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INVITED REVIEW ARTICLE

Semen Analysis

Recent advances in bird sperm morphometric analysis and its role in male gamete characterization and reproduction technologies

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Postcopulatory sexual selection through sperm competition may be an important evolutionary force affecting many reproductive traits, including sperm morphometrics. Environmental factors such as pollutants, pesticides, and climate change may affect different sperm traits, and thus reproduction, in sensitive bird species. Many sperm-handling processes used in assisted reproductive techniques may also affect the size of sperm cells. The accurately measured dimensions of sperm cell structures (especially the head) can thus be used as indicators of environmental influences, in improving our understanding of reproductive and evolutionary strategies, and for optimizing assisted reproductive techniques (e.g., sperm cryopreservation) for use with birds. Computer-assisted sperm morphometry analysis (CASA-Morph) provides an accurate and reliable method for assessing sperm morphometry, reducing the problem of subjectivity associated with human visual assessment. Computerized systems have been standardized for use with semen from different mammalian species. Avian spermatozoa, however, are filiform, limiting their analysis with such systems, which were developed to examine the approximately spherical heads of mammalian sperm cells. To help overcome this, the standardization of staining techniques to be used in computer-assessed light microscopical methods is a priority. The present review discusses these points and describes the sperm morphometric characteristics of several wild and domestic bird species.

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INTRODUCTION

Avian spermatogenesis

Avian spermatozoa are filiform, which limits their examination by computer-assisted sperm morphology analysis (CASA-Morph) systems, as these systems were designed to investigate the approximately spherically headed spermatozoa of mammalian species. Filiform spermatozoa are the product of a complicated process involving a chronological sequence of cellular interactions modulated by endocrine, autocrine, and paracrine hormones, cytokines, and growth factors. The main stages of spermatogenesis include the proliferation and renewal of spermatogonia, the meiotic events in primary spermatocytes, and their morphological transformation during spermiogenesis. The spermatogonia are found at the periphery of the seminiferous tubules, in contact with the latter's basement membranes; elliptical in shape, they contain large, round nuclei.¹ The primary spermatocytes are the largest germ cells present in the testis and contain chromatin in the form of dispersed, thin filaments.¹ The secondary spermatocytes are smaller than primary spermatocytes, but slightly larger than spermatogonia;² their nuclei contain thick clumps of chromatin.¹ Spermatids, which emerge after the second meiotic division, are comparatively small cells with a small and spherical

nucleus³ located above or between bundles of filiform spermatids.¹ Spermiogenesis also involves the formation of a pro-acrosomal granule that attaches to the anterior pole of the nucleus, forming the acuminate spine of the mature sperm cell. At the opposite end of the nucleus, the proximal and distal centrioles form the flagellum. The formation of a microtubule system (or “manchette”) around the nucleus allows its elongation into the narrow, cylindrical, worm-like shape characteristic of mature sperm cells. The improper formation of this microtubule system results in malformed spermatids. The final step in spermiogenesis includes the sloughing of the cytoplasmic remnant. Abnormal spermatids are destroyed by the Sertoli cells via induced apoptosis, followed by the engulfing and digestion of the debris. In turkeys suffering from yellow semen syndrome, abnormal spermatids appear in the semen;⁴ in partridges and roosters, spermatids are not seen in normal ejaculates,⁵ but in falcons, nonapoptotic spherical spermatids (immature spermatids) are habitually seen in the lumen of the seminiferous tubule and in normal ejaculates, although their physiological role in reproduction is unknown.

The duration and characteristics of spermatogenetic activity vary with season. Gonadotropins and testosterone levels show seasonal alterations that determine cellular changes in the testes. During the

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breeding period, when the levels of these hormones rise, the testes undergo a proliferative phase. In contrast, when gonadotropin and testosterone levels fall, testicular size decreases via the inhibition of cell proliferation and the apoptosis of the germinal cells.^{6,7} In very seasonal species (the majority of wild birds), spermatogenic activity is abolished when basal levels of testosterone are reached (azoospermic conditions).⁸ However, in species such as the chicken (*Gallus gallus domesticus*) and turkey (*Meleagris gallopavo*), spermatogenesis does not stop. Nonetheless, it is strongly affected by low gonadotropin and testosterone levels, leading to ejaculates with low sperm concentrations⁹ and morphologically abnormal spermatozoa.¹⁰

Assisted reproduction in birds

Assisted reproduction techniques for use with birds have largely been developed for poultry. The design of sperm recovery techniques, the formulation of semen diluent, and the establishment of artificial insemination procedures with fresh and chilled spermatozoa, all initially developed for chicken and turkey production.^{11–13} Recently, however, the risk of extinction of numerous wild species and even domestic breeds of poultry has encouraged the scientists and governments to look for ways of using assisted reproduction technologies developed for mammals with threatened birds.

Around the world, over 1300 bird species are threatened (www.birdlife.org). Captive breeding and assisted reproduction can both be used to help avert extinction.¹⁴ However, given the space limitations faced by zoos and animal parks, long-term germplasm storage would appear to offer a means of preserving genetic diversity while minimizing space requirements. A number of national programs (e.g., in France, the USA, The Netherlands, and Spain) are currently trying to cryopreserve chicken spermatozoa.^{15,16} Although the conservation of primordial germ cells (PGCs) would open up new possibilities, a number of technical limitations have prevented this being feasible. Thus, to date, it has only been possible to cryopreserve male gametes – a drawback since in birds the female is the heterogametic sex.¹⁷ Spermatozoa are, of course, the most accessible sex cells.¹⁸ Unfortunately, the fertility rates of cryopreserved avian spermatozoa are dramatically lower than those recorded for domestic mammalian species. For example, cryopreserved rooster sperm may retain <2% of the fertilizing capacity of fresh semen.¹⁹ Sperm quality assays can, however, be used to predict the fertilizing capacity of spermatozoa and to evaluate the damage caused to their function by cryoprotectants and the freezing-thawing process. In such testing, the variables usually assayed include motility (either subjectively or objectively via computerized sperm motion analysis), sperm viability, membrane function, acrosomal integrity, sperm morphological abnormalities, and sperm-egg interactions.^{20–22} Recently, the analysis of DNA integrity has also been reported as feasible.²³ Other variables, such as sperm head size, and the size of sperm subpopulations categorized by sperm cell dimensions, are beginning to be used in birds, as in mammals.^{24,25}

The ability of spermatozoa to survive a freeze/thaw cycle and the value of different freezing-thawing protocols may be predicted by examining morphometric variables before and after cryopreservation.^{24–26} The sperm head size influences the volume of water carried by the cell, as well as the permeability of the cell membrane to water and cryoprotectant, in turn affecting the survival rates of the cells. Thus, the expected cryodamage to spermatozoa might be directly related to sperm head dimensions,²⁵ and a better ability of spermatozoa to survive the freezing process should be expected for the chicken (sperm head area 13.9 μm^2) than for that of species with larger sperm heads (e.g., king penguins, 19.7 μm^2). In

other species with small sperm heads, such as falcons, the response to the freeze-thaw process appears to be affected by the presence of large numbers of immature sperm cells.²⁷ Guinea fowl²⁸ and gander²⁹ semen contain many more pleomorphic cells than that of chickens, turkeys, and ducks (although not as many as in falcon semen; see³⁰ for a review); this could reduce the cryopreservation efficacy associated with their spermatozoa.

SPERM SIZE AND REPRODUCTIVE FUNCTION IN BIRD SPECIES

Knowledge of sperm head dimensions may provide important information on the evolutionary adaptation and function of the spermatozoa from different species. Variation in the size and shape of cells or their nuclei (pleomorphism) in ejaculates may be related to the presence of different subpopulations of mature spermatozoa with direct roles in fertilizing capacity, as reported for mammals.³¹ Pleomorphism may also be related to the presence of cells showing different degrees of maturity (primary spermatocytes, secondary spermatocytes, spermatids in their different stages of maturation).²⁷ External factors such as environmental pollutants and pesticides might also modify sperm head dimensions via disturbances in the chromatin package or by affecting acrosomal integrity.³²

Sperm size and sperm competence

Sexual strategies such as polygyny and monogamy also influence the morphological and functional characteristics of spermatozoa. In polygynous avian species, such as the majority of galliformes, sperm competition dictates ejaculates be of high quality, i.e., of high motility and with low sperm abnormality. In contrast, the spermatozoa of monogamous species (e.g., falcons, eagles, and certain penguin species) usually exhibit high sperm abnormality.³³ The high incidence of immature spermatozoa (about 60%) in falcons suggests that these creatures may show a degree of monogamy unusual even among birds. Differences in the degree of pleomorphy are also seen in smaller birds. In the Eurasian bullfinch (*Pyrrhula pyrrhula*), a more monogamous species in which sperm competition is low, 8%–18% of sperm cells may be immature or show head abnormalities, while in the polygamous dunnock (*Prunella modularis*), in which sperm competition is intense, only 4%–5% may be so affected.³⁴ Pleomorphism is easily observed by standard microscopy and is related to the presence of spermatocytes or spermatids that have reached different points in their transformation. Sometimes, subtle variations in the head size may comprise subpopulations identifiable only by methods that can accurately record sperm morphometry. It has been suggested that morphometric characteristics are regulated genetically.³¹

Postcopulatory sexual selection via sperm competition may be an important evolutionary force affecting different reproductive traits including sperm morphometrics.³⁵ Sperm competition is a strong selective force that promotes, among other things, a larger and faster-swimming gamete. Although larger sperm heads might be considered a handicap to rapid swimming,³⁶ recent data show that red-legged partridge spermatozoa with longer heads swim faster.⁸ Other authors have reported similar observations in other species.^{37,38} The length and area of the sperm head of the red-legged partridge are smaller in pure (*Alectoris rufa*) than in hybrid (*A. rufa* × *Alectoris chukar*) birds. *A. chukar* is more promiscuous than *A. rufa*,³⁹ and thus more polyandry might be expected of the hybrids than of pure birds. Similarly, several authors report that the males of species with polyandrous females have longer and faster spermatozoa than species with sexually monogamous females.^{33,40,41} Female reproductive

biology (i.e., clutch size and spread of laying) has been suggested to affect sperm size and the evolution of sperm morphometrics in several pheasant species.³⁵ In the latter study, sperm size traits were negatively associated with the duration of sperm storage. In other species, such as the Gouldian finch (*Erythrura gouldiae*), sperm morphometric values may show plasticity within the same breeding season if the social environment is modified. Males may produce an increased sperm midpiece size when in a highly competitive environment and an increased sperm tail length when competition is weak.⁴²

External factors affecting sperm size

Sperm characteristics may be used as biological markers of environmental influences (pollutants, pesticides, and climate change) since germinal cells are very sensitive to them. Birds are found in forest, urban, mountain, marine, desert, and even polar habitats, with some species migrating between continents. Bird spermatozoa, therefore, provide an interesting model for evaluating the impact of environmental stresses.

Toxins and pollutants may disturb different stages of spermatogenesis and, thus, the final size of avian sperm cells. In mammals, it is well established that high concentrations of estrogenic chemical residues (e.g., DDT) in water, sediment, and tissue impair spermatogenesis in humans and certain wild animals,⁴³ and certain pesticides are known to have harmful effects on sperm function and DNA.³² The pesticide phosphamidon has been reported to increase the proportion of sperm cells with aberrant head morphology in mice,⁴⁴ and organophosphorus pesticides can cause the formation of diploid or even polyploid spermatids, which have abnormal head sizes.^{45,46} In humans, exposure to ethylene dibromide – an active component of several pesticide fumigants – has been related to a narrowing of sperm heads.⁴⁷ Although far fewer studies of their effects in birds have been undertaken, it might be expected that such pesticides have similar effects on them.

Birds have shown clear ecological and evolutionary responses to recent climate change,⁴⁸ and further changes may be expected in their sexual behavior, breeding activity and migratory behavior in the medium and long term. It may be that these responses affect future reproductive function related to polygyny, polyandry, and monogamy, and in turn sperm morphological characteristics. Further, bird species can be affected by heat stress.⁴⁹ This might impair spermatogenesis by reducing testicular germ cell proliferation and increasing apoptosis.⁵⁰ Certainly, environmental heat stress has been reported to affect sperm head ellipticity in rams.⁵¹

The manipulation of spermatozoa in the laboratory may also affect sperm cell morphometry. For example, the choice of fixative (e.g., methanol, glutaraldehyde) employed when stains are used is known to modify the size of avian spermatozoa.⁵ The osmotic stress resulting from the use of hyper- or hypo-tonic solutions to dilute semen may also affect sperm size via the dehydration or swelling of cells. The freezing-thawing of spermatozoa can also influence sperm cell size; in falcons, this is caused by the loss of the acrosome or changes in chromatin structure.⁵

Finally, diseases (e.g., yellow semen syndrome in turkeys) can directly or indirectly affect the morphometric values of sperm cells.⁴

SPERM MORPHOMETRY

Conventional method

Sperm morphology and morphometry have been deemed important in sperm evaluation since artificial reproduction techniques were first developed. Studies on the function of the different structures in

sperm cells and their importance in sperm quality and fertilization capacity have long been reported. The first were mainly descriptive. For example, in 1888, Ballowitz⁵² described sperm cells to be composed of a head, midpiece, and tail, and described an intensely stained region in the apex of the head which would be identified later as the acrosome. In the 1940s, the electron microscope was used to examine the spermatozoa of several species including roosters.⁵³ The authors described the different regions of these cells after treating them enzymatically or with distilled water, and measured their length and width. Detailed electron microscope-based information is available on the appearance, measurements, and classification of normal and defective chicken spermatozoa,⁵³ as well as on their ultrastructure.⁵⁴ Scanning and transmission electron microscopy have since been used to analyze the ultrastructure of ostrich, guinea fowl, turkey, and Japanese quail sperm cells.⁵⁵⁻⁵⁷ However, this usually requires the use of phosphate-buffered glutaraldehyde solutions to fix the sample, followed by dehydration in ascending concentrations of ethanol,⁵⁷ and this can strongly affect sperm morphometric variables. Indeed, shrinkage often occurs when biological material is prepared for electron microscopy.⁵⁸ This may explain why both chicken and turkey sperm morphometric variables are usually recorded as smaller when examined in the electron microscope^{53,54} than in the light microscope.^{5,57}

Morphometric analyses of avian spermatozoa by light microscopy are, however, relatively scarce.⁵⁹ Most studies have involved conventional techniques and the subjective assessment of sperm variables;^{60,61} unfortunately, these methods can return significantly different results when performed in different laboratories or by different operators.⁶²⁻⁶⁴ Eosin-nigrosin staining has been used to measure sperm traits in duck spermatozoa,⁶⁵ and amido black, Spermac^{®57} and other stains^{55,66} in the morphometric analysis of quail and rooster sperm, but they provide conflicting results.

Computer-assisted sperm morphometry (CASA-Morph)

A number of reliable and accurate CASA-Morph systems have been developed for the assessment of sperm morphometry.^{24,67,68} The problem of subjectivity associated with visual assessment methods is significantly reduced by these systems,⁶⁹ which have been standardized for use with semen from different animal species, including humans.^{70,71} In each case, the most appropriate staining and sampling techniques have been established.^{72,73} CASA-Morph systems are now commonly used to examine mammalian spermatozoa,⁷⁴ and the head morphometry results they provide have been used to predict fertilization rates⁷⁵⁻⁷⁸ and sperm cryodamage.^{25,26,79} However, they have been little used with avian semen, a consequence of the filiform shape of bird spermatozoa.

For mammalian spermatozoa, the most commonly used stains employed with CASA-Morph systems are Hemacolor[®], Diff-Quick[®], Haematoxylin, and SpermBlue[®].^{68,80,81} Hemacolor[®] is the most suitable for sperm head morphometry assessment in ibexes (*Capra pyrenaica*)⁶⁸ and humans.⁸² A recent study compared the performance of Hemacolor[®] and aniline blue staining as part of a computer-assessed, light microscopic method for measuring avian sperm head characteristics.⁵ Semen from roosters (*G. g. domesticus*) and red-legged partridges (*A. rufa*) was used. Both stains clearly distinguished the end of the head from the midpiece and flagellum: accurate measurements of the sperm head were therefore guaranteed. The percentage of measurable spermatozoa was higher with the Hemacolor[®] staining technique than with the aniline blue technique, both for the rooster and partridge spermatozoa. The reasons that some spermatozoa could not be measured included the background particles around them preventing their certain identification, and the stain being too faint to

differentiate some cells from the background (this occurred only with the aniline blue technique). Unfortunately, in those samples with large numbers of particles, the Hemacolor® technique was problematical since these particles were also stained, making it difficult to identify the sperm cells. However, the Hemacolor® technique might be deemed more appropriate for computerized sperm assessment systems since it provides larger percentages of measurable cells and shows greater repeatability.⁵

Aniline blue staining has been used to assess chromatin condensation status in human spermatozoa^{83–85} because the stain indicates the persistence of histones. It is commonly used to stain avian spermatozoa since it reveals morphological abnormalities well and renders acrosomal integrity easy to examine.⁹ However, it has never been used for morphometric purposes since its stain intensity is rather low, although studies conducted with rooster, partridge,^{5,8} and golden eagle⁸⁶ spermatozoa have shown an intensity of staining sufficient to be detected by the computerized system. Again, the proportion of measurable spermatozoa was lower than that with Hemacolor®. Aniline blue staining, however, allows sperm abnormalities to be recognized easily, and facilitates acrosomal imaging when using phase contrast microscopy.⁹ The better definition of the acrosome provided by the latter stain may explain the difference in rooster and partridge sperm head lengths recorded with it and with Hemacolor®.

When using CASA-Morph, staining can influence the sperm morphometric results obtained.^{64,87} The different fixatives required (i.e., methanol for Hemacolor® and 2% (v/v) glutaraldehyde for aniline blue) may be the reason, dehydrating or swelling the sperm cells to different extents.^{68,81} Certainly, sperm head size would seem to be subject to such effects.⁶⁹ Formaldehyde,⁸⁸ Hancock's solution,⁸⁹ and glutaraldehyde⁵⁹ have all been used in sperm assessment, with the last of these reported to reduce the cell shrinkage observed in air-dried semen smears, thus allowing greater structural detail to be observed by phase contrast microscopy.^{90,91} In roosters, Hemacolor® returns significantly larger sperm head widths and areas than the aniline blue technique does while the latter results in greater sperm head lengths.⁵ In the red-legged partridge, no differences are seen in the results for sperm head width and area provided by these two techniques, but aniline blue staining is associated with larger length measurements.⁵ These findings may reflect a species-specific response to the methanol fixative. Similar results have been observed in other species.^{10,80,92}

Morphometric characteristics in domestic and wild species

Sperm morphometric data have been reported for only a few bird species, with most recorded for roosters, turkeys, and quails.^{53,57,93} Only, recently, some studies have focused on wild species. Although all bird sperm heads are filiform, the exact shape varies between Orders, and there are appreciable differences in the shape of spermatozoa from galliformes, passeriformes, and falconiformes. A vermiform sperm head morphology (long and narrow) is very apparent in galliformes (chicken, quail, and partridges), while in falconiformes (eagles and falcons), the sperm heads are wider (**Figure 1**). Morphologically, avian spermatozoa have been classified as of the “sauropsid” and “spiral shaped” types.⁵⁷ Sauropsid-type spermatozoa, which include those of galliformes and most nonpasserine birds, are smooth and elongated. The spiral type shows an external, helically wound, undulating membrane, which is very characteristic of passerines.⁹⁴ The length of bird spermatozoa is very variable (30–300 μm), with no correlation between sperm dimensions and species size and weight.⁹⁵ For example, within Galliformes, roosters (with a body weight of 3.0–3.5 kg for native breeds) have smaller spermatozoa than red legged-partridges (with a

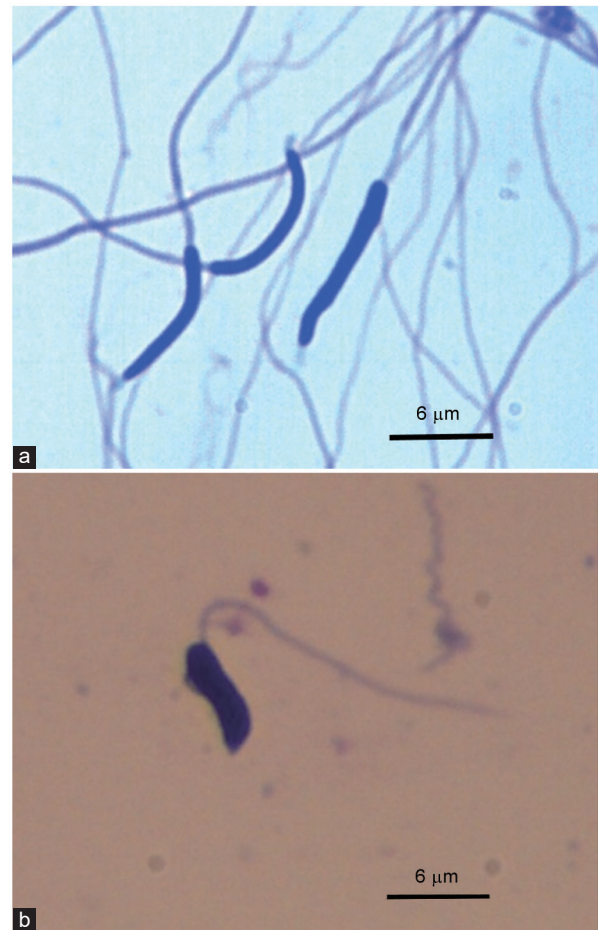


Figure 1: Chicken sperm (vermiform sperm head morphology – sauropsid type [a]) and peregrine falcon sperm (short and wide sperm head morphology [b]). Sperm stained with Hemacolor®. Scale bar = 6 μm .

body weight of 0.4–0.5 kg) do (**Table 1**). The size of the different parts of the sperm (head, midpiece [especially], total tail, etc.) is also very variable between species (**Table 1**).

Falcons have smaller spermatozoa than galliformes, with the sperm heads shorter but wider. Sperm head length in fowl ranges from 11 to 21 μm , while in falcons, it is usually 6–7 μm (**Table 1**). Falcon ejaculates characteristically contain pleomorphic spermatozoa whereas sperm morphology in the golden eagle is very heterogeneous. The most common abnormal sperm morphology is a rounded head with a triangular acrosome although commonly observed wasp-stinger-shaped acrosomes are deemed normal on the basis of the intactness of the organelle. The percentage of abnormal spermatozoa in golden eagle ejaculates is also higher than that in ostriches and the white-backed vulture.^{97,98} Pleomorphy, a characteristic of falcon spermatozoa, is particularly notable in fresh gyrfalcon (*Falco rusticolus*) sperm cells. The immature sperm cells in such ejaculates include type I or type II spermatocytes, spermatids in different phases of development, and quasi-mature spermatozoa still with the residual body attached to the head.²⁷ No spermatogonia are observed, however, unlike that reported earlier for the peregrine falcon (*Falco peregrinus*).⁹⁹ The mean percentage of immature spermatozoa is about 55%–65% in peregrine falcon and gyrfalcon ejaculates (Villaverde-Morcillo *et al.*, unpublished data). This high percentage agrees with that reported earlier for peregrine falcon ejaculates⁹⁹ although the latter authors

Table 1: Summary of sperm morphometric measurements (expressed as mean±s.d. when available) in avian species

Species	Measurements				Midpiece length (µm)	Tail length (µm)	Technique ASMA*/fixative/stain	References
	Head							
	Length (µm)	Width (µm)	Perimeter (µm)	Area (µm ²)				
Chicken (<i>G. domesticus</i>)	14	0.5	-	-	4	82	e.m*/formalin	53
Chicken (<i>G. domesticus</i>)	13.5±3.5	1.3±1.7	-	13.9±0.7	-	-	Motic®/Hemacolor®	5
Turkey (<i>M. gallopavo</i>)	10.4–11.9				5.2–6.0	66–72	l.m*/formalin/Spermac®	57
Japanese quail (<i>C. japonica</i>)	20.8–23.8				160–170	40–60	l.m*/formalin/Spermac®	57
Red-legged partridge (<i>A. rufa</i>)	14.4±0.2	1.3±0.0	-	14.7±0.3	-	-	Motic®/Hemacolor®	8
Emu (<i>D. novaehollandiae</i>)	11.7±0.9	-	-	-	2.9±0.4	47.0±2.8	Soft Imaging System iTEM-Olympus®/ glutaraldehyde/Wright	59
Golden eagle (<i>A. chrysaetos</i>)	7.4±0.8	1.1±0.2	-	6.8±1.7	-	-	Motic®/glutaraldehyde/ aniline blue	5
F. peregrinus Brookei (<i>F. peregrinus Brookei</i>)	7.0±1.2	1.7±0.3	16.5±2.8	10.0±2.4	-	-	Motic®/Hemacolor®	Villaverde-Morcillo <i>et al.</i> unpublished data
F. peregrinus Scottish (<i>F. peregrinus peregrinus</i>)	6.3±1.0	1.6±0.2	14.8±2.1	8.5±1.4	-	-	Motic®/Hemacolor®	Villaverde-Morcillo <i>et al.</i> unpublished data
Duck, synthetic line (<i>A. platyrhynchos</i>)	13.9±0.6				3.5±0.2	55.6±2.5	LuciaG5®/eosin nigrosin	65
Gyrfalcon (<i>F. rusticolus</i>)	6.3±1.4	2.1±0.5	16.0±3.2	11.6±4.0	-	-	Motic®/Hemacolor®	Villaverde-Morcillo <i>et al.</i> unpublished data
Barn swallow (<i>H. rustica</i>)	13.4±0.5				62.5±2.5	15.0±2.2	ZeissAxioVision®-Leica®/ formalin	96
Ostrich (<i>S. camelus</i>)	12.8±1.1				3.1±0.3	56.7±3.7	e.m.*/glutaraldehyde, ethanol	56
Gentoo penguin (<i>P. papua</i>)	12.5±1.0	1.6±0.2	28.9±2.5	18.2±4.1	-	-	Motic®/Hemacolor®	Present study
King penguin (<i>A. patagonicus</i>)	13.8±0.4	1.6±0.2	30.9±0.5	19.7±3.0	-	-	Motic®/Hemacolor®	Present study

*Is included when other method is used instead of ASMA; e.m: scanning electron microscope; l.m: light microscopy without computerized system. s.d.: standard deviation; ASMA: assisted sperm head morphometry analysis; *G. domesticus*: *Gallus domesticus*; *M. gallopavo*: *Meleagris gallopavo*; *C. japonica*: *Coturnix japonica*; *A. rufa*: *Alectoris rufa*; *D. novaehollandiae*: *Dromaius novaehollandiae*; *A. chrysaetos*: *Aquila chrysaetos*; *F. peregrinus*: *Falco peregrinus*; *A. platyrhynchos*: *Anas platyrhynchos*; *F. rusticolus*: *Falco rusticolus*; *H. rustica*: *Hirundo rustica*; *S. camelus*: *Struthio camelus*; *P. papua*: *Pygoscelis papua*; *A. patagonicus*: *Aptenodytes patagonicus*

identified the immature sperm cells they saw as spermatogonia. It may be that the high percentage of spermatocytes and spermatids in falcon ejaculates has a physiological role in the reproduction of these species. Indeed, four sperm subpopulations, based on sperm head size, have been identified in falcon ejaculates.²⁷ The proportion of each is similar in the brookei peregrine falcon (*F. peregrinus brookei*), Scottish falcon (*F. p. peregrinus*), and gyrfalcon, but the role of these subpopulations in their reproductive biology and fertility remains unknown.

Penguin species are southern hemisphere birds found from the Equator to Antarctica; they are thus subjected to different ecological constraints on the reproductive strategy followed. Breeding activity is very sensitive to environmental conditions, and therefore highly responsive to climate change.¹⁰⁰ For example, in the equatorial Galapagos penguin (*Spheniscus mendiculus*), the onset of breeding is closely linked to mean sea surface temperature.¹⁰¹ The breeding patterns and reproductive characteristics (e.g., functional and morphometric sperm parameter values) of penguins may, therefore, provide useful indicators of oceanographic conditions. With the use of Hemacolor® and a CASA-Morph system,⁵ morphometry of sperm samples from gentoo (*Pygoscelis papua*) and king penguins (*Aptenodytes patagonicus*) revealed similar sperm head lengths to those of the rooster, but greater head widths than those recorded for the galliformes (and therefore more like those of falcons) (Table 1). The sperm head area was, in fact, greater than that of most bird sperm cells studied. Although penguins are monogamous, interannual fidelity varies according to species,¹⁰⁰

and polygynous trios (one male with two females) have been observed in emperor penguins (*A. patagonicus*). The larger sperm heads of the king penguin, and a lower count of immature cells, suggest a certain degree of polyandry is possible.

CONCLUSIONS

Given the many factors that affect the morphology and morphometrics of avian sperm cells, the standardization of staining techniques to be used in computer-assessed light microscopy methods is a priority.

COMPETING INTERESTS

None of the authors have any financial or personal relationship that could inappropriately influence or bias the content of the paper.

AUTHOR CONTRIBUTIONS

JSM conceived the study and wrote the basis of the article; MCE designed experiments, performed data analysis, and drafted the manuscript; SVM performed data analysis and drafted the manuscript; ATD and CC conducted bird management and sperm collection; ALS and RV performed data analysis; ALG and JGM took charge of penguin sperm collection and sperm analysis.

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