Light microscopy of emu (*Dromaius novaehollandiae*) sperm: preparatory technique, morphological features and morphometry

Lizette du Plessis[1*] and John T. Soley[2]

[1]Electron Microscope Unit, Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa

[2]Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa

*Corresponding author

e-mail: lizette.duplessis@up.ac.za

Telephone: +27 12 5298212

Abstract

A comprehensive morphological description of emu sperm at the light microscopy level, an essential prerequisite for the routine evaluation of semen quality in this species, is not currently available. In this study sperm structure was visualized by making conventional semen smears from samples collected from the distal *ductus deferens* and fixed in 2.5% glutaraldehyde. This was followed by air-drying the smears and staining with a Romanowsky type stain. Examination of the smears using phase contrast illumination and a 100x oil immersion lens readily resolved the various components of the cell, namely, the acrosome, nucleus, midpiece, principal piece and endpiece. This technique was simple to use and produced consistently reproducible results. Normal emu sperm were typically filiform in appearance and closely resembled sperm of the ostrich and other non-passerine species, particularly poultry. A previously undescribed cytoplasmic appendage, associated with the

base of the head, was a novel morphological feature. Sperm dimensions in the emu were similar to those of other ratites. This was reflected by a head:tail ratio of 1:4 which is in accordance with the value reported for the ostrich.

Keywords: emu, *Dromaius novaehollandiae*, light microscopy, sperm morphology, morphometry

Introduction

Sperm morphology, sperm motility and sperm concentration are considered to be the three most important parameters when assessing semen quality [1-5]. Morphological features serve as a reliable indicator in predicting the fertilizing capacity of sperm and also reflect certain disorders of spermatogenesis [2]. In humans the accurate assessment of morphological abnormalities is of prognostic value in predicting the results of assisted reproduction and fertilization when male fertility is compromised. Likewise, artificial insemination (AI), an important and widely used technique in ensuring and maintaining genetic traits in domestic animals such as the bull and stallion, relies on a clear understanding of normal sperm structure [2]. The value of sperm morphology in determining reproductive success is reflected in the numerous studies carried out, for example, on man [5-7] and domesticated animals such as the bull [2,8,9], stallion [10-12], boar [13] and dog [14-16].

Avian sperm structure, in particular that of non-passerine birds of economic importance, has likewise received considerable attention. Numerous studies have detailed the morphological features of both normal and defective sperm in the chicken [4,17-20], turkey [1,21-23], duck [24], goose [25,26], Japanese quail [27,28], pintailed duck [29] and guinea fowl [30,31]. The commercial exploitation of ratite species such as the ostrich, rhea and emu, has prompted a renewed interest in ratite sperm structure. Since artificial insemination has been suggested as a means of improving the economic viability of these niche industries [32], a thorough

knowledge of normal sperm structure in these birds is of paramount importance. This view was further emphasized by Bertschinger et al. [33] who considered sperm morphology to be one of the most important factors in predicting fertility in the ostrich. The morphology of normal sperm has been well documented for the ostrich by both light and electron microscopy [33-39], while the ultrastructure of rhea sperm and aspects of their development were reported by Phillips and Asa [40]. Apart from a brief ultrastructural description of the development and morphology of emu sperm [34], little definitive information has been published on the structure of the normal male gamete in this species.

Recent detailed publications on the classification and description of defective sperm in the emu [41-44] have emphasized the need to re-evaluate the dearth of published information on normal emu sperm, particularly at the LM level. This information is required to provide comparative data for the accurate identification of abnormal forms. This paper provides a detailed account of the normal morphology of emu (*Dromaius novaehollandia*) sperm as observed by light microscopy. It documents a simple and cost-effective technique for preparing and evaluating emu semen smears which provides excellent morphological detail and consistent results. Comparative morphometric data on linear sperm dimensions are also presented.

Materials & Methods

Semen samples were collected during mid-breeding season (May – July) from 15 healthy (animals approved for slaughter) and sexually active emus following slaughter at commercial abattoirs (Protocol V070/11, Faculty of Veterinary Science, University of Pretoria). The birds ranged in age from 22 months to five years. Two groups of birds were sampled. One group (n=5) was sourced from the Rustenburg district in the North West Province, South Africa and

slaughtered at the Emu Ranch Abattoir. The second group (n=10) was from the Grahamstown district, Eastern Cape Province, South Africa, and were slaughtered at the Grahamstown Ostrich Abattoir. Samples were collected approximately 60 minutes after the birds had been slaughtered. Drops of semen were gently squeezed from the distal *ductus deferens* into plastic test tubes containing 2.5% glutaraldehyde in 0.13M Millonig's phosphate-buffer.

Smears for light microscopy (LM) were routinely prepared from the fixed cell suspensions, air-dried for a minimum period of 24 hours and stained with Wrights' stain (Rapidiff®, Clinical Sciences Diagnostics, Johannesburg, South Africa) in Coplin jars. The dried smears were fixed in methanol for 20 seconds, stained with eosin for 30 seconds, blotted and stained with methylene blue solution for 60 seconds. The smears were then gently rinsed with distilled water and allowed to air dry before mounting with Entellan® and a coverslip. One smear from each bird was examined with an Olympus BX63 light microscope (Olympus Corporation, Tokyo, Japan) using a 100x oil immersion objective (bright field as well as phase contrast microscopy) to evaluate normal sperm morphology. The incidence of normal sperm was determined for each bird by counting the number of normal/abnormal sperm present in a total of 300 cells. Images of sperm cells were digitally recorded using the Olympus cellSens Imaging Software (Olympus Corporation, Tokyo, Japan).

The linear dimensions of the various segments of the sperm (acrosome, nucleus, midpiece, principal piece and end piece), as well as the total length of the cells, were determined by measuring a minimum of twenty cells from each bird. The measurements were processed using the Soft Imaging System iTEM software (Olympus, Műnster, Germany) and expressed as the average ± SD. Statistical analysis of the different parameters assessed was performed using a computer package (Sigma Plot 12.0) and descriptive statistics for each of the sperm

segments as well as the total sperm length were generated. Respective data sub-sets were tested for normality (Shapiro-Wilk) and equal variance, and subsequently, averaged linear sperm dimensions for individuals of the two groups of birds (Grahamstown and Rustenburg) were compared using non-parametric Mann-Whitney Rank Sum Test. All tests were two tailed, with the α-level of significance set at 0.05.

Results

Morphology

When viewed by LM, spermatozoa were typically filiform-shaped, consisting of a head and tail connected by an indistinct neck (Fig.1). The relatively straight or gently convoluted head tapered anteriorly and consisted of a clearly defined nucleus capped by a small conical acrosome which occasionally appeared pointed, particularly when using bright field illumination. The nuclear material was homogeneous in appearance with no obvious evidence of vacuolation. When using phase contrast microscopy the nucleus was starkly illuminated in white, unequivocally demonstrating the full extent of the nuclear contents (Fig.1b,c). In most sperm a thin, thread-like appendage could be discerned at the base of the head (Fig.1c). This appendage varied in length and generally extended vertically from the cell surface. The tail was composed of three distinct segments, namely, the midpiece, principal piece and endpiece. The base of the head was continuous with the midpiece, the first segment of the tail. The nuclear base and the proximal end of the midpiece were of similar diameter although the midpiece as a unit was thinner than the head. The midpiece revealed no specific features on LM and was often difficult to distinguish from the rest of the tail on the Rapidiff® smears using bright field microscopy (Fig.1a). However, the various components of the sperm could clearly be distinguished from each other using phase contrast microscopy (Fig.1b,c). After termination of the midpiece, the tail continued as the long undulating principal piece. The

principal piece was thinner than the midpiece and terminated in a short, visibly thinner and non-conspicuous endpiece of variable length (Fig.1). In the 15 birds studied, 86.4% of the sperm displayed the normal morphological features outlined above.

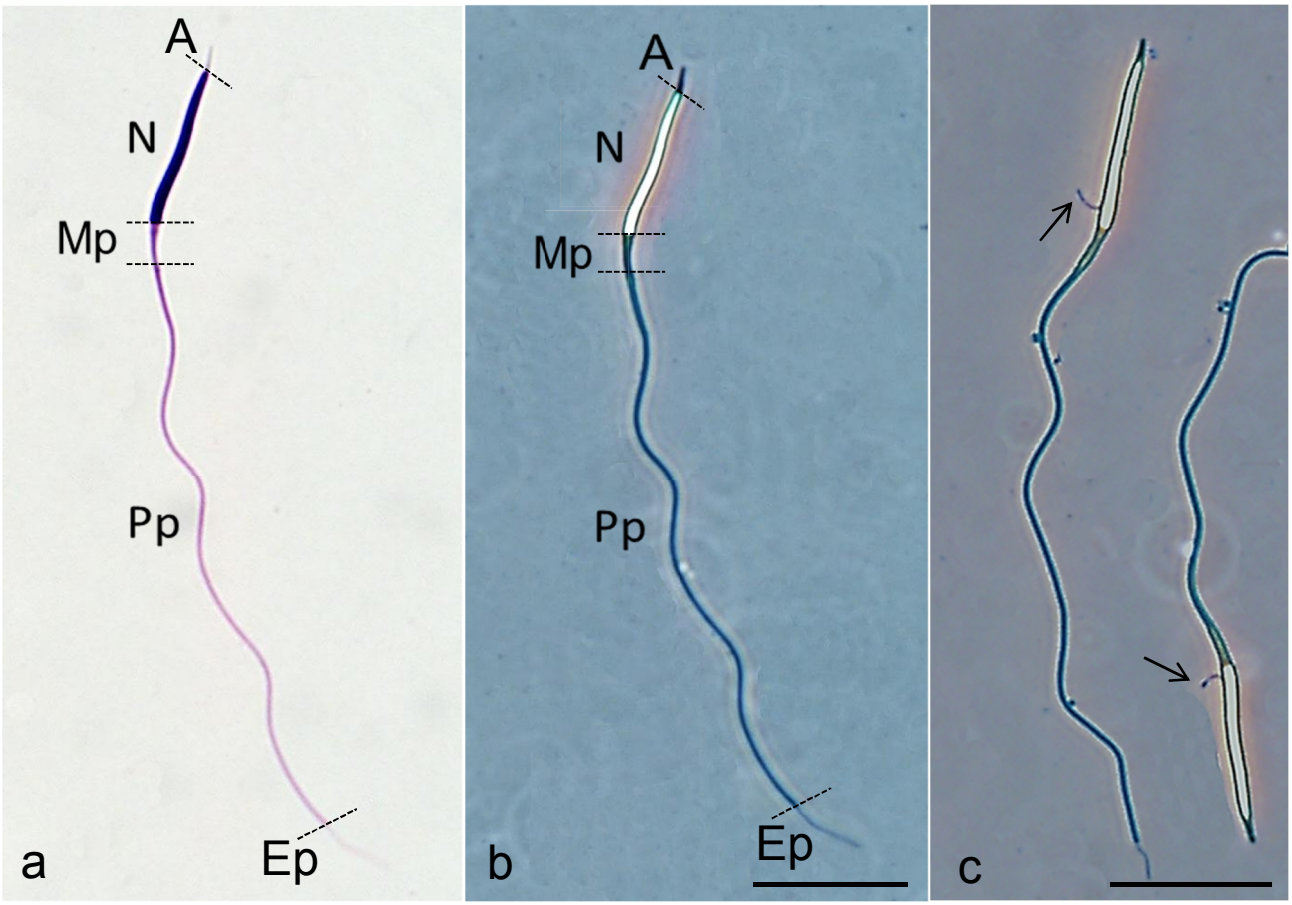

Figure 1. Light microscopy, Wright's stain. The various components of the sperm, namely, the acrosome (A), nuclear region of the head (N), midpiece (Mp), principal piece (Pp) and endpiece (Ep) are more clearly resolved using phase contrast microscopy (b,c) than normal bright field illumination (a). In (c) the thread-like appendage situated near the nuclear base can be seen (arrows). Bar = 10µm.

Morphometry

The acrosome was short, measuring on average 1.84 ± 0.31µm (range 0.94 - 3.01µm), while the nucleus measured 11.77 ± 0.93µm in length (range 9.09 - 14.49µm). The average lengths of the segments of the flagellum were 2.91 ± 0.4µm (range 1.27 - 4.16µm) for the midpiece, 47.45 ± 2.8µm (range 41.04 - 59.25µm) for the principal piece and 3.69 ± 0.82µm (range 1.38

- 6.33µm) for the endpiece. The average total sperm length was 67.64 ± 3.13µm (range 60.14 - 79.49µm). The dimensions of the various segments of the sperm are presented in Table 1 which reflects the measurements for each individual bird. Based on extrapolation from the above measurements, the total head length was determined to be 13.61µm and the total tail length 54.05µm, giving a head:tail ratio of 1:4. There were no statistically significant differences (T=33-56, P>0.05) observed between any of the measurements for the same sperm segment when comparing the Rustenburg and Grahamstown birds.

Table 1: Average ± sd measurements (µm) of the various sperm segments as determined for individual birds as well as for the two different groups of birds (G= Grahamstown birds; R= Rustenburg birds) and all birds combined.

Bird	*Acrosome*	*Nucleus*	*Midpiece*	*P/piece*	*Endpiece*	*Total length*
G1	1.76 ± 0.26	12.09 ± 0.48	3.08 ± 0.44	47.47 ± 1.47	4.02 ± 0.61	68.43 ± 1.62
G2	1.94 ± 0.25	12.24 ± 0.35	2.66 ± 0.52	50.27 ± 1.52	3.91 ± 0.45	71.04 ± 1.68
G3	1.73 ± 0.22	12.4 ± 0.41	3.05 ± 0.27	45.04 ± 1.77	3.34 ± 0.44	64.55 ± 1.84
G4	1.98 ± 0.31	12.4 ± 0.65	3.14 ± 0.34	48.73 ± 1.79	2.66 ± 0.52	68.9 ± 2.89
G5	1.83 ± 0.24	12.93 ± 0.84	2.75 ± 0.22	48.76 ± 2.82	2.87 ± 0.56	69.13 ± 3.14
G6	1.86 ± 0.26	12.69 ± 0.66	2.9 ± 0.31	45.66 ± 1.4	3.68 ± 0.68	66.79 ± 1.69
G7	1.8 ± 0.2	11.05 ± 0.34	2.72 ± 0.3	49.96 ± 1.72	3.22 ± 0.6	68.75 ± 1.64
G8	1.68 ± 0.31	11.93 ± 0.59	2.83 ± 0.39	44.65 ± 1.85	4.45 ± 1.0	65.54 ± 1.96
G9	1.67 ± 0.22	11.21 ± 0.59	3.22 ± 0.43	46.68 ± 1.13	3.23 ± 0.51	65.41 ± 1.4
G10	1.65 ± 0.3	10.83 ± 0.63	2.78 ± 0.28	47.24 ± 1.96	4.04 ± 0.85	66.54 ± 2.06
Total G	1.79 ± 0.11	11.98 ± 0.72	2.91 ± 0.19	47.44 ± 1.98	3.54 ± 0.57	67.5 ± 2.05
R1	2.3 ± 0.34	11.97 ± 0.57	2.93 ± 0.39	50.41 ± 2.2	3.94 ± 0.68	71.54 ± 2.52
R2	1.87 ± 0.23	11.86 ± 0.54	2.94 ± 0.45	50.04 ± 2.03	4.24 ± 0.52	70.96 ± 2.33
R3	1.83 ± 0.27	12.05 ± 0.81	3.17 ± 0.41	46.09 ± 1.76	3.86 ± 0.63	67.01 ± 2.06
R4	1.78 ± 0.22	10.62 ± 0.62	2.81 ± 0.36	43.48 ± 1.33	4.21 ± 0.7	62.75 ± 1.49
R5	1.86 ± 0.3	10.8 ± 0.54	2.74 ± 0.46	48.46 ± 2.26	3.57 ± 0.67	67.43 ± 2.7
Total R	1.93 ± 0.21	11.46 ± 0.69	2.91 ± 0.16	47.69 ± 2.9	3.96 ± 0.28	67.93 ± 3.54
All birds	1.84 ± 0.31	11.77 ± 0.93	2.91 ± 0.4	47.54 ± 2.8	3.69 ± 0.82	67.64 ± 3.13

Discussion

Technique

Whereas sophisticated and presumably more objective methods such as computer-aided sperm analysis (CASA) and flow cytometry are used by larger laboratories for sperm analysis, these techniques are costly and not always economically feasible for application in smaller laboratories or in the field. Conventional light microscopy is a relatively cheap, accessible option and will remain the most practical and cost effective manner of evaluating sperm morphology, particularly when used in conjunction with appropriate staining and illumination techniques.

Various methods have been used to prepare and examine avian sperm at the light microscope level [1,19,25,33,35,48]. Despite the use of these techniques, however, published light micrographs on avian sperm morphology are not always convincing particularly in respect of revealing structural detail. For example, in a light microscopy study of chicken sperm, Lake et al. [19] reported that it was difficult to discern the different segments of the cell with the limited magnification and resolution of the light microscope while Soley [45] reported that the endpiece in ostrich sperm was difficult to resolve by light microscopy irrespective of which imaging technique was used. Similarly, the use of wet mount protocols, although necessary for motility studies, is limited in scope for the morphological examination of sperm [46] and fewer normal sperm are identified using this method than with stained smears [47]. Likewise there are some studies on avian sperm that have used air-dried fresh semen smears [4,20,48,49]. However, the use of fixed avian semen samples for light microscopic evaluation has been found to be advantageous [1,25,33,35]. Although various fixatives have been employed, for example, formaldehyde [25] and Hancock's solution [1], the current study used 2.5% glutaraldehyde in 0.13M Millonig's phosphate-buffer. Fixing semen immediately

after collection with buffered glutaraldehyde, preserves overall sperm morphology and the specimens can then be stained and examined at the investigator's convenience. It also has the added benefit of reducing cell shrinkage (possibly due to the osmolarity of the fixative) that results from air-drying fresh semen smears. In respect of ostrich sperm, Gee et al. [35] compared non-fixed eosin/nigrosin-stained semen smears and unstained glutaraldehyde fixed semen smears and found that fixing with glutaraldehyde gave the best structural detail. This was also the method of choice of Bertschinger et al. [33] in an earlier study of ostrich sperm morphology.

Use of stains, particularly the Romanowsky type stains, for revealing structural detail of mammalian sperm has been practiced for a number of years. For example, Mota and Ramalho-Santos [50] found the Diff-Quick® stain useful in revealing head abnormalities in cat sperm. This stain is similar to the Wright's stain used in the present study and is employed routinely in human reproduction laboratories, usually in conjunction with bright field microscopy. Similarly, the SpermBlue® stain, developed by Van der Horst and Maree [51] and tested on a number of mammalian and non-mammalian species, also differentially stains the various sperm components with the added benefit that the stained smears are suitable for automated analysis. Contrary to the practice of using stains for evaluating sperm structural details, Specher and Coe [9], in their study on bull sperm, found that phase contrast microscopy of unstained smears was a more accurate method of studying sperm morphology as it revealed a greater number of cytoplasmic droplets and head abnormalities than standard bright field microscopy of eosin/nigrosin-stained semen smears. This study however demonstrated that the characteristic filiform shape of emu sperm was particularly amenable to the use of phase contrast microscopy with glutaraldehyde-fixed and Wright's-stained smears. Examining stained smears with phase contrast microscopy proved to be a rapid and

convenient method for clearly distinguishing the various components of emu sperm and also clearly discriminated between normal and abnormal gametes in this species [41-44].

Sperm structure

Avian sperm can be divided into two basic types according to their structure, namely, the more 'primitive' filiform sperm typical of non-passerine birds and the 'complex' helical sperm of passerine species [66]. The primitive filiform sperm is 'worm-like' in appearance and is also referred to as 'sauropsid' since it resembles reptilian sperm [37]. A degree of overlap between the two basic morphological sperm types has been observed, based mainly on the tendency towards spiralization seen in some non-passerine birds [67]. Sub-oscine species are also reported to display morphological features of both passerine and non-passerine bird sperm [52]. Emu sperm are typically filiform and on LM closely resemble those of other ratites such as the ostrich [45] as well as galliform birds, notably the chicken [18], goose [25] and turkey [23]. As demonstrated in these studies [18,23,25,45] and confirmed in the emu, non-passerine bird sperm reflect five clearly defined segments, namely, the acrosome and nucleus forming the head and the midpiece, principal piece and endpiece constituting the tail. The head of emu sperm is generally straight and only occasionally displays the comma-shaped and convoluted forms identified in the ostrich [45]. The densities described in the ostrich sperm at each end of the midpiece, and which are considered to represent the proximal centriole and annulus, respectively [45], are not obvious in emu sperm and have not been reported in other non-passerine birds studied by LM.

The presence of a thread-like appendage near the base of the nucleus has not previously been described in any other bird sperm. The appearance of this particular structure using phase contrast illumination clearly indicates that it is not nuclear material therefore suggesting that

it represents a remnant of residual cytoplasm resulting from the release of the mature sperm from the surrounding Sertoli cells during spermiation. This appendage should be viewed as a normal feature of emu sperm.

Morphometry

Although based on a limited number of species, the length of non-passerine bird sperm appears to be highly variable [49,53-55]. However, a comparison of the dimensions of sperm from three ratite species (Table 2) demonstrates a remarkable uniformity. The average length

Table 2: Comparison of ratite sperm length (μm).

	Acrosome	*Nucleus*	*Head*	*Midpiece*	*Principal piece*	*Endpiece*	*Total length*	*Technique*	*Reference*
	2.0	(14)	16.0	3.0	40.0	1.0	60.0	SEM	34
Ostrich	1.91	10.95	12.86	3.16	51.18	2.39	69.58	SEM	39,45
Rhea				3.0				SEM	40
	1.5	(10.5)	12.0	3.0	47.0	3.0	65.0	SEM	34
Emu	1.8 ± 0.3	11.8 ± 0.9	(13.6)	2.9 ± 0.4	47.5 ± 2.8	3.7 ± 0.8	67.64 ± 3.1	LM	Present study

Values in brackets are extrapolated.

of emu sperm (67μm) in the present study was similar (65μm) to that previously reported by Baccetti et al. [34]. It is noteworthy that Baccetti and co-workers [34] based their measurements on scanning electron microscope (SEM) images whereas the present study used light micrographs. It is generally acknowledged that a high degree of shrinkage id associated with the preparation of biological material for SEM [56], including sperm [57]. As the semen samples in both studies were fixed with glutaraldehyde, it would therefore appear that there was little additional shrinkage as a result of the samples being dehydrated and critical point dried for SEM. No obvious difference in total sperm length or head length

was observed between the birds from the Eastern Cape and North-West Province. In contrast, Soley and Roberts [39] distinguished two subgroups of domesticated South African ostriches based on total sperm length, suggesting that "the two different ranges of sperm size observed may reflect persistent genetic (subspecies) variations in the domestic ostrich population". Farmed South African ostriches are known to be hybrids resulting from the crossing of local *S.camelus australis* stock with North African (*S.camelus camelus*) and Syrian *(S.camelus syriacus*) birds for the improvement of feather quality [45]. Emus on the other hand have only recently been introduced into South Africa and the provenance of these birds is unknown. If sperm head length and total length are indicators of genetic origin, as appears to be the case with domesticated South African ostriches, the emus investigated in the present study could conceivably be of the same genetic stock. It is interesting to note that three subspecies of emu have been recognised in Australia, namely, *Dromaius navaehollandiae novaehollandiae, D. novaehollandiae woodwardi* and *D. novaehollandaie rothschildi* [58]. Whether differences in head length would reflect the identity of these subspecies as reported in sandhill cranes [59] remains speculative. From a practical or applied perspective, known head length dimensions would assist in accurately identifying micro- and macrocephalic sperm in the emu.

With a total length of approximately 68µm, ratite sperm are appreciably shorter than sperm from a number of other non-passerine birds. Chicken sperm, for example, range in total length from 90µm [31] to 109µm [19] while turkey sperm measure from 75 - 80µm [22,31]. Sperm of another galliform bird, the guinea fowl, also display similar dimensions, varying in length between 75 - 87µm [30,31]. On the other hand, pigeon [60] and quail [28] sperm are appreciably longer with lengths of approximately 165µm and greater than 227µm, respectively. There are also non-passerine birds with sperm that fall within the range of ratite

sperm. For instance, Ferdinand [25] reported an average sperm length of 67µm in geese. Budgerigar sperm, which have features of both passerine and non-passerine birds, are slightly shorter with a total average length in the region of 62µm [61].

The avian acrosome differs markedly from that of mammals in respect of its size relative to the rest of the head. In the emu it is a relatively short structure (1.8µm present study; 1.5µm reported by Baccetti et al. [34]) being similar in length to that of the ostrich (maximum length of 2 µm) [34,38]. The acrosome of other non-passerine birds such as the chicken [19,31] and duck [24] are marginally longer. Nuclear length in the emu (11.8 ± 0.9µm) corresponds closely to that reported for other non-passerine birds, such as the ostrich [39], chicken [19], duck [24] and guinea fowl [30]. It should be noted, however, that nuclear length as measured in ratites and tinamous is not an accurate representation of this segment of the head due to the nuclear rostrum in these species extending deep within the acrosome [34,38,52,62].

The midpiece of emu sperm and other ratites [34,38,40] measures approximately 3µm in length. Similar values have been presented for the chicken [18,19], pigeon [60] and turkey [22]. However, the midpiece of non-passerine sperm can vary from very short (in the trogon) to very long (in doves) as illustrated by McFarlane [53] and Jamieson [37]. Some extremely long midpiece lengths have also been reported, for example, that of the domestic pigeon (98µm) [63] and Japanese quail (161µm) [28]. To what extent midpiece length in non-passerine birds reflects the number of mitochondria required to provide the necessary energy for forward motility remains unknown, although studies in passerine birds and mammals have shown that essential biological functions, such as the provision of energy, determine gross sperm morphology [64,65].

The only comparative data available on the length of the principal piece and endpiece in ratites is that for the ostrich and emu [34,39,45]. The present study revealed that the dimensions of these two regions are comparable to those reported by Baccetti et al. [34]. Moreover, the values are similar to those found in the ostrich [39,45].

The head:tail ratio of 1:4 for emu sperm calculated in the present study is similar to the deduced value of 1:4.4 for the emu [34] and the reported value of 1:4.4 for the ostrich [39,45]. This ratio further indicates the uniformity of both head and tail lengths in ratite sperm. It remains unclear why a discrepancy exists regarding the head:tail ratio of 1:2.7 (extrapolated from data reported by Baccetti et al. [34]) for the ostrich as opposed to the value of 1:4.4 reported by Soley [45] and Soley and Roberts [39]. Of the non-passerine birds, the head:tail ratio of the guinea fowl (1:4.3, extrapolated from data given by Thurston et al. [30]) is similar to that of the ratites [30,31]. However, the head:tail ratio in most other non-passerines is much higher [39], due to the greater length of the principal piece in these birds. Sperm tail lengths in the chicken exceed 70μm [19,30,60] while pigeon sperm tails are reportedly longer than140μm [60], resulting in head:tail ratios of 1:6 (or higher) for the chicken and 1:9.4 for the pigeon. Studies on sperm dimensions have mainly focused on the benefits of increased length as a way of adapting to variations in the female reproductive tract. For instance, longer heads have been proven to positively influence fertility [61] while the general assumption is that longer sperm tails will increase the swimming potential of sperm [66,67]. Humphries et al. [67], working on various animal species, came to the conclusion that, although there are a number of factors which may influence the swimming speed of sperm, the head:tail ratio provides a simple measurement to assess potential speed. Kleven and co-workers [68] came to a similar conclusion, in their study on passerine birds, that swimming speed and sperm length develop independently as a result of sperm

competition in birds. Reportedly polygamous species (primates and rodents) have longer and therefore faster swimming sperm than monogamous species [69]. A similar situation, due to the influence of sperm competition, is reported in promiscuous birds [54]. Ratites, including the emu, are considered to be monogamous species [70,71]. It is therefore tempting to suggest that ratites (with relatively short sperm and a low head:tail ratio) do not require fast-swimming sperm to compete with those of other males, unlike in the shorebirds [72] that are considered to be promiscuous. This question can probably be resolved by the application of CASA to determine the comparative swimming speed of sperm.

This study demonstrated that the characteristic filiform shape of emu sperm was particularly amenable to the use of phase contrast microscopy with glutaraldehyde-fixed and Wright's-stained smears. Examining stained smears with phase contrast microscopy proved to be a rapid and convenient method for clearly distinguishing the various components of emu sperm which showed morphological features consistent with non-passerine bird sperm in general.

Acknowledgements

The authors thank Mr P. Bezuidenhout, Chairman of the Eastern Cape Emu Breeders Association and Mrs P. Rough for making material available and the Grahamstown Ostrich Abattoir and Emu Ranch, Rustenburg, Northwest Province, South Africa for use of their facilities. The authors would further like to thank Prof A. Ganswindt, Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, for help with the statistical analysis of data.

References

[1] Alkan S, Baran A, Özdaş ŐB, Evecen M. Morphological defects in turkey semen. Turk J Vet Anim Sci 2002;26:1087-92.

[2] Barth AD, Oko RJ. Abnormal morphology of bovine spermatozoa. Ames: Iowa State University Press; 1989.

[3] Malmgren L. Assessing the quality of raw semen: a review. Theriogenology 1997; 48:523-30.

[4] Tabatabaei S, Batavani RA, Talebi AR. Comparison of semen quality in indigenous and Ross broiler breeder roosters. J Anim Vet Adv 2009;8:90-3.

[5] Zamboni Z. Sperm structure and its relevance to infertility: an electron microscopic study. Arch Pathol Lab Med 1992;116: 325-44.

[6] Holstein AF, Roosen-Runge EC. Atlas of human spermatogenesis. Berlin: Grosse Verlag; 1981.

[7] Menkveld R, Oettlé EE, Kruger TF, Swanson RJ, Acosta AA, Oehninger S. Atlas of human sperm morphology. Baltimore: Williams & Wilkins; 1991.

[8] Freneau GE, Chenoweth PJ, Ellis R, Rupp G. Sperm morphology of beef bulls evaluated by two different methods. Anim Reprod Sci 2010;118:176-81.

[9] Specher DJ, Coe PH. Differences in bull spermiograms using eosin-nigrosin stain, Feulgen stain and phase contrast microscopy methods. Theriogenology 1996;45:757-64.

[10] Brito LFC. Evaluation of stallion sperm morphology. Clin Tech Eq Prac 2007;6: 249-64.

[11] Dowsett KF, Osborne HG, Pattie WA. Morphological characteristics of stallion spermatozoa. Theriogenology 1984;22:463-72.

[12] Jasko DL, Lein DH, Foote RH. Determination of the relationship between sperm morphologic classifications and fertility in stallions: 66 cases (1987-1988). J Am Vet Med Assoc 1990;197:389-94.

[13] Briz MD, Bonet S, Pinart B, Camps, R. Sperm malformations throughout the boar epididymal duct. Anim Reprod Sci 1996;43:221-39.

[14] Martínez AI Peña. Canine fresh and cryopreserved semen evaluation. Anim Reprod Sci 2004;82:209-24.

[15] Oettlé EE. Sperm morphology and fertility in the dog. J Reprod Fertil Suppl 1993;47:257-60.

[16] Oettlé EE, Soley JT. Sperm abnormalities in the dog: a light and electron microscopic study. Vet Med Rev 1988;59:28-70.

[17] Bakst MR, Howarth B. The head and midpiece of cock spermatozoa examined with the

transmission electron microscope. Biol Reprod 1975;12:632-40.

[18] Grigg GW, Hodge AJ. 1949. Electron microscopic studies of spermatozoa. I. The morphology of the spermatozoon of the common domestic fowl (*Gallus domesticus*). Aust. J. Sci. Res. Ser. B 1949;2:271-86.

[19] Lake PE, Smith W, Young D. The ultrastructure of the ejaculated fowl spermatozoan. Q J Exp Physiol 1968;53:356-66.

[20] Łukaszewicz E, Jerysz A, Partyka A, Siudzińska A. Efficacy of evaluation of rooster sperm morphology using different staining methods. Res Vet Sci 2008;85:583-8.

[21] Kamar GAR, Rizik MAA. Semen characteristics of two breeds of turkeys. J Reprod Fertil 1972;29:317-25.

[22] Marquez BJ, Ogasawara FX. Scanning electron microscope studies of turkey semen. Poultry Sci 1975;54:1139-43.

[23] Wakely WJ, Kosin IL. A study of the morphology of the turkey spermatozoa with special reference to the seasonal prevalence of abnormal types. Am J Vet Res 1951;12:240-45.

[24] Maretta M. The ultrastructure of the spermatozoon of the drake. I. Head. Acta Vet Acad Sci H 1975;25:47-52.

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

[26] Marvan F, Rob O, Janeckova E. Classification of morphological abnormalities of gander sperm. Zuchthygiene 1981;16:176-83.

[27] Chelmońska B., Jerysz A, Łukaszewicz E, Kowalczyk A, Malecki I. Semen collection from Japanese Quail (*Coturnix japonica*) using a teaser female. Turk J Vet Anim Sc 2008;32:19-24.

[28] Wooley DM. The structure of the spermatozoon of the Japanese quail, *Coturnix coturnix* L. var. *japonica*. Acta Zool Stockholm 1995;76:45-50.

[29] Penfold LM, Wildt DE, Herzog TL, Lynch W, Ware L, Derrickson SE, Monfort SL. Seasonal patterns of LH, testosterone and semen quality in the Northern pintail duck (*Anas acuta*). Reprod Fert Develop 2000;12:229-35.

[30] Thurston RJ, Hess RA, Hughes BL, Froman DP. Ultrastructure of the guinea fowl (*Numidia meleagris*) spermatozoan. Poultry Sci 1982;61:1738-43.

[31] Thurston RJ, Hess RA. Ultrastructure of spermatozoa from domesticated birds: Comparative study of turkey, chicken and guinea fowl. Scanning Microscopy 1987;1:1829-38.

[32] Malecki IA, Rybnick PK, Martin GB. 2008. Artificial insemination technology for ratites: a review. Aust J Exp Agr 2008;48:1284-92.

[33] Bertschinger HJ, Burger WP, Soley JT, de Lange JH. Semen collection and evaluation of the male ostrich. Grahamstown: Proc Biennial Congr S Afr Vet Assoc 1992; p154-8.

[34] Baccetti B, Burrini AG, Falchetti E. Spermatozoa and relationships in Paleognath birds. Biol Cell 1991;71:209-16.

[35] Gee GF, Bertschinger H, Donoghue AM, Blanco J, Soley JT. Reproduction in nondomestic birds: Physiology, semen collection, artificial insemination and cryopreservation. Avian Poult Biol Rev 2004;15:47-101.

[36] Irons PC, Bertschinger HJ, Soley JT, Burger WP. Semen collection and evaluation in the ostrich. In: Deeming DC, editor. Improving our understanding of ratites in a farming environment. Manchester: Ratite Conference; 1996, p.157-99.

[37] Jamieson BGM. Avian spermatozoa: Structure and Phylogeny. In: Jamieson, BGM, editor. Reproductive Biology and Phylogeny of Birds Part A. Jersey: Science Publishers; 2007, p.349–511.

[38] Soley JT. Ultrastructure of ostrich (*Struthio camelus*) spermatozoa: I. Transmission electron microscopy. Onderstepoort J Vet Res 1993;60:119-30.

[39] Soley JT, Roberts D.C. Ultrastructure of ostrich (*Struthio camelus*) spermatozoa: II. Scanning electron microscopy. Onderstepoort J Vet Res 1994;61:239-46.

[40] Phillips DM, Asa CS. Development of spermatozoa in the rhea. Anat Record 1989;223:276–82.

[41] Du Plessis L, Soley JT. Incidence, structure and morphological classification of abnormal spermatozoa in the emu (*Dromaius novaehollandiae*). Theriogenology 2011;75: 589-601.

[42] Du Plessis L, Soley JT. Head-base bending and disjointed spermatozoa in the emu (*Dromaius novaehollandiae*): a morphological comparison of two closely related defects. Theriogenology 2011;76:1275-83.

[43] Du Plessis L, Soley JT. Abaxial tail implantation in the emu, *Dromaius novaehollandiae*: morphological characteristics and origin of a rare avian sperm defect. Theriogenology 2012;77:1137-43.

[44] Du Plessis L, Soley JT. Structural peculiarities associated with multiflagellate sperm in the emu, *Dromaius novaehollandiae.* Theriogenology 2012;78:1094-101.

[45] Soley JT. A histological study of spermatogenesis in the ostrich (*Struthio camelus*). Ph.D. Thesis, University of Pretoria, Pretoria, 1992.

[46] Mocé E, Graham JK. In vitro evaluation of sperm quality. Anim Reprod Sci 2008;105:104-18.

[47] Meschede D, Keck C, Zander M, Cooper TG, Yeung C-H, Nieschlag E. Influence of three different preparation techniques on the results of human sperm morphology analysis. Int J Androl 1993;16:362-9.

[48] Hemberger MY, Hospes R, Bostedt H. Semen collection, examination and spermiogram in ostriches. Reprod Domest Anim 2001;36:241-3.

[49] Humphreys PN. Brief observations on the semen and spermatozoa of certain passerine and non-passerine birds. J Reprod Fertil 1972;29:327-36.

[50] Mota PC, Ramalho-Santos J. Comparison between different markers for sperm quality in the cat: Diff-Quick as a simple optical technique to assess changes in the DNA of feline epididymal sperm. Theriogenology2006;65:1360-75.

[51] Van der Horst G, Maree L. SpermBlue®: A new universal stain for human and animal sperm which is also amenable to automated sperm morphology analysis. Biotech Histochem 2009;84:299-308.

[52] Asa CS, Phillips DM. Ultrastructure of avian spermatozoa: A short review. In: Mohri H, editor. New Horizons in Sperm Cell Research. New York: Gordon and Breach Science Publishers; 1987, p.365-73.

[53] McFarlane R W. The taxonomic significance of avian sperm. In: Sibley GC, editor. Proceedings of the XIII International Ornithological Congress. American Ornithologists'

Union. New York: Ithaca; 1963, p.91-102.

[54] Briskie JV, Montgomerie R, Birkhead TR. The Evolution of Sperm Size in Birds. Evolution 1997;51:937-45.

[55] Briskie JV, Montgomerie R. Testis size, sperm size and sperm competition. In: Jamieson BGM, editor. Reproductive Biology and Phylogeny of Birds Part A. Jersey: Science Publishers 2007, p.513-51.

[56] Boyde A, Wood C. Preparation of animal tissues for surface scanning electron microscopy. J Microsc 1969; 90:221-249.

[57] Van der Horst G, Curry PT, Kitchin RM, Burgess W, Thorne ET, Kwiatkowski D, Parker M, Atherton RW. Quantitative light and scanning electron microscopy of ferret sperm. Mol Reprod Dev 1991;30:232-240.

[58] Sales J. The emu (*Dromaius novaehollandiae*): a review of its biology and commercial products. Avian Poult Biol Rev 2007;18:1-20.

[59] Sharlin JS, Shaffner CS, Gee GF. Sperm head length as a predictor of fecundity in the sandhill crane, *Grus canadensis*. J Reprod Fertil 1979;55:411-3.

[60] Szumowski P, Theret M, Denis B. Semen and artificial insemination of pigeons. VIIIth Int Congr Animal Reproduction and Artificial Insemination, Cracow, 1976; p.1086-89.

[61] Samour JH, Smith CA, Moore HD, Markham JA. Semen collection and spermatozoa characteristics in budgerigars (*Melopsittacus undulates*). Vet Rec 1986;118: 397-9.

[62] Asa CS, Phillips DM, Stover J. Ultrastructure of spermatozoa of the crested tinamou. J Ultra Mol Struct R 1986;94:170-5.

[63] Vernon GG, Woolley DM. Three-dimensional motion of avian spermatozoa. Cell Motil Cytoskel 1999;42:149-61.

[64] Cardullo RA, Baltz JM. Metabolic regulation in mammalian sperm: mitochondrial volume determines sperm length and flagellar beat frequency. Cell Motil Cytoskel 1991;19: 180-8.

[65] Immler S, Birkhead TM. Sperm competition and sperm midpiece size: no consistent pattern in passerine birds. P Roy Soc Lond B 2007;274:561-8.

[66] Calhim S, Double MC, Margraf N, Birkead TR, Cockburn A. Maintenance of sperm variation in a highly promiscuous wild bird. PLoS One 2011;6:e28809.

[67] Humphries S, Evans JP, Simmons LW. Sperm competition: linking form to function. BMC Evol Biol 2008;8:319.

[68] Kleven O, Fossøy Laskemoen T, Robertson RJ, Rudolfsen G, Lifjeld JT. Comparative evidence for the swimming speed by sperm competition and female sperm storage duration in passerine birds. Evolution 2009;63:2466-73.

[69] Gomendio M, Roldan ERS. Sperm size and sperm competition in mammals. P Roy Soc Lond B 1991;243:181-5.

[70] Malecki IA, Cummins JM, Martin GB, Lindsay DR. Effect of collection frequency on semen quality and the frequency of abnormal forms of spermatozoa in the emu. P Aus S Ani 1998;22:406.

[71] Malecki IA, Beesley JR, Martin GB. Changes in the characteristics of emu semen with season. Brit Poultry Sci 2000;41:S18.

[72] Johnson DDP, Briskie JV. Sperm competition and sperm length in shorebirds. Condor 1999;101:848-54.