Animal Reproduction Science xxx (xxxx) xxx

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

What is the importance of sperm subpopulations?

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ARTICLE INFO

Keywords:
Sperm subpopulations
Spermatogenesis
Sperm motility
Sperm morphometry
CASA
Post-meiotic transcription

ABSTRACT

The study of sperm subpopulations spans three decades. The origin, meaning, and practical significance, however, are less clear. Current technology for assessing sperm morphology (CASA-Morph) and motility (CASA-Mot) has enabled the accurate evaluation of these features, and there are many options for data classification. Subpopulations could occur as a result of the stage of development of each spermatozoon in the subpopulation. Spermatogenesis might contribute to the production of these subpopulations. Insights from evolutionary biology and recent molecular research are indicative of the diversity among male gametes that could occur from unequal sharing of transcripts and other elements through cytoplasmic bridges between spermatids. Sperm cohorts exiting the gonads would contain different RNA and protein contents, affecting the spermatozoon physiology and associations with the surrounding environmental milieu. Subsequently, these differences could affect how spermatozoa interact with the environmental milieu (maturation, mixing with seminal plasma, and interacting with the environmental milieu, or female genital tract and female gamete). The emergence of sperm subpopulations as an outcome of evolution, related to the reproductive strategies of the species, genital tract structures, and copulatory and fertilization processes. This kind of approach in determining the importance of sperm subpopulations in fertilization capacity should have a practical impact for conducting reproductive technologies, inspiring and enabling new ways for the more efficient use of spermatozoa in the medical, animal breeding, and conservation fields. This manuscript is a contribution to the Special Issue in memory of Dr. Duane Garner.

1. Introduction

The consideration that ejaculated semen as a uniform, homogeneous population of cells is a thing of the past. Results from several key studies decades ago indicated there was great diversity in spermatozoa within a semen sample (Katz et al., 1979; Robertson et al., 1988; Davis et al., 1991). These authors reported that this heterogeneity was not solely due to the presence of malformed or defective spermatozoa (e.g., abnormal forms, asthenozoospermic or dead) but to differences between otherwise normal spermatozoa. While these first studies focused on sperm motility and morphological classifications, results from subsequent research indicated this diversity results from differences in physiological and molecular mechanisms. For example, sperm subpopulations could be defined according to energetics (substrate use, mitochondrial activity, or ATP content) (Nesci et al., 2020), capacitation-related changes (Kumaresan et al., 2014), membrane composition (Beer-Ljubić et al., 2012), and compartmentalization (e.g., channels and receptors

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https://doi.org/10.1016/j.anireprosci.2021.106844
Received 16 June 2021; Received in revised form 30 August 2021; Accepted 31 August 2021
Available online 11 September 2021

distribution) (González-Arto et al., 2016; Prieto-Martínez et al., 2017). Furthermore, results from other studies indicated spermatozoa could vary in protein and RNA content (Boerke et al., 2007; Hosken and Hodgson, 2014). Sperm subpopulations, therefore, can be defined by molecular and cytometric techniques, and the study of sperm chromatin allows for there to be differentiating of germ cells based on differential protamination, DNA compactness, or fragmentation (Gosálvez et al., 2014; García-Molina et al., 2020).

A primary aim of this review is to explore sperm subpopulations as an emerging property based on cellular and molecular intrinsic variability. In this regard, whereas the words "semen" or "ejaculate" will frequently appear in the text, the utilization of terms such as "sperm sample" or "sperm set" would be more appropriate because sperm subpopulations could be defined in testicular spermatozoa or the oviductal reservoir, where utilization of the terms semen or ejaculate would make little sense. Furthermore, sperm subpopulations have been defined in different samples such as epididymal (Esteso et al., 2009) or cryopreserved (Peña et al., 2012). This subpopulation-type seems to re-emerge, even after imposing methods that apparently homogenize the sperm population (Macías García et al., 2009).

This review is initiated by revisiting how sperm subpopulations are analyzed using image analysis and statistical techniques, but this is not the primary focus of this review. There are many comprehensive papers on the analysis of sperm subpopulations, and the reader is invited to evaluate the contents of these manuscripts for technical details on this topic (Martínez-Pastor et al., 2011; Amann and Waberski, 2014; Yániz et al., 2015b; Ramón and Martínez-Pastor, 2018; Valverde et al., 2019). Instead, the aim of this review is to provide important information regarding the origins of sperm subpopulations and putative importance in the ejaculate. If subpopulations are a natural feature of a given sperm set, the expectation might be that evolutionary forces have shaped reproductive mechanisms to produce gametes that result in enhanced reproductive efficiency. Some of this information is relatively recent or there has been minor integration in the field of animal science, although there are some interesting comparative reports aimed at this essential interdisciplinary view (Lüpold and Pitnick, 2018; Evans et al., 2019; Roldan, 2019, 2020).

If the ejaculate is heterogeneous, this heterogeneity must have important effects on the practical use of spermatozoa in artificial reproductive techniques, either in human medicine as well as in livestock breeding. In this context, the research on sperm subpopulations would have economic and social importance with the potential for enhancing assisted reproduction and animal production (Ibanescu et al., 2020; Barquero et al., 2021; Terán et al., 2021; Valverde et al., 2021). It is important to consider that the study of sperm subpopulations is still limited. Characterization, assessment of significance, and practical application of sperm subpopulations can and must be improved.

2. Identification of sperm subpopulations from motility and morphometry data is an ongoing endeavor

The subjective evaluation of a sperm sample suggests the presence of cells with different characteristics and functionality, from subtle shape variation to differences in the time frame for activation and motility pattern, such as in fish. Image analysis has enabled an increasingly more rapid and accurate assessment of sperm morphology and motility. These systems were once termed CASMA (Computer-Assisted Sperm Morphology Analysis) and CASA (Computer-Assisted Sperm Analysis), but CASA-Morph and CASA-Mot are currently preferred terms (Santolaria et al., 2016). Once these automated systems allowed detailed analysis of hundreds or thousands of spermatozoa per sample, the study of sperm subpopulations resulted for managing the vast amount of data and as a need for precisely evaluating previous unrealized aspects of these data.

There is a fascinating evolution of the CASA, from self-made systems to commercial and open-software-based options (Wilson-Leedy and Ingermann, 2007; Purchase and Earle, 2012; Giaretta et al., 2017; Alquézar-Baeta et al., 2019). With the current hardware, there has been an overcoming of the technical limitations of the earlier-developed systems, both in terms of image definition and capture speed (for motility analysis). In addition, the efforts of dozens of researchers have resulted in the refinement of staining, image quality, protocols, and data reliability. In the last few years, new approaches have allowed for expandeding the capabilities of this kind of sperm analysis, such as the combination of fluorescence techniques that have resulted in more accurate morphometry or motility assessment (Yániz et al., 2016, 2018) or stain-free morphometry analysis (Soler et al., 2016).

Sperm motility and morphometry data from CASA were first evaluated using simple statistics such as the mean of the different variables. It was soon evident that this simple approach did not take advantage of the amount of data available, and some of the relevant information could not be evaluated when using these types of analyses (Ramón et al., 2014). The size of databases, however, is too large for simple approaches, both in the number of observations and variables, resulting in matrices of thousands and even millions of cells. The need for obtaining manageable results provided the impetus for the development of multivariate analysis, mainly descriptive approaches such as PCA (principal component analyses) or grouping such as data clustering. Interestingly, some patterns emerged despite there being a large amount of heterogeneity between CASA settings, experimental protocols, and statistical approaches. For example, with research on sperm motility subpopulations there is usually reporting of three to four subpopulations, typically differing in sperm speed and linearity, often including "weak" and "vigorous" subpopulations (Martínez-Pastor et al., 2011). Clustering of morphometry data generally focused on the sperm head, and in most studies there were a similar number of populations, defined by head size and with some subpopulations being separated by the head ellipticity. Nonetheless, a relevant line of research focuses on evolutionary biology and between-species comparisons, with a different scope and richer data variability. Variables when conducting these studies include not only head morphometry but also the dimensions of the midpiece or principal piece of the flagellum (Varea-Sánchez et al., 2014).

In most studies, there has been use of unsupervised classification algorithms (Martínez-Pastor et al., 2011; Ramón and Martínez-Pastor, 2018). That is, the algorithms adjust to the patterns present in the raw data. Whereas this approach helps discover such patterns, findings from these studies are affected by the variability between experiments and there is considerable difficulty when attempting to compare and standardize protocols. This is one reason why, even though there has been a considerable number of studies

in which there was evaluation of sperm subpopulations, sperm subpopulation analyses have not been applied in semen evaluations of commercial AI enterprises. Another problem is that the initial expectations with sperm subpopulation development were that there would a close association between a specific subpopulation and sperm fertility or other economic/medical endpoints but these outcomes have not eventuated. Nevertheless, there are some promising findings (Yániz et al., 2015a; Dorado et al., 2017; Nagata et al., 2018), although results from other reports indicate a lack of such a relationship (Santolaria et al., 2015), and some have proposed that utilization of findings from subpopulation analysis does not improve the predictive value of the classical sperm variables (Ibanescu et al., 2020).

Whereas unsupervised classification is a top-down approach (from data to reality), with supervised classification algorithms, there is use of a bottom-up approach (using real-life parameters to define groups in the data). More commonly termed "machine learning algorithms," these methods require prior information (Ramón and Martínez-Pastor, 2018). Whereas this could be considered a disadvantage, adding complexity to the process compared with the straightforward unsupervised analysis, it makes sense to proceed in this manner. A training dataset with accompanying information (e.g., fertility ratios, freezability, or other relevant data) allows for obtaining a prediction model or "learner" (Ramón et al., 2013). If this learner is a effective model for this purpose, it could be used to sort spermatozoa according to morphology or motility with great efficiency and obtain subpopulations related to the variable of interest. These desirable features are consistent with the methodology termed support vector machines (SVM), which seems to be the most promising for conducting these procedures and these approaches have been used in several studies (Goodson et al., 2011; Ramón et al., 2012; Mirsky et al., 2017). Other similar methods, however, are available (Sahoo and Kumar, 2014; Chang et al., 2017). Goodson et al. (2011, 2017) made available a learner for classifying human or mouse spermatozoa according to a prior classification of spermatozoa movement (https://csbio.unc.edu/CASAnova/index.py).

Subpopulation analysis based on supervised methods bears promise for more consistent and applied results. Primary data are available elsewhere for developing efficient learners in very different fields. These methods, therefore, have been successfully applied for relating kinetics or morphological data to sperm cryopreservation capacity (Ramón et al., 2012), fertility (Ramón et al., 2013), or toxicant contaminations of semen (Vieira et al., 2019).

3. Subpopulations as an "emerging property": heterogeneity of spermatozoa and some hypotheses for sperm subpopulation development and purpose

Subpopulations of sperm are now realized to be an intrinsic feature of any sperm population. It, therefore, is proposed that these sperm subpopulations result from integrating many factors during the formation and period when spermatozoa are viable in the gonads as well as male and female reproductive tract. This is a more complex problem when there is consideration of some of these factors that are deterministic (e.g., haploid complement), but many of these factors could be stochastic (e.g., mixing with seminal plasma fractions), and the consequence of this occurrence is not clear. For example, the allelic complement resulting from meiosis could partly define the fate of a spermatid, but the chromosomal fragments are randomly distributed between the daughter spermatids.

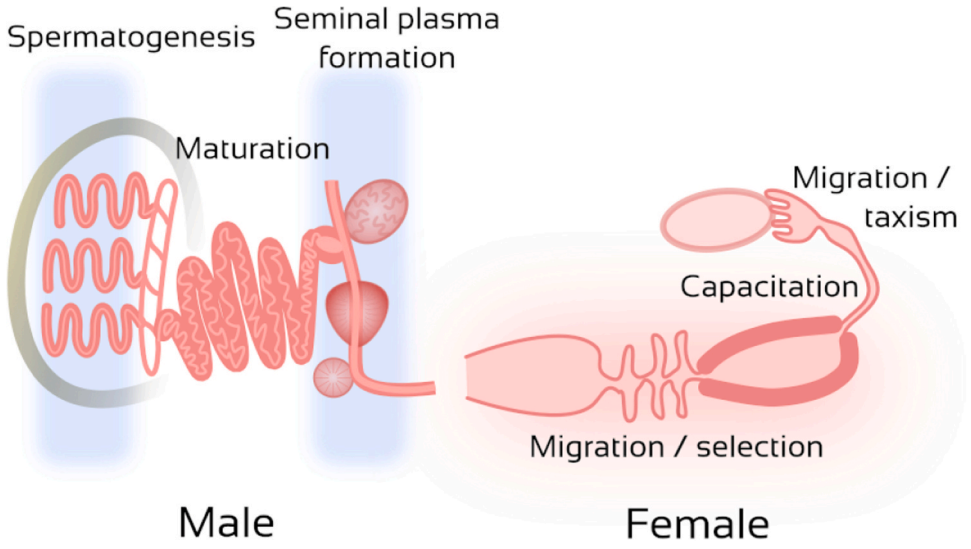

Fig. 1. A schematic and incomplete relation of the spatiotemporal processes affecting spermatozoa when in different physiological milieus (mammalian male/female genital tracts) that could contribute to the heterogeneity resulting in the formation of sperm subpopulations. Blue-shaded areas (spermatidogenesis/spermiogenesis and ejaculate formation, with seminal plasmamixing) are the most likely sources for spermatozoa phenotypical diversification, conditioning the subpopulation structure. Additional processes (maturation, selection, and capacitation) would lead to modification of primordial subpopulations, and therefore different subpopulation patterns mightarise at various times and locations in the testicles and male and female reproductive tracts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Sperm subpopulations could be considered as an emergent property of the sperm set. Emergent properties result from the synergic composition of many factors and hierarchies of spatial and temporal factors (Mast et al., 2014). The study of sperm subpopulations including the importance and origin of these subpopulations are part of the field of systems biology, and are an emerging property of biological organisms still too complex to be entirely understood. Many factors could cause the emergence of sperm subpopulations resulting in greater heterogeneity or homogeneity between spermatozoa populations (Fig. 1). Research in evolutionary biology has been integral in understanding the forces and processes resulting in sperm heterogeneity in the different animal groups (and indeed in other kingdoms (Till-Bottraud et al., 2005)), and why there is this heterogeneity and associations with evolutionary pressure and reproductive strategies (Tourmente et al., 2016; Parker, 2020). The biology of each species and especially the predominance of pre- or post-copulatory competition greatly influences how spermatozoa are produced and transported to the site of ovum fertilization in the oviduct (energy investment, spermatogenic efficiency, development of alternative morphology) (Dixson and Anderson, 2004; Montoto et al., 2012; Lüpold et al., 2020). The presence of sperm subpopulations could be a consequence of the evolutionary history of the species (or at least, a side effect of evolution-affected processes). This area of study constitutes an exciting turn of events for spermatology in other fields, including human medicine and animal science. This is not a novel proposal (Holt and Van Look, 2004; Roldan, 2019), but it has received little recognition in general reproductive research. Recent developments could stimulate the interdisciplinary study in this field of research.

Even though there is a multiplicity of factors, the most indisputable aspect of heterogeneity among spermatozoa is the genetic variability resulting from meiosis and how this occurs as a result of post-meiotic processes (spermiogenesis) (Fig. 2). This could result

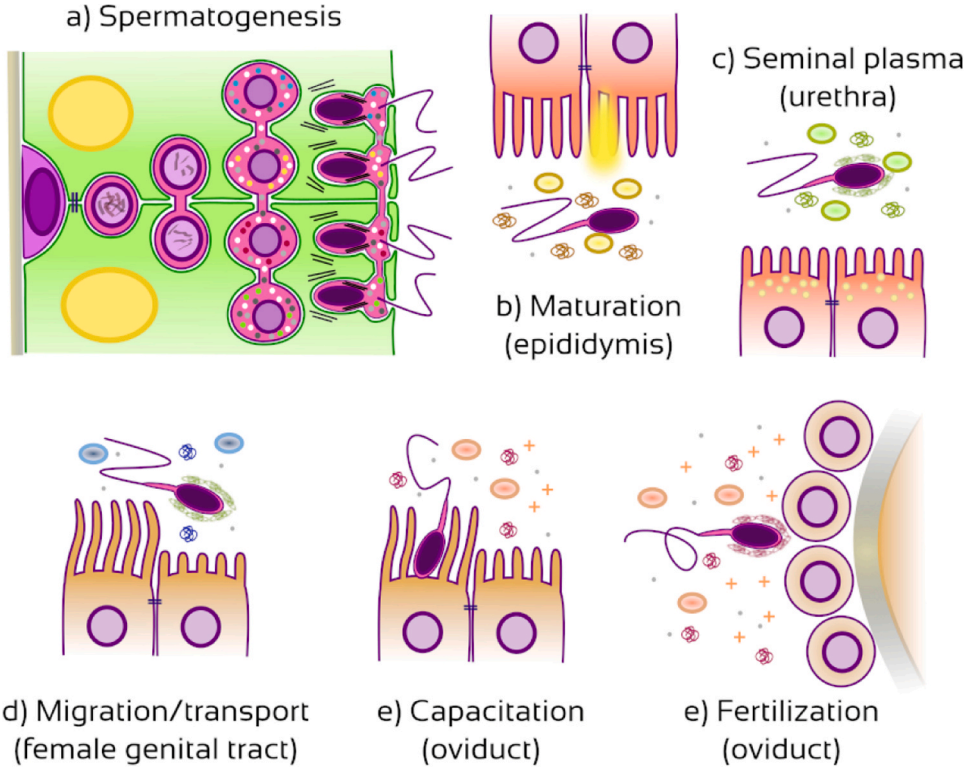

Fig. 2. Sources of the RNA and protein contents of spermatozoa, and possible reasons for spermatozoa homogeneity "within-subpopulations" and heterogeneity "between-subpopulations." (a) Homogenization and heterogenization of transcripts and maybe other elements during late stages of spermatogenesis; after meiosis, transcripts and other factors (colored dots) cross cytoplasmic bridges and homogenize between sister spermatids (blue-grayish dots), therefore, negating haploid diversity; however, others might be retained by the producing spermatid (blue-yellow-red-green dots), resulting in a different RNA and protein contents (Bhutani et al., 2021; Sutter and Immler, 2020) and possibly reflecting in features such as plasmalemma composition, metabolism, and energetics, and sperm morphology (dark lines in Sertoli cells and late spermatids representing cytoskeleton and related structures involved in sperm shaping (Teves and Roldán, 2021). These subpopulations might interact differently with the male reproductive tract secretions (extracellular vesicles as ovoid objects, proteins as coiled threads, and other elements as dots) such as (b) epididymal products (notably, epididymosomes), and (c) products of different glands forming the seminal plasma. The emission of the semen in different fractions could promote the formation of new sperm subpopulations with different capacities and functions. Similarly, sperm subpopulations might then also interact differently with the various secretions and environments in the female tract: (d) transport and selection (cervix and uterus); attachment to the oviductal lining and formation of subpopulation-wise capacitation waves (chemotactic molecules as plus signs); (e) fertilization. This schematic is based on processes in mammals. Some stages, however, are analogous to what occurs in external fertilizing species (sperm activation upon release into the external environment, interaction with the egg micropile in fish, etc.). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in different sperm cohorts and possibly be important in haploid selection (Immler, 2019). Furthermore, the physiological processes in the spermatogenic epithelium could affect how slightly different spermatozoa interact with subsequent environments (epididymis, seminal plasma, female reproductive fluids, or epithelia). For example, modifications of the plasmalemma in the developing spermatid, including the lipidic composition or the presence or distribution of membrane proteins, could alter the capacity for receiving material from epididymosomes or how seminal plasma proteins coat the spermatozoon, processes that are very important in sperm biology (Foot and Kumar, 2021; Rodriguez-Martinez et al., 2021). Due to heterogeneity emerging after completion of the late stages of spermatogenesis, changes in these processes might modulate (enhance or decrease) between-sperm differences.

This hypothesis has recently received crucial experimental support after there was discovery that there are processes that promote germ cell diversity during spermiogenesis (Gòdia et al., 2020; Sutter and Immler, 2020; Bhutani et al., 2021). Meiosis results in production of spermatids with a different haploid genotype, potentially resulting in different sperm phenotypes (thus there is development of sperm subpopulations). This simple idea, which would be consistent with many observations on sexual selection and sperm competition, however, has been repeatedly disputed (Sutter and Immler, 2020). Spermatids were supposedly transcriptionally silent, with there only being a specific subset of mRNA transcripts for histone to protamine transition, and at the same time, the characteristics of the condensed nucleus would prevent the production of a specific RNA transcripts. Furthermore, there is an active transport system between cytoplasmic bridges of developing spermatids that provide for an association that results in sharing of RNA to organelles (Ventelä et al., 2003), helping to synchronize development and apparently homogenizing contents and thus reducing the variability due to meiotic redistribution of the haploid genome. Evidence for post-meiotic rich and active transcription (Soumillon et al., 2013) and unequal distribution of part of the transcripts through the cytoplasmic bridges (Bhutani et al., 2021), however, has emerged. This might result in phenotypic variability between these developing germ cells and may be the primary cause of natural sperm subpopulation emergence (Fig. 2a). An early proof and very extreme example of the consequences of the unequal distribution of transcripts among linked spermatids and the generation of within-ejaculate diversity is the t-phenotype in mice (Véron et al., 2009). In this case, spermatids contain a "toxic" element (deregulation of signaling cascades involved in motility control by genetic *t complex distorters*, *Tcd*), and only those spermatids containing the *t* element in the haploid genome (*Smok1*^{Tcr} allele, namely *Tcr*, *t complex responder*) result in normal spermatozoa. Briefly, *Tcd* expresses pre-meiotically, and therefore, all spermatids contain these toxic elements, whereas the *Tcr* is transcribed post-meiotically, but the *Tcr* mRNA is not equally distributed to sister spermatids (+), being sequestered in *t* spermatids. This results in an extreme case of subpopulation emergence, with + spermatozoa being motility defective, resulting in a case of non-Mendelian inheritance (transmission ratio distortion, TRD) of the *Tcr*. Bhutani et al. (2021) proposed that this case of unequal distribution between sister spermatids would be widespread regarding both transcripts and species. In some recent reviews, there is further discussion of the relevance of these molecular processes (Sutter and Immler, 2020; Teves and Roldán, 2021) and there is greater consideration of spermatozoa homogenization or diversification mechanisms during spermatogenesis. The TRD phenotype has also been reported in domestic species (Casellas et al., 2014; Id-Lahoucine et al., 2019). Results from a recent study indicated there was an association of TRD in pigs to post-meiotic stages of spermatogenesis, potentially affecting sperm function (Gòdia et al., 2020). A fascinating hypothesis is the possible formation of detectable sperm subpopulations with different capacities, resulting in TRD.

Interestingly, there may be greater differences among spermatozoa resulting from sister spermatids than spermatozoa from other spermatid sets. There, therefore, may be occurrences where sperm subpopulations might contain clusters of spermatozoa from different spermatogonia, with spermatozoa from sister spermatids (and, therefore, different features) distributed in different sets. This is a daring hypothesis that could be tested by RNA analysis of the different sperm subpopulations. Current technologies could be used for conducting this type of study, combining sperm separation and molecular analysis techniques (Nagata et al., 2018; Alvarez-Rodriguez et al., 2020; Zhu et al., 2021).

These differences in the post-meiotic transcriptional and translational activities would result in differences in the signaling, energetics, head shape, and axonemal architecture, contributing to the emergence not only of sperm subpopulations differing in motility (Ramón and Martínez-Pastor, 2018; Roldan, 2020) but also subpopulations defined by different susceptibility to undergoing capacitation (Holt and Van Look, 2004; Luque et al., 2018), response to external stimuli (Gimeno-Martos et al., 2019), and even viability resilience to assisted reproductive techniques (ART; Ortega-Ferrusola et al., 2017).

It is probable that the differences in the RNA and protein content between spermatids also affect the intricate mechanisms giving rise to sperm morphology during late spermiogenesis, including coordination with Sertoli cell functions, and thus to morphology-defined subpopulations. Subtle modifications in the histone to protamine replacement, nuclear, acrosomal, and flagellar shaping, and communication with the Sertoli cells would translate into a different size or shape of the developing cell (Teves and Roldán, 2021). The field of evolutionary biology has extensively considered the topic of sperm morphology, with there being many examples of variation between and within species that are modulated by reproductive strategies and selective environmental pressures (van der Horst and Maree, 2014; Roldan, 2019; Fitzpatrick, 2020). The emergence of these sperm subpopulations (as mentioned for other features), therefore, is possibly a by-product of the evolutionary history of the species. This would have resulted in an abundance of heterogeneity (including clearly abnormal forms) in species where there is little or no sperm competition (van der Horst and Maree, 2014).

Nevertheless, while this review focuses on the unequal sperm content hypothesis as a significant factor for the generation of the sperm subpopulations, there are many other factors contributing to these subpopulations. From photoperiodic cues to intraspecific competition, environmental forces affecting spermatogenesis could modulate resulting sperm subpopulations (Malo et al., 2005; Flowers, 2015; delBarco-Trillo et al., 2016). Furthermore, past effects on previous generations can have effects, via epigenetic modifications of the inherited DNA or histones (modulating gene expression in the embryo). Spermatogenesis is affected by these epigenetic factors (Ben Maamar et al., 2020; Marcho et al., 2020), adding complexity to the study of the testicular emergence of sperm

subpopulations.

A second factor relevant in the emergence of sperm subpopulations could be the seminal plasma (Fig. 2c) or, more specifically, the fractions of this fluid originating from the different glands in the male reproductive tract (Rodriguez-Martinez et al., 2021). This could be of minor relevance in species emitting the ejaculate in a single fraction (e.g., ruminants). In this case, most spermatozoa (from the testicles, sperm ducts, or epididymides, depending on the taxon) would be simultaneously mixed with the seminal plasma (or analogous fluid) and emitted. Spermatozoa, however, would be affected differently in species emitting a fractionated ejaculate (García et al., 2009; Rodríguez-Martínez et al., 2009), effectively modifying spermatozoa physiology, motility, and the capacity for traversing the female reproductive tract and capacitation. If the sperm fractions, therefore, are allowed to mix before evaluation, it could be expected that there would be a modified subpopulation structure which is quite different from that derived from spermatogenesis.

Sperm subpopulations are subsequently modified by the milieu of the female reproductive tract (both in external and internal fertilization), the interaction with the female reproductive tract (including attachment to the oviductal lining and analogous structures in the case of internal fertilization), and, of course, the application of assisted reproductive techniques (especially those related to sperm storage and cryostorage). Because the aim of this review is to reflect on the ultimate origin of the sperm subpopulations (which would condition the subpopulation structures subsequently emerging from these processes), the reader is encouraged to evaluate the rich literature base related to this fascinating topic (Santiago-Moreno et al., 2016; Yeste, 2016; Amann et al., 2018; Roldan, 2020; Saint-Dizier et al., 2020; Barquero et al., 2021).

4. Current shortcomings of subpopulation analysis and future perspectives

It is time that those conducting studies of the sperm subpopulations take advantage of new developments in sperm analysis. These new technologies can be used for evaluation of spermatozoa functions in a complex environment while spermatozoa are continuously being affected by selective forces, could transcend current approaches for analyzing sperm motility and morphology (CASA-Mot and CASA-Morph). The use of some of these alternative approaches might result in more useful data being collected, such as those where there is evaluation of the combination of physiological measurements with motility assessment (Yániz et al., 2017), or geometric morphometrics to study sperm morphology (Tourmente et al., 2016). Furthermore, other improvements could enhance the study of these populations. Whereas typical studies consider hundreds of spermatozoa per sample, increasing the sample size would lead to enhancement of the reliability of the data collected in these studies. These approaches are now realistic with the more advanced hardware and software that is available, allowing for assessment of the motility and morphology, combined with other measurements, and for processing these very large datasets.

Nevertheless, the classical approach based on 2-D measurements of motility or morphology with conventional light, phase-contrast, or fluorescent microscopy enables obtaining only an incomplete understanding of these features. Spermatozoa have functions in a 3-D environment, when from species which are both external and internal fertilizers with these spermatozoa interacting with surfaces (being these the egg's surface and micropile or the epithelial lining of the female reproductive tract) (Elgeti et al., 2015; Ishimoto et al., 2016). Also, the female reproductive tract of internal fertilizing species has developed with there being diverse and intricate structures that spermatozoa must navigate or interact with in many ways (Huang et al., 2020; Suarez, 2016). There are promising technologies that could fully acquire data associated with sperm swimming patterns which would result in obtaining a more accurate 3-D description of spermatozoa morphometry (e.g., holographic techniques) (Daloglu and Ozcan, 2017; Nygate et al., 2020). Combined with the design of customized measurement chambers for simulating different environments (Huang et al., 2020), this technology could enable a more realistic study of sperm subpopulations, both based on motility and morphometry data.

Additionally, current advances allow for determining functional features of spermatozoa and morphological or kinematic characteristics of the same cell. Techniques such as MSOME (motile sperm organelle morphology examination) have been used mainly for human ART (Setti et al., 2013), enabling evaluation of different variables in real-time. Currently, more robust strategies are in development, combining specific labeling with morphology or motility analysis. While requiring a fluorescence microscope with a suitable camera, these techniques allow for the capacity to evaluate sperm viability or organelle integrity or functionality to the subpopulation analysis (Yániz et al., 2018). Combining this strategy with high-throughput techniques such as image cytometry (Roldan, 2020) might allow for simultaneous analysis of sperm morphology together with evaluation of a wide range of functional markers. Whereas the image resolution might not equal that of a conventional microscope, improvements could soon be available.

These new developments could be combined with sperm separation techniques and chemo/thermotaxis, fields that have seen significant advances in the last years (Perez-Cerezales et al., 2015; Li et al., 2016; Chinnasamy et al., 2018; Rodríguez-Gil, 2019). Connecting with our hypothesis of a genetic basis for the emergence of sperm subpopulations (at least partially), the detailed analysis of sperm motility and morphology followed by the separation of the resulting subpopulations (e.g., by microfluidics) would allow for confirmation if spermatozoa have a different RNA/protein content and the significance of these differences.

Recognizing sperm subpopulations as the phenotypical expression of meiotic and post-meiotic processes in spermatogenesis should also contribute to a more biologically sensible and possibly applicative interpretation. Species with a large smount of post-copulatory sperm competition seem to have more homogeneity in the sperm population of the ejaculate, maybe by repressing post-meiotic transcription and promoting between-spermatid interchanges of contents (Ramm et al., 2014). Equally compelling, a small extent of between-ejaculate competition would lead to haploid selection, resulting in a larger intra-ejaculate variability of spermatozoa (Ezawa and Innan, 2013; Ramm et al., 2014). The study of sperm subpopulations coupled with molecular analyses could shed light on some interesting evolutive biology problems, and at the same time, result in solutions to some problems in human andrology and domestic animal breeding. Regarding the latter, there are some species (mainly cattle and pigs) where males are also intensively selected for semen quality or storage resilience. In other cases, this selection might exist, but it is less intense (sheep), or selection for

productive features could inadvertently result in lesser sperm quality. How human intervention has affected the sperm subpopulations in these cases is a relevant problem, and there could be benefits from information about sperm competition of non-domesticated species (Roldan, 2019; Firman, 2020; Kustra and Alonzo, 2020). Subsequent research could result in the development of ART in animal breeding. In this regard, considering subpopulations and the phenotypical diversity of spermatozoa from different points of view could improve the understanding of male fertility and facilitate improvements of current technologies (Roldan, 2019).

5. Conclusions

There are, without question, different sperm subpopulations, and the study of these subpopulations is a promising and rapidly advancing field. The lack of consistent findings when studies have been conducted, mainly the relationship of these subpopulations with values for fertility variables provides an impetus for developing new approaches for evaluations of these relationships. In this review there was consideration of novel research dealing with spermatogenesis and evolutionary biology for evaluation of physiological processes resulting in heterogeneity in the spermatid population ultimately resulting in these sperm subpopulations that might have developed as a result of selection forces applied to the species.

Adequate research techniques are already available for testing this hypothesis, maybe resulting in practical applications. Furthermore, the development of devices realistically simulating the different environmental milieus might allow for the accurate evaluations of sperm subpopulations in relevant situations, improving the predictive value of these analyses for sperm performance and for enhancing fertility when these approaches are utilized with the different ART.

Author contributions

Felipe Martínez-Pastor was the sole author of this manuscript.

Competing interests

None declared.

Acknowledgments

To the memory of Dr. Duane L. Garner. This article is also dedicated to Dr. S. Pérez and A. Bisbal, promising young souls taken too early. The author thanks Dr. Manuel Ramón and Dr. Aurelio Malo for their insightful inspiration on this topic.

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