Cellular and Molecular Medicine, Faculty of Health Sciences, SAMRC Extramural Unit for Stem Cell Research and Therapy, University of Pretoria, South Africa.

[D]Directorate Animal Sciences: Elsenburg, Private Bag X1, Elsenburg 7607, South Africa.

[E]Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa.

[F]Corresponding author. Email: lizette.duplessis@up.ac.za

Abstract

Sperm macrocephaly syndrome (SMS) is characterised by a high percentage of spermatozoa with enlarged heads and multiple tails, and is related to infertility. Although this multiple sperm defect has been described in other mammalian species, little is known about this anomaly in birds. Morphological examination of semen from nine South African black ostriches (*Struthio camelus* var. *domesticus*) involved in an AI trial revealed the variable presence of spermatozoa with large heads and multiple tails. Ultrastructural features of the defect were similar to those reported in mammals except that the multiple tails were collectively bound within the plasmalemma. The tails were of similar length and structure to those of normal spermatozoa, and the heads were 1.6-fold longer, emphasising the uniformity of the anomaly across vertebrate species. Flow cytometry identified these cells as diploid and computer-aided sperm analysis revealed that they swim slower but straighter than normal spermatozoa, probably due to the increased drag of the large head and constrained movement of the merged multiple tails. The high incidence of this defect in one male ostrich indicates that, although rare, SMS can occur in birds and may potentially have an adverse effect on breeding programs, particularly for endangered species.

Keywords: CASA, large heads, multiple tails, ultrastructure.

Introduction

Macrocephalic spermatozoa are defined as spermatozoa with a head larger than the heads of a normal population of spermatozoa for that species (Salisbury and Baker 1966; Barth and Oko 1989). In addition, a high proportion of macrocephalic spermatozoa is associated with the presence of multiple flagella (Bertschinger 1975; Nistal *et al.* 1977; Escalier 1984; Weissenberg *et al.* 1998; Benzacken *et al.* 2001; Lewis-Jones *et al.* 2003; Kopp *et al.* 2007; Coutton *et al.* 2015; Ray *et al.* 2017). This particular defect is commonly found in mammals, although the incidence is generally low (Nistal *et al.* 1977; Molinari *et al.* 2013) and rarely the cause of infertility (Barth and Oko 1989). However, when a high incidence is reported, the prognosis for fertility is generally poor (Devillard *et al.* 2002; Molinari *et al.* 2013). In man, this multiple defect (a high incidence of spermatozoa with enlarged heads and multiple tails) has been termed the macrocephalic sperm head syndrome (Nistal *et al.* 1977; Perrin *et al.* 2008), sperm macrocephaly syndrome (SMS; Molinari *et al.* 2013) or meiotic division deficiency (Escalier 2002). The ultrastructural features of the defect have been thoroughly described, demonstrating the uniformity of the anomaly across different mammalian species (Nistal *et al.* 1977; Escalier 1984; Barth and Oko 1989; Bonet and Briz 1991).

Macrocephalic spermatozoa have been identified in several avian species (Wakely and Kosin 1951; Nwakalor *et al.* 1988; Ferdinand 1992; Hartley 1999; Lindsay *et al.* 1999; Wishart *et al.* 1999; Barna and Wishart 2003; Sontakke *et al.* 2004; Klimowicz *et al.* 2005; Chelmońska *et al.* 2008; Tabatabaei *et al.* 2009), including ratites such as the ostrich (du Plessis *et al.* 2014) and emu (Malecki *et al.* 1998; du Plessis and Soley 2011). The incidence of this particular defect in birds, expressed as a percentage of the total sperm count, is generally low, ranging from 2.8% in the quail (Chelmońska *et al.* 2008) to 7.4% in the Indian white-backed vulture (Umapathy *et al.* 2005). Similarly, in the ostrich, the overall incidence of the defect is low, although the anomaly represents the most commonly observed head defect, constituting 7.7% of total sperm defects (du Plessis *et al.* 2014). However, subsequent studies on ostrich semen have revealed an exceptionally high individual incidence of this defect (an average of almost 30% of total sperm count over the winter and spring seasons) in a specific breeding male (L. du Plessis, unpubl. data). Despite the numerous accounts of macrocephaly in birds, little reference has been made to the combination of giant heads and multiple tails that characterise SMS. Some macrocephalic spermatozoa in the Houbara bustard (which shows a high incidence of macrocephalic sperm ranging from 5% to 40% of total sperm count) reportedly exhibit multiple tails (Lindsay *et al.* 1999). Due to the commercial importance of ratites such as the ostrich and emu, and considering the ongoing research on the application of AI in this farming enterprise (Malecki *et al.* 2008; Bonato and Cloete 2013), attention has focused on documenting abnormal sperm morphology in both species (du Plessis and Soley 2011; du Plessis *et al.* 2014). Whereas in the emu relatively large numbers of spermatozoa conforming to the morphological criteria for SMS have been reported (du Plessis and Soley 2012), in the ostrich an unusual phenomenon is described by light microscopy whereby spermatozoa with giant heads often exhibit a thickened tail that appears, although not yet confirmed ultrastructurally, to represent closely apposed multiple tails (du Plessis *et al.* 2014). Can such spermatozoa, if present in sufficient numbers, be considered to represent this syndrome?

Macrocephalic spermatozoa have been shown to exhibit polyploidy in both mammals (Salisbury and Baker 1966; Carothers and Beatty 1975; Viville *et al.* 2000; Devillard *et al.* 2002; Lewis-Jones *et al.* 2003; Guthauser *et al.* 2006; Kopp *et al.* 2007; Coutton *et al.* 2015) and birds (Barna and Wishart 2003; Lindsay *et al.* 1999). This phenomenon has been demonstrated using a variety of techniques, including Feulgen staining, flow cytometry, fluorescence *in situ* hybridisation (FISH) analysis and 4'-6-diamidino-2-phenylindole (DAPI) staining (Salisbury and Baker 1966; Bertschinger 1975; Carothers and Beatty 1975; Weissenberg *et al.* 1998; Barna and Wishart 2003; Sun *et al.* 2006; Revay *et al.* 2010). Information based on these techniques has identified diploid, triploid, tetraploid and hyperploid forms of macrocephalic spermatozoa (Sun *et al.* 2006; Coutton *et al.* 2015). However, some reports suggest that diploid spermatozoa appear to be the most common form of polyploidy (Salisbury and Baker 1966; Bertschinger 1975; De Braekeleer *et al.* 2015). Whether this trend holds true for ratite spermatozoa remains to be determined.

Information on motility parameters for SMS is limited. Escalier (1984) notes that affected spermatozoa exhibit variable motility with no definite trajectory being observed due to the uncoordinated beating of the multiple tails. Conflicting information has been presented on birds. In the Guinea fowl it was reported that the curvilinear velocity of large-nuclei spermatozoa (with suspected multiple axonemes) was markedly lower than that of normal spermatozoa (Barna and Wishart 2003). However, in the Houbara bustard, the velocity of spermatozoa with large nuclei (but not necessarily associated with multiple flagella) was reported to be greater than that of normal spermatozoa (Lindsay *et al.* 1999). Although the motility of ostrich spermatozoa has been assessed subjectively (Rybnik *et al.* 2007; Bonato *et al.* 2011) and by computer-aided sperm analysis (CASA; Ciereszko *et al.* 2010; Bonato *et al.* 2012; Smith *et al.* 2016, 2018*a*, 2018*b*), no association between motility parameters and SMS has been reported for ratites.

In view of the paucity of information on SMS in birds, this paper reports on the fine structure of this multiple defect in the ostrich, elaborates on the unusual tail arrangement, examines the DNA content of affected spermatozoa by flow cytometry and compares motility parameters between aberrant and normal spermatozoa using CASA.

Materials and methods

Animals

Semen samples were collected using the dummy female method (Rybnik *et al.* 2007; Malecki *et al.* 2008) from nine sexually active and fully trained South African black ostriches, ranging from 3 to 10 years of age. Birds were maintained at the Oudtshoorn Research Farm of the Western Cape Department of Agriculture (global positioning system (GPS) coordinates – 33°37'54.1848"S, 22° 15'25.6932"E) in the Little Karoo region of South Africa as part of a project on AI in ostriches. In all, 171 ejaculates were collected during the winter (June) and spring (September) seasons. Aliquots of neat semen from all nine birds were used for CASA immediately after collection. The remaining semen was mixed with 4%

phosphate-buffered glutaraldehyde in equal volumes for the examination of sperm morphology and for flow cytometric analysis.

In addition, data on sperm dimensions (normal and macrocephalic) generated during a previous study (du Plessis *et al.* 2014) provided complementary evidence for the present study. Data are expressed as the mean ± s.d.

Morphology

Smears of the fixed semen samples were stained with Wright's stain (Rapidiff; Clinical Sciences Diagnostics) for the morphological evaluation of spermatozoa on an Olympus BX63 light microscope using a 100× oil immersion objective (phase contrast illumination) and Olympus cellSens Imaging Software version 1.5 (du Plessis *et al.* 2014). Based on the light microscopic evaluation, selected samples from birds exhibiting a high incidence of macrocephalic spermatozoa were routinely prepared for transmission electron microscopy (TEM; du Plessis and Soley 2011). The incidence of the defect for the nine birds sampled is expressed as the median value and interquartile range (IQR, 25% and 75%).

Motility

Sperm cell motility was evaluated using the method described by Smith *et al.* (2016). Briefly, neat semen was resuspended to a final concentration of 20×10^6 spermatozoa mL^{-1} in a standard motility buffer, using sodium chloride (150 mM) and TES (20 mM) with 2% of the male-specific seminal plasma. The sample was incubated at 38°C for 1 min, after which 2 µL diluted semen was placed on a prewarmed slide, covered with a coverslip (22 × 22 mm) and allowed to settle for a few seconds before recording. Random images of 5 to 10 different fields were captured to obtain a total of at least 300 images of motile spermatozoa using the Sperm Class Analyzer (SCA) version 5.3 (Microptic) with a Basler A312fc digital camera mounted on an Olympus BX41 microscope equipped with phase contrast optics. Only images containing both normal and macrocephalic spermatozoa were used for subsequent analysis. Each macrocephalic and normal sperm cell was then individually identified and their motility recordings extracted for analysis (n = 6087). The same investigator (MB) performed all motility analyses for consistency.

Sperm cell motility variables included amplitude of lateral displacement (ALH), beat-cross frequency (BCF), linearity (LIN) and wobble (WOB). Various measures of velocity can be calculated, such as average path motility (VAP), curvilinear velocity (VCL) and straight line velocity (VSL), but VAP was used in the present study because it has been shown to correlate well with fertility in other bird species like the fowl (Wishart and Palmer 1986; Cornwallis and O'Connor 2009) and was highly correlated with VCL (r = 0.93) and VSL (r = 0.75) in the present study. Generalised linear mixed models (GLMM) were then performed using Genstat version 18 (VSN International) to investigate whether sperm cell motility was influenced by head morphology. Head morphology (normal vs macrocephalic), season (winter or spring) and male age were entered as fixed factors, whereas male identity was entered as a random factor. LIN and WOB variables were arcsin transformed to achieve normal distribution before the analysis. Furthermore, to control for potential inbreeding depression, inbreeding coefficients were estimated for each bird using the MTDFNRM

software developed by Boldman *et al.* (1995), and included as a covariate in all GLMM analyses.

Flow cytometry

For flow cytometry, 12 randomly selected samples (of the 171 ejaculates) from the nine birds were used for blind analysis. To determine DNA ploidy, 100 µL cell lysis buffer (LPR buffer, DNA Prep Reagents Kit, Beckman Coulter) was added to 100 µL fixed sperm solution and briefly vortexed. After 5 min incubation at room temperature, 1 mL DNA staining solution (10 mg mL^{-1} propidium iodide (PI); Sigma Aldrich) was added to the lysed cell suspension, which was further incubated for 30 min in the dark at room temperature. Samples were analysed using a Gallios flow cytometer (Beckman Coulter). The PI fluorescent signal was detected using the FL3 (620/30 nm) bandpass filter. During analysis, larger aggregates were removed using an FL3 log width versus FL3 log area plot. To establish the relationship between relative DNA concentration and relative forward scatter (FS; cell size), 1 mL DNA staining solution was added to 100 µL fixed sperm suspension and briefly vortexed. The cell suspension was incubated for 30 min in the dark at room temperature, after which flow cytometric analysis was performed. During analysis, nuclei aggregates were excluded using an FL3 width versus FL3 area plot.

Postacquisition data analysis was performed using Kaluza Analysis software (version 2.1; Beckman Coulter) consistently employing the same gating strategy. The median FS area signal was used as an indication of size differences observed between the diploid and haploid populations (Standerholen *et al.* 2014). The instrument configuration and settings used are summarised in Table S1, available as Supplementary Material to this paper.

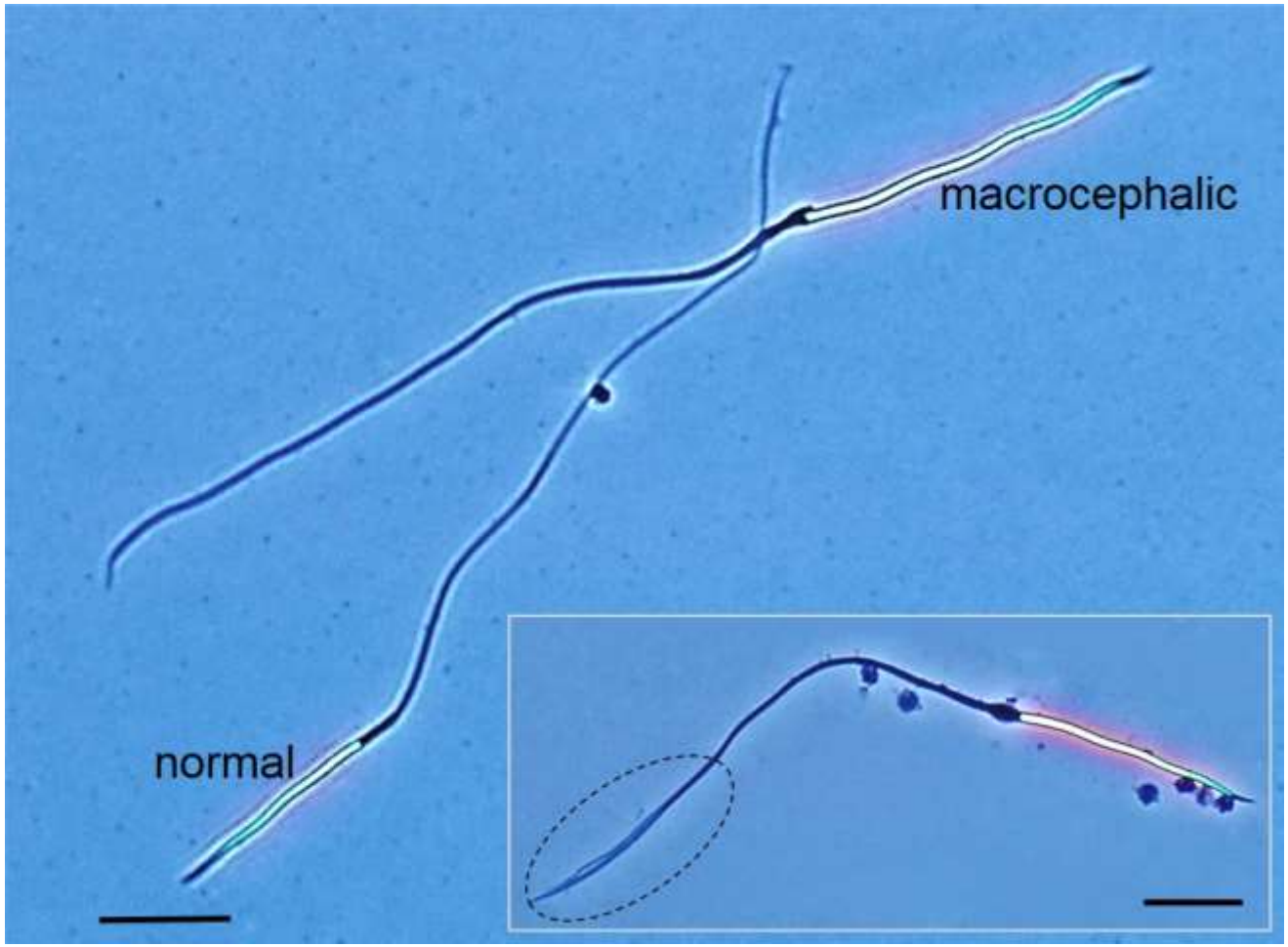
Results

Morphology

For the nine birds in the present study, the incidence of the defect was variable, ranging from 0% to 29.6% (median 0.9%, IQR 0.3%, 1.6%) of the total sperm count. One bird consistently revealed a higher percentage of giant heads, ranging from 8.8% to 29.6% (median 13.8%, IQR 12.5%, 17.9%).

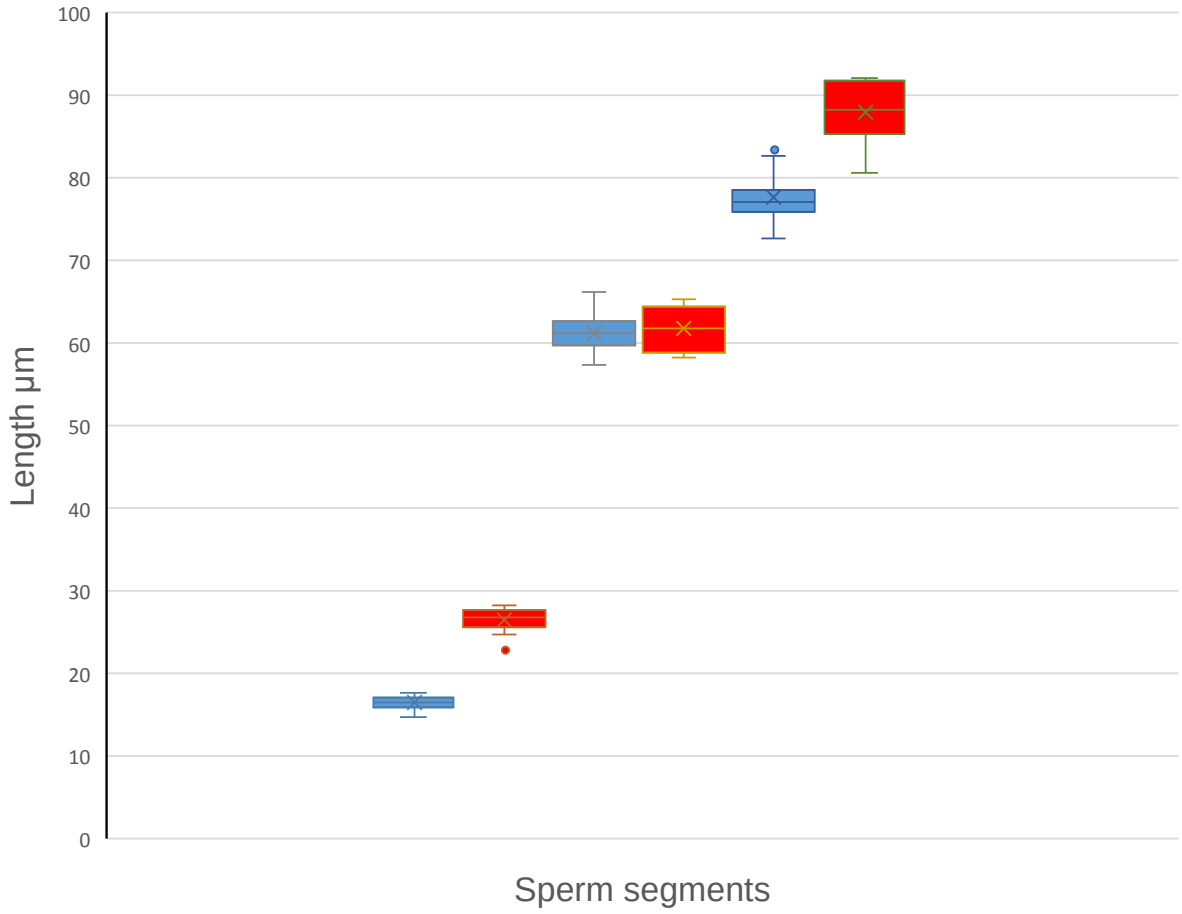
On light microscopy, macrocephalic spermatozoa were readily identified by the presence of a visibly longer head than that in normal spermatozoa (26.5 ± 1.6 vs 16.7 ± 0.6 µm respectively; Figs 1, 2; du Plessis *et al.* 2014). The base of the head often appeared bulbous, indicating widening to accommodate the supernumerary centriolar complexes (CC) observed by TEM (see below). In addition, aberrant spermatozoa exhibited an apparently single but noticeably thicker flagellum of similar length to that in normal spermatozoa (61.7 ± 2.6 vs 60.9 ± 2.8 µm respectively; Fig. 2). However, due to the longer head length, total sperm length was greater in macrocephalic spermatozoa (Fig. 2). In macrocephalic spermatozoa, the compound tail (revealed by electron microscopy) generally formed a complete unit involving all segments of the tail. However, in some cells the unitary tail was

Fig. 1. Light micrograph showing the difference in head length between normal and macrocephalic spermatozoa. Note the apparently single but thickened tail characteristic of most macrocephalic spermatozoa. Inset: unravelling of the end-piece and distal part of the principal piece of a macrocephalic spermatozoon (circled). Several free cytoplasmic droplets are also obvious. Glutaraldehyde-fixed spermatozoa; Wright's stain; Phase contrast microscopy. Scale bars = 10 µm.



partially separated, generally in the vicinity of the end-piece, but also in the more distal parts of the principal piece (Fig. 1 inset), revealing its true multiflagellate nature.

Fig. 2. Box plot showing head, tail and total sperm lengths of normal (*n* = 125; blue) and macrocephalic (*n* = 26; red) spermatozoa. The obvious difference in total length is almost entirely due to the longer head length of macrocephalic spermatozoa (extrapolated from the original data of du Plessis *et al.* (2014)). Boxes show the interquartile range, with the median value indicated by the horizontal line and the mean value shown by an 'X'. Whiskers indicate minimum and maximum values and outliers are indicated by circles.



At the ultrastructural level, normal ostrich spermatozoa exhibited the typical morphological features previously reported for this species (Soley 1993). In contrast, macrocephalic spermatozoa were characterised by a unitary midpiece housing a variable number (two being the most common variant) of CC. Each CC was surrounded by a mitochondrial sheath that generally incorporated only a single row of mitochondria between adjacent CCs (Figs 3, 4). In some defective cells, mitochondrial cement replaced the mitochondria between adjacent CCs (Fig. 3*b*, *c*). Although in most instances the widened nuclear base accommodated the supernumerary CCs, peripheral attenuation of the nuclear base was observed in some cells. When three or more CCs were present, they were circularly arranged beneath the nuclear base (Fig. 4*d*), although triplet CCs were occasionally linearly arranged. Cytoplasmic droplets were frequently associated with the multicentriolar midpiece (Figs 3, 4).

Fig. 3. (*a*–*c*) The neck region and proximal midpiece of ostrich spermatozoa revealing twin centriolar complexes. In (*a*), mitochondria and patches of inter-mitochondrial cement fill the space between the centriolar complexes, whereas in (*b*) and (*c*) inter-mitochondrial cement occupies this position (stars). A nuclear spine (white arrow) projects between the centriolar complexes in (*a*) and (*b*). PC, proximal centriole; DC, distal centriole; Cd, cytoplasmic droplet. Scale bars = 0.5 µm.

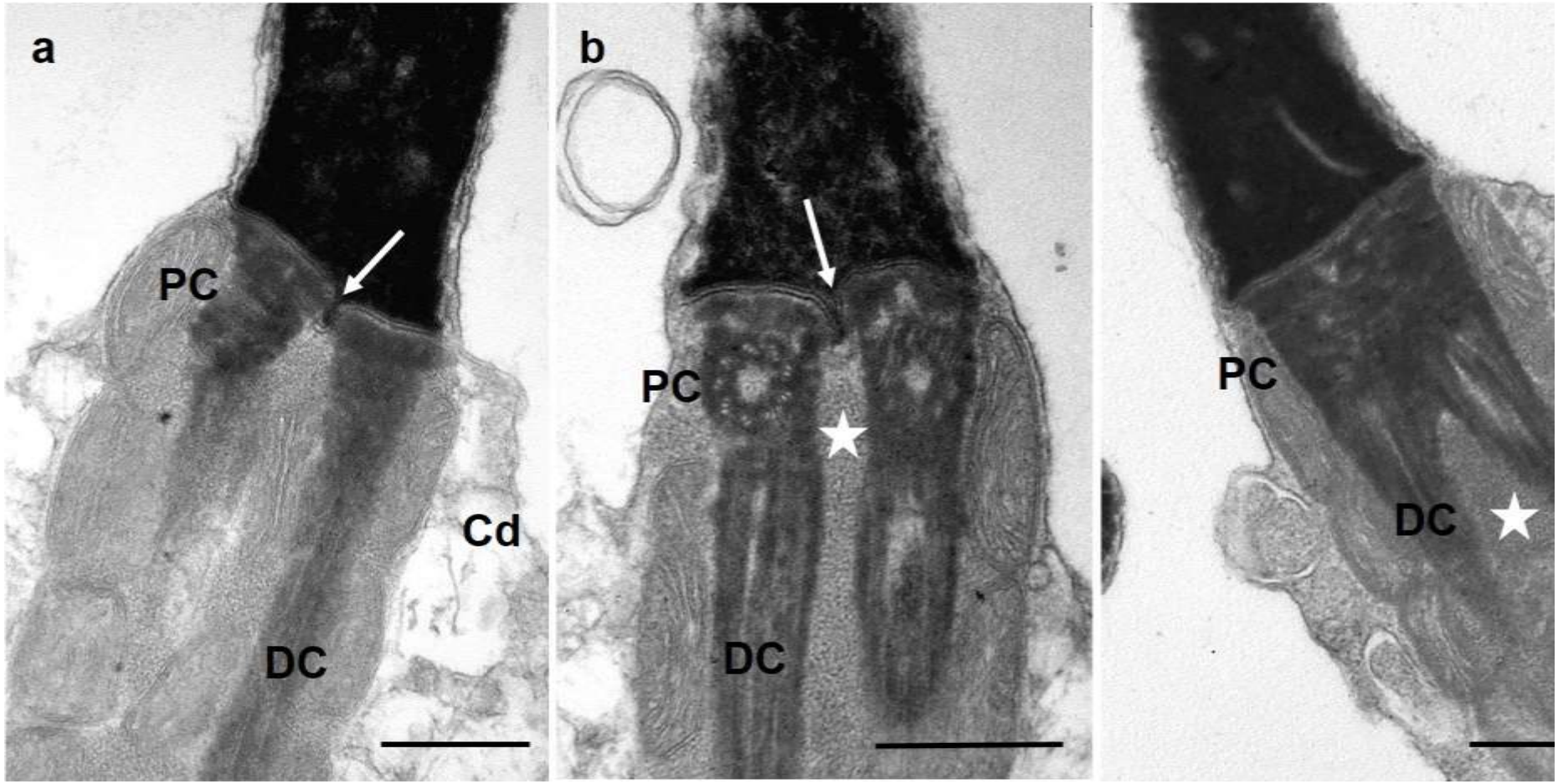
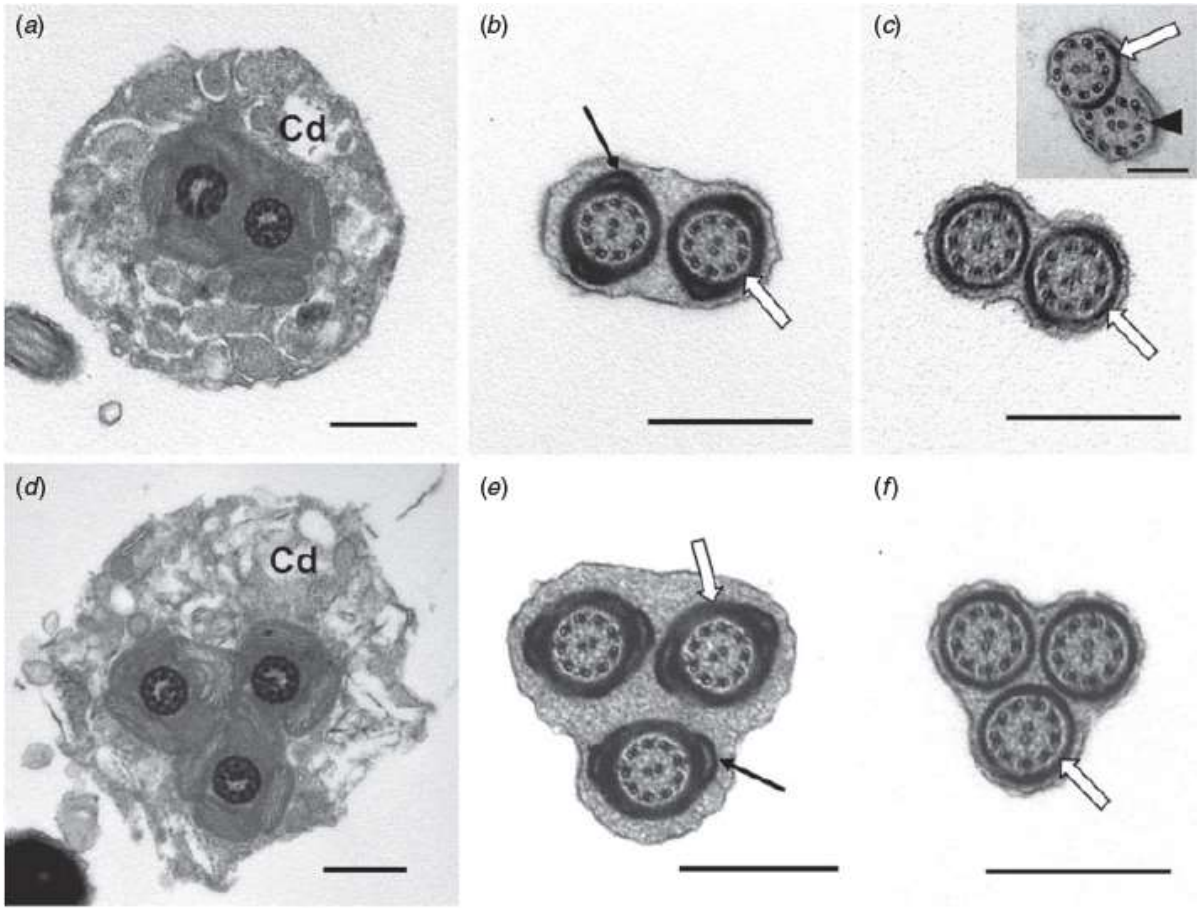
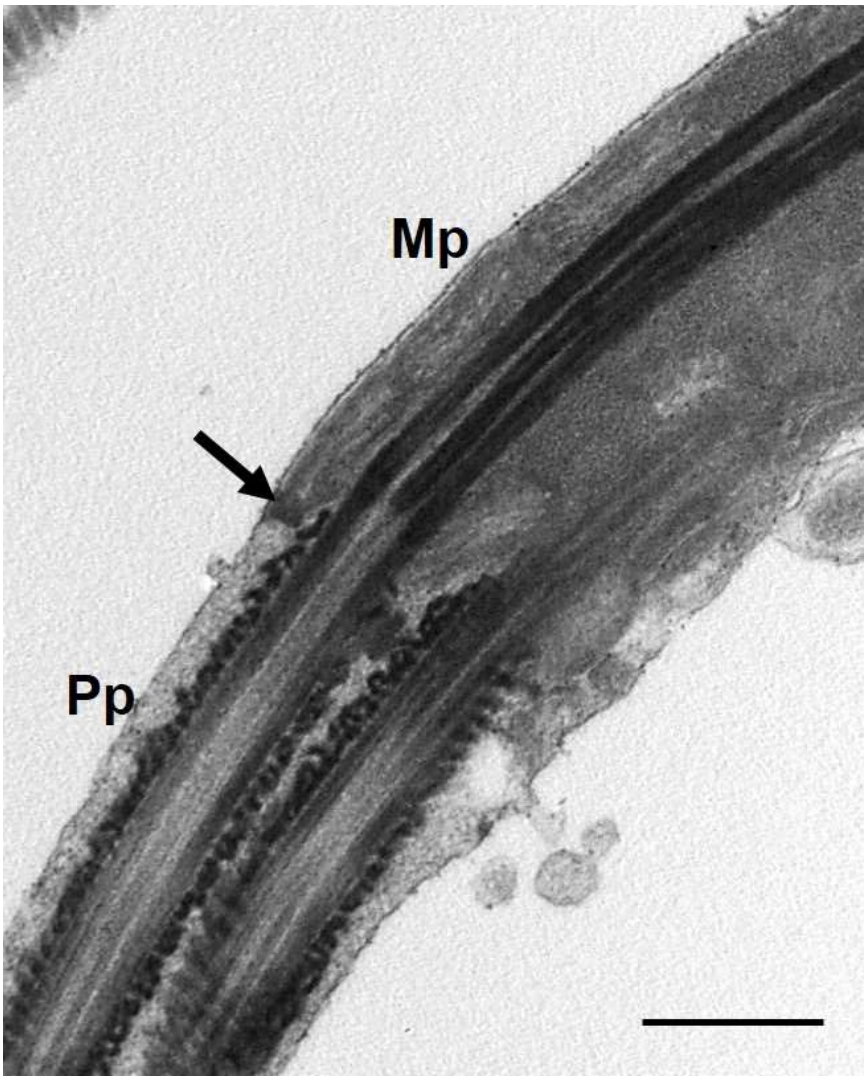


Fig. 4. Series of micrographs from proximal to distal demonstrating transverse sections of ostrich spermatozoa with double (*a*–*c*) and triple (*d*–*f*) tails. Note the cytoplasmic droplet (Cd) surrounding the midpiece (*a*, *d*) and, in the principal piece (*b*, *c*, *e*, *f*), the normal axoneme surrounded by longitudinal columns (thin black arrows) and ribs of the fibrous sheath (white arrows). The inset in (*c*) shows the end-piece of a twin tail demonstrating remnants of the fibrous sheath (white arrow) and disruption of some microtubular doublets (arrowhead). Scale bars = 0.5 µm (*a*–*f*); 0.25 µm (inset, *c*).



A conspicuous annulus defined the border between the midpiece and principal piece of the flagellum and was present around each of the multiple tails (Fig. 5). This transition between the two parts of the flagellum was precise and identical for each of the flagella involved (Fig. 5). The thickened tail observed by light microscopy was shown ultrastructurally to consist of multiple principal pieces (generally two, although as many as five were observed) collectively bound within the plasmalemma (Fig. 4). Each principal piece was independent and clearly separated from adjoining tails by a conspicuous layer of cytoplasm (Fig. 4). This part of the tail was composed of a typical axoneme surrounded by a fibrous sheath consisting of rudimentary longitudinal columns connected by rib-like extensions (Figs 4, 5). The duplicated axonemes continued to the end of the tail to form the end-piece, which exhibited varying degrees of microtubular disruption (Fig. 4). Therefore, based on these observations, macrocephalic spermatozoa in the ostrich also possess multiple but unseparated flagella.

Fig. 5. Longitudinal profile of a spermatozoon sectioned at the midpiece (Mp)–principal piece (Pp) junction showing the emergence of identically formed twin tails collectively bound within a common cell membrane. The arrow indicates the annulus. Scale bar = 0.5 μm.



Motility

In all, 6087 spermatozoa from the nine birds were recorded for the motility analysis during the 2016 winter and spring seasons, of which 191 were identified as macrocephalic and 5896 were identified as normal. No macrocephalic spermatozoa were observed in the semen of one bird. Both CASA and light microscopy independently revealed the incidence of macrocephalic spermatozoa, which, expressed as a percentage, were similar. Therefore, the two techniques supported each other with regard to the incidence of the defect.

Macrocephalic spermatozoa showed an increase in BCF and LIN and a decrease in VAP and WOB compared with normal spermatozoa ($P < 0.05$; Tables 1, 2). An effect of season was also detected for all variables except for BCF and VAP ($P < 0.05$), with higher values being observed for ALH and LIN in winter and a higher value for WOB in the spring. Male age also affected all motility variables measured, but to a different extent: ALH, VAP and WOB

Table 1. Least square mean (± s.e.m.) and s.d. and CV for the effect of head morphology, season and male age on sperm kinematic traits of spermatozoa from nine South African black ostriches

*P < 0.05, **P < 0.01, ***P < 0.001. ALH, amplitude of lateral displacement; BCF, beat-cross frequency; LIN, linearity; VAP, average path velocity; WOB, wobble

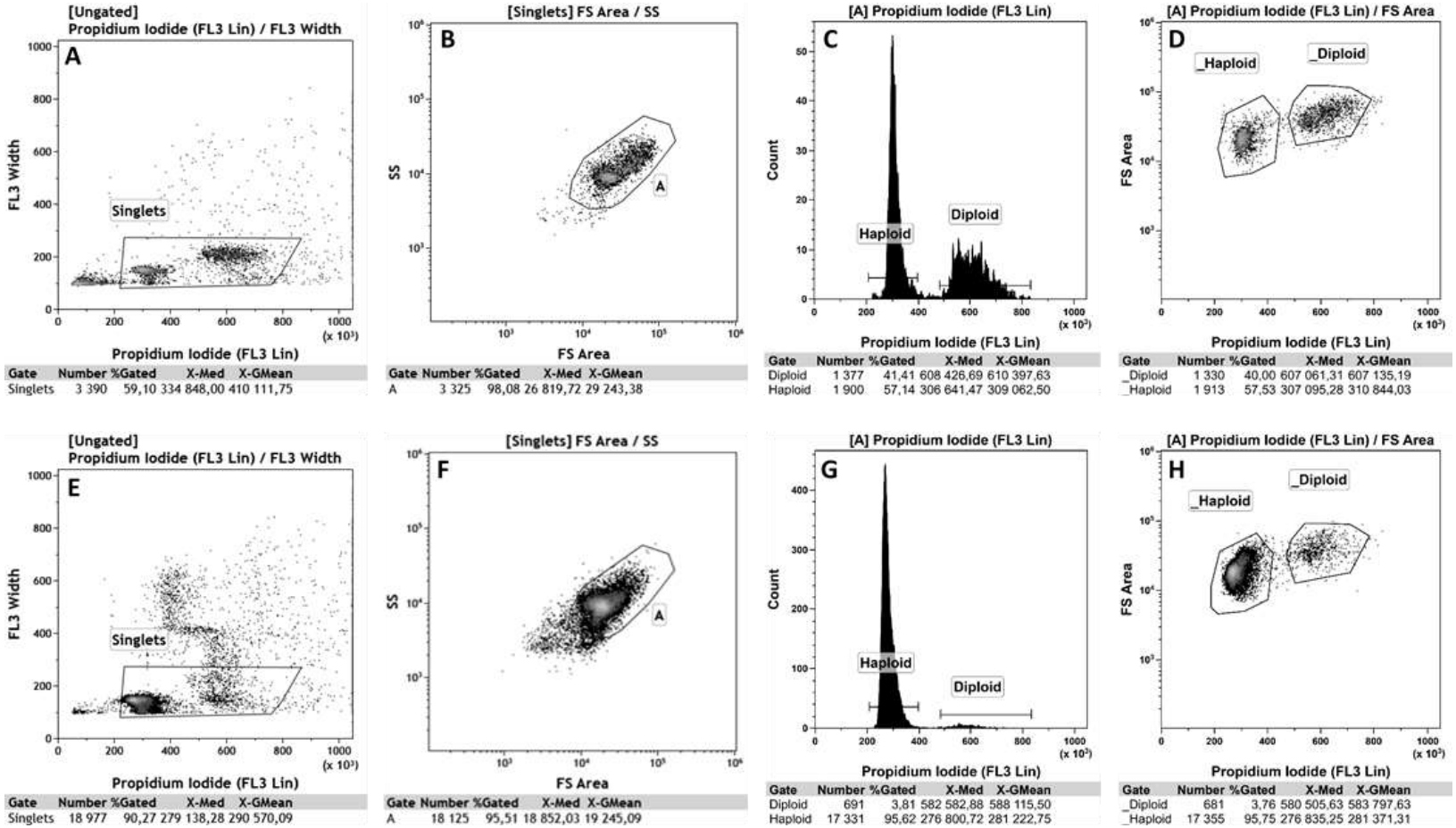
Variation source	ALH (μm s^{-1})			BCF (Hz)			LIN (%)			VAP (μm s^{-1})			WOB (%)		
	Mean	s.d.	CV	Mean	s.d.	CV	Mean	s.d.	CV	Mean	s.d.	CV	Mean	s.d.	CV
Head morphology	$P > 0.05$			***			***			**			*		
Macrocephalic	2.58 ± 0.07	0.94	36.47	7.23 ± 0.22	3.09	42.71	54.91 ± 2.08	29	52.13	48.16 ± 2.06	28.44	59.05	69.93 ± 1.46	20	28.78
Normal	2.64 ± 0.02	1.14	43.06	5.71 ± 0.04	3.12	54.58	44.51 ± 0.38	29	64.93	54.86 ± 0.38	29.49	53.76	73.45 ± 0.25	19	26.78
Season	***			$P > 0.05$			***			*			***		
Winter	2.83 ± 0.22	1.22	42.92	5.73 ± 0.06	3.04	53.08	47.00 ± 0.475	26	55.43	55.43 ± 0.55	30.02	54.15	72.50 ± 0.34	18.9	26.06
Spring	2.45 ± 0.02	1.06	41.09	5.79 ± 0.06	3.21	55.44	42.80 ± 0.567	31.4	73.43	53.86 ± 0.52	28.92	53.7	74.20 ± 0.36	19.9	26.79
Male age (years)	*			***			***			***			***		
3	2.56 ± 0.02	1.03	40.14	5.99 ± 0.06	3.11	51.9	47.40 ± 0.57	27.3	57.69	52.54 ± 0.62	30.07	57.23	72.00 ± 0.43	20.7	28.73
4	2.69 ± 0.03	1.18	43.73	5.95 ± 0.07	3.08	51.81	46.40 ± 0.61	25.1	54.1	53.99 ± 0.79	27.29	50.55	74.30 ± 0.44	18.2	24.51
6	2.47 ± 0.03	1.26	42.98	5.22 ± 0.09	3.31	63.38	38.70 ± 1.08	36.9	55.39	58.63 ± 1.07	27.29	50.55	76.10 ± 0.51	17.4	22.89
10	2.93 ± 0.04	1.1	44.49	5.52 ± 0.09	2.97	52.84	43.50 ± 0.88	26.7	61.38	53.75 ± 0.79	32.58	55.57	71.50 ± 0.66	20.2	28.22

Table 2. Effects of head morphology, season, male age and their interactions on amplitude of lateral displacement (ALH), beat-cross frequency (BCF), linearity (LIN), average path velocity (VAP) and wobble (WOB) of spermatozoa from nine South African black ostriches

Significant *P*-values are bolded. HM, head morphology; S, season; A, male age

Variables	ALH (μm s^{-1})			BCF (Hz)			LIN (%)			VAP (μm s^{-1})			WOB (%)		
	d.f.	*F*	*P*-value	d.f.	*F*	*P*-value	d.f.	*F*	*P*-value	d.f.	*F*	*P*-value	d.f.	*F*	*P*-value
Fixed factors															
HM	1, 6070	0.63	0.427	1, 6070	45.67	**<0.001**	1, 6070	24.89	**<0.001**	1, 6070	9.73	0.002	1, 6070	6.22	0.013
S	1, 6070	186.96	**<0.001**	1, 6070	0.33	0.565	1, 6070	34.93	**<0.001**	1, 6070	4.20	0.04	1, 6070	12.44	**<0.001**
A	3, 6070	33.05	**<0.001**	3, 6070	18.49	**<0.001**	3, 6070	24.32	**<0.001**	3, 6070	9.57	**<0.001**	3, 6070	15.38	**<0.001**
HM × S	1, 6070	1.97	0.161	1, 6070	6.28	**0.012**	1, 6070	1.55	0.213	1, 6070	8.22	0.004	1, 6070	12.79	**<0.001**
HM × A	3, 6070	0.47	0.701	3, 6070	3.10	**0.025**	3, 6070	0.63	0.593	3, 6070	2.60	0.055	3, 6070	2.68	0.046
S × A	3, 6070	16.12	**<0.001**	3, 6070	42.06	**<0.001**	3, 6070	36.01	**<0.001**	3, 6070	12.51	**<0.001**	3, 6070	8.96	**<0.001**
HM × S × A	3, 6070	3.05	**0.028**	3, 6070	1.46	0.223	3, 6070	0.86	0.463	3, 6070	1.81	0.144	3, 6070	0.64	0.591
Covariate															
Inbreeding coefficient	1, 6070	0.00	0.983	1, 6070	7.12	**0.008**	1, 6070	8.76	**0.003**	1, 6070	0.37	0.545	1, 6070	6.90	**0.009**
Random factor															
Male ID	4, 6070	3.08	**0.015**	4, 6070	24.52	**<0.001**	4, 6070	9.66	**<0.001**	4, 6070	13.51	**<0.001**	4, 6070	11.16	**<0.001**

Fig. 6. Flow cytometry histograms obtained by propidium iodide staining of selected glutaraldehyde-fixed ostrich semen samples. (*a*–*d*) Semen sample (#139) from a bird with a known high concentration of macrocephalic spermatozoa; (*e*–*h*) semen sample (#176) from a bird with a low concentration of aberrant spermatozoa. (*a*, *e*) Ungated sperm population; (*b*, *f*) gated cell population (large aggregates removed) showing two subpopulations of spermatozoa; (*c*, *g*) histogram plot indicating the levels of fluorescence (*x*-axis) emitted from the two subpopulations; (*d*, *h*) a relationship is demonstrated between cell size (forward scatter (FS)) and DNA content, with the larger spermatozoa (diploid) revealing double the DNA content of the smaller (haploid) sperm population.



tended to increase with age, whereas BCF and LIN tended to decrease with age. Furthermore, significant interactions between sperm morphology and season were detected for BCF, VAP and WOB (Table 2; $P < 0.05$). Although both normal and macrocephalic spermatozoa had higher VAP values in winter (55.43 ± 0.56 and 55.66 ± 3.16 µm s^{-1} respectively) compared with spring (54.27 ± 0.53 and 42.26 ± 2.59 µm s^{-1} respectively; $P < 0.01$), BCF and WOB values for normal and macrocephalic spermatozoa followed different trends with regard to season: normal spermatozoa had higher BCF and WOB values in spring than in winter (BCF: 5.75 ± 0.06 vs 5.67 ± 0.06 Hz respectively; WOB: 74.5 ± 0.4% vs 72.4 ± 0.4% respectively; $P < 0.05$), whereas macrocephalic spermatozoa had higher values in winter than in spring (BCF: 7.81 ± 0.34 vs 6.77 ± 0.29 Hz respectively; WOB: 74.4 ± 0.2% vs 66.4 ± 0.2% respectively; $P < 0.05$). Furthermore, significant interactions were detected between head morphology and male age for BCF and WOB ($P < 0.05$), between season and male age for all motility variables ($P < 0.001$) and between head morphology, season and age for ALH ($P < 0.05$; Table 2).

Flow cytometry

Flow cytometric analysis indicated two distinct populations within the samples tested based on the fluorescence intensity of nuclei after staining with the stoichiometric DNA stain PI (Fig. 6). In gated samples, the population on the right represented diploid spermatozoa, whereas the population on the left signified haploid cells (Fig. 6*c*, *g*). The diploid population (Fig. 6*c*, *d*, *g*, *h*) exhibited a twofold increase in fluorescence intensity (indicating DNA content) compared with the haploid population (mean fluorescence intensity 328 917 ± 13 257 vs 161 746 ± 11 977 respectively). The diploid population also showed an increase in forward light scatter compared with the haploid population (Fig. 6*d*, *h*), indicating that diploid cell nuclei were larger than haploid cell nuclei. FS is proportional to the cell surface area or diameter of the cells or particles being measured. Flow cytometry proved sensitive enough to clearly distinguish between two sperm populations (normal and macrocephalic) even when the incidence of macrocephalic spermatozoa was low (Fig. 6g, *h*).

Discussion

This study revealed that a variable number of abnormal ostrich spermatozoa exhibit structural peculiarities similar to those of SMS described in mammals. Ultrastructural features of the defect were similar except that the characteristic multiple tails were collectively bound within the plasmalemma. Flow cytometry identified these cells as diploid and CASA revealed that they swim slower but straighter than normal spermatozoa.

Morphology

With regard to head size, macrocephalic spermatozoa in the ostrich are 1.6-fold longer (extrapolated from du Plessis *et al.* 2014) and marginally thicker (subjective observations from the present and previous studies on ostrich spermatozoa) than normal spermatozoa. A similar increase in length has been reported for macrocephalic spermatozoa in the emu (du

Plessis and Soley 2011), and the heads of spermatozoa in triploid ZZZ fowls are 1.4-fold longer than those of the normal ZZ genotype (Lin *et al.* 1995*b*). Similarly, in a study on passerine spermatozoa, it was reported that sperm head area was 1.6-fold greater in triploid ZZZ zebra finches than in normal diploid males (Girndt *et al.* 2014). These figures compare favourably with affected human spermatozoa, where an increase in surface area of 1.5-fold has been recorded (Guthauser *et al.* 2013). However, macrocephalic spermatozoa in the Houbara bustard had a mean length twice that of normal spermatozoa (Lindsay *et al.* 1999), whereas another human study observed a threefold increase in nuclear volume (Escalier 1984). It should be noted that when comparing sperm head dimensions in birds, particularly with regard to their length, the effect of various fixatives and dyes used during sample preparation for light microscopy should be taken into account (Santiago-Moreno *et al.* 2016). In contrast, evidence from passerine species suggests that long-term storage of sperm samples in 5% formaldehyde solution, or prior exposure to various solvent media, has no effect on sperm length (Schmoll *et al.* 2016). Of possibly greater significance is the potential effect of the collection method on sperm length. As demonstrated in *Passer domesticus*, spermatozoa collected following faecal massage were significantly shorter than those collected by abdominal massage. The female dummy method (successfully used in the present study for obtaining physiological ejaculates) could not be compared due to the failure of male house sparrows to accept the dummy (Girndt *et al.* 2017). Although exhibiting markedly longer heads than normal spermatozoa, macrocephalic spermatozoa in the ostrich did not demonstrate the lack of chromatin condensation reported for spermatozoa with elongated heads (Prisant *et al.* 2007) or in macrocephalic spermatozoa (Escalier 1984) from some human patients.

Macrocephalic spermatozoa in mammals may typically exhibit two or more flagellae (Nistal *et al.* 1977; Benzacken *et al.* 2001; Devillard *et al.* 2002), with an average of 3.6 being observed in one instance in man (Escalier 1984). In contrast, very little has been published on this phenomenon in birds. An unusual finding in the present study was the occurrence of multiple tails bound together by the plasmalemma in association with the majority of macrocephalic spermatozoa. Whereas in most reports on the occurrence of multiple flagellae in mammals and birds separation of the duplicate principal and end-pieces is apparent (Nistal *et al.* 1977; Escalier 1984; Lindsay *et al.* 1999; du Plessis and Soley 2012), in the ostrich, with few exceptions, all components of the multiple tails are closely bound together by a common cell membrane. A similar phenomenon has been described in the boar (Bonet and Briz 1991) and duck (Maretta 1979), although no association was made between this anomaly and macrocephalic heads. The collective multiple tails were identical with regard to their dimensions and structure to normal tails and to the separate multiple flagellae described in other species (Nistal *et al.* 1977; du Plessis and Soley 2012). As in the emu (du Plessis and Soley 2011, 2012) and duck (Maretta 1979), most multiflagellate spermatozoa in the ostrich were biflagellate in nature, although triplet forms were also commonly observed. Based on these observations it is clear that the thickened tails of macrocephalic ostrich spermatozoa are indeed multiflagellate. Why the multiple tails fail to separate, with the exception of the short midpiece, during spermiogenesis as happens, for example, in the emu (du Plessis and Soley 2012), remains unknown, but poses interesting questions pertinent to the normal development of the flagellum.

Motility

In the present study, the increased values of motility parameters such as LIN and STR for macrocephalic spermatozoa suggest that they swim in a straighter line than normal spermatozoa, which reportedly exhibit a low linearity of movement in the ostrich (Ciereszko *et al.* 2010). Although it is conceded that the present study was performed on a relatively small number of males (n = 9), the lower values observed for VAP indicate that macrocephalic spermatozoa swim slower than normal spermatozoa. Reduced velocity appears to be a consistent feature of macrocephalic spermatozoa in birds and has been demonstrated in the guinea fowl (Barna and Wishart 2003). A contradictory reinterpretation (see Introduction) of the earlier work of Lindsay *et al.* (1999) by the same authors (Wishart *et al.* 2002) suggests that the motility of macrocephalic spermatozoa in the Houbara bustard is only 30% that of spermatozoa with normal nuclear dimensions. A similar trend has been reported for human spermatozoa, with morphologically normal spermatozoa being observed to swim faster (and in this instance straighter) than spermatozoa with elongated heads and megalocephalic sperm (Katz *et al.* 1982). Similarly, spermatozoa with large heads and multiple tails show low mobility (Nistal *et al.* 1977) or varying degrees of motility (Escalier 1984).

Humphries *et al.* (2008) note that the velocity of spermatozoa moving through a medium will be 'proportional to the balance between drag from the head and thrust from the flagellum', and suggest further that 'the ratio between flagellum and head length may provide a reasonable predictor for swimming speed'. Whereas this basic concept may be applicable for non-passerine species, in passerine birds sperm head morphology (in particular the helical nature of the sperm head and width of the helical membrane) rather than sperm head length has been associated with sperm swimming speed (Støstad *et al.* 2018). Macrocephalic ostrich spermatozoa exhibit a markedly lower head : tail ratio (1 : 2.3) compared with that of normal ostrich spermatozoa (1 : 3.7; du Plessis *et al.* 2014). This would suggest that they swim slower than normal spermatozoa, a phenomenon confirmed by the CASA data generated in the present study. It could be argued that the combined beating of the multiple flagella of the macrocephalic ostrich spermatozoa, if synchronised, should provide additional thrust. However, because the flagella are collectively bound within a common plasmalemma, and the individual principal pieces are firmly embedded in a cytoplasmic matrix (see Fig. 4*b*, *e*), the free (independent) movement required by the multiple tails to potentially generate additional propulsion is lacking. The fixed, constricted arrangement of the multiple flagella to form a single, combined structure would appear to explain the sluggish movement of the thickened tails of affected spermatozoa observed by CASA (M. Bonato, pers. obs.). The confined movement of the thickened tails may also explain the straighter trajectory of macrocephalic spermatozoa. This contrasts sharply with the observation on SMS in men that 'no definite trajectory could be defined for those spermatozoa with uncoordinated beating of their multiple tails' (Escalier 1984).

Because fertilisation success has been demonstrated to correlate well with sperm velocity and sperm morphology in several disparate species, such as red deer (Malo *et al.* 2005), green swordtail (Gasparini *et al.* 2010) and domestic fowl (Wishart and Palmer 1986; Birkhead *et al.* 1999), male ostriches exhibiting a higher proportion of slower-moving large-headed spermatozoa may demonstrate a lower rate of fertilisation success than males with

a higher proportion of normal spermatozoa. This is particularly important because most ostrich farmers use large multimale–multifemale groups in breeding camps (Cloete and Malecki 2011) and several studies have confirmed that specific males tend to sire significantly more chicks than others (Essa *et al.* 2004; Bonato *et al.* 2015). Hence, the reproductive skew observed in this promiscuous species could be explained, in part, by differences of sperm quality in males. Although this may be a valid argument for the ostrich, the presence of a relatively high proportion of macrocephalic spermatozoa in ejaculates of the Houbara bustard has been shown to have no apparent effect on fertility (Lindsay *et al.* 1999; Wishart *et al.* 2002). Further compounding the above argument is the observation in semen of red-legged partridge hybrids that spermatozoa with longer heads appear to swim faster (VSL) than do spermatozoa of normal head length found in pure red-legged partridges, possibly as an adaptation to sperm competition (Santiago-Moreno *et al.* 2015).

The effect of season on sperm motility variables observed in the present study is consistent with previous findings in ostriches (Bonato *et al.* 2014; Smith *et al.* 2016, 2018*a*) whereby some motility variables showed greater values either in spring or winter seasons. Although the reasons for this are still not fully understood, a change in daylight length in the spring season associated with elevated androgen concentrations, such as testosterone, which affects cell production and maturation, could potentially explain these variations (Degen *et al.* 1994). Furthermore, Smith *et al.* (2018*b*) demonstrated that day of collection influences the composition of seminal plasma and consequently sperm motility in ostriches. Thus, further investigations are needed to determine whether potential seasonal variation of the seminal plasma composition could shed light on the significant interactions between head morphology and sperm motility variables observed in the present study. In particular, increasing the number of males used could potentially help confirm or refute the effect of male age, as well as its interactions with head morphology and season observed on some of the motility variables.

Flow cytometry

In the present study, flow cytometric analysis of ostrich semen revealed a clear distinction between spermatozoa with heads of normal dimensions and those showing macrocephaly, and that the larger heads contained twice the normal DNA content, thus establishing their diploid nature. This association between diploidy and macrocephaly has been well established. The larger heads observed in triploid ZZZ fowls reportedly indicate that they may be diploid in nature (Lin *et al.* 1995*b*), supporting a previous study on testicular material (Lin *et al.* 1995*a*) suggesting that spermatids with a large nucleus, twin CCs and acrosomes and two tails may represent near diploid cells. The obvious size difference and intense DAPI staining of the nucleus of macrocephalic spermatozoa demonstrating two flagellae in the Houbara bustard would also suggest that they 'contain more than the haploid content of genetic material and that some were actually diploid' (Lindsay *et al.* 1999). The diploid nature of macrocephalic guinea fowl spermatozoa has also been reported (Barna and Wishart 2003). In men, macrocephalic spermatozoa have been associated with a diploid chromosomal content (Yurov *et al.* 1996; In't Veld *et al.* 1997), a phenomenon also apparent in the bull (Salisbury and Baker 1966; Revay *et al.* 2010).

The link between macrocephaly and polyploidy has led to the general consensus that both meiotic divisions are affected by incomplete partitioning (chromosome non-disjunction) coupled with a failure of nuclear cleavage (Devillard *et al.* 2002; Escalier 2002; Coutton *et al.* 2015; De Braekeleer *et al.* 2015; Gatimel *et al.* 2017; Ray *et al.* 2017). An association has been established between mutations of the aurora kinase C (*AURKC*) gene and macrocephalic spermatozoa (Chianese *et al.* 2015; Coutton *et al.* 2015; Gatimel *et al.* 2017; Ray *et al.* 2017), with evidence from *AURKC*-mutated patients indicating that DNA synthesis occurs unhindered in the developing germ cells, which, however, fail to complete any of the two meiotic divisions (Dieterich *et al.* 2007; Ben Khelifa *et al.* 2011). Whether a similar relationship exists between a specific gene mutation and SMS in the ostrich (and other birds) remains to be determined.

Conclusion

The increased head size, double DNA content and multiple tails observed in macrocephalic ostrich spermatozoa would indicate that such spermatozoa, if present in sufficient numbers, represent the SMS described in men. Should this syndrome in birds, although not proven, adversely affect fertility, as demonstrated in human clinical studies, its identification in commercial operations (poultry, ostrich and emu production) and breeding programs for endangered species would be essential. The thickened single yet composite flagellum characterising this syndrome in the ostrich is an interesting finding with the potential to provide additional data on tail development during spermiogenesis. The combination of enlarged sperm head (increasing drag) and merged multiple tails of normal length (adversely affecting thrust) would appear to account for the loss of motility exhibited by macrocephalic spermatozoa in the ostrich.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgement

This research did not receive any specific funding.

References

Barna, J., and Wishart, G. J. (2003). Excess nuclear DNA in spermatozoa of guinea fowl. *Theriogenology* **59**, 1685–1691.

Barth, A. D., and Oko, R. J. (1989). ‘Abnormal Morphology of Bovine Spermatozoa.’ (Iowa State University Press: Ames.)

Ben Khelifa, M., Zouari, R., Harbuz, R., Halouani, L., Arnoult, C., Lunardi, J., and Ray, P. F. (2011). A new *AURKC* mutation causing macrozoospermia: implications for human spermatogenesis and clinical diagnosis. *Mol. Hum. Reprod.* **17**, 762–768.

Benzacken, B., Gavelle, F. M., Martin-Pont, B., Dupuy, O., Lièvre, N., Hugues, J.-N., and Wolf, J.-P. (2001). Familial sperm polyploidy induced by genetic spermatogenesis failure. *Hum. Reprod.* **16**, 2646–2651.

Bertschinger, H. J. (1975). The hereditary occurrence of diploid spermatozoa in the semen of Brown Swiss bulls. Ph.D. Thesis, University of Zurich.

Birkhead, T. R., Martinez, J. G., Burke, T., and Froman, D. P. (1999). Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proc. Biol. Sci.* **266**, 1759–1764.

Boldman, K. G., Kriese, L. A., Van Vleck, L. D., Van Tassell, C. P., and Kachman, S. D. (1995). 'A Manual for Use of MTDFREML. A Set of Programs to Obtain Estimates of Variances and Covariances.' (United States Department of Agriculture, Agricultural Research Service.)

Bonato, B., and Cloete, S. W. P. (2013). Artificial insemination technology for ostriches: a way forward. *AgriPROBE* **10**, 24–26.

Bonato, M., Rybnik-Trzaskowska, P. K., Malecki, I. A., Cornwallis, C. K., and Cloete, S. W. P. (2011). Twice daily collection yields greater semen output and does not affect male libido in the ostrich. *Anim. Reprod. Sci.* **123**, 258–264.

Bonato, M., Cornwallis, C. K., Malecki, I. A., Rybnik-Trzaskowska, P. K., and Cloete, S. W. P. (2012). The effect of temperature and pH on the motility and viability of ostrich sperm. *Anim. Reprod. Sci.* **133**, 123–128.

Bonato, M., Malecki, I. A., Rybnik-Trzaskowska, P. K., Cornwallis, C. K., and Cloete, S. W. P. (2014). Predicting ejaculate quality and libido in male ostriches: effect of season and age. *Anim. Reprod. Sci.* **151**, 49–55.

Bonato, M., Cherry, M. I., and Cloete, S. W. P. (2015). Mate choice, maternal investment and implications for ostrich welfare in a farming environment. *Appl. Anim. Behav. Sci.* **171**, 1–7.

Bonet, S., and Briz, M. (1991). New data on aberrant spermatozoa in the ejaculate of *Sus domesticus. Theriogenology* **35**, 725–730.

Carothers, A. D., and Beatty, R. A. (1975). The recognition and incidence of haploid and polyploid spermatozoa in man, rabbit and mouse. *J. Reprod. Fertil.* **44**, 487–500.

Chelmońska, B., Jerysz, A., Łukaszewicz, E., Kowalczyk, A., and Malecki, I. (2008). Semen collection from Japanese quail (*Coturnix japonica*) using a teaser female. *Turk. J. Vet. Anim. Sci.* **32**, 19–24.

Chianese, C., Fino, M. G., Riera Escamilla, A., López Rodrigo, O., Vinci, S., Guarducci, E., Daguin, F., Muratori, M., Tamburrino, L., Lo Giacco, D., Ars, E., Bassas, L., Costa, M., Pisatauro, V., Noci, I., Coccia, E., Provenzano, A., Ruiz-Castañé, E., Giglio, S., Piomboni, P., and Krausz, C. (2015). Comprehensive investigation in patients affected by sperm macrocephaly and globozoospermia. *Andrology* **3**, 203–212.

Ciereszko, A., Rybnik, P. K., Horbanczuk, J. O., Dietrich, G. J., Deas, A., Slowinska, M., Liszewska, E., and Malecki, I. A. (2010). Biochemical characterization and sperm motility parameters of ostrich (*Struthio camelus*) semen. *Anim. Reprod. Sci.* **122**, 222–228.

Cloete, S. W. P., and Malecki, I. A. (2011). Breeder welfare: the past, present and future. In 'The Welfare of Farmed Ratites'. (Eds P. Glatz, C. Lunam, and I. Malecki.) pp. 13–44. (Springer-Verlag: Heidelberg.)

Cornwallis, C. K., and O'Connor, E. (2009). Sperm: seminal fluid interactions and the adjustment of sperm quality in relation to female attractiveness. *Proc. Biol. Sci.* **276**, 3467–3475.

Coutton, C., Escoffier, J., Martinez, G., Arnoult, C., and Ray, P. F. (2015). Teratozospermia: spotlight on the main genetic actors in the human. *Hum. Reprod. Update* **21**, 455–485.

De Braekeleer, M., Nguyen, M. H., Morel, F., and Perrin, A. (2015). Genetic aspects of monomorphic teratozoospermia: a review. *J. Assist. Reprod. Genet.* **32**, 615–623.

Degen, A. A., Weil, S., Rosenstrauch, A., Kam, M., and Dawson, A. (1994). Seasonal plasma levels of luteinizing and steroid hormones in male and female domestic ostriches (Struthio camelus). *Gen. Comp. Endocrinol.* **93**, 21–27.

Devillard, F., Metzler-Guillemain, C., Pelletier, R., DeRobertis, C., Bergues, U., Hennebicq, S., Guichaoua, M., Sèle, B., and Rousseaux, S. (2002). Polyploidy in large-headed sperm: FISH study of three cases. *Hum. Reprod.* **17**, 1292–1298.

Dieterich, K., Soto Rifo, R., Faure, A. K., Hennebicq, S., Ben Amar, B., Zahi, M., Perrin, J., Martinez, D., Sèle, B., Jouk, P., Ohlmann, T., Rousseaux, S., Lunardi, J., and Ray, P. F. (2007). Homozygous mutation of *AURKC* yields large-headed polyploid spermatozoa and causes male infertility. *Nat. Genet.* **39**, 661–665.

du Plessis, L., and Soley, J. T. (2011). Incidence, structure and morphological classification of abnormal sperm in the emu (*Dromaius novaehollandiae*). *Theriogenology* **75**, 589–601.

du Plessis, L., and Soley, J. T. (2012). Structural peculiarities associated with multiflagellate sperm in the emu, *Dromaius novaehollandiae. Theriogenology* **78**, 1094–1101.

du Plessis, L., Malecki, I., Bonato, M., Smith, M., Cloete, S., and Soley, J. (2014). A morphological classification of sperm defects in the ostrich (*Struthio camelus*). *Anim. Reprod. Sci.* **150**, 130–138.

Escalier, D. (1984). Human spermatozoa with large heads and multiple flagella: a quantitative ultrastructural study of 6 cases. *Biol. Cell* **48**, 65–74.

Escalier, D. (2002). Genetic approach to male meiotic division deficiency: the human macronuclear spermatozoa. *Mol. Hum. Reprod.* **8**, 1–7.

Essa, F., Cloete, S. W. P., and Fossey, A. (2004). Parentage determination of ostriches in breeding flocks using microsatellite markers. In 'Proceedings of the 3rd International Ratite Science Symposium of the World's Poultry Science Association (WPSA) and 12th World Ostrich Congress'. (Ed. E. Carbajo) pp. 29–33. (World's Poultry Science Association: Beekbergen, The Netherlands.)

Ferdinand, A. (1992). Licht- und elektronenmikroskopische Untersuchungen zur Morphologie von Ganterspermatozoen. Ph.D. Thesis, University of Veterinary Medicine, Hannover.

Gasparini, C., Simmons, L. W., Beveridge, M., and Evans, J. P. (2010). Sperm swimming velocity predicts competitive fertilization success in the green swordtail *Xiphophorus helleri. PLoS One* **5**, e12146.

Gatimel, N., Moreau, J., Parinaud, J., and Léandri, R. D. (2017). Sperm morphology: assessment, pathophysiology, clinical relevance, and state of the art in 2017. *Andrology* **5**, 845–862.

Girndt, A., Knief, U., Forstmeier, W., and Kempenaers, B. (2014). Triploid ZZZ Zebra finches *Taeniopygia guttata* exhibit abnormal sperm heads and poor reproductive performance. *Ibis* **156**, 472–477.

Girndt, A., Cockburn, G., Sánchez-Tójar, A., Løvlie, H., and Schroeder, J. (2017). Method matters: experimental evidence for shorter avian aperm in faecal compared to abdominal massage samples. *PLoS One* **12**, e0182853.

Guthauser, B., Vialard, F., Dakouane, M., Izard, V., Albert, M., and Selva, J. (2006). Chromosomal analysis of spermatozoa with normal-sized heads in two infertile patients with macrocephalic sperm head syndrome. *Fertil. Steril.* **85**, 750.e5–750.e7.

Guthauser, B., Boitrelle, F., Albert, M., Ketata, F., Meynant, C., Ferfouri, F., Selve, J., and Vialard, F. (2013). Contraindication of ART following a sperm FISH analysis, even though only 12% of the spermatozoa had large heads. *Syst. Biol. Reprod. Med.* **59**, 214–217.

Hartley, P. S. (1999). The cryopreservation of semen from non-domestic avian species. M.Phil. Thesis, University of Abertay, Dundee.

Humphries, S., Evans, J. P., and Simmons, L. W. (2008). Sperm competition: linking form to function. *BMC Evol. Biol.* **8**, 319.

In't Veld, P. A., Broekmans, F. J., de Frans, H. F., Pearson, P. L., Pieters, M. H., and van Kooij, R. J. (1997). Intracytoplasmic sperm injection (ICSI) and chromosomal abnormal spermatozoa. *Hum. Reprod.* **12**, 752–754.

Katz, D. F., Diel, L., and Overstreet, J. W. (1982). Differences in the movement of morphologically normal and abnormal human seminal spermatozoa. *Biol. Reprod.* **26**, 566–570.

Klimowicz, M., Łukaszewicz, E., and Dubiel, A. (2005). Effect of collection frequency on quantitative and qualitative characteristics of pigeon (*Columba livia*) semen. *Br. Poult. Sci.* **46**, 361–365.

Kopp, C., Sukura, A., Tuunainen, E., Gustavsson, I., Parvinen, M., and Andersson, M. (2007). Multinuclear–multiflagellar sperm defect in a bull – a new sterilizing sperm defect. *Reprod. Domest. Anim.* **42**, 208–213.

Lewis-Jones, I., Aziz, N., Seshadri, S., Douglas, A., and Howard, P. (2003). Sperm chromosomal abnormalities are linked to sperm morphologic deformities. *Fertil. Steril.* **79**, 212–215.

Lin, M., Thorne, M. H., Martin, I., and Jones, R. C. (1995a). Electron microscopy of the seminiferous epithelium in the triploid (ZZZ and ZZW) fowl, *Gallus domesticus. J. Anat.* **186**, 563–576.

Lin, M., Thorne, M. H., Martin, I. C. A., Sheldon, B. L., and Jones, R. C. (1995b). Development of the gonads in triploid (ZZ and ZZZ) fowl, *Gallus domesticus*, and comparison with normal diploid males (ZZ) and females (ZV). *Reprod. Fertil. Dev.* **7**, 1185–1197.

Lindsay, C., Staines, H. J., McCormick, P., McCullum, C., Choulani, F., and Wishart, G. J. (1999). Variability in the size of the nucleus in spermatozoa from the Houbara bustards, *Chlamydotis undulata undulata. J. Reprod. Fertil.* **117**, 307–313.

Malecki, I. A., Cummins, J. M., Martin, G. B., and Lindsay, D. R. (1998). Effect of collection frequency on semen quality and the frequency of abnormal forms of spermatozoa in the emu. *P. Aus. S. Ani.* **22**, 406.

Malecki, I. A., Rybnik, P. K., and Martin, G. B. (2008). Artificial insemination technology for ratites: a review. *Aust. J. Exp. Agric.* **48**, 1284–1292.

Malo, A. F., Garde, J. J., Soler, A. J., Garcia, A. J., Gomendio, M., and Roldan, E. R. S. (2005). Male fertility in natural populations of red deer is determined by sperm velocity and the proportion of normal spermatozoa. *Biol. Reprod.* **72**, 822–829.

Maretta, M. (1979). The ultrastructure of double and multiple flagella of the spermatozoa of a drake. *Vet. Med. (Praha)* **24**, 679–689.

Molinari, E., Mirabelli, M., Raimondo, S., Brussino, A., Gennarelli, G., Bongioanni, F., and Revelli, A. (2013). Sperm macrocephaly syndrome in a patient without *AURKC* mutations and with a history of recurrent miscarriage. *Reprod. Biomed. Online* **26**, 148–156.

Nistal, M., Paniagua, R., and Herruzo, A. (1977). Multi-tailed spermatozoa in a case with asthenospermia and teratospermia. *Virchows Arch. B Cell Pathol.* **26**, 111–118.

Nwakalor, L. N., Okeke, G. C., and Njoku, D. C. (1988). Semen characteristics of the guinea fowl *Numida meleagris meleagris. Theriogenology* **29**, 545–554.

Perrin, A., Morel, F., Moy, L., Colleu, D., Amice, V., and de Braekeleer, M. (2008). Study of aneuploidy in large-headed, multiple-tailed spermatozoa: case report and review of the literature. *Fertil. Steril.* **90**, 1201.e13–1207.e17.

Prisant, N., Escalier, D., Soufir, J.-C., Morillon, M., Schoevaert, D., Misrahi, M., and Tachdjian, G. (2007). Ultrastructural nuclear defects and increased chromosome aneuploidies in spermatozoa with elongated heads. *Hum. Reprod.* **22**, 1052–1059.

Ray, P. F., Toure, A., Metzler-Guillemain, C., Mitchell, M. J., Arnoult, C., and Coutton, C. (2017). Genetic abnormalities leading to qualitative defects of sperm morphology or function. *Clin. Genet.* **91**, 217–232.

Revay, T., Kopp, C., Flyckt, A., Taponen, J., Ijäs, R., Nagy, S., Kovacs, A., Rens, W., Rath, D., Hidas, A., Taylor, J. F., and Anderson, M. (2010). Diploid spermatozoa caused by failure of the second meiotic division in a bull. *Theriogenology* **73**, 421–428.

Rybnik, P. K., Horbanczuk, J. O., Naranowicz, H., Lukaszewicz, E., and Malecki, I. A. (2007). Semen collection in the ostrich (*Struthio camelus*) using a dummy or a teaser female. *Br. Poult. Sci.* **48**, 635–643.

Salisbury, G. W., and Baker, F. N. (1966). Frequency of occurrence of diploid bovine spermatozoa. *J. Reprod. Fertil.* **11**, 477–480.

Santiago-Moreno, J., Castaño, C., Toledano-Díaz, A., Esteso, M. C., López-Sebastián, A., Gañán, N., Hierro, M. J., Campo, J. L., and Blesbois, E. (2015). Characterization of red-legged partridge (*Alectoris rufa*) sperm: seasonal changes and influence of genetic purity. *Poult. Sci.* **94**, 80–87.

Santiago-Moreno, J., Esteso, M. C., Villaverde-Morcillo, S., Toledano-Díaz, A., Castaño, C., Velázquez, R., López-Sebastián, A., Goya, A. L., and Martínez, J. G. (2016). Recent advances in bird sperm morphometric analysis and its role in male gamete characterization and reproduction technologies. *Asian J. Androl.* **18**, 882–888.

Schmoll, T., Sanciprian, R., and Kleven, O. (2016). No evidence for effects of formalin storage duration or solvent medium exposure on avian sperm morphology. *J. Ornithol.* **157**, 647–652.

Smith, A. M. J., Bonato, M., Dzama, K., Malecki, I. A., and Cloete, S. W. P. (2016). Classification of ostrich sperm characteristics. *Anim. Reprod. Sci.* **168**, 138–150.

Smith, A. M. J., Bonato, M., Dzama, K., Malecki, I. A., and Cloete, S. W. P. (2018a). Ostrich specific semen diluent and sperm motility characteristics during *in vitro* storage. *Anim. Reprod. Sci.* **193**, 107–116.

Smith, A. M. J., Bonato, M., Dzama, K., Malecki, I. A., and Cloete, S. W. P. (2018b). Mineral profiling of ostrich (*Struthio camelus*) seminal plasma and its relationship with semen traits and collection day. *Anim. Reprod. Sci.* **193**, 98–106.

Soley, J. T. (1993). Ultrastructure of ostrich (*Struthio camelus*) spermatozoa: I. Transmission electron microscopy. *Onderstepoort J. Vet. Res.* **60**, 119–130.

Sontakke, S. D., Umapathy, G., Sivaram, V., Kholkute, S. D., and Shivaji, S. (2004). Semen characteristics, cryopreservation, and successful artificial insemination in the blue rock pigeon (*Columba livia*). *Theriogenology* **62**, 139–153.

Standerholen, F. B., Myromslien, F. D., Kommisrud, E., Ropstad, E., and Waterhouse, K. E. (2014). Comparison of electronic volume and forward scatter principles of cell selection using flow cytometry for the evaluation of acrosome and plasma membrane integrity of bull spermatozoa. *Cytometry* **85**, 719–728.

Støstad, H. N., Johnsen, A., Lifjeld, J. T., and Rowe, M. (2018). Sperm head morphology is associated with sperm swimming speed: a comparative study of songbirds using electron microscopy. *Evolution* **72**, 1918–1932.

Sun, F., Ko, E., and Martin, R. H. (2006). Is there a relationship between sperm chromosome abnormalities and sperm morphology? *Reprod. Biol. Endocrinol.* **4**, 1.

Tabatabaei, S., Batavani, R. A., and Talebi, A. R. (2009). Comparison of semen quality in indigenous and Ross broiler breeder roosters. *J. Anim. Vet. Adv.* **8**, 90–93.

Umapathy, G., Sontakke, S., Reddy, A., Ahmed, S., and Shivaji, S. (2005). Semen characteristics of the captive Indian white-backed vulture. *Biol. Reprod.* **73**, 1039–1045.

Viville, S., Mollard, R., Bach, M. L., Falquet, C., Gerlinger, P., and Waters, S. (2000). Do morphological anomalies reflect chromosomal aneuploidies? *Hum. Reprod.* **15**, 2563–2566.

Wakely, W. J., and Kosin, I. L. (1951). A study of the morphology of the turkey spermatozoa with special reference to the seasonal prevalence of abnormal types. *Am. J. Vet. Res.* **12**, 240–245.

Weissenberg, R., Aviram, A., Golan, R., Lewin, L. M., Levron, J., Madgar, I., Dor, J., Barkai, G., and Goldman, B. (1998). Concurrent use of flow cytometry and fluorescence *in-situ* hybridization techniques for detecting faulty meiosis in a human sperm sample. *Mol. Hum. Reprod.* **4**, 61–66.

Wishart, G. J., and Palmer, F. H. (1986). Correlation of the fertilising ability of semen from individual male fowls with sperm motility and ATP content. *Br. Poult. Sci.* **27**, 97–102.

Wishart, G. J., Lindsay, M., and Knowles-Brown, A. (1999). Sperm quality in a captive-bred golden eagle. *J. Reprod. Fertil. Abstr. Ser.* **24**, 6.

Wishart, G. J., Lindsay, C., Staines, H. J., and McCormick, P. (2002). Semen quality in captive Houbara bustard, *Chlamydotis undulata undulata. Reprod. Fertil. Dev.* **14**, 401–405.

Yurov, Y. B., Saias, M. J., Vorsanova, S. G., Erny, R., Soloviev, I. V., Sharonin, V. O., Guichaoua, M. R., and Luciani, J. M. (1996). Rapid chromosomal analysis of germ-line cells by FISH: an investigation of fertile male large-headed spermatozoa. *Mol. Hum. Reprod.* **2**, 665–668.