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INVITED REVIEW

Semen Analysis

Current status and potential of morphometric sperm analysis

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The spermatozoon is the most diverse cell type known and this diversity is considered to reflect differences in sperm function. How the diversity in sperm morphology arose during speciation and what role the different specializations play in sperm function, however, remain incompletely characterized. This work reviews the hypotheses proposed to explain sperm morphological evolution, with a focus on some aspects of sperm morphometric evaluation; the ability of morphometrics to predict sperm cryoresistance and male fertility is also discussed. For this, the evaluation of patterns of change of sperm head morphometry throughout a process, instead of the study of the morphometric characteristics of the sperm head at different stages, allows a better identification of the males with different sperm cryoconservation ability. These new approaches, together with more studies employing a greater number of individuals, are needed to obtain novel results concerning the role of sperm morphometry on sperm function. Future studies should aim at understanding the causes of sperm design diversity and the mechanisms that generate them, giving increased attention to other sperm structures besides the sperm head. The implementation of scientific and technological advances could benefit the simultaneous examination of sperm phenotype and sperm function, demonstrating that sperm morphometry could be a useful tool for sperm assessment.

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INTRODUCTION

Sperm research has received increased attention in recent decades, given the peculiarities of this cell: it develops part of its lifespan outside the male body, often in a hostile environment, and it carries the genetic material from the male to the oocyte. The differences in sperm morphology and physiology, even between related species, and the presence of highly specialized structures, have led to questions on the reasons for this diversity and specialization. Considering different sperm features, the study of sperm morphology has been considered an essential part of sperm research. Two main questions arise: how did the diversity in sperm morphology arise during speciation (a backward perspective) and what role do the different specializations play in sperm function (a forward perspective)?

Several studies have addressed the first question, many of them from an evolutionary point of view, and the majority being descriptive.[1–3] These studies have put considerable effort into finding the ultimate cause of this sperm diversification, and how within- and between-male variation and within- and between-species variation contribute to sperm performance and behavior.[4,5] Adaptation to specific fertilization environments and the fertilization process itself have been proposed as the main selection forces.[4,6,7] With these forces, sperm competition seems to play a major role, influencing not only sperm morphology but also sperm length and sperm numbers.[8,9]

Considering the forward perspective, most studies have tried to establish functional relationships between sperm traits during the fertilization process and their performance in assisted reproductive techniques (ARTs), with regard to sperm resilience or fertility. Typically, traits such as motility, viability (either plasma-lemma integrity or the hyper-osmotic shock test [HOST] responsiveness), acrosomal integrity, or absence of abnormalities have been used as endpoints for predicting sperm fertility or, rather, discarding potentially low-fertility semen doses.[10–13] Availability of advanced techniques and hardware such as computer-assisted sperm analysis (CASA),[14–16] fluorescence probes and ultimately flow cytometry,[17,18] and new endpoints, e.g. capacitation and chromatin assessment,[19,20] have allowed more objective and faster analysis, but the predictive power of laboratory sperm assessment still needs improvement.[21] The routine evaluation of semen has traditionally included the assessment of normal sperm morphology, but the important subjective component has limited its practical use.[22] The development of automatic image-processing systems has displaced classical methods and is a major advance in sperm analysis. Computer-assisted sperm morphometric analysis (CASA-Morph) systems have been successfully used to determine the relationships between sperm shape and fertility of males[23,24] or sperm freezability.[25,26]

There is a need to develop new analytical tools to capture sperm diversity better, and to improve data analysis methods. New equipment

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should take advantage of high-resolution optics and image analysis programs to increase accuracy and precision of laboratory tests; the implementation of this new equipment should allow the simultaneous assessment of morphological and functional sperm features. Given the high amount of information that is expected in the mid-term future, the development of new statistical methods is also necessary for the joint analysis of all these sources of information, which will allow a better assessment of relationships among sperm features and their functional meaning.

MORPHOLOGICAL SPERM DIVERSITY

The spermatozoon is the most diverse cell type known. This diversity is thought to reflect adaptation to conditions under which the cells function, as a way to ensure their survival in different fertilization environments and to maximize their fertilizing capacity.[4,6] The spermatozoon of thousands of species has been described including insects,[27] crustaceans,[28] fish,[29] birds,[30] mammals,[31,32] and many other groups. The existence of morphological diversity among species is widely recognized, with a high degree of variation in size, shape, and behavior,[3,32,33] reflected in main structures (i.e., head, midpiece, and principal piece) and overall size. Different specializations have been observed, such as the absence of a flagellum or multiple flagella, gigantism, the presence of an apical hook, bristles or brushtail, etc. In some species, the shape of the spermatozoon allows it to cooperate with others, as in the case of sperm conjugation, a phenomenon in which two or more spermatozoa are physically united during transport through female reproductive tract.

A first attempt to explain this diversity was conducted by Franzén[4,6] who proposed that sperm modifications are adaptations to their specific fertilization environment. Thus, pre- and post-copulatory selection such as mate choice, mode of fertilization, cryptic female choice, sexual conflict, and sperm competition may influence the evolution of sperm traits.[5] Two of these traits are likely to play an important role as selective forces: female selection and sperm competition.[9] Numerous studies have focused on the correlated evolution of sperm length and female reproductive tract design,[34–38] suggesting a sexual coevolution of the fertilization system.[39] Selection would favor very different sperm traits depending on whether spermatozoa are released into open water (e.g., external fertilizers), have to remain for longer periods of time in female storage organs (e.g., birds, bats and insects), or have a short time window to fertilize after being transferred to the female tract (e.g., mammals).

Sperm competition has also been found to be a significant source of sperm variability.[40–44] High levels of sperm competition are associated with increases in testicular mass relative to body size[5,41,45,46] and with high relative sperm numbers[45,47,48] in many taxa. While initial hypotheses were that an energy trade-off exists between sperm numbers and sperm size,[49] and consequently sperm competition would result in a reduction in sperm size, later studies demonstrated that higher sperm size represented an advantage by increasing sperm velocity[43,44,48,50–52] and that sperm competition, therefore, resulted in an increase of sperm size.[41,53,54]

Evolutionary forces

Morphological variation has been driven by evolutionary forces. However, the development of assisted reproductive techniques (ARTs) has introduced a new – artificial – source of variation in sperm morphology.[55] The *in vitro* fertilization process includes stages outside the male and female reproductive tracts, during which spermatozoa are subjected to procedures aimed at maximizing reproductive success. The sperm characteristics that determine which are the best for ART may not be the same as those determined from a physiological assessment. Therefore, it may be expected that those males having a higher proportion of the relevant kind of spermatozoa would produce more offspring, ultimately leading to a selection toward more favorable sperm design for ART.

A number of studies have reported that spermatozoa with smaller heads withstand the cryopreservation process better[56,57] and that the sperm head morphometry–cryoresistance relationship is in part genetically determined.[26] The use of selection media before AI could also select cells of a specific morphology[58] and could favor a certain shape although these methods are expected to work in a similar manner to the selection processes occurring in the female reproductive tract.[59,60] It is too soon to assess the role that ART plays in the evolution of sperm morphology, but it might be advisable to follow up morphological changes derived from the use of these techniques, and their consequences.

ASSESSMENT OF SPERM MORPHOMETRY I – TECHNOLOGICAL ASPECTS

Many techniques can assess sperm morphometry[61,62] but CASA-Morph has become the main choice, because it provides increased reliability and repeatability, and reduced subjectivity.[62–64] Studies that assess the different sources of variation affecting CASA-Morph are critical for guaranteeing its repeatability and consistency among laboratories. The main sources of variation of CASA-Morph, other than the software and data analysis, are the sample preparation, fixation method, staining method, microscopic system (optics and camera), and technician. All these steps can affect not only the repeatability of the experiments but also the reproducibility and the comparison of results among laboratories, which are necessary for the practical use of sperm morphometric analyses. These aspects have been studied by several authors,[64–71] and they have not yet been completely resolved.

Sample preparation

The preparation of the sample, its concentration, and the fixation procedure are the first steps to consider in a CASA-Morph protocol. Varying the sperm concentration may affect CASA-Morph performance.[66,72–74] Fixation, together with drying of the sample, has received little attention, but they are both critical steps, and it has been demonstrated that they affect CASA-Morph results.[75,76] During slide preparation, it is advisable to make at least one replicate so that inter-slide variability can be assessed,[77,78] and that replicates that fall outside certain thresholds can be rejected, because of unacceptable variability in slide preparation.

The choice of staining protocol is the issue on which most authors have conducted their studies[63,65,66,68,70–73,79,80] since it influences background noise, sperm contrast, and the identification of different areas within the cell. To prevent these problems during morphometric sperm analysis, fluorescence-based methods in combination with a CASA-Morph system have been developed for a more precise measurement of the nucleus, acrosome, and sperm head, yielding promising results.[64,81] In addition, a new system has been developed (Trumorph®, Proiser R+D, Paterna, Spain) that avoids fixing and staining of the sample, and in combination with phase-contrast microscopy, allows assessment of sperm size and shape in wet-mount preparations.[82,83]

The higher the quality of the hardware (microscope, lenses, and camera), the more reliable the analysis, at least for experimental work. At high magnification, lenses capable of providing a sharp,

aberration-free and bright image, and a camera capable of high resolution will reduce the errors of the CASA-Morph software and will allow a more reliable analysis.[69,74] However, widespread use of CASA-Morph systems in clinical or production environments may require the use of cheaper equipment. In such cases, efforts should be directed at improving staining results and optimizing software to cope with the limitations due to the use of basic equipment.

Regarding software, automatic methods should be preferred, to save analysis time and to reduce operator errors. Goulart *et al.*[69] suggested using semi-automatic methods, with some operator intervention. When the interaction of the technician with the software is limited to removing misdigitized spermatozoa, the effect of the technician in the analysis is relatively low, compared with subjective morphology assessment.[25,78,84]

Replication and statistical procedures

Some authors have tried to establish the minimum number of spermatozoa necessary for obtaining reliable and stable morphometry parameter values. A general recommendation is that at least 200 spermatozoa should be analyzed per sample[85] although some authors have suggested that lower numbers could be adequate for some species and experiments.[25,68,70,72,84,86] If the aim of the analysis is to obtain a proportion, usually the percentage of normal spermatozoa, the confidence intervals vary with the value of that proportion. If the proportion of abnormal forms is close to the extremes of the range (0%–100%), a situation that is fairly common in animal reproduction, the coefficient of variation (CV%) increases considerably.[85,87] Currently, the capabilities of computers, sophistication of CASA-Morph software, and availability of automatic acquisition systems (e.g., microscopes with motorized stages and autofocus) allow the acquisition of large amounts of data with little operator time. Moreover, if the aim of CASA-Morph is subpopulation analysis, the number of spermatozoa analyzed needs to be high, because the total number analyzed (the sample size) must be divided among the subpopulations, thereby decreasing the statistical confidence of the statistics defining each population. This would be aggravated if there were subpopulations of relatively low size (thus receiving a small percentage of the total number of spermatozoa analyzed).

In addition, it is also advisable to assess a high number of individuals, to have an adequate representation of the species, allowing conclusive results to be obtained. Thus, the main problem of most CASA-Morph studies so far is the use of a low sample size.[23,26,88–91] Only a few authors[24,92–94] have conducted large-scale studies to assess, in the same species, the sperm head morphometry and also to study its relationship with sperm function.

It must be pointed out that most studies have used incorrect statistical techniques to compare protocols, not only regarding the number of spermatozoa analyzed but also technician effect, stains, etc. These statistics, generally based on a means comparison or correlations/regressions, are not appropriate for assessing differences between methods.[95,96] Thus, the conclusions are usually limited and should be reevaluated using the appropriate methodology. Only a few studies[84] have applied correct statistical methods (e.g. Bland and Altman agreement coefficients[97]).

Regarding the consistency and repeatability of analyses, laboratories should set up a quality assurance system for CASA-Morph. The use of latex beads of a determined size[69] or standard sperm doses could be combined in a quality assurance protocol, which could also be used for assessing inter-laboratory variability.

As a final point, CASA-Morph protocols and software should be validated, a process that is not always straightforward. In general, protocols have been validated for reduced intra-slide and between-slide (for the same sample) variability, while enhancing between-male or between-treatment variability and reducing digitization or analytical errors.[66,68,78] Some studies have compared different protocols, describing their respective strengths and weaknesses. However, most validations lack the definition of a "gold standard" that would allow a broader comparison among studies and protocols. Examples of such a "gold standard" would be morphometric data obtained from electron microscopy (much more resolution and thus more reliable, although not fit for routine use) or a previously validated technique. Some authors have used other methods, such as measuring the heads directly on screen with calipers, and comparing these measurements with those provided by the CASA-Morph software.[70]

ASSESSMENT OF SPERM MORPHOMETRY II – FUNCTIONAL ASPECTS

Studies on the relationships between sperm design and sperm function have often yielded contradictory results.[24,44,89,94,98–100] Most of the research made in this aspect has usually been conducted at an interspecific level[44,101] since finding differences between species is easier than intraspecific or intra-male levels. Studies at the intraspecific level are quite limited and most of them have used a low sample size (fewer than 25 individuals)[23,88,89,91] making robust conclusions difficult to obtain.

Recently, some authors have reported that some sperm characteristics are genetically determined,[26,102] sperm morphometry being one of them. Thus, it is expected that sperm morphometry reflects differences in sperm function. Indeed, different sperm morphometric features have been identified between breeds[103] but also between animals from the same breed belonging to different herds (i.e., reflecting their origin).[92]

Ignoring the differences between the morphometric dimensions of X- and Y-bearing spermatozoa due to their DNA content,[104] most differences detected between spermatozoa are probably caused by changes in the media in which spermatozoa are suspended, which could modify the sperm volume. Some authors have explored the morphological response to diluting or washing the sperm sample,[105,106] to capacitation[107] or to cryopreservation.[26,108–110]

Sperm cryopreservation and morphology

The effects of cryopreservation on sperm head morphometry have been studied in numerous species: humans,[111] bulls,[105,112] boars,[113,114] rams,[26] goats,[115,116] stallions,[117] dogs,[118] bears,[119] and red deer.[56,57] All these studies have reported a significant reduction in sperm head dimensions by cryopreservation of freshly extended samples. This reduction in sperm head dimension is reflected not only in the size of sperm head but also in its shape. Different hypotheses have been proposed to explain the reasons for the sperm head dimension decrease after cryopreservation, including osmotic changes,[117,120] alterations of some cell compartments,[25,117] damage or loss of the sperm acrosome,[121,122] and over-condensation of sperm nuclear chromatin.[74,119]

The spermatozoa from different individuals may exhibit significantly different responses to the same freezing treatment.[123–125] Thus, males can be classified as "good" or "bad" freezers on the basis of their sperm cells' resistance to the cryopreservation process, and sperm morphometry is a useful tool to this end. For example, Hidalgo

et al.[115] observed that in the goat, sperm morphometric changes after cryopreservation were lower in semen samples showing better quality in fresh semen, while a further reduction in sperm dimensions was observed for those semen samples with initial poor semen quality. Esteso *et al*.[126] observed that deer with better cryoresistant spermatozoa were characterized by a low sperm head area and a large sperm head shape factor (defined as the length/width ratio). These authors also observed that semen samples with lower intravariability for sperm morphometric measurements showed smaller changes in their morphometry.[56] Moreover, they reported that those males with no or small changes in sperm dimensions after freezing-thawing showed a low sperm head dimension in the extended samples. These authors thus suggested that in semen samples of better quality the sperm cryodamage was less and the effect of cryopreservation on the sperm head morphometry was also less. Ramon *et al*.[26] have gone a little further on the prediction of sperm cryoresistance. Whereas a general study of the morphometric characteristics of the ram sperm head at each stage of the freezing process did not allow an adequate identification of the males with better sperm cryopreservation, the study of patterns of change throughout the cryopreservation process led to the identification of differently defined patterns clearly related to cryopreservation ability. Furthermore, these authors showed that each male retained its pattern of response for all ejaculates examined, and that those males sharing the same pattern of response were more closely related, suggesting the possibility of a genetic control of the response.

Therefore, the study of the morphological changes in response to cryopreservation may be presented as an opportunity to improve the reproductive ability of individuals. First, as a way to indicate what changes happen and how they occur, and second, as a tool to develop better ART methodologies to prevent undesirable response patterns, or for designing selection programs toward fitter sperm designs.

Despite most of the studies on the effect of cryopreservation on sperm morphology have been focused on the evaluation of sperm head features, Ros-Santaella *et al*.[109] recently reported that stags not differing in sperm head dimensions showed significant differences in sperm cryoresistance that were strongly related to the volume of the sperm principal piece. This study indicates a key role of the sperm flagellum during cryopreservation and suggests new approaches for the characterization of those spermatozoa with good cryoresistance.

Sperm physiology and morphology

Other aspects of sperm physiology and morphology may be considered; spermatozoa actively migrate through the female genital tract. In many species, the first barrier is cervical mucus, which only allows the advance to the uterus of progressively motile sperm with normal morphology and through which they migrate (with the aid of myometrial contractions) to the oviduct, where fertilization will take place.[127] In attempts to mimic this process of *in vivo* selection, different sperm selection methods are routinely used in *in vitro* protocols, such as *in vitro* fertilization (IVF) or sperm sorting, to enrich the sample with morphologically normal and motile spermatozoa.[128] During this process, not only morphologically normal and progressively motile spermatozoa are selected, but also the male germ cells undergo physiological changes, termed capacitation, which are fundamental prerequisites for the acquisition of functional sperm competence to undergo the acrosome reaction and hence fertilize the oocyte.[129] However, it is not yet known in detail how these processes affect sperm morphology; more importantly, the morphometric characteristics of the cells that eventually fertilize the oocyte are unknown. Recently, García-Vázquez *et al*.[130] reported how boar spermatozoa in the female reproductive tract are selected on the basis of their size and shape, with those with a larger head and longer tail being those reaching the fertilization site. For sperm morphometric changes during capacitation, García-Herreros and Leal[107] reported that the induction of *in vitro* capacitation in bovine spermatozoa modified sperm head morphometry. As capacitated spermatozoa showed a decrease in all sperm head size and shape parameters, the authors concluded that sperm head morphometry is an objective diagnostic tool for sperm assessment during capacitation.

During the migration of spermatozoa through the female genital tract toward the fertilization site, sperm motility is essential.[131] Several studies have addressed how sperm morphology and sperm velocity may be related,[23,44,51,99,109,132] and their impact on reproductive performance.[23,98,133] However, results are contradictory on how sperm diversity translates into variation in sperm velocity.[43,99,132] Only a few studies have been made in the same species to study directly sperm design and motility. Ramon *et al*.[23] reported, for the first time, the relationships between stag sperm design and velocity (in the same sample) in a species with internal fertilization, and the role that both may play in male fertility. These researchers observed that males with ejaculates containing a high percentage of spermatozoa with fast and linear motility also had small and elongated heads and yielded higher fertility rates. These relationships were also reported by Fitzpatrick *et al*.[134] in externally fertilizing species (fish). Sperm head elongation may play an important role by making sperm more hydrodynamically efficient, which, in turn, may influence sperm fertilizing ability. Indeed, other authors have reported that spermatozoa with elongated heads may be faster[23,44,98] because of lower resistance to forward progression. This could compensate evolutionary constraints to increases in sperm length by allowing increased swimming efficiency, or for the same reason, it might increase sperm lifespan if energy reserves last longer or are used more efficiently, which would result in more spermatozoa reaching the fertilization site.[43]

In addition, because most of the sperm head is occupied by the nucleus, its compactness can influence sperm head shape. Some authors have presented evidence supporting the involvement of protamines in sperm head shaping, leading to smaller and more elongated sperm heads when they are present in high proportions.[135,136] Similar results have been reported by Gómez Montoto *et al*.[137] who observed in rodents that an increase in sperm swimming speed is also associated with elongated sperm heads. On the other hand, they also found that an increase in total sperm size maximized the chances that sperm cells would reach the ova in a sperm competition context. On whether sperm shape and dimensions reflect defects in the structure and integrity of the sperm chromatin, Sailer *et al*. (1996) reported that variations in sperm head morphometry were related to abnormal chromatin structure in the bull. However, many researchers have not found these relationships in other species.[103,138,139]

There are few intraspecific studies on the role of sperm morphometry and reproductive performance, and they have provided contradictory results.[23,24,94,100,112] For this reason, it is advisable to settle these questions using more sophisticated techniques that define which sperm morphometric parameters are crucial for breeding success.

PERSPECTIVES ON THE FUTURE OF SPERM MORPHOMETRIC STUDIES

Normal sperm morphology is a major criterion in sperm quality evaluation. However, although there is evidence of relationships between sperm morphometric characteristics and fertility, results vary widely and are sometimes contradictory. Future studies should

aim at understanding the causes of sperm design diversity and the mechanisms that generate them, with emphasis on intraspecific variability. In addition, more attention should be focused on other sperm structures besides the head. The implementation of scientific and technological advances could benefit the simultaneous examination of sperm phenotype and sperm function. Such technology could combine high-resolution optics and image analysis programs to increase the accuracy and precision of laboratory tests, and these should go hand-in-hand with the advance in new statistical methods that allow the analysis of large amounts of data given for these novel technologies.

Statistical approaches

Since the importance of sperm flagellar dimensions on sperm function has been demonstrated,[98,109] CASA-Morph systems should be modified to incorporate new tools that allow an automatic and accurate evaluation of these sperm structures. These advances will allow the undertaking of basic studies of sperm morphometry since most of the research done so far has focused only on the sperm head. Therefore, future studies of sperm morphometry should not overlook the role of the flagellum as a modulator of sperm function.

Regarding the accuracy of the evaluation of the sperm shape, CASA-Morph systems only provide an approximation of head shape on the basis of sperm head linear dimensions.[26,55,73,92] Elliptic Fourier analysis is one method, based on the use of successive points located by a coordinate system that fits the cell perimeter to a Fourier function, and that can identify more features of morphological variation in spermatozoa than manual methods.[140,141] However, it has not been used by many researchers. Moreover, geometric morphometrics has been developed to avoid the shortcomings of CASA-Morph morphometric parameters and was recently used by Varea Sánchez *et al.*[142] to evaluate sperm head morphometry in rodents. These authors obtained a better characterization of sperm shape, finding some regions in the sperm head that were not characterized by the linear descriptors, and which were nevertheless susceptible to change. Furthermore, geometric morphometrics should allow the assessment of size and shape separately, and the exact definition of where the main shape differences are located in the sperm head. Nevertheless, that study dealt with different mouse species, whose sperm heads have convoluted shapes and vary noticeably between species. However, it is possible that in species with spermatozoa of more simple shape (ungulates, primates, and humans), classical CASA-Morph systems can provide enough information to define sperm head shape adequately.

Sperm preparation

Other new methods have been developed to evaluate sperm morphometry without the need for sperm staining.[143] These methods are focused on humans and allow the use of the observed spermatozoon to be used in assisted reproductive techniques such as ICSI after the measurement. However, since chromatin and DNA integrity are not always related to sperm head size or shape,[103,138,139] further studies should be done to find phenotypic sperm parameters providing accurate information about sperm function, allowing the selection of spermatozoa which will generate healthy offspring. Along this line, the study of proteins associated with sperm head shape[144] would offer an important new tool to deepen knowledge of sperm shape and sperm function.

The combination of flow cytometry with single quantitative image analysis will provide new and interesting capabilities. This type of analysis will couple the collection of high-throughput data with streamlined image analysis. Sperm features such as size and shape, granularity, intensity, radial distribution, and texture could be obtained[145] in a large sperm population. In addition, the main advantage of this technique, which makes it unique, is the ability simultaneously to evaluate the morphometric and physiological parameters in the same sperm cell.

AUTHOR CONTRIBUTIONS

AMM, OGA, MR, FMP, MRFS, AJS, and JJG conceived the study, wrote the manuscript, and reviewed drafts of the manuscript. All authors read and approved the final version of the manuscript, and agree with the order of presentation of the authors.

COMPETING INTERESTS

The authors declared no competing interest.

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