

# Semen evaluation: can we forecast fertility?



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## Abstract

This review critically comments on established and emerging methods of semen evaluation that would foresee the fertility of the ejaculate (neat or processed) or the male. Functional sperm testing through biomimetic *in vitro* assays are priority since these, 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. The review also focuses on the exploration of the genome, the transcriptome and the proteome

of both spermatozoa and the seminal plasma which may help to unveil how spermatozoa and seminal plasma components would signal to the female and link to fertility and even prolificacy. Specific seminal plasma components, both among individuals and portions of the ejaculate, not only relate to sperm survival but also to signal the female immune system towards a differential immune tolerance warranting fertility.

**Key words:** *semen analysis; sperm quality; in vitro methods; fertility estimation*

## Introduction

Over the past decades, we have experienced an explosive development of *in vitro* assays to determine sperm intactness and measurement of sperm function as summarised in multiple reviews (Rodriguez-Martinez and Larsson, 1998; Graham, 2001; Rodriguez-Martinez, 2003; Parkinson, 2004; Graham and Moce, 2005; Guillan et al., 2005; Rijsselaere et al., 2005; Rodriguez-Martinez, 2006, 2007a; Petrunkina et al., 2007; Rodriguez-Martinez and Barth, 2007; Moce and Graham, 2008; Hossain et al., 2011; Petrunkina and Harrison, 2013; Sanchez et al., 2013; Barazani et al., 2014). However, conventional semen evaluation

is restricted to determinations of sperm numbers, sperm motility and only sometimes, sperm morphology, following the paradigm 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 (Rodriguez-Martinez et al., 2005; Rodriguez-Martinez, 2007b; 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

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of spermatozoa (mostly related to their membranes but also their metabolomics) to interact with the surrounding fluids (SP, female genital fluids and *in vitro* culture media), cells (epithelia, cumulus cells, oocytes), or extracellular material (hyaluronan coating, the zona pellucida) 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 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 (CASA) or morphology (ASMA) to bench-model flow cytometers (FCs), are making assays accessible for clinical diagnostics and semen processing. However, considering many of these methods are only of limited value for prediction of fertility, this review aims to critically review methodological advances in the assessment of semen and the capacity different assays have to really prognose fertility.

## Outcomes of the spermogram and fertility

Current examination of the ejaculate includes aspect, volume and pH, sperm numbers (concentration or total per ejaculate) and sperm motility (often including kinematics when using CASA instruments). 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). 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), while

studies in other species yield variable results mostly owing to operator bias and differences in numbers of females bred when accounting fertility (Rodriguez-Martinez, 2006). Kinematic analyses (CASA) of linearity correlate with field fertility but with large variation among studies (Bailey et al., 1994; Zhang et al., 1998; Hirai et al., 2001; Januskauskas et al., 2001, 2003; Broekhuijse et al., 2012). Fertility estimation in AI dairy sires was stronger when motility patterns were combined with other parameters of sperm function (Januskauskas et al., 2001) or gene analyses (Iso-Touru et al., 2019). Percentages of morphologically normal bull ejaculated spermatozoa strongly relates to post-AI fertility (Phillips et al., 2004; Al-Makhzoomi et al., 2008; Nagy et al., 2013) reflecting normality of spermatogenesis and sperm maturation. Morphological abnormalities differ in their impact on fertility, some being specific defects (Nagy et al., 2018) that hamper fertilization while others (as pear-shaped sperm head deviation), impair embryo development (Rodriguez-Martinez and Barth, 2007). The ASMA instruments analyse sperm head dimensions but not yet sperm abnormalities of other nature (Auger, 2010) and lack clear relationships to fertility (Saravia et al., 2007b; Gravance et al., 2009).

## Functional sperm testing and fertility prognosis

In specialized laboratories, intactness of sperm function towards fertilization is studied *in vitro* and outcomes related to fertility (Rodriguez-Martinez, 2007a). Integrity and stability of the plasma membrane are paramount, studied via a multitude of methods (microscopy in wet smears, membrane impermeable dye eosin (eosin-nigrosin test), exposure to a hypo-osmotic saline solution (HOS-test), or single or multiple fluorophores and FC

with significant –albeit variable– correlations to fertility (rev by Rodriguez-Martinez and Barth, 2007). 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 (Mero-cyanine-540, YO-PRO-1 and Hoechst 33342) or phospholipid asymmetry (Annexin-V/PI) (Januskauskas et al., 2003; 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 affecting sperm motility under the control of specific membrane receptors as Catsper (Vicente-Carrillo et al., 2017) or opioid receptors (Vicente-Carrillo et al., 2016). The  $Ca^{2+}$  influx also influences the generation of controlled amounts of ROS, as well as the phosphorylation of protein residues eventually associated to fertility (Harrison and Gadella, 2005; O'Flaherty et al., 2006; Fabrega et al., 2011). 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).

Correlations between mitochondria status and fertility are variable, mostly due to changes in mitochondria functionality over time (Martinez-Pastor et al., 2004; Hallap et al., 2005a; 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) (Roca et al., 2016). A certain level of ROS is essential for sperm function, including fertilising capacity, but only when it is kept at

optimal levels by the antioxidant capacity of the seminal plasma (Awda et al., 2009; Barranco et al., 2015; Žura Žaja et al., 2019), via antioxidant enzymes such as paraoxonase-1 (PON-1) or the sperm-present PON-2 (Barranco et al., 2014). Levels of ROS are variable, making their proper determination difficult, albeit yet possible using the probe hydro-ethidine or measuring lipid peroxidation (LPO) levels in the membrane lipid bilayer by using the 5-iodoacetamidofluorescein probe family (BODIPY- $C_{11}$ ) (Ortega-Ferrusola et al., 2009a; Hossain et al., 2011). Acrosome intactness, a prerequisite for fertilization, can be readily examined *in vitro* using phase contrast microscopy (Rodriguez-Martinez and Larsson, 1998) or be examined by fluorophore-linked lectins by multiparametric analysis (Nagy et al., 2004). Yet, correlation between acrosome status and fertility are variable (Rodriguez-Martinez, 2007). 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; Alvarez-Rodriguez et al., 2018) which binds to HA in the cumulus cloud. Use of the PCSI Sperm Selection device, USA (Huszar et al., 2007), to trap only mature spermatozoa and select best spermatozoa for ICSI has been used for stallion spermatozoa (Colleoni et al., 2011), but numbers are low to determine a true relation to fertility. However, HA has per se a doubtful influence to sperm motility (Alvarez-Rodriguez et al., 2018).

Simple methods for *in vitro* separation of spermatozoa for robustness are available, from washing/centrifugation to density gradients (Rodriguez-Martinez et al., 1997; Samardzija et al., 2006a). Swim-assays select for sperm motility and membrane integrity, essential parameters for fertilization (Rodriguez-Martinez et al., 1997) and has proven valuable for fertility prognosis, with

numbers of viable spermatozoa post swim-up reflecting innate fertilizing capacity (Zhang et al., 1998, Hallap et al., 2005a, b, 2006b; Samardzija et al., 2006b), particularly when hyaluronan (HA) has been added (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). Discontinuous density gradients of silate coated silica spheres provide higher sperm yield (Rodriguez-Martinez et al., 1997) and centrifugation through a single column of species-specific formulations of silate-coated colloid spheres (the SLC method) has proven successful to harvest the most robust spermatozoa from any (raw or serially processed) semen suspension, in all species tested so far (Morrell and Rodriguez-Martinez, 2009, 2010; Morrell et al., 2011), but selection *per se* has a low relation to fertility.

Sperm-ZP binding is a critical step for fertilization being used *in vitro* with either whole ZP (oocytes), or hemi ZPs (cleaved oocytes) (Rodriguez-Martinez, 2006). Outcomes from ZP binding tests yield significant correlations with AI-fertility in bulls (Zhang et al., 1998) and pigs (Ardon et al., 2005). The oocyte penetration test implies testing the ability of presumably capacitated spermatozoa to penetrate into homologous oocytes *in vitro* (Oh et al., 2010) which seem to relate to fertility (Henault and Killian, 1995), albeit 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). Most studies looking for a relationship between *in vitro* outcome/s and field fertility are retrospective 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. Significant relations appear when the sires differed widely 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 (Zhang et al., 1997, 1999). Unfortunately, most studies (Rodriguez-Martinez, 2007b) had only low to medium relationships with fertility, lowest for morula/blastocyst rates.

## Sperm genomics

Sperm chromatin can show spermiogenesis-related compaction abnormalities, with DNA fragmentation being considered the most frequent cause of paternal DNA anomaly transmitted to progeny (Johnson et al., 2011). Pertaining to its relevance, evaluation of the degree of DNA integrity has increased over the years (Barratt et al., 2010, Sanchez et al., 2013). Sperm DNA fragmentation can be studied with many techniques, including staining with the DNA fluorophore Propidium Iodide (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, Halosperm) 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 FC is used for SCSA and TUNEL. 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). Other methods, less cumbersome and waiving the need of FC, as SCD appears to provide a similar outcome to SCSA, at least in human (Liffner et al., 2019).

## Sperm epigenetics

Both DNA quality and the packaging of the paternal genome (epigenome) are 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, and use of global RNA profiles of spermatozoa from fertile and infertile men (García-Herrero et al., 2010), or bulls with different NRRs, could lead to the identification of transcripts (protein kinase and ADAM5P) associated with high sperm motility (Bissonnette et al., 2009). Levels of miRNAs relate to sperm functional attributes in bull (Govindaraju et al., 2012), stallion (Das et al., 2013) or pig (Curry et al., 2009, 2011). In bull sperm, miRNA profiles show differential expression in relation to the fertility levels of the sires (Govindaraju et al., 2012). Exploration of the miRNAome in sperm and SP, in relation to sperm quality and fertility, is relevant to establish molecular biomarkers.

## 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 (Barazani et al., 2014). Despite these advances, proteomic studies of spermatozoa are still limited (Oliva et al., 2009; Arnold and Frohlich, 2011; Barazani et al., 2014), but building comprehensive sperm protein databases (de Mateo et al., 2011; Perez-Patiño et al., 2018; 2019a, b). The proteins identified cover a broad spectrum of function (from energy production to cell recognition), a relation to the portion of

the ejaculate (Perez-Patiño et al., 2019a) particularly in relation to seminal plasma exposure (Perez-Patiño et al., 2018a) or cryopreservation (Perez-Patiño et al., 2019b) and a clear relation to fertility (Novak et al., 2010a, b; Perez-Patiño et al., 2018a, 2019a, b).

## The seminal plasma, relevant for fertility prognosis?

The main proteins of the SP belong to one of three groups: proteins carrying fibronectin type II (Fn-2) modules (boar, stallion, bull, buck), spermadhesins (boar) or cysteine rich secretory proteins (CRISPs, stallion). 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 sperm quality and reproductive outcome (Drabovich et al., 2011; Milardi et al., 2012, Gonzalez-Cadavid et al., 2014), 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). OPN has been related to fertility in pig (Hao et al., 2008) and stallion (Brandon et al., 1999) while lipocalins are present among the fertility-related proteins in the pig ejaculate (Rodriguez-Martinez et al., 2009, 2011). 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., 2010a) with SP-1 being positively (Brandon et al., 1999) or negatively (Novak et al., 2010a) correlated with fertility. Proteins explored in pig seminal plasma, using

novel proteomic techniques has provided clear evidence of the relation of these proteins to the fertility of the males, including both farrowing rates and prolificacy (Perez-Patiño et al., 2016, 2018b).

Abundance of CRISP3 in equine SP is positively correlated to 1st-cycle conception rate (Novak et al., 2010a) 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., 2010b). SP cytokine levels vary among males, but levels are not related to fertility, including the most abundant TGF- $\beta$ , which lacks a linear relation to fertility (Loras et al., 1999; O'Leary et al., 2011). Specific enzymes, often with strong antioxidant capacity, as Paraoxonase type 1 (PON-1) and Glutathione Peroxidase-5, are present in pig seminal plasma, with a clear relationship to fertility (Barranco et al., 2015, 2016, 2017).

## Can we then prognose male fertility by semen assessment?

Assays and/or attributes tested differ in relation to fertility. For instance, membrane integrity evaluated via FC appears more closely related to semen fertility than sperm motility. Sample power is most relevant; assessing hundred spermatozoa per sample 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 (Rodri-

guez-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., 2000; 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% non-return rate), while the other young bulls predicted to have satisfactory fertility had non-return rates of  $\geq 65\%$ . 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.

## Are we looking at the wrong outcome?

Entry of spermatozoa and of seminal plasma during mating or artificial insemination elicit changes in the expression of genes related to sperm survival but also of genes involved in immune processes at the sperm reservoirs in animals as distant phylogenetically as chicken and pigs, indicating the genes affected are conserved over evolution (Atikuzzaman et al., 2017). Experimental evidence of female internal genital tract samples collected 24 h after exposure to semen, isolated spermatozoa or sperm-free seminal plasma and examined via microarrays confirmed the effects are

differential towards immune-related genes, including those related to the establishment of a status of maternal tolerance to paternal antigens present in spermatozoa or embryos (Alvarez-Rodriguez et al., 2019). Whether these gene expression changes can be monitored towards fertility of the females, or males, implies we are closer to new indicators which might be more relevant than the classical sperm attributes were classically explore.

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## References

1. AL-MAKHZOOMI, A., N. LUNDEHEIM, M. HÅÅRD and H. RODRIGUEZ-MARTINEZ (2008): Sperm morphology and fertility of progeny-tested AI dairy bulls in Sweden. *Theriogenology* 70, 682-691.
2. AL NAIB, A., J. P. HANRAHAN, P. LONERGAN and S. FAIR (2011): In vitro assessment of sperm from bulls of high and low fertility. *Theriogenology* 76, 161-167.
3. ALVAREZ-RODRIGUEZ, M., A. VICENTE-CARRILLO and H. RODRIGUEZ-MARTINEZ (2018): Hyaluronan does not improve long-term storage nor cryo-survival of CD44-bearing boar spermatozoa. *J. Reprod. Dev.* 64, 351-360.
4. ALVAREZ-RODRIGUEZ, M., M. ATIKUZZAMAN, H. VENHORANTA, D. WRIGHT and H. RODRIGUEZ-MARTINEZ (2019): Expression of immune regulatory genes in the porcine internal genital tract is differentially triggered by spermatozoa and seminal plasma. *Int. J. Mol. Sci.* 20, 513; doi: 10.3390/ijms20030513.
5. ARDON, F., M. EVERT, M. BEYERBACH, K. F. WEITZE and D. WABERSKI (2005): Accessory sperm: a biomonitor of boar fertilization capacity. *Theriogenology* 63, 1891-1901.
6. ARNOLD, G. J. and T. FROHLICH (2011): Dynamic proteome signatures in gametes, embryos and their maternal environment. *Reprod. Fertil. Dev.* 23, 81-93.
7. ATIKUZZAMAN, M., M. ALVAREZ-RODRIGUEZ, A. VICENTE-CARRILLO, M. JOHNSSON, D. WRIGHT and H. RODRIGUEZ-MARTINEZ (2017): Conserved gene expression in sperm reservoirs

- between birds and mammals in response to mating. *BMC Genomics* 18, 98; doi: 10.1186/s12864-017-3488-x.
8. AUGER, J. (2010): Assessing human sperm morphology: top models, underdogs or biometrics? *Asian J. Androl.* 12, 36-46.
  9. AWDA, B. J., M. MACKENZIE-BELL and M. M. BUHR (2009): Reactive oxygen species and boar sperm function. *Biol. Reprod.* 81, 553-561.
  10. BAILEY, J. L., L. ROBERTSON, and M. M. BUHR (1994): Relations among in vivo fertility, computer-analysed motility and Ca<sup>++</sup> influx in bovine spermatozoa. *Can. J. Anim. Sci.* 74, 53-58.
  11. BARAZANI, Y., A. AGARWAL and E. S. SABANEKH (2014): Functional sperm testing and the role of proteomics in the evaluation of male infertility. *Urology* 84, 255-261
  12. BARRANCO, I., A. TVARIJONAVICIUTE, C. PEREZ-PATIÑO, M. A. GIL, J. J. CERON, E. A. MARTINEZ, H. RODRIGUEZ-MARTINEZ and J. ROCA (2014): Paraoxonase type 1 in boar seminal plasma and its relationship with sperm quality and functionality. *Reprod. Dom. Anim.* 49S3, 57.
  13. BARRANCO, I., A. TVARIJONAVICIUTE, C. PEREZ-PATIÑO, I. PARRILLA, J. J. CERON, E. A. MARTINEZ, H. RODRIGUEZ-MARTINEZ and J. ROCA (2015): High total antioxidant capacity of the porcine seminal plasma (SP-TAC) relates to sperm survival and fertility. *Scientific Reports-UK* 5, 18538.
  14. BARRANCO, I., A. TVARIJONAVICIUTE, C. PEREZ-PATIÑO, D. V. ALKMIN, J. J. CERON, E. A. MARTINEZ, H. RODRIGUEZ-MARTINEZ and J. ROCA (2015): The activity of Paraoxonase type 1 (PON-1) in boar seminal plasma and its relationship with sperm quality, functionality and in vivo fertility. *Andrology* 3, 315-320; doi: 10.1111/andr.309).
  15. BARRANCO, I., A. TVARIJONAVICIUTE, C. PEREZ-PATIÑO, A. VICENTE-CARRILLO, A. PARRILLA, J. J. CERON, E. A. MARTINEZ, H. RODRIGUEZ-MARTINEZ and J. ROCA (2016): Glutathione Peroxidase-5 is expressed by the entire pig male genital tract and once in the seminal plasma contributes to sperm survival and in vivo fertility. *PLoS One* DOI:10.1371/journal.pone.0162958, September 14, 2016.
  16. BARRANCO, I., C. PEREZ-PATIÑO, A. TVARIJONAVICIUTE, I. PARRILLA, A. VICENTE-CARRILLO, M. ALVAREZ-RODRIGUEZ, J. J. CERON, E. A. MARTINEZ, H. RODRIGUEZ-MARTINEZ and J. ROCA (2017): Active paraoxonase-1 is synthesised throughout the internal boar genital tract. *Reproduction* 154, 137-143; doi: 10.1530/REP-17-0300. Epub 2017 Jun 13.
  17. BARRATT, C. L. R., R. J. AITKEN, L. BJÖRNDAHL, D. T. CARRELL, P. DE BOER, U. KVIST, S. E. M. LEWIS, S. D. PERREAULT, M. J. PERRY, L. RAMOS, B. ROBAIRE, S. WARD and A. ZINI (2010): Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications- a position report. *Hum. Reprod.* 25, 824-838.
  18. BERGQVIST, A. S., J. BALLESTER, A. JOHANNISSON, M. HERNÁNDEZ N. LUNDEHEIM and H. RODRÍGUEZ-MARTÍNEZ (2006): In vitro capacitation of bull spermatozoa by oviductal fluid and its components. *Zygote* 14, 259-273.
  19. BISSONNETTE, N., J. P. LÉVESQUE-SERGERIE, C. THIBAUT and G. BOISSONNEAULT (2009): Spermatozoal transcriptome profiling for bull sperm motility: a potential tool to evaluate semen quality. *Reproduction* 138, 65-80.
  20. BRANDON, C. I, G. L. HEUSNER, A. B. CAUDLE and R. A. FAYRER-HOSKEN (1999): Two-dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their correlation with fertility. *Theriogenology* 52, 863-873.
  21. BROEKHUIJSE, M. L., E. SOSTARIC, H. FEITSMA and B. M. GADELLA (2012): Application of computer-assisted semen analysis to explain variations in pig fertility. *J. Anim. Sci.* 90, 779-789.
  22. CANCEL, A. M., D. A. CHAPMAN and G. J. KILLIAN (1999): Osteopontin localization in the Holstein bull reproductive tract. *Biol. Reprod.* 60, 454-460.
  23. CARRELL, D. T. (2008): Contributions of spermatozoa to embryogenesis: assays to evaluate their genetic and epigenetic fitness. *Reprod. Biomed. Online* 16, 474-484.
  24. CHRISTENSEN, P., R. LABOURIAU, A. BIRCK, G. B. BOE-HANSEN, J. PEDERSEN and S. BORCHERSEN (2011): Relationships among seminal quality measures and field fertility of young dairy bulls using low-dose inseminations. *J. Dairy Sci.* 94, 1744-1754.
  25. COLLEONI, S., I. LAGUTINA, G. LAZZARI, H. RODRIGUEZ-MARTINEZ, C. GALLI and J. M. MORRELL (2011): New methods for selecting stallion spermatozoa for assisted reproduction. *J. Equine Vet. Sci.* 31, 536-541.
  26. CREMADES, T., J. ROCA, H. RODRIGUEZ-MARTINEZ, T. ABAIGAR, J. M. VAZQUEZ and E. A. MARTINEZ (2005): Kinematic changes during the cryopreservation of boar spermatozoa. *J. Androl.* 26, 610-618.
  27. CURRY, E., S. E. ELLIS and S. L. PRATT (2009): Detection of porcine sperm microRNAs using a heterologous microRNA microarray and reverse transcriptase polymerase chain reaction. *Mol. Reprod. Dev.* 76, 218-219.
  28. CURRY, E., T. J. SAFRANSKI and S. L. PRATT (2011): Differential expression of porcine sperm microRNAs and their association with sperm morphology and motility. *Theriogenology* 76, 1532-1539.
  29. DAS, P. J., F. MCCARTHY, M. VISHNOI, N. PARIJA, C. GRESHAM, G. LI, P. KACHROO, A. K. SUDDERTH, S. TEAGUE, C. C. LOVE, D. D. VARNER, B. P. CHOWDHARY and P. RAUDSEPP (2013): Stallion sperm transcriptome comprises functionally coherent coding and regulatory RNAs



- as revealed by microarray analysis and RNA-seq. *PLoS One* 8, e56535.
30. D'OCCHIO, M. J., K. J. HENGSTBERGER, D. TUTT, R. G. HOLROYD, G. FORDYCE, G. B. BOEHANSEN and S. D. JOHNSTON (2013): Sperm chromatin in beef bulls in tropical environments. *Theriogenology* 79, 946-952.
  31. DRABOVICH, A. P., K. JARVI and E. P. DIAMANDIS (2011): Verification of male infertility biomarkers in seminal plasma by multiplex selected reaction monitoring assay. *Mol. Cell Proteomics* 10, M110.004127.
  32. EVENSON, D. and R. WIXON (2006): Clinical aspects of sperm DNA fragmentation detection and male infertility. *Theriogenology* 65, 979-991.
  33. FABREGA, A., M. PUIGMULE, M. YESTE, I. CASAS, S. BONET and E. PINARD (2011): Impact of epididymal maturation, ejaculation and in vitro capacitation on tyrosine phosphorylation patterns exhibited of boar (*Sus domesticus*) spermatozoa. *Theriogenology* 76, 1356-1366.
  34. FRASER, L. (2004): Structural damage to nuclear DNA in mammalian spermatozoa: its evaluation techniques and relationship with male fertility. *Polish J. Vet. Sci.* 7, 311-321.
  35. GARCÍA-HERRERO, S., M. MESEGUER, J. A. MARTÍNEZ-CONEJERO, J. REMOHI A. PELLICER and N. GARRIDO (2010): The transcriptome of spermatozoa used in homologous intrauterine insemination varies considerably between samples that achieve pregnancy and those that do not. *Fertil. Steril.* 94, 1360-1373.
  36. GERENA, R. L., D. IRIKURA, Y. URADE, N. EGUCHI, D. A. CHAPMAN and G. J. KILLIAN (1998): Identification of a fertility-associated protein in bull seminal plasma as lipocalin-type prostaglandin D synthase. *Biol. Reprod.* 58, 826-833.
  37. GIL, J., A. JANUSKAUSKAS, M. C. H. HÅÅRD, M. G. M. HÅÅRD, A. JOHANNISSON, L. SADERQUIST and H. RODRIGUEZ-MARTINEZ (2000): Functional sperm parameters and fertility of bull semen extended in Biociphos-Plus® and Triladyl®. *Reprod. Domest. Anim.* 35, 69-77.
  38. GONZALEZ-CADAVID, V., J. A. M. MARTINS, F. B. MORENO, T. S. ANDRADE, A. C. L. SANTOS, A. C. O. MONTEIRO-MOREIRA, R. A. MOREIRA and A. A. MOURA (2014): Seminal plasma proteins of adult boars and correlations with sperm parameters. *Theriogenology* 82, 697-707.
  39. GOVINDARAJU, A., A. UZUN, L. ROBERTSON, M. O. ATLI, A. KAYA, E. TOPPER, E. A. CRATE, J. PADBURY, A. PERKINS and E. MEMILI (2012): Dynamics of microRNAs in bull spermatozoa. *Reprod. Biol. Endocrinol.* 10, 82.
  40. GRAHAM, J. K. (2001): Assessment of sperm quality: a flow cytometric approach. *Anim. Reprod. Sci.* 68, 239-247.
  41. GRAHAM, J. K. and E. MOCÉ (2005): Fertility evaluation of frozen/thawed semen. *Theriogenology* 64, 492-504.
  42. GRAVANCE, C. G., M. E. CASEY and P. CASEY (2009): Pre-freeze bull sperm head morphometry related to post-thaw fertility. *Anim. Reprod. Sci.* 114, 81-88.
  43. GUILLAN, L., G. EVANS and W. M. C. MAXWELL (2005): Flow cytometric evaluation of sperm parameters in relation to fertility potential. *Theriogenology* 63, 445-457.
  44. HAO, Y., C. N. MURPHY, L. SPATE, D. WAX, Z. ZHONG, M. SAMUEL, N. MATHIALAGAN, H. SCHATTEN and R. S. PRATHER (2008): Osteopontin improves in vitro development of porcine embryos and decreases apoptosis. *Mol. Reprod. Dev.* 75, 291-298.
  45. HALES, B. F., L. GRENIER, C. LALANCETTE and B. ROBAIRE (2011): Epigenetic programming: from gametes to blastocyst. *Birth Def. Res., A, Clin. & Mol. Teratol.* 91, 652-665.
  46. HALLAP, T., M. HÅÅRD, Ü. JAAKMA, B. LARSSON and H. RODRIGUEZ-MARTINEZ (2004): Variations in quality of frozen-thawed semen from Swedish Red and White AI sires at 1 and 4 years of age. *Int. J. Androl.* 27, 166-171.
  47. HALLAP, T., S. NAGY, U. JAAKMA, A. JOHANNISSON and H. RODRIGUEZ-MARTINEZ (2005a): Mitochondrial activity of frozen-thawed spermatozoa assessed by MitoTracker Deep Red 633. *Theriogenology* 63, 2311-2322.
  48. HALLAP, T., S. NAGY, M. HÅÅRD, Ü. JAAKMA, A. JOHANNISSON and H. RODRIGUEZ-MARTINEZ (2005b): Sperm chromatin stability in frozen-thawed semen is maintained over age in AI bulls. *Theriogenology* 63, 1752-1763.
  49. HALLAP, T., Ü. JAAKMA and H. RODRIGUEZ-MARTINEZ (2006a): Changes in semen quality in Estonian AI bulls at 3, 5 and 7 years of age. *Reprod. Domest. Anim.* 41, 214-218.
  50. HALLAP, T., S. NAGY, Ü. JAAKMA, A. JOHANNISSON and H. RODRIGUEZ-MARTINEZ (2006b): Usefulness of a triple fluorochrome combination Merocyanine 540/Yo-Pro 1/Hoechst 33342 in assessing membrane stability of viable frozen-thawed spermatozoa from Estonian Holstein AI bulls. *Theriogenology* 65, 1122-1136.
  51. HARRISON, R. and B. GADELLA (2005): Bicarbonate-induced membrane processing in sperm capacitation. *Theriogenology* 63, 342-351.
  52. HENAULT, M. A. and G. J. KILLIAN (1995): Effects of sperm preparation and bull fertility on in vitro penetration of zona-free bovine oocytes. *Theriogenology* 43, 739-749.
  53. HIRAI, M., A. BOERSMA, A. HOEFELICH, E. WOLF, J. FOLL, T. R. AUMÜLLER and J. BRAUN (2001): Objectively measured sperm motility and sperm head morphometry in boars (*Sus scrofa*): relation to fertility and seminal plasma growth factors. *J. Androl.* 22, 104-110.
  54. HOLT, W. V. (2011): Mechanisms of sperm storage in the female reproductive tract: an interspecies comparison. *Reprod. Domest. Anim.* 46S2, 68-74.

55. HOSSAIN, M. D. S., A. JOHANNISSON, M. WALLGREN, S. NAGY, A. PIMENTA SIQUEIRA and H. RODRIGUEZ-MARTINEZ (2011): Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. *Asian J. Androl.* 13, 406-419.
56. HUNTER, R. H., P. COY, J. GADEA and D. RATH (2011): Considerations of viscosity in the preliminaries to mammalian fertilisation. *J. Assist. Reprod. Genet.* 28, 191-197.
57. HUSZAR, G., C. C. OZENCI, S. CAYLI, Z. ZAVACZKI, E. HANSCH and L. VIGUE (2003): Hyaluronic acid binding by human sperm indicates cellular maturity, and unreacted acrosomal status. *Fertil. Steril.* 79, 1616-1624.
58. HUSZAR, G., A. JAKAB, D. SAKKAS, C. C. OZENCI, S. CAYLI, E. DELPIANO and S. OZKAVUKCU (2007): Fertility testing and ICSI sperm selection by Hyaluronic acid binding: clinical and genetic aspects. *Reprod. Biomed. Online* 14, 650-663.
59. ISO-TOURU, T., C. WURMSER, H. VENHORANTA, M. HILTPOLD, T. SAVOLAINEN, A. SIRONEN, K. FISCHER, K. FLISIKOWSKI, R. FRIES, A. VICENTE-CARRILLO, M. ALVAREZ-RODRIGUEZ, S. NAGY, M. MUTIKAINEN, J. PEIPPO, J. TAPONEN, G. SAHANA, B. GULDBRANTSEN, H. SIMONEN, H. RODRIGUEZ-MARTINEZ, M. ANDERSSON and H. PAUSCH (2019): A splice donor variant in *CCDC189* is associated with asthenospermia in Nordic Red dairy cattle. *BMC Genomics* 20, 286 (<https://doi.org/10.1186/s12864-019-5628-y>).
60. JANUSKAUSKAS, A., A. JOHANNISSON and H. RODRIGUEZ-MARTINEZ (2001): Assessment of sperm quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen-thawed semen from Swedish AI bulls. *Theriogenology* 55, 947-961.
61. JANUSKAUSKAS, A., A. JOHANNISSON and H. RODRIGUEZ-MARTINEZ (2003): Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. *Theriogenology* 60, 743-758.
62. JENKINS, T. G. and D. T. CARRELL (2011): The paternal epigenome and embryogenesis: poisoning mechanisms for development. *Asian J. Androl.* 13, 76-80.
63. JOHNSON, G. D., C. LALANCETTE, A. K. LINNEMANN, F. LEDUC, G. BOISSONEAULT and S. A. KRAWETZ (2011): The sperm nucleus: chromatin, RNA, and the nuclear matrix. *Reproduction* 141, 21-36.
64. KILLIAN, G. J., D. A. CHAPMAN and L. A. ROGOWSKI (1993): Fertility-associated proteins in Holstein bull seminal plasma. *Biol. Reprod.* 49, 1202-1207.
65. KOPPERS, A., G. DE IULIS, J. FINNIE, E. MCLAUGHLIN and R. AITKEN (2008): Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *J. Clin. Endocrinol. Metab.* 93, 3199-3207.
66. KRAWETZ, S. A., A. KRUGER, C. LALANCETTE, R. TAGETT, E. ANTON, S. DRAGHICI and M. P. DIAMONG (2011): A survey of small RNAs in human sperm. *Hum. Reprod.* 26, 3401-3412.
67. LIFFNER, S., I. PEHRSON, L. GARCIA-CALVO, E. NEDSTRAND, S. ZALAVARY, M. HAMMAR, H. RODRIGUEZ-MARTINEZ and M. ALVAREZ-RODRIGUEZ (2019): Diagnostics of DNA fragmentation in human spermatozoa: are Sperm Chromatin Structure Analysis (SCSA) and Sperm Chromatin Dispersion tests (SCD-HalospermG2) comparable? *Andrologia* e13316; doi org/10.1111/and.13316.
68. LORAS, B., F. VETELE, A. EL MALKI, J. ROLLET, J. C. SOUFIR and M. BENAHMED (1999): Seminal transforming growth factor- $\beta$  in normal and infertile men. *Hum. Reprod.* 14, 1534-1539.
69. MACÍAS GARCÍA, B., A. MIRÓ MORAN, L. GONZÁLEZ FERNÁNDEZ, C. ORTEGA FERRUSOLA, A. MORILLO RODRIGUEZ, J. M. GALLARDO BOLAÑOS, C. M. BALAO DA SILVA, H. RODRÍGUEZ-MARTÍNEZ, J. A. TAPIA and F. J. PEÑA (2012): The mitochondria of stallion spermatozoa are more sensitive than the plasmalemma to osmotic induced stress: role of c-Jun N-terminal Kinase (JNKs) pathway. *J. Androl.* 33, 105-113.
70. MARTINEZ-PASTOR, F., A. JOHANNISSON, J. GIL, M. KAABI, L. ANEL, P. PAZ and H. RODRIGUEZ-MARTINEZ (2004): Use of chromatin stability assay, mitochondrial stain JC-1, and fluorometric assessment of plasma membrane to evaluate frozen-thawed ram semen. *Anim. Reprod. Sci.* 84, 121-133.
71. MILARDI, D., G. GRANDE, F. VINCENZONI, I. MESSANA, A. PONTECORVI, L. DE MARINIS, M. CASTAGNOLA and R. MARANA (2012): Proteomic approach in the identification of fertility pattern in seminal plasma of fertile men. *Fertil. Steril.* 97, 67-73.
72. MILLER, D., M. BRINKWORTH and D. ILES (2010): Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction* 139, 287-301.
73. MOCE, E. and J. K. GRAHAM (2008): In vitro evaluation of sperm quality. *Anim. Reprod. Sci.* 105, 104-118.
74. MORRELL, J. M., A. JOHANNISSON, A. M. DALIN, L. HAMMAR, T. SANDEBERT and H. RODRIGUEZ-MARTINEZ (2008): Sperm morphology and chromatin integrity in Swedish warmblood stallions and their relationship to pregnancy rates. *Acta. Vet. Scand.* 50, 2.
75. MORRELL, J. M. and H. RODRIGUEZ-MARTINEZ (2009): Biomimetic techniques for improving sperm quality in animal breeding: a review. *The Open Androl. J.* (Open access) 1, 1-9.
76. MORRELL, J. M. and H. RODRIGUEZ-MARTINEZ (2010): Practical applications of sperm selection techniques as a tool for improving reproductive efficiency. *Vet. Med. Int.* 2011, 894767.

77. MORRELL, J. M., H. RODRIGUEZ-MARTINEZ and A. JOHANNISSON (2010): Single layer centrifugation of stallion spermatozoa selects the most robust spermatozoa from the rest of the ejaculate in a large sample size: data from three breeding seasons. *Equine Vet. J.* 42, 579-585.
78. MORRELL, J. M., A. JOHANNISSON and H. RODRIGUEZ-MARTINEZ (2011a): Effect of osmolarity and density of colloid formulations on the outcome of SLC-selection of stallion spermatozoa. *ISRN Vet. Sci.* 2011,128984.
79. MURATORI, M., S. MARCHANI, L. TAMBURRINO, V. TOCCI, G. FORTI and E. BALDI (2008): Nuclear staining identifies two populations of human sperm with different DNA fragmentation extent and relationship with semen parameters. *Hum. Reprod.* 23, 1035-1043.
80. NAGY, S., T. HALLAP, A. JOHANNISSON and H. RODRIGUEZ-MARTINEZ (2004): Changes in plasma membrane and acrosome integrity of frozen-thawed bovine spermatozoa during a 4h incubation as measured by multicolor flow cytometry. *Anim. Reprod. Sci.* 80, 225-235.
81. NAGY, S., A. JOHANNISSON, T. WAHLSTEN, R. IJÁS, M. ANDERSSON and H. RODRIGUEZ-MARTINEZ (2013): Sperm chromatin structure and sperm morphology: their association with fertility in AI-dairy Ayrshire sires. *Theriogenology* 79, 1153-1161.
82. NAGY, S., T. TAMMINEN, M. ANDERSSON and H. RODRIGUEZ-MARTINEZ (2018): Ejaculated boar spermatozoa displaying a rare multivesicular defect. *Acta Vet. Scand.* 60, 21. doi: 10.1186/s13028-018-0375-7.
83. NOVAK, S., T. A. SMITH, F. PARADIS, L. BURWASH, M. K. DYCK, G. R. FOXCROFT and W. T. DIXON (2010a): Biomarkers of in vivo fertility in sperm and seminal plasma of fertile stallions. *Theriogenology* 74, 956-967.
84. NOVAK, S., A. RUIZ-SANCHEZ, W. T. DIXON, G. R. FOXCROFT and M. K. DYCK (2010b): Seminal plasma proteins as potential markers of relative fertility in boars. *J. Androl.* 31, 188-200.
85. O'FLAHERTY, C., E. DE LAMIRANDE and C. GAGNON (2006): Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. *Free Rad. Biol. Med.* 41, 528-540.
86. OH, S. A., Y. A. YOU, Y. J. PARK and M. G. PANG (2010): The sperm penetration assay predicts the litter size in pigs. *Int. J. Androl.* 33, 604-612.
87. O'LEARY, A., D. T. ARMSTRONG and S. A. ROBERTSON (2011): Transforming growth factor- $\beta$  (TGF $\beta$ ) in porcine seminal plasma. *Reprod. Fertil. Dev.* 23, 748-758.
88. OLIVA, R., S. DE MATEO and J. M. ESTANYOL (2009): Sperm cell proteomics. *Proteomics* 9, 1004-1017.
89. ORTEGA FERRUSOLA, C., L. GONZÁLEZ FERNÁNDEZ, J. M. MORRELL, C. SALAZAR SANDOVAL, B. MACÍAS GARCÍA, H. RODRÍGUEZ-MARTINEZ, J. A. TAPIA and F. J. PEÑA (2009): Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. *Reproduction* 138, 55-63.
90. PACHECO, S. E., E. A. HOUSEMAN, B. C. CHRISTENSEN, C. J. MARSIT, K. T. KELSEY, M. SIGMAN and K. BOEKELHEIDE (2011): Integrative DNA methylation and gene expression analyses identify DNA packaging and epigenetic regulatory genes associated with low motility sperm. *PLoS One* 6, e20280.
91. PARKINSON, T. J. (2004): Evaluation of fertility and infertility in natural service bulls. *Vet. J.* 168, 215-229.
92. PEREZ-PATIÑO, C., I. BARRANCO, I. PARRILLA, E. A. MARTINEZ, H. RODRIGUEZ-MARTINEZ and J. ROCA (2016): Characterization of the porcine seminal plasma proteome comparing ejaculate portions. *J. Proteomics* 142, 15-23.
93. PÉREZ-PATIÑO, C., I. PARRILLA, J. LI, I. BARRANCO E. A. MARTÍNEZ, H. RODRÍGUEZ-MARTÍNEZ and J. ROCA (2018a): The proteome of pig spermatozoa is remodeled during ejaculation. *Mol. Cell Proteomics*, September 26 2018, mcp.RA118.000840; <https://doi.org/10.1074/mcp.RA118.000840>.
94. PEREZ-PATIÑO, C., I. PARRILLA, I. BARRANCO, M. VERGARA-BARBERÁN, S. SIMO-ALFONSO, J. HERRERO-MARTÍNEZ, H. RODRÍGUEZ-MARTÍNEZ, E. MARTINEZ and J. ROCA (2018b): A new in-depth analytical approach of the porcine seminal plasma proteome reveals potential fertility biomarkers. *J. Proteome Res.* 17, 1065-1076; doi: 10.1021/acs.jproteome.7b00728.
95. PEREZ-PATIÑO, C., J. LI, I. BARRANCO, E. MARTINEZ, H. RODRIGUEZ-MARTINEZ, J. ROCA and I. PARRILLA (2019a): The proteome of frozen-thawed pig spermatozoa is dependent on ejaculate fraction source. *Scientific Reports-UK (Nature PG)* 9, 705, doi:10.1038/s41598-018-36624-5.
96. PEREZ-PATIÑO, C., I. BARRANCO, J. LI, L. PADILLA, E. A. MARTINEZ, H. RODRIGUEZ-MARTINEZ, J. ROCA and I. PARRILLA (2019b): Cryopreservation differently alters the proteome in epididymal and ejaculated pig spermatozoa. *Int. J. Mol. Sci.* 20, 1791; doi:10.3390/ijms20071791.
97. PEÑA, F. J., A. JOHANNISSON, M. WALLGREN and H. RODRIGUEZ-MARTINEZ (2003): Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: a new method to evaluate sperm membrane integrity. *Theriogenology* 60, 677-689.
98. PEÑA, F. J., A. JOHANNISSON, M. WALLGREN and H. RODRIGUEZ-MARTINEZ (2005): A new and simple method to evaluate early membrane changes in frozen-thawed boar spermatozoa. *Int. J. Androl.* 28, 107-114.
99. PEÑA, F. J., F. SARAVIA, A. JOHANNISSON, M. WALLGREN and H. RODRIGUEZ MARTINEZ (2007): Detection of early changes in sperm

- membrane integrity pre-freezing can estimate post-thaw quality of boar spermatozoa. *Anim. Reprod. Sci.* 97, 74-83.
100. PEÑA, F. J., H. RODRÍGUEZ-MARTINEZ, J. A. TAPIA, C. ORTEGA FERRUSOLA, L. GONZALEZ FERNÁNDEZ and B. MACÍAS GARCÍA (2009): Mitochondria in mammalian sperm physiology and pathology: a mini-review. *Reprod. Domest. Anim.* 44, 345-349.
  101. PETRUNKINA, A. M. and R. A. P. HARRISON (2013): Fluorescence technologies for evaluating male gamete (dys)function. *Reprod. Domest. Anim.* 48, S11-24.
  102. PETRUNKINA, A. M., D. WABERSKI, A. R. GÜNZEL-APEL and E. TÖPFFER-PETERSEN (2007): Determinants of sperm quality and fertility in domestic species. *Reproduction* 134, 3-17.
  103. PHILLIPS, N. J., M. R. MCGOWAN, S. D. JOHNSTON and D. G. MAYER (2004): Relationship between thirty post-thaw spermatozoal characteristics and the field fertility of 11 high-use Australian dairy AI-sires. *Anim. Reprod. Sci.* 81, 47-61.
  104. PONTING, C. P., P. L. OLIVER and W. REIK (2009): Evolution and functions of long noncoding RNAs. *Cell* 136, 629-641.
  105. RIJSSELAERE, T., A. VAN SOOM, S. THANGHE, M. CORYN, D. MAES and A. DE KRUIF (2005): New techniques for the assessment of canine semen quality: a review. *Theriogenology* 64, 706-719.
  106. ROCA, J., I. PARRILLA, M. A. GIL, C. CUELLO, E. A. MARTINEZ and H. RODRIGUEZ-MARTINEZ (2016): Non-viable sperm in the ejaculate: lethal escorts for contemporary viable sperm. *Anim. Reprod. Sci.* 169, 24-31.
  107. RODRIGUEZ-MARTINEZ, H. (2003): Laboratory semen assessment and prediction of fertility: still utopia? *Reprod. Domest. Anim.* 38, 312-318.
  108. RODRIGUEZ-MARTINEZ, H. (2006): Can we increase the estimative value of semen assessment? *Reprod. Domest. Anim.* 41S2, 2-10.
  109. RODRIGUEZ-MARTINEZ, H. (2007a): State of the art in farm animal sperm evaluation. *Reprod. Fertil. Dev.* 19, 91-101.
  110. RODRIGUEZ-MARTINEZ, H. (2007b): Role of the oviduct in sperm capacitation. *Theriogenology* 68, 138-146.
  111. RODRIGUEZ-MARTINEZ, H. and A. D. BARTH (2007): In vitro evaluation of sperm quality related to in vivo function and fertility. In: *Reproduction in Domestic Ruminants VI*. Edited by Juengel, J. I., Murray J. F., Smith, M. F. Nottingham University Press, Nottingham, UK, pp. 39-54.
  112. RODRIGUEZ-MARTINEZ, H. and B. LARSSON (1998): Assessment of sperm fertilizing ability in farm animals. *Acta Agric. Scand.* 29, 12-18.
  113. RODRIGUEZ-MARTINEZ, H., B. LARSSON and H. PERTOFT (1997): Evaluation of sperm damage and techniques for sperm clean-up. *Reprod. Fertil. Dev.* 9, 297-308.
  114. RODRIGUEZ-MARTINEZ, H., F. SARAVIA, M. WALLGREN, P. TIENHAI, A. JOHANNISSON, J. M. VÁZQUEZ, E. MARTÍNEZ, J. ROCA, L. SANZ and J. J. CALVETE (2005): Boar spermatozoa in the oviduct. *Theriogenology* 63, 514-535.
  115. RODRIGUEZ-MARTINEZ, H., U. KVIST, F. SARAVIA, M. WALLGREN, A. JOHANNISSON, L. SANZ, F. J. PEÑA, E. A. MARTINEZ, J. ROCA, J. M. VÁZQUEZ and J. J. CALVETE (2009): The physiological roles of the boar ejaculate. In: "Control of Pig Reproduction VIII" eds. Rodriguez-Martinez, H., J. L. Vallet, A. J. Ziecik. Nottingham University Press, UK, 2009, pp. 1-21.
  116. RODRIGUEZ-MARTINEZ, H., U. KVIST, J. ERNERUDH, L. SANZ and J. J. CALVETE (2011): Seminal plasma proteins: what role do they play? *Am. J. Reprod. Immunol.* 66, S1, 11-22.
  117. RUIZ-SANCHEZ, A. L., R. O'DONOGHUE, S. NOVAK, M. K. DYCK, J. R. COSGROVE, W. T. DIXON and G. R. FOXCROFT (2006): The predictive value of routine semen evaluation and IVF technology for determining relative boar fertility. *Theriogenology* 66, 736-748.
  118. SAMARDZIJA, M., M. KARADJOLE, M. MATKOVIC, M. CERGOJ, I. GETZ, T. DOBRANIC, A. TOMASKOVIC, J. PETRIC, J. SURINA, J. GRIZELJ and T. KARADJOLE (2006a): A comparison of BoviPure® and Percoll® on bull sperm separation protocols for IVF. *Anim. Reprod. Sci.* 91, 237-247.
  119. SAMARDZIJA, M., M. KARADJOLE, I. GETZ, Z. MAKEK, M. CERGOJ and T. DOBRANIC (2006b): Effects of bovine spermatozoa preparation on embryonic development in vitro. *Reprod. Biol. Endocrinol.* 4:58.
  120. SANCHEZ, V., J. WISTUBA and C. MALLIDIS (2013): Semen analysis: update on clinical value, current needs and future perspectives. *Reproduction* 146, R249-R258.
  121. SARAVIA, F., M. HERNÁNDEZ, M. K. WALLGREN, A. JOHANNISSON and H. RODRÍGUEZ-MARTÍNEZ (2007a): Cooling during semen cryopreservation does not induce capacitation of boar spermatozoa. *Int. J. Androl.* 30, 485-499.
  122. SARAVIA, F., I. NÚÑEZ-MARTÍNEZ, J. M. MORÁN, C. SOLER, A. MURIEL, H. RODRÍGUEZ-MARTÍNEZ and F. J. PEÑA (2007b): Differences in boar sperm head shape and dimensions recorded by computer-assisted sperm morphometry are not related to chromatin integrity. *Theriogenology* 68, 196-203.
  123. SHAMSUDDIN, M. and H. RODRIGUEZ-MARTINEZ (1994): A simple, non-traumatic swim-up method for the selection of spermatozoa for in vitro fertilization in the bovine. *Anim. Reprod. Sci.* 36, 61-75.
  124. SUH, R. S., X. ZHU, N. PHADKE, D. A. OHL, S. TAKAYAMA and G. D. SMITH (2006): IVF within microfluidic channels requires total numbers and lower concentrations of sperm. *Hum. Reprod.* 21, 477-483.
  125. TAMBURRINO, L., S. MARCHIANI, M. MONTOYA, F. E. MARINO, I. NATALI, M. CAMBI, G. FORTI, E. BALDI and M. MURATORI

- (2012): Mechanisms and clinical correlates of sperm DNA damage. *Asian J. Androl.* 14, 24-31.
126. TARDIF, S., J. P. LAFOREST, N. CORMIER and J. L. BAILEY (1999): The importance of porcine sperm parameters on fertility in vivo. *Theriogenology* 52, 447-459. 125.
127. THUNDATHIL, J., J. GIL, A. JANUSKAUSKAS, B. LARSSON, L. SADERQUIST, R. MAPLETOFT and H. RODRÍGUEZ-MARTÍNEZ (1999): Relationship between the proportion of capacitated spermatozoa present in frozen-thawed semen and fertility with artificial insemination. *Int. J. Androl.* 22, 366-373.
128. TIENHAI, P., M. YOKOO, N. KIMURA, P. HELDIN, E. SATO and H. RODRÍGUEZ-MARTÍNEZ (2003): Immunohistochemical localization and expression of the hyaluronan receptor CD44 in the porcine oviductal epithelium during oestrus. *Reproduction* 125, 119-132.
129. VICENTE-CARRILLO, A., M. ÁLVAREZ-RODRÍGUEZ and H. RODRÍGUEZ-MARTÍNEZ (2016): The mu ( $\mu$ ) and delta ( $\delta$ ) opioid receptors modulate boar sperm motility. *Mol. Reprod. Dev.* 83, 724-734.
130. VICENTE-CARRILLO, A., M. ÁLVAREZ-RODRÍGUEZ and H. RODRÍGUEZ-MARTÍNEZ (2017): The CatSper channel modulates boar sperm motility during capacitation. *Reprod. Biol.* 17, 69-78. doi: 10.1016/j.repbio.2017.01.001.
131. WANG, W., G. T. LIANG, Y. Y. PENG, D. Y. LIU and X. M. ZHOU (2011): Effects of a microfluidic sperm sorter on sperm routine parameters and DNA integrity. *Zhonghua Nan Ke Xue* 17, 301-304.
132. YAMAUCHI, Y., J. A. SHAMAN and W. S. WARD (2011): Non-genetic contributions of the sperm nucleus to embryonic development. *Asian J. Androl.* 13, 31-35.
133. ZHANG, B. R., B. LARSSON, N. LUNDEHEIM and H. RODRÍGUEZ-MARTÍNEZ (1997): Relation between embryo development in vitro and 56-day non-return rates of frozen-thawed semen from dairy AI bulls. *Theriogenology* 48, 221-231.
134. ZHANG, B. R., B. LARSSON, N. LUNDEHEIM and H. RODRÍGUEZ-MARTÍNEZ (1998): Sperm characteristics and zona pellucida binding in relation to field fertility of frozen-thawed semen from dairy AI bulls. *Int. J. Androl.* 21, 207-216.
135. ZHANG, B. R., B. LARSSON, N. LUNDEHEIM, M. G. HÅÅRD and H. RODRÍGUEZ-MARTÍNEZ (1999): Prediction of bull fertility by combined in vitro assessments of frozen-thawed semen from young dairy bulls entering an AI-program. *Int. J. Androl.* 22, 253-260.
136. ŽURA ŽAJA, I., A. SLUGANOVIĆ, M. SAMARDŽIJA, S. MILINKOVIĆ-TUR, T. DOBRANIĆ, S. STRELEC, D. ĐURIČIĆ, H. VALPOTIĆ and S. VINCE (2019): The effects of oxidative stress on the male reproductive system and mechanisms of antioxidant protection. *Vet. stn.* 50, 43-54. (In Croatian).

## Ocjena ejakulata: može li se procijeniti plodnost?

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Ovaj pregledni članak pruža kritički osvrt na standardne i inovativne metode za procjenu kakvoće sjemena kako bi se predvidio oplodni potencijal ejakulata (nativnog ili obrađenog) i mužjaka. Funkcionalna testiranja spermija u *in vitro* uvjetima su nužna, budući da se slični procesi odvijaju tijekom transporta spermija, pohrane i interakcije sa ženskim spolnim sustavom i oocitama, jer pružaju uvid u ulogu pojedinih subpopulacija spermija u ejakulatu i time pomažu kod odabira spermija. Nadalje, ovaj se članak isto tako

usredotočuje na istraživanje genoma, transkriptoma i proteoma spermija i sjemene plazme što može pomoći pri otkrivanju kako spermiji i molekule sjemene plazme mogu dati signal ženki i povezati to s plodnošću pa čak i s brojem potomaka. Specifične molekule sjemene plazme koje se razlikuju između jedinki između pojedinih frakcija ejakulata, povezane su s preživljavanjem spermija kao i s majčinom imunomnogom tolerancijom prema spermijima što omogućuje plodnosti.

**Ključne riječi:** analiza sjemena, kakvoća sperme, metode *in vitro*, procjena plodnosti