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Semen evaluation techniques and their relationship with fertility

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Abstract

This review summarizes those methods- established and emerging- of semen assessment whose outcome intents revealing its potential fertility and, as a carry-over concept, that of the sire whose semen we examined. The review does not, however, focus on the wide display of current techniques designed to explore specific or multiple sets of sperm attributes essential for fertilization but on two basic concerns present: the *heterogeneity* of the sperm suspension and the *multitude of attributes required* for each spermatozoon to be fertile; concepts that shadow our diagnostic capabilities. The review points out advancements in the exploration of the genome, the transcriptome, and the proteome of both spermatozoa and the seminal plasma which unveil how spermatozoa modulate their own survival and signal to the environment when displaying degenerative changes. Specific seminal plasma components, both among individuals and portions of the ejaculate, not only relate to survival but also signal differential immune tolerance by the female with a previously unattended linkage to fertility. Lastly it foresees how Cytomics, combining novel designed motility analyzers, flow cytometers and enhanced digital imaging shall dominate the landscape of andrological laboratories and enable quick determinations on huge sperm numbers for markers highly relevant to sperm function and hence, for fertility.

Keywords: comparative sperm evaluation, fertility estimation, *in vitro* methods, semen analysis, sperm quality.

Introduction

Over the past decade, we have experienced an explosive development of *in vitro* assays to determine sperm intactness and measurement of sperm function that helped andrological diagnosis and the optimization of semen processing methods, as summarized in multiple reviews (Rodriguez-Martinez and Larsson, 1998; Graham, 2001; Katila, 2001; Rodriguez-Martinez, 2003, 2006, 2007b; Parkinson, 2004; Graham and Moce, 2005; Guillan *et al*., 2005; Rijsselaere *et al*., 2005; Petrunkina *et al*., 2007; Rodriguez-Martinez and Barth, 2007; Moce and Graham, 2008). However, conventional semen evaluation is still often restricted to determinations of sperm numbers, sperm motility and sometimes, but rather sparsely, sperm morphology. The main reason behind this restriction is the fundamental axiom that an ejaculate must contain above a certain number of motile, morphologically 'normal' spermatozoa to achieve minimum sperm numbers reaching the oviducts for eventual participation in the complex process of fertilization, finally leading to the safe development of the embryo(s) (Rodriguez-Martinez *et al*., 2005; Rodriguez-Martinez, 2007a; Holt, 2011).

More and more methods are now available for semen evaluation that not only make it possible to disclose the level of 'normality' of the male genital organs but also the capability of spermatozoa (mostly related to their membranes but also their metabolomics) to interact with the surrounding fluids (seminal plasma [SP], female genital fluids, and *in vitro* culture media), cells (epithelia, cumulus cells, oocytes), or extracellular material (hyaluronan coating, the zona pellucida [ZP]) before fertilization. Methods are also available to disclose the status of the different organelles, the intactness of the nuclear genome and of the available transcriptome; all related to the capability to initiate early embryo development. Although some of these methods, particularly those of an '*omic*' nature, are yet restricted to the research bench, the accompanying development of relevant instruments, from Computer Assisted Sperm Analysers for motility or morphology (CASA respectively ASMA) to bench-model flow cytometers (FCs), are making assays accessible for clinical diagnostics and for semen processing for assisted reproduction. Yet, many of these methods are only of limited value for prediction of fertility (Rodriguez-Martinez, 2003). This review aims to critically review advances in the methodology to assess semen and the capacity different assays have to prognose fertility. Particular attention is paid to new methods to determine DNA and transcript intactness; also to those biomimetic *in vitro* assays that, by resembling events during sperm transport, storage, and interaction with the female genital tract and the oocyte, best provide clues for sperm selection and the role of sperm sub-populations in the ejaculate.

Conventional semen assessment and fertility

The currently used spermiogram of ejaculated spermatozoa focuses (besides the aspects of pH and volume of the ejaculate) solely on the number of

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spermatozoa (per unit of volume, i.e. concentration or as total per ejaculate) and its motility (including sometimes its kinematic patters, if a CASA instrument is used). Sperm numbers are a blunt variable in relation to fertility, and only when below possible 'threshold numbers' do we see a proven relation between sperm numbers and fertility (Tardif *et al*., 1999; Christensen *et al*., 2011).

The subjectively measured sperm motility has been statistically related to fertility even for post-thawed semen in bulls (Rodriguez-Martinez, 2003), and in pigs (Cremades *et al*., 2005). Studies in other species yield erratic results, with large variation between laboratories, owing to operator bias and differences in numbers of breeding/female numbers used to determine fertility (Rodriguez-Martinez, 2006). Kinematic analyses using CASA have shown variable correlations between particular motility patterns, such as linearity, and field fertility (Bailey *et* al., 1994; Holt *et al*., 1997; Zhang *et al*., 1998; Hirai *et al*., 2001; Januskauskas *et al*., 2001; 2003; Broekhuijse *et al*., 2012). Combining motility patterns with other parameters of sperm function in AI dairy sires allowed, however, for fertility estimation (Januskauskas *et al*., 2001). Major constraints for conventional CASA instrumentation relate to the few spermatozoa analyzed/sample, the variability between users (Ehlers *et al*., 2011) which, combined with the high cost of the instrumentation, jeopardize their wider use (Feitsma *et al*., 2011). Alternative instrumentation is now available (Qualisperm™, Biophos, Switzerland) based on another principle than the classical digitalization of centroids over time. This novel technology is based on correlation analysis of single particles (spermatozoa) in confocal volume elements. Individual spermatozoa are projected on a pixel grid of a CMOS camera and the algorithm analyzes the number of fluctuations by correlation function instead of trajectories. This system benefits from a high throughput (usually 4 fields per minute), analyzing >2,000 spermatozoa/sample, and has been thoroughly tested for several species (Tejerina *et al*., 2008, 2009; Johannisson *et al*., 2009).

Most often, the proportion of morphologically normal spermatozoa in the ejaculate of a bull is related to its fertility post-AI (Phillips *et al*., 2004; Al-Makhzoomi *et al*., 2008; Nagy *et al*., 2013) reflecting, together with sperm numbers and sperm motility, the degree of normality of spermatogenesis and sperm maturation within a cohort of sires. Morphological abnormalities are always present in any ejaculate, but differ in their impact on fertility. Some are specific defects that hamper fertilization while others, such as the pear-shaped sperm head deviation, impair proper embryo development (Rodriguez-Martinez and Barth, 2007), thus calling in AI stud sires for frequent (2-month interval) detailed assessments of sperm morphology using wet and stained smears. The reliability of such analyses requires large numbers of spermatozoa accounted for per sample, i.e. 200 per wet smear and 500 for stained sperm heads. The latter allows for determinations of defects with clear relation to fertility for their uncompensable nature such as pyriform sperm head shape as an expression of a defective chromatin condensation during spermiogenesis (Al-Makhzoomi *et al*., 2008). Software for ASMA have been developed since the 1980´s, and have now reached an acceptable reliability for the analyses of sperm head dimensions, although they cannot yet determine sperm abnormalities of other nature (Auger, 2010); nor are there clear relationships with fertility (Peña *et al.,* 2005b; Saravia *et al*., 2007b; Gravance *et al*., 2009).

Sophisticated tests of specific sperm attributes and function, do they prognose fertility?

At specialized laboratories, biomarkers of sperm intactness of function proven relevant for fertilization are studied (Silva and Gadella, 2006), mostly to explore *in vitro* how relevant the interactions between the spermatozoa and the female genital tract, the oocyte vestments and the process of fertilization in itself are, including the early development of the embryo. Finally, the different outcomes are related to fertility (Rodriguez-Martinez, 2007b).

Integrity and stability of the plasma membrane is paramount, and methods vary, from microscopy in wet smears, the use of the membrane impermeable dye eosin (eosin-nigrosin test), exposure to a hypo-osmotic saline solution (HOS-test) to use of single or multiple fluorophores (reviewed by Rodriguez-Martinez and Barth, 2007). Either method has indicated significant correlations to fertility and can either use microscopy or flow cytometry (FC) can be used for screening (Kavak *et al*., 2003; Nagy *et al*., 2004; Saravia *et al*., 2007a, Martinez-Pastor *et al*., 2010; Hossain *et al*., 2011; Petrunkina and Harrison, 2011; Balao da Silva *et al*., 2013). Fluorophores are most advantageously used combined, for instance to determine subtle changes in permeability using SNARF-1, YO-PRO-1 and Ethidium homodimer, the so-called triple stain (Peña *et al*., 2005a, 2007), related to phospholipid scrambling (Merocyanine-540, YO-PRO-1 and Hoechst 33342) or phospholipid asymmetry (Annexin-V/PI; Januskauskas *et al*., 2003; Hallap *et al.,* 2006b; Peña *et al*., 2003, 2005a, 2007; Saravia *et al*., 2007a), all related to capacitation in several species and with a good correlation with fertility (Hossain *et al*., 2011). Sperm capacitation includes, moreover, an influx of Ca^{2+} to the sperm perinuclear and neck regions and flagellum, the generation of controlled amounts of ROS, as well as the phosphorylation of protein residues (Gadella and Van Gestel, 2004; Harrison and Gadella, 2005; O´Flaherty *et al*., 2006; Tulsiani *et al*., 2007; Fabrega *et al*., 2011), steps that can be measured *in vitro* and, eventually, associated with the fertility of the males. Mapping of

intracellular Ca^{2+} levels in spermatozoa and of Ca^{2+} displacement, for instance using the CTC-technique has helped discriminate fertility among bull sires (Thundathil *et al*., 1999; Gil *et al*., 2000). The accompanying hyperactivated motility has however, shown a low relationship with fertility (Zhang *et al*., 1998; Januskauskas *et al*., 2001; Rodriguez-Martinez *et al*., 2008).

Correlations between mitochondria status and fertility are variable, mostly owing to the changes in mitochondria functionality over time (Martinez-Pastor *et al*., 2004; Hallap *et al*., 2005b; Peña *et al*., 2009) and sperm handling (Macias Garcia *et al*., 2012). Besides energy, sperm mitochondria produce by-products of the metabolism of oxygen, including superoxide which converts into the damaging hydrogen peroxide, a Reactive Oxygen Species (ROS), which is mostly, but not completely, converted to oxygen and water by the enzymes catalase or superoxide dismutase (also known as antioxidants or scavengers). A certain level of ROS is essential for sperm function, including fertilizing capacity, but only when it is kept at optimal levels by the antioxidant capacity of the seminal plasma (Awda *et al*., 2009; Mancini *et al*., 2009; Am-in *et al*., 2011), via antioxidant enzymes such as paraoxonase-1 (PON-1, Verit *et al*., 2009) or the sperm-present PON-2 (Vicente-Carrillo *et al*., 2013, Linköping University, Sweden; unpublished). However, when excessive numbers of leukocytes are present in the ejaculate, or the semen is subjected to oxidative stress (as during cooling in the absence of SP or other natural scavengers), increased ROS generation, either extrinsic (leukocytes) or intrinsic (sperm neck cytoplasm in immature or morphologically abnormal mitochondria), causes a deterioration in sperm motility (Guthrie *et al*., 2008), sperm membrane integrity through peroxidation of its lipids (LPO) as well as DNA breakage and cross linking of the chromatin (Aitken and West, 1990; Koppers *et al*., 2008) all leading to fertility deterioration. ROS levels are therefore very variable, making their proper determination difficult, albeit yet possible using the probe hydro-ethidine or through measurement lipid peroxidation (LPO) levels in the membrane lipid bilayer by using the 5-iodoacetamidofluorescein probe family (BODIPY-C_{11}®; Guthrie and Welch, 2007; Aitken *et al*., 2007; Ortega-Ferrusola *et al*., 2009a).

Acrosome intactness, a pre-requisite for fertilization, can be readily examined *in vitro* using phase contrast microscopy (Rodriguez-Martinez *et al*., 1998) or be examined by fluorophore linked lectins by multi-parametric analysis (Nagy *et al*., 2003, 2004). Yet, correlation between acrosome status and fertility are variable (Rodriguez-Martinez, 2007b).

Spermatozoa from human, boars, and bulls contain the hyaluronan (HA) receptor CD44 in their plasma membrane (Huszar *et al*., 2003; Tienthai *et al*., 2003; Bergqvist *et al*., 2006, Vicente-Carrillo *et al*., 2013, Linköping University, Sweden; unpublished) and should thus bind to solid state HA depots (PICSI, Sperm Selection device, USA, Huszar *et al*., 2007), a technique to trap only mature spermatozoa that are able to react with the HA and depict some degree of hyperactivated-like motility pattern, ideal to select best spermatozoa for ICSI in human and was later used for stallion spermatozoa (Colleoni *et al*., 2011), but numbers are low to determine a true relation to fertility.

The effective binding of the spermatozoon to the ZP is a critical step in the process of fertilization. The binding is species specific, only elicited by capacitated spermatozoa and it precedes acrosome reaction (AR) occurrence. Since ZP binding can easily be performed *in vitro*, several sperm ZP binding tests have been designed since the 1980´s, either using whole ZP (oocytes), or hemi ZPs (cleaved oocytes; Rodriguez-Martinez, 2006). Although outcomes from ZP binding tests yielded significant correlations with AI-fertility in pigs (Lynham and Harrison, 1998; Ardon *et al*., 2005) and bulls (Zhang *et al*., 1998), the biological significance of the assay is questioned, mainly due to the fact that physiological sperm capacitation, and hence AR, do not involve all spermatozoa at a given time.

An alternative usually tested by many laboratories is the ability of presumably capacitated spermatozoa to penetrate into homologous oocytes *in vitro*, the so called oocyte penetration test, under conditions of *in vitro* oocyte maturation (Henault and Killian, 1995; Brahmkshtri *et al*., 1999; Oh *et al*., 2010) which seem to relate to fertility (Henault and Killian, 1995). However, since oocytes maturity level varies as well as not all spermatozoa at a given time are capacitated and prompted to engage in ZP penetration; there is variation in penetration rates which do not mirror possible fertility differences among sires.

Different end points in fertilization and subsequent early embryo development can be determined using *in vitro* fertilization (IVF); spermatozoa of various species have been repeatedly examined looking for a relationship between *in vitro* outcome(s) and field fertility when the same semen (or males) was used for AI. In most cases, the approaches were retrospective, i.e. the fertility levels of the semen or males used were already known and only a few were really made prospective, i.e. the semen was coded, used *in vitro* and the outcomes used to calculate an '*in vitro* fertility' that was thereafter contrasted to the 'real' fertility in the field. It was soon apparent that significant relations appeared when the semen used had wide variations in fertility, and results could be accepted as reliable when the conditions for IVF were of a certain stringency and stability, i.e. low sperm numbers used, same levels of success in a control line over time, not major differences between cleavage and morula/blastocyst yields. Unfortunately, most studies (Rodriguez-Martinez, 2007b) had only low to medium relationships with fertility, being lowest for

morula/blastocyst rates.

Di-thio-treitol (DTT) and detergents (such as sodium dodecylsulphate) have been used to study the relative capacity of sperm nuclei to decondense *in vitro*, attempting to establish a method that resembles the process needed to form a male pronucleus during fertilization. The degree of decondensation can be assessed microscopically (Rodriguez *et al*., 1985) or via Flow cytometry (FC) after Propidium-iodide (PI)-loading (Cordova-Izquierdo *et al*., 2006) and has been related to fertility in sheep and pigs, respectively. Apoptotic-like changes and the presence of apoptotic markers have been detected in species where retained cytoplasmic droplets are common, such as the equine (Ortega Ferrusola *et al*., 2009a, b, 2010). Although clearly related to storage and cooling, it remains unclear whether the presence of caspases is biologically relevant for male fertility.

Methods for *in vitro* separation of spermatozoa for robustness have been described (Rodriguez-Martinez *et al*., 1997) with a major focus on the fact that spermatozoa in a normal semen sample usually show a typical progressive, innate linear motility; linearity that is used to surpass natural barriers such as the cervix, where they migrate along sialic acid rich mucus filled deep furrows. Assays exploiting the fact that spermatozoa have an innate tendency to migrate into most media (often culture medium but also more complex preparations of varying viscosity) brought in contact with a semen sample (swim-over, swim-down, swim-up) have been used to mimic *in vivo* events. This simple procedure has proven to select for sperm motility and membrane integrity, essential parameters for fertilization (Rodriguez-Martinez *et al*., 1997) and has proven valuable for fertility prognosis, since the number of viable spermatozoa post swim-up reflected the innate fertilizing capacity of the tested semen sample (Zhang *et al*., 1998; Hallap *et al*., 2005a, b, 2006a). Viscosity, often associated with additives of the swim-up media has improved the results, basically by mimicking the *in vivo* situation (Rodriguez-Martinez, 2007b; Hunter *et al*., 2011). Hyaluronan, a component of the oviductal fluid and the cumulus cell cloud (Rodriguez-Martinez *et al*., 2001) has proven an excellent additive since it increased viscosity to the right proportion *in vivo* and selected for fertilizing capacity (Shamsuddin and Rodriguez-Martinez, 1994). As a follow-up, artificial (hyaluronate-based, not sialic-based) cervical mucus has also been tested, albeit with less discriminative results (Al Naib *et al*., 2011).

Novel methods have recently been developed using alternative multiple micro fluidic flow streams for sperm self-migration which allow for the sorting of motile spermatozoa in a similar fashion as *in vivo* (Wang *et al*., 2011), although not suitable for the isolation of large sperm numbers, these latter methods appear promising when adapted for IVF, where low, quasi physiological sperm numbers are co-incubated with oocytes (Suh *et al*., 2006). Other methods have been put forward as substitutes for farm animals, where a higher output of an intact population is selected (Rodriguez-Martinez *et al*., 1997; Samardzija *et al*., 2006). Examples of these methods are the centrifugation through columns of adherent particles, Sephadex or glass wool, (Januskauskas *et al*., 2005) or through discontinuous density gradients of silate coated silica spheres (Rodriguez-Martinez *et al*., 1997). Centrifugation through a single column of species specific formulations of colloid (based on silate-coated spheres, the SLC method) has proven successful to harvest the most robust spermatozoa from any (raw or serially processed) semen suspension, in most species tested so far (Morrell and Rodriguez-Martinez, 2009, 2010; Morrell *et al.,* 2010). The selective power, which is clearly related to species differences in osmolarity and density of the colloid (Morrell *et al*., 2011), is equally present in different volumes and sperm preparations, but -once again-, the selection is simply a mirror of the proportion of robust spermatozoa in a semen sample, and thus with low relationship to fertility.

Sperm ´omics

The exponential advances in analytical molecular biochemistry, also named the ´omics revolution, have even involved sperm assessment. The ´omics revolution refers to the study of genes (genomics), and the function of their products (functional genomics) either as RNA transcripts (transcriptomics), proteins (proteomics) and the various metabolites (Aitken, 2010), opening our possibilities to determine how their presence or changes relate to cell function including fertility. Such endeavor is being made possible by the application of DNA sequencing, DNA microarrays, mass spectrometry, and protein arrays which, when proper interfaces and bioinformatic tools are available, may provide cues for sperm function (Carrell, 2008).

Sperm genomics

Spermatozoa provide during fertilization a haploid genome with intact coding regions and regulatory regions for essential genes, copies that must be intact (i.e. should not contain single or double stranded DNA breaks). Mammalian spermatozoa have the most tightly compacted eukaryotic DNA, built up upon transformations during spermiogenesis where the sperm chromatin replaces histones first by transient proteins and then by protamines (Oliva and Castillo, 2011). Sperm chromatin can show different abnormalities related to compaction; from damage to the actual DNA physical integrity as single or double stranded DNA strand breaks, nuclear protein defects interfering with histone or protamine conversion and

DNA compaction; to chromatin structural abnormalities such as defective tertiary chromatin configuration. While the last named can imply defects in the decondensation of the nucleus before building the male pronucleus and impair fertilization, the other two can jeopardize embryonic development since the oocyte (albeit being able to repair a limited amount of sperm DNA damage) would not be able to correct those damages (Johnson *et al*., 2011). Sperm DNA disorders also include mutations, epigenetic modifications, base oxidation and DNA fragmentation, the latter also related to sperm handling. Pertaining to its relevance, evaluation of the degree of DNA integrity has increased over the years (Barratt *et al*., 2010). DNA fragmentation, by being considerably present in subfertile males, is considered the most frequent cause of paternal DNA anomaly transmitted to progeny. Damaged sperm DNA may be incorporated into the genome of the embryo, and participate or lead to errors in DNA replication, transcription or translation during embryo development, ultimately contributing to diseases in future generations (Katari *et al*., 2009). Moreover, DNA damage may remain in the germ line for generations, a matter of concern related to the increasing use of ICSI (today even used in horses or pets; Aitken *et al*., 2009). Sperm DNA fragmentation can be studied with many techniques, including staining with the DNA fluorophore PI which, in species where DNA compaction is not high, can present two types of staining, a dimmer (related to low sperm quality) and a brighter version (normal spermatozoa, Muratori *et al*., 2008). Other classical methods to determine DNA damage are: (a) the single-cell gel electrophoresis assay (COMET), (b) the terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labelling (TUNEL), (c) the acridine orange test (AOT), (d) the tritium-labelled 3H-actinomycin D (3H-AMD) incorporation assay, (e) the in situ nick translation (ISTN), (f) the DNA breakage detection fluorescence in-situ hybridizations (DBD-FISH), (g) the sperm chromatin dispersion test (SCD, Halo) or the evaluation of (h) the degree of induced denaturation of the DNA (the so called Sperm Chromatin Structure Assay, SCSA) (Fraser, 2004; Evenson and Wixon, 2006; Tamburrino *et al*., 2012). Most of the above methods can use fluorescence microscopy while SCSA and TUNEL are usually explored via FC. Although SCSA has been extensively used, the outcome provided conflicting relations to fertility in selected bull and boar sires (Rodriguez-Martinez and Barth, 2007; Christensen *et al*., 2011; D´Occhio *et al*., 2013) or unselected stallions (Morrell *et al*., 2008). SCSA does not specifically identify the amount of DNA damage but rather its susceptibility to harsh treatment, whereas TUNEL does. A TUNEL/PI procedure is now available combining the accuracy of TUNEL and the differentiation of two sperm populations depending on PI intensity, of which one is probably participating in fertilization since the observed damage has no relation to motility or morphology (Muratori *et al*., 2008). Alternatively, use of dithiotreitol (DTT) to decondense sperm nuclei and inclusion of a stain for dead cells provides a higher accessibility to the TDNT enzyme of the TUNEL, alongside with the detection of DNA fragmentation in live spermatozoa (Mitchell *et al*., 2011). Considering the above, TUNEL appears to be a more sensitive method to predict infertility than SCSA, as determined in a recent meta-analysis (Zini *et al*., 2008).

Sperm epigenetics

Noteworthy, not only DNA quality but also the packaging of the paternal genome (epigenome) is essential to embryonic development and fertility (Miller *et al*., 2010; Jenkins and Carrell, 2011). Alongside genetic material, the spermatozoon also contributes with epigenetic components (i.e. other than DNA-coding changes that can alter or regulate gene expression) that affect early embryo development (Hales *et al*., 2011). Processes such as DNA methylation, selective histone retention, sperm specific histones with tail modifications, other chromatin associated proteins, perinuclear theca proteins, organization of the DNA loop domain by the sperm nuclear matrix and of sperm born RNAs are included (Pacheco *et al*., 2011; Yamauchi *et al*., 2011). Microarray- and serial- analyses of gene expression assays of spermatozoa from several species have shown differential presence of regulatory non-coding RNAs (either long [lncRNAs] or short [microRNAs, small interfering isRNAs and Piwi-associated piRNAs; Ponting *et al*., 2009) which provide the zygote with a unique set of paternal mRNAs (Krawetz *et al*., 2011). These provide variable array signals, which correspond to the inherent variability among spermatozoa within an ejaculate, between ejaculates and individuals. Despite this, use of suppressive substraction hybridization (Lalancette *et al*., 2008), or of global RNA profiles of spermatozoa from fertile and infertile men (García-Herrero *et al*., 2010), or bulls (using a cDNA collection on DNA microarrays) with different NRRs, could lead to the identification of transcripts (protein kinase and ADAM5P) associated with high sperm motility (Bissonnette *et al*., 2009). More recently, semen from high- respectively low-fertility bulls provided, when examined with Affymetrix bovine gene chips, significant differences of specific transcripts associated with fertility (Feugang *et al*., 2010). It is foreseen that microarrays shall be a determinant for future diagnostics.

Sperm proteomics

The study of protein products expressed by the genome has dramatically expanded over the past decade, owing to multidisciplinary methodological and instrumental developments, but also due to the central

role of protein interactions in cell function (Cox and Mann, 2007; Brewis and Gadella, 2010; Baker, 2011; du Plessis *et al*., 2011). Spermatozoa are, by being so highly differentiated, advantageous cells to study proteomics of specific compartments such as the membrane, which basically is the area of major importance for their role in interacting with their surroundings and the oocyte (Arnold and Frohlich, 2011). Despite this methodological development, proteomic studies of spermatozoa are still limited (Oliva *et al*., 2009), yet leading to comprehensive sperm protein databases (Duncan and Thompson, 2007; de Mateo *et al*., 2011) with numbers of proteins and fragments exponentially increasing over time towards several thousands. The proteins identified thus far cover the expected spectrum of function (from energy production to cell recognition), but few are accurately linked to (in)fertility, most of them being enzymes (Novak *et al*., 2010a, b).

The seminal plasma, a forgotten cue for fertility prognosis?

The main proteins of the SP belong to one of three groups: proteins carrying fibronectin type II (Fn-2) modules (as present in boar, stallion, bull or buck), spermadhesins (boar) or cysteine rich secretory proteins (CRISPs, stallion) and their bulk is, in most species, of vesicular gland origin (Kelly *et al*., 2006). SP proteins, acting as adsorbed proteins to the plasma membrane, modulate several essential steps preceding fertilization, regulating capacitation, the establishment of the oviductal sperm reservoir, the modulation of the uterine immune response, and sperm transport through the female genital tract, as well as in gamete interaction and fusion. Therefore, SP proteomes have been assessed in relation to reproductive outcomes (either fertility levels or (in) fertility (Drabovich *et al*., 2011; Milardi *et al*., 2012), in several species. SP proteins have been identified as associated with high/low fertility in bulls (Killian *et al*., 1993), isolated as Osteopontin (OPN) and Lipocalin-Type Prostaglandin D synthase (Gerena *et al*., 1998; Cancel *et al*., 1999). The latter has been always observed in the sperm-rich spurts of ejaculates in species with fractionated ejaculation, including the pig (Rodriguez-Martinez *et al*., 2009, 2010, 2011). The OPN has been related to fertility in pig (IVF, Hao *et al*., 2006, 2008) and stallion (Brandon *et al*., 1999). Some SP proteins (SP-2, SP-3, SP-4, and clusterin) have been found in higher concentrations in stallions with low fertility scores (Novak *et al*., 2010b). SP-1 is positively (Brandon *et al*., 1999) or negatively (Novak *et al*., 2010b) correlated with fertility and was suggested to be homologous to a bovine fertility associated protein described by Killian *et al*. (1993), probably OPN. Moreover, the abundance of CRISP3 in equine SP was positively correlated to 1st-cycle conception rate (Novak *et al*., 2010b) suggesting the protein family might have a role in fertility, as suggested for rodents and humans (Koppers *et al*., 2011). The spermadhesin PSP-I, seems to be negatively related to pig fertility (Novak *et al*., 2010a). SP cytokine levels vary among males. Variation in SP contents of TGF-β lacks a linear relation to fertility (Loras *et al*., 1999; O´Leary *et al*., 2011). However, a female could express different levels of endogenous cytokines (relevant for embryo survival) depending on the exposure to SP from different males, which might thus relate to the often observed differences in embryo survival among sires (e.g. innate fertility; Robertson, 2007, 2010).

Have we reached full diagnostic and prognostic value?

Assays and/or attributes tested differ in relation to fertility. For instance, membrane integrity evaluated via fluorometry (FC) appeared more closely related to semen fertility than sperm motility. Sample power is most relevant; assessing a hundred spermatozoa per sample or exploring thousands of them lead to unsecure relationship to fertility. Strength can be gained also by adjoining assays, even when this implies that some attributes are repeatedly measured. There is no risk in this, since spermatozoa that are tested with one assay are different from all others, so a battery of tests is always advantageous (Rodriguez-Martinez, 2003). Following that path, several groups have combined the results of *in vitro* tests of the same semen samples in analyses of multiple regression (Rodriguez-Martinez and Barth, 2007), yielding higher correlations with fertility even when being retrospective. Calculations of predicted fertility combining the outcomes of various methods of semen evaluation *in vitro* in multivariate analysis, before the fertility of the donor males was tested in the laboratory or the field, has proven valuable (Zhang *et al*., 1999; Gil *et al*., 2005; Ruiz-Sanchez *et al*., 2006). This approach enabled identification of sub-fertile bulls, whose expected and real fertility was below the limit considered for sub-fertility (62% nonreturn rate), while the other young bulls predicted to have satisfactory fertility had nonreturn rates of ≥65%. Identification of sub-fertile sires had been obtained with other bull (Hallap *et al*., 2004) and boar stud populations (Ruiz-Sanchez *et al*., 2006). Interestingly, most sperm parameters (and to some extent even fertility) appeared maintained over the functional age of the sires, provided no pathologies are acquired between measurements (Zhang *et al*., 1997, 1998; Hallap *et al*., 2005b, 2006a). However, intrinsic variation between ejaculates within sire was always present, which requires analyses of many ejaculates.

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