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Review article

Reproductive physiology of the boar: What defines the potential fertility of an ejaculate?

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ABSTRACT

Despite decades of research and handling of semen for use in artificial insemination (AI) and other assisted reproductive technologies, 5–10% of selected boar sires are still considered sub-fertile, escaping current assessment methods for sperm quality and resilience to preservation. As end-product, the ejaculate (emitted spermatozoa sequentially exposed to the composite seminal plasma, the SP) ought to define the homeostasis of the testes, the epididymis, and the accessory sexual glands. Yet, linking findings in the ejaculate to sperm production biology and fertility is suboptimal. The present essay critically reviews how the ejaculate of a fertile boar can help us to diagnose both reproductive health and resilience to semen handling, focusing on methods -available and under development- to identify suitable biomarkers for cryotolerance and fertility. Bulk SP, semen proteins and microRNAs (miRNAs) have, albeit linked to sperm function and fertility after AI, failed to enhance reproductive outcomes at commercial level, perhaps for just being components of a complex functional pathway. Hence, focus is now on the interaction sperm-SP, comparing *in vivo* with *ex vivo*, and regarding nano-sized lipid bilayer seminal extracellular vesicles (sEVs) as priority. sEVs transport fragile molecules (lipids, proteins, nucleic acids) which, shielded from degradation, mediate cell-to-cell communication with spermatozoa and the female internal genital tract. Such interaction modulates essential reproductive processes, from sperm homeostasis to immunological female tolerance. sEVs can be harvested, characterized, stored, and manipulated, e.g. can be used for andrological diagnosis, selection of breeders, and alternatively be used as additives to improve cryosurvival and fertility.

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1. Introduction

The meeting entitled "Deep Freezing of Boar Semen" (Johnson and Larsson, 1985) held at SLU-Uppsala on August 25–27, 1985 gathered researchers aiming to disclose why fertility with cervically artificially inseminated (AI) frozen-thawed boar ejaculated spermatozoa could not be improved despite fourteen very active years of research, following the birth of living offspring by three geographically distant groups (Crabo and Einarsson, 1971; Graham et al., 1971; Pursel and Johnson, 1971). The participants were sadly aware of the fact that unless fertility was improved, research in the area was to decrease, despite industry demands of using frozen semen for gene spreading. They knew the problem was dual; the freezing process was complex and led to low survival rates of the thawed spermatozoa (25–30%) which, in turn, resulted in low fertility after AI, obviously requiring a review of the methods involved. The goal of reaching similar fertility as with liquid semen is still far, or even utopic, but already at that initial meeting some cues were put forward to study: the interactions between spermatozoa, seminal plasma (SP) and extenders during cooling, and the overall need to maintain membrane structure and function as intact as possible. Sounds familiar? It should, and the series of meetings of the International Conference on Boar Semen Preservation (ICBSP) following that initial conference (II-1990-Beltsville, III-1995-Mariensee, IV-1999-Beltsville, V-2003-Doorwerth, VI-2007-Ontario, VII-2011-Bonn, VIII-2015-Champaign, IX-2019-New South Wales-Australia), have highlighted the same problems, yet with just few of them being solved, at least partially, over time. One take home-message by the organizers of the 1985-meeting had a striking impact on us: "*Always adjust techniques to basic biology*" (Johnson and Larsson, 1985). Pointing out that "*Once the biological limitations are known, a working procedure can be designed*" implied that we need to know more about the production of mature spermatozoa and the building of semen, to wide diagnostics of sperm function in relation to semen handling pressure. Moreover, with fertility depending on the interaction of semen with the female genital tract, exploring this interplay was mandatory. Alternative methods had to be further studied and improved, including the use of chilled semen, of vitrified spermatozoa or even of coated-cells, and they projected a period of 5–10 years for glimpses of success ahead, a goal yet to be reached.

2. The past 40 years of boar semen research: back to basics

Research during this interval provided interesting results along with the development of novel technologies as sperm cleansing, sexing, manipulation, etc. Do we know better? Yes, but not in all aspects. People still consider freezing-thawing of boar semen as *quasi experimental*, with low sperm cryosurvival and a shortened life-span among surviving cells (Roca et al., 2006). It is thus not surprising that <1% of boar semen is used as frozen AI-doses, while above 93% of females are inseminated with liquid semen (Mellagi et al., 2023). Breeding boars considered good and bad freezers as well as siblings with varying fertility have always been present, and we need to detect those most fertile. Is it then wise to think we can use the same technique to freeze semen from any boar, as we do with liquid semen? Do we need to know more about how that handled semen interacts with the female during and beyond insemination?

The overwhelming fact, perhaps pivotal, is that we are still looking for markers in a physiologically fractioned ejaculate that could foresee both the normality of the gonads and the post-gonadal maturation organs, as well as the fertility after AI. The goal is to distinguish the better boars from the good ones. Many have been the attempts to describe the pig ejaculate (Rodríguez-Martínez et al., 2009), and the relevance of using specific fractions against the increasing use of bulk-ejaculate collection (Alkmin et al., 2014; Pérez-Patiño et al., 2019a), but… has any of the -omics (proteomics, genomics, epigenomics, transcriptomics, metabolomics, etc.) provided better answers explaining fertility after AI and/or other assisted reproductive technologies (ARTs)? Has the interplay between semen and female genital tract delivered suitable biomarkers? Difficult is to grasp practices to ameliorate the man-made climatic changes affecting pig reproduction, including how to best raise, select and handle the sires as well as to prepare and use their AI-semen under this context. Perhaps most interesting is the evident track-back to basics in many of the areas displayed as concerns for nearly 40 years ago: how do we judge normality of semen production and make best use of the indicators our results provide? Answers reside in disclosing where the biological limitations for potential fertility are. Surely, we need to look back to the ejaculate and decipher how components interplay in relation to sperm resilience to *ex-corpore* handling and fertility.

The present invited essay, therefore, aims to critically review how the ejaculate of a fertile boar can help us decipher diagnostics of reproductive health, focusing on methods, available and on development, that could advance identification of suitable biomarkers for (dys)function and fertility prognosis, applicable at commercial level. Not less relevant, we focus on sperm resilience after handling and cryopreservation (Yeste, 2016). One of the aspects considered is the interaction of spermatozoa with SP, comparing *in vivo* with *ex vivo* -omics screenings. The update is, obviously, not exhaustive enough to allocate the enormous amount of research performed during the past 40 years, most of which has been presented -one way or another- in the past nine ICBSP-meetings, and which shall likely renew and advance in the current volume.

3. The normality of boar genital anatomy and physiology is reflected in the ejaculate

The most relevant pre-requisite for a genetically selected stud boar is the production and delivery of large numbers of spermatozoa in the ejaculate depicting attributes required to attain high fertility, two inherent concepts, not necessarily positively related to each other. In general, sires undergo rigorous reproductive controls to ensure they deliver ejaculates with a mean of 50×10^9 total spermatozoa, most cells depicting linear progressive motility (>70%) and normal morphology (>75%). With this basic information in the ejaculate we assume the boar has normal spermatogenesis and sperm maturation, thus being potentially fertile (Rodriguez-Martinez, 2014), but -as we shall describe latter- without knowing to what level. In fact, 5–10% of the highly selected breeding boars show fertility outcomes below breed average and are thus considered sub-fertile (Roca et al., 2015). This evidences our inability to reliably

prognose levels of fertility among stud boars, often due to its multifactorial nature and the additional confounding factors involved, from boar management to environmental stud conditions. More comprehensive semen analyses, including omics are apparently needed.

3.1. Testicular sperm production

The anatomy of the organs involved in sperm production and the endocrinological interplay controlling male behavior has been extensively reviewed (Bonet et al., 2013). Likewise, has Flowers recently made an excellent review of the factors affecting ejaculate production (season, photoperiod, nutrition, semen collection frequency, management, etc.) (Flowers, 2022). Boar sperm production obviously concerns the complex process of spermatogenesis (Swierstra, 1968). The seminiferous tubules, surrounded by a basal lamina and peritubular myoid cells are embedded in a capillary-rich interstitial stroma of connective tissue including macrophages and cells of the immune system and massive conglomerates of Leydig cells (LC). The pulsatile production/delivery of testosterone and other androgens by the LCs floods into the seminiferous tubules to modulate spermatogenesis (Berndtson, 2014). The looped tubules contain the seminiferous epithelium with somatic Sertoli cells (SC) and different stages of spermatogenic germ cells, from basally located diploid stem spermatogenic cells (SSCs) to ad-luminal haploid elongated spermatids (Swierstra, 1968). Spermatogenesis is cellularly highly organized within a section of the tubule, with a high degree of interaction between the SC and the spermatogenic cells, alongside all stages of the process (Fig. 1 a-b). The SC supports germ cells, both architectonically (allocating cells within branches of cytoplasmic projections) and building unique microchambers for each cell stage. Adjacent SC build lateral junctional complexes (tight junctions, desmosomes, gap junctions, ectoplasmic specializations, tubulo-bulbar complexes) separating two compartments: a basal containing the SSCs, committed undifferentiated spermatogonia and early primary spermatocytes; and an ad-luminal, containing meiotic/post-meiotic differentiation stages with spermatocytes (I and II) and spermatids (round to elongated). The tight junctions thus define a SC-barrier for immunologically foreign meiotic cells, constituting the main component of the hemo-testicular barrier (HTB) together with the endothelial continuous capillary bed (Luaces et al., 2023). Spermatogenesis can be separated into three processes where different events occur in different metabolic milieu: spermatocytogenesis, meiosis (I and II) and spermiogenesis, which shall end up in the delivery of testicular spermatozoa to the lumen (spermatoteleosis), all guided by the SCs. The SCs are responsible for the nutrition and hormonal control of the process of meiosis and subsequent differentiation, and for the selective regulation of the transport of relevant growth factors (i.e. GDNF, bFGF, IGF1, CSF1, WNT5A, LIF, retinoic acid, etc.) and other molecules (Garcia and Hofmann, 2015; Voigt et al., 2023) through the lateral junctional complexes. The niches they form are also characterized by different pathways controlling cellular metabolism, the basal ones being mainly glycolytic (anaerobic, converting glucose to pyruvate) contrasting to the ad-luminal niche where oxidative phosphorylation (i.e., aerobic) dominates (see the recent excellent article by (Voigt et al., 2023). Not only do these different metabolic pathways seem to promote the survival of SCCs and SC under hypoxic situations, but they also warrant the highly polarized SC to rule a constant flow of glycolysis-derived lactate/pyruvate to the developing germ cells, creating a metabolically unique, aerobic mitochondrial oxidative phosphorylation ad-luminal milieu (Voigt et al., 2023).

Spermatogenesis is thus a complex process that includes mitotic proliferation of spermatogonia and formation of early spermatocytes, production of haploid round spermatids, chromatin condensation and nuclear shaping, removal of excess cytoplasm, and formation of the acrosome and sperm tail (Salilew-Wondim et al., 2020). A histological section of a boar testis depicts eight different stages of spermatogenesis (França et al., 2005; Swierstra, 1968), where each stage (in a transversal section, Fig. 1 a-b) contains four or five layers of germ cells associated according to a specific pattern, each representing a cell generation derived from a B spermatogonia. Each committed spermatogonium entering spermatogenesis gives rise to 64 testicular spermatozoa, 50 µm-long cells consisting of a head and a tail with specific structures (Briz and Fàbrega, 2013). Spermatozoa enter the lumen of the dual-opened seminiferous tubules

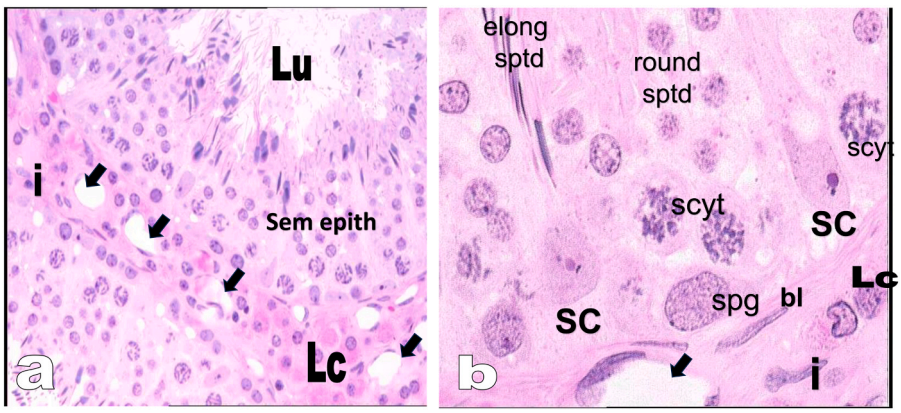

Fig. 1. a-b: Histological sections of boar testis. In a, a partial view of two tubuli separated by the interstice (i) with numerous Leydig cells (Lc) and empty blood vessels (arrows, the testis was fixed by vascular perfusion). Sem epith: seminiferous epithelium, Lu: tubule lumen. In b, a higher magnification of the seminiferous epithelium showing the major cell types (SC: Sertoli cells, spg: spermatogonia, scyt: spermatocytes, round sptd: round spermatids, elong sptd: elongated spermatids; arrow: blood vessel; bl: basal lamina; Lc: Leydig cell (H&E, Photo: H. Rodriguez-Martinez).

Table 1
Some significant biomarkers for sperm function and fertility in the boar ejaculate.

Biomarker	*Source*	*Function*	*Reference*
Proteins			
Acrosin-binding	Sperm	Plays a role in the capacitation pathway and	(Vilagran et al., 2013)
Ras-related protein	Sperm	Regulates sperm capacitation	(Kwon et al., 2014)
Osteopontin 70-	Accessory	During ejaculation binds to sperm interacting with	(Hao et al., 2006)
Heat Shock Protein	Sperm	Increases sperm thermal resistance and protects	(Casas et al., 2010)
Porcine seminal plasma proteins (PSP I, PSP II)	SP	Prevent premature capacitation and the acrosome reaction	(Rutherfurd et al., 1992)
Spermadhesin	SP	Mediates sperm-zona pellucida binding	(Rodríguez-Martinez et
Spermadhesin AQN-	SP	Mediates sperm–oviduct interactions	(Manásková et al.,
βmicroseminoprotein	SP	Potential sperm motility inhibitor	(Manásková et al.,
Lipocalin-type prostaglandin D-synthase (L-PGD-S)	SP-SPF	Relevant for sperm maturation	(Rodriguez-Martinez et al., 2011)
Enzymes			
Glutathione S-	Sperm	Prevents lipid membrane peroxidation, a process	(Kwon et al., 2015)
Glutathione	Sperm	Inhibits acrosome exocytosis during sperm storage	(Michos et al., 2021)
Glutathione	SP	Positively related to *in vivo* (AI) fertility	(Barranco et al., 2016)
TAC/total antioxidant capacity	SP	Positively related to *in vivo* (AI) fertility	(Barranco et al., 2015d)
Immune system components			
Interferon-gamma (IFN-γ)	SP	Positively related to increased sperm membrane permeability	(Barranco et al., 2019b)
Interleukine-6 (IL-6)	SP	Positively related to sperm ROS generation	(Barranco et al., 2019b)
Hormones			
Oxytocin	SP	Positively related to in vivo (AI) fertility	(Padilla et al., 2021)
miRNAs			
Ssc-miR-182	Sperm-SPF	Associated to high sperm motility and intact structure	(Curry et al., 2011)
Ssc-miR-1285	Sperm-SRF	Related to sperm production	(Alvarez-Rodriguez et al., 2020)
Ssc-miR-191	Sperm-EpiTS	Possible modulation of intercellular communication within the epididymis	(Martinez et al., 2022)
Ssc-miR-92	Sperm-SRF	Potential implication in maternal reproductive tract response	(Alvarez-Rodriguez et al., 2020)
Ssc-miR-122	SP exosomes	Influence spermatocyte development and maturation	(Zhao et al., 2024)
DNA methylation			
ROPN1L	Sperm	Related to sperm motility	(Pértille et al., 2021)
LMX1A	Sperm	Related to sperm motility	(Pértille et al., 2021)
FOX11	Sperm	Sperm numbers, motility, capacitation	(Pértille et al., 2021)
IFT172	Sperm	Spermiogenesis defects	(Pértille et al., 2021)
ULK2 & TMEM126B	Sperm	Anti-apoptotic functions	(Pértille et al., 2021)
ITMB2B	Sperm	Sperm capacitation	(Pértille et al., 2021)

Abbreviations: SP: seminal plasma, SPF: sperm-peak fraction, EpiTS: epididymis terminal segment, SRF: sperm-rich fraction. (Barranco et al., 2019; Casas et al., 2010; Hao et al., 2006; Kwon et al., 2014; Manásková et al., 2003; Manjarín et al., 2015; Michoset al., 2021; Rutherfurd et al., 1992; Vilagran et al., 2013; Zhang et al., 2023; Zhao et al., 2024; Zhu et al.,2021).

asynchronously, providing an overall continuous sperm release. This cyclic release of spermatozoa of each segment along the duct defines the spermatogenetic wave, with spermatozoa passively transported in a constant flow of testicular fluid. Boar testicular spermatozoa leave the epithelium every 8.6 days, thus taking 44 days for a spermatozoon to develop from a B spermatogonia (França et al., 2005; Malmgren et al., 1996).

Such delicate processes as the recombination of genomic material during meiosis or the dramatic differentiation changes exposed by the haploid spermatids during spermiogenesis (Berndtson, 2014) can deviate from normal. A faulty spermiogenesis can result in morphological abnormalities easily recognized among ejaculated spermatozoa (Rodriguez-Martinez and Barth, 2007). Other modifications escape morphological evaluations, being sub-cellular as the intactness of the chromatin (Didion et al., 2009) or defects in the masking of accessibility of the DNA for transcription. The latter include changes in chromatin configuration, DNA methylation, histone modifications, and the action of non-coding RNAs (ncRNAs), all regulating gene expression at fertilization -and beyond -without disrupting the DNA sequence and defining the epigenome, which "simply" regulates transcription (L. Zhu et al., 2021). Up to 90% of the genome is transcribed to some extent, depending on the availability of protein-coding messenger RNAs (mRNAs). These mRNAs are barely 1–2% of the total RNAs present in the spermatozoon, the rest being ncRNAs either classified into "housekeeping" RNAs (e.g., ribosomal RNAs, transfer RNAs, small nuclear RNAs, small nucleolar RNAs), as "regulatory" RNAs (e.g., small non-coding RNAs (sncRNAs), or as long non-coding RNAs (lncRNAs) involved in modulating gene expression both during spermatogenesis and after fertilization (Alvarez-Rodriguez et al., 2021; L. Zhu et al., 2021). The sncRNAs involved in sperm production include endogenous small interfering RNAs (endo-siRNAs), PIWI-interacting RNAs (piRNAs) and microRNAs (miRNAs, single-strand RNAs of 20–25 nucleotides). These sncRNAs are categorized depending on the involvement of the RNase III endonuclease DICER in their biogenesis. Endo-siRNAs and miRNAs are DICER-dependent, whereas piRNAs are DICER-independent, but predominantly expressed in tissues involved in male germ line development. DICER is essential for miRNA processing during haploid differentiation of germ cells, being relevant for stage-specific transcription during spermatogenesis, but also as post-transcriptional developmental regulators by binding -fully or partially- to the 3'-UTR of target mRNA. becoming associated to fertility (Martinez et al., 2022; Salilew-Wondim et al., 2020; Yadav and Kotaja, 2014).

Ejaculated spermatozoa carries intact, healthy attributes (proteins, mRNAs, DNA methylation, histone modification, and miRNAs) that can be retrospectively related to the observed sire fertility (Rodriguez-Martinez, 2019; Rodriguez-Martinez and Larsson, 1998). Genomic-Wide Association Studies (GWAS) have aided to identify candidate genes associated with semen traits, including sperm numbers, motility and morphology (Gao et al., 2019; Y. Zhang et al., 2023; Zhao et al., 2020), rightly the same traits we currently routinely evaluate and that often reflect an acceptable level of sperm chromatin intactness (Khezri et al., 2019). The identification of miRNAs in ejaculated spermatozoa would both help disclose the roles of specific miRNAs during spermatogenesis as well as to indicate relations to fertility.

Fertility, a major phenotypic difference among males, has increased during decades of selective breeding. Fertility depends on genetic variation (i.e., via single nucleotide polymorphisms, SNPs) (Krupa et al., 2023; Wang et al., 2023) as well as modifications of epigenetic factors. The latter include mostly environmentally induced (including management factors) changes at histone level, DNA methylation of CpG dinucleotides and/or the action of ncRNAs which are "memorized" by the offspring (Casas and Vavouri, 2014). We have recently identified genetic variation through GWAS in parallel with epigenetic differences of Differentially Methylated Regions (DMR) combining Genotyping by sequencing and methylated DNA immunoprecipitation in the genome of spermatozoa from stud boars with good semen attributes and specific and well-documented differences in fertility (farrowing rate) and prolificacy (litter size), categorized as with high (HF), low (LF) and unknown fertility (Pértille et al., 2021). A total of 165,944 SNPs were identified and explained 14–15 % of variance among selection lines, while 58 % of the variance between HF and LF could be explained by 169 SNPs at $P \leq 0.00015$ level. The greatest differences in DMRs between HF- and LF-boars across the genome appeared located in chromosomes 3, 9, 13, and 16; most DMRs being hypermethylated in LF-boars, with significant seasonal variation. These non-invasive methylome analyses on ejaculated spermatozoa discerning fertility levels in stud boars can be applied for detailed andrological diagnosis to aid sire selection via genomic differences, even among breeding lines (Table 1). It clearly waives using repeated biopsies damaging the well-vascularized testicular capsule (Ohanian et al., 1979) and the HTB, affecting sperm production.

We have characterized the differential abundance of miRNAs in ejaculated pig spermatozoa collected from three different fractions of the pig ejaculate comparing breeding HF- and LF-boars after AI, using high-output small RNA sequencing. Four sperm miRNAs were identified (miR-182, miR-1285, miR-191, and miR-96) which target genes playing key roles in fertility, sperm survival or immune tolerance, and whose expression differed between HF- and LF-boars (Martinez et al., 2022). Again, we seem now capable of monitoring spermatogenesis by screening RNA material present in ejaculated spermatozoa and relevant for their fertility (Table 1). Furthermore, recent relevant findings have summarized the screening of blood-circulating miRNAs specifically linked to pig spermatogenesis (Y. Zhang et al., 2023). Some of the miRNAs (miR-10a, miR-125b, let-7 f, miR-186) were highly expressed in pig spermatogonia, pachytene spermatocytes, round spermatids, and spermatozoa (Chen et al., 2017). Likewise, SC could be followed by not less than 18 miRNAs, including miR-7173, miR-217, miR-362, miR-202, and miR-149 (Chen et al., 2020), reinforcing our capacity to non-invasively monitor spermatogenesis in boars.

3.2. Post-gonadal sperm maturation

The so-called testicular spermatozoa (free elongated spermatids) have negligible motility and capacity to fertilize. Their sperm head (1/10th of the length) is flat (racket-like) and consists, apart from the nucleus, of an acrosome containing molecules involved in the binding to and the penetration of the glycoprotein-rich zona pellucida (ZP) during fertilization. The tail, which attaches to the sperm head via an implantation fossa (where an undifferentiated centriole is located), consists of a flagellum surrounded in the mid-

piece of about 80 mitochondria of a fibrous column-like sheath in the principal piece, to become “naked” by the tapered end piece (Briz and Fàbrega, 2013). The entire spermatozoon is covered by a "regionalized" plasma membrane, defining specific and distinguishable subdomains of a lipid bilayer including structural and attached proteins that can anchor and adsorb external proteins, peptides and other molecules (Tsai and Gadella, 2009). As soon as the testicular spermatozoa freely enter the tubular lumen, the membrane sub-domains are subjected to modifications of surface proteins, in preparation for events during fertilization. The most dramatic changes occur in the subdomains of the sperm head (Tsai and Gadella, 2009) particularly the apical ridge, considered to be in capacitated spermatozoa bearing an intact acrosome the sole area that -via specific receptors- able to recognize the ZP three constituent glycoproteins (ZPGs, ZP2, ZP3, ZP4), binding to a heterocomplex of ZP3 and ZP4) (Yonezawa et al., 2012). Such concept has been challenged by experiments using intact cumulus-cell coated oocytes inseminated with acrosome-reacted spermatozoa retrieved from the peri-vitelline space of zygotes, implying that other subdomains can be involved (Buffone et al., 2014). Once bound, the acrosome reaction occurs, which facilitates ZP-penetration and entry to the peri-vitelline space and binding to the oolemma. The large pre-equatorial domain, covering the front 2/3 parts of the acrosome participates in the acrosome reaction, while the adjacent equatorial segment recognizes and fuses to the oolemma during fertilization. The rest of the plasmalemma covering the neck, the midpiece and the rest of the sperm tail is also heterogeneous and participates in other events related to interaction with the surroundings and the regulation of sperm motility (Gautier and Aurich, 2022; Lehti and Sironen, 2017). Hence, testicular spermatozoa are membrane-interactive under the influence of numerous molecules, free or included in extracellular vesicles (EVs), from the testis to the ejaculate (Roca et al., 2022). The major process of maturation towards fertilization capacity is registered along the epididymis.

3.3. *Sperm maturation and storage in the epididymis*

The testicular spermatozoa are transported to the *rete testis* as immature cells, unselected in terms of defective morphology, negligible motility, and lack of fertilizing capacity (Crabo, 1985). They thereafter enter the 60–65 m long, highly convoluted epididymal duct. The organ is anatomically divided into caput, corpus and cauda areas which only partially relate to the functional events better defined as initial, middle, and terminal segments; where testicular fluid resorption, sperm maturation respectively storage of mature spermatozoa occur (Glover and Nicander, 1971) (Fig. 2). While the major rate of removal of defective spermatozoa is done in the rete testis and the initial segment, it continues along the epididymis alongside increases of the spermatocrit. The increasing bolus of the so-far immotile spermatozoa is moved forward by peristaltic contractions issued by the smooth muscle beneath the epithelium. We have recently reviewed the development, anatomy, and overall physiology of the boar epididymis (Rodriguez-Martinez et al., 2022). Very remarkable modifications occur during this10–12 days long transport all conspicuously occurring in the middle epididymal segment (Glover and Nicander, 1971) (Fig. 2). Here, the spermatozoa mature morphologically, which includes nuclear compaction by inherent reduction of disulfide bridges between cysteine residues of protamines, cytoskeletal rearrangements, and displacement of the cytoplasmic droplet from a proximal neck location to a distal annular placement. As well, subtle membrane modifications occur with evident changes in the composition of proteins, leading to the achievement of forward motility and, particularly, the acquisition of fertilizing capacity (Rodriguez-Martinez et al., 1990a). The estrogen- and androgen-dependent pseudostratified lining epithelium of the duct builds a protective epididymal-blood barrier, providing peripheral immune tolerance to testicular spermatozoa despite displaying xeno-antigens (Pleuger et al., 2020). In spite of its apparent morphological simplicity, the

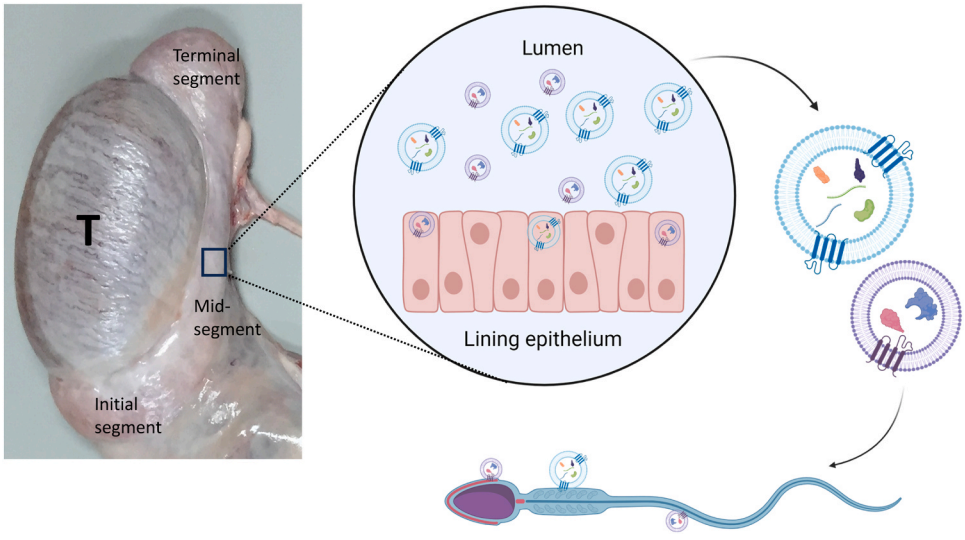

Fig. 2. Sperm maturation in the boar epididymis occurs mainly in the mid-segment, where the lining epithelium releases extracellular nanovesicles, containing lipids, proteins and nucleic acids that can interact with the epithelium itself (autocrine action), bind to maturing spermatozoa or stay in the epididymal fluid. T: testis, initial segment at the epididymis head, terminal segment at the cauda and the adjacent first segment of the ductus deferens. Organs in scrotal position, with the epididymis tail dorsally (Created with BioRender.com).

epithelium rules electrolyte and pH luminal fluid changes, and produces novel proteins and sncRNAs (Rodriguez-Martinez et al., 2022), modifying luminal spermatozoa during epididymal transit. Also in this segment, the cholesterol content of the sperm membrane decreases along with an increase in the amounts of long-chain polyunsaturated fatty acids (PUFAs), changes related to the increase in membrane fluidity that characterizes mature spermatozoa (Gautier and Aurich, 2022). Through mechanisms of both merocrine and apocrine secretion, the lining epithelium sequentially modifies the multiple generations of spermatozoa that leave the testis to accumulate as "mature" spermatozoa in the terminal segment (cauda epididymis and the adjacent first convoluted segment of the ductus deferens). In this terminal segment (Glover and Nicander, 1971) (Fig. 2), spermatozoa are kept metabolically quiescent and immotile, having their inbuilt instability of the plasmalemma/acrosome suppressed (Rodriguez-Martinez et al., 2022). The terminal segment displays a different protein and electrolyte composition than the rest of the epididymis, blood plasma, or testicular fluid (Einarsson, 1971). For example, osmotic pressure is high (330–360 mOsm) (Einarsson, 1971), pH is low (ca. 6) and bicarbonate levels are 10-fold lower than in blood (Rodriguez-Martinez et al., 1990b) contributing, together with a low oxygen content and high spermatocrit, to immobilize spermatozoa until ejaculation (Rodriguez-Martinez, 1991).

In pig epididymal spermatozoa, many sncRNAs, including miRNAs, piRNAs, yRNA and tRNA-derived small RNAs (tsRNAs), are transcripts originating in the testes selectively retained in the epididymis (Martinez et al., 2022). These transcripts are maintained in ejaculated spermatozoa (Alvarez-Rodriguez et al., 2020; Curry et al., 2011; Kasimanickam and Kastelic, 2016) and beyond (through pregnancy days 9–15) as miR-92b-3p and miR-17–5p (Zhou et al., 2020).

Further, the epididymal lining epithelium can deliver EVs (also called epididymosomes) to the lumen. These lipid bilayer nanovesicles, still ill-characterized in the pig and other species (Parra et al., 2023), are considered a major mechanism for the transfer of biomolecules, including proteins, to the epididymal spermatozoa (Barrachina et al., 2022) (Fig. 2). Among other known biomolecules affecting maturing spermatozoa, we can include the cytokine macrophage migration inhibitory factor (MIF) as a ruler of motility; lipoproteins, hyaluronan receptor CD44, tetraspanins, nucleic acids and many specific miRNAs, promoting oxidation-reduction and metabolic changes in spermatozoa (Alvarez-Rodriguez et al., 2021, 2019b; Barranco et al., 2019a; Roca et al., 2022; Rodriguez-Martinez et al., 2022). Epididymosomes follow the emitted spermatozoa at ejaculation, included in the small (2–5 %) contribution of caudal fluid to the ejaculate. Once a part of the ejaculate, epididymosomes might play another role, affecting sperm motility and fertilizing capacity. For instance epididymosomes could, by transferring their contents to the lining epithelium of the internal genital tract of the female (Padilla et al., 2023) influence the local immune responsiveness towards tolerance of sperm-xeno antigens (Alvarez-Rodriguez et al., 2019a; Rodriguez-Martinez et al., 2022, 2021) or even modulate post-translational events of epigenetic nature in relation to pregnancy and fertility (Martinez et al., 2020, 2019; Martinez and Rodriguez-Martinez, 2022). As we shall describe later, the presence and fold-change of specific miRNAs associated with caudal or ejaculated spermatozoa suggest these would be also valuable as non-invasive biomarkers for sperm maturation, function and, ultimately, for fertility.

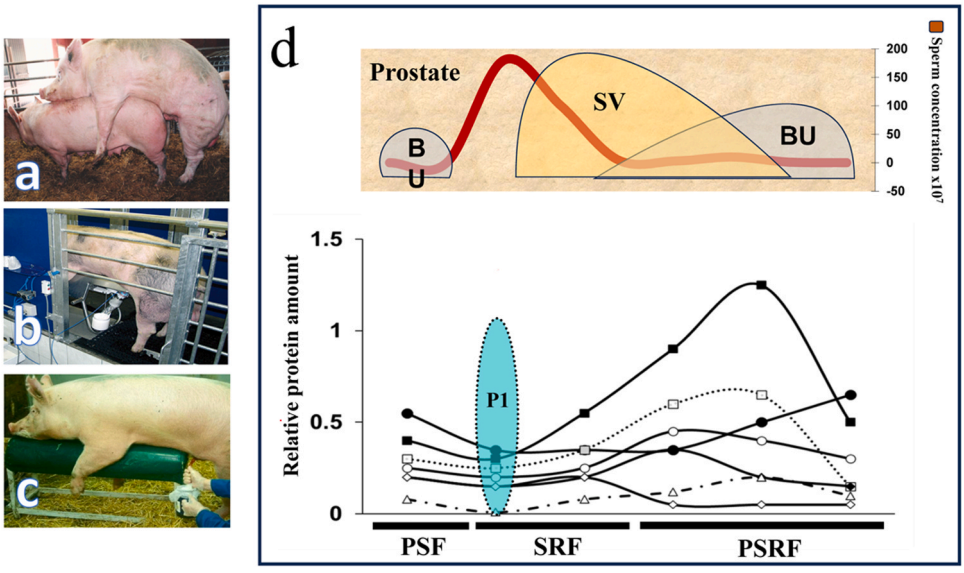

Fig. 3. a-d: The boar emits an ejaculate in fractions, even during natural mating (a), but the ejaculate can be collected while mounting a dummy, either as bulk (b) using a semi-automatic device or in three major separated fractions during operator-manual collection (c). Fractions are named pre-sperm (PSF), sperm-rich (SRF), where its initial 10–12 mL constitutes the sperm-peak portion (P1, ca. 25% of the total sperm numbers) and the postSRF (PSRF), where a clotting-gel secretion of the bulbourethral glands (BU) is finally voided (d, upper panel). The accessory sexual glands secrete variable amounts during ejaculation (d, upper panel), some constantly (prostate) and some in specific fractions (e.g. seminal vesicles (SV, protein-rich) and the BUs, in relation to the emission of spermatozoa (red line). The relative amounts of large proteins (mostly spermadhesins) in seminal plasma are presented in (d, lower panel) (●: PSP-I, ■: PSP-II, ▫: AQN-1, Δ: AQN-3, ◆: AWN-1, O: AWN-2, ◇: inhibitor of acrosin/trypsin) as collected in consecutive samples of the boar ejaculate.

4. The ejaculate: how composition of fractions rules sperm function and resilience

The voluminous boar ejaculate (250–300 mL for an adult boar) consists of spermatozoa emitted in small amounts of caudal fluid (2–5%) and suspended in a fluid derived from secretions of the accessory glands where seminal vesicles provide 15–20%, the bulbourethral glands 10–25% and the prostate the rest of the gel-free part of the SP. Different substances in the ejaculate can be used as markers to trace back origin and function. For example, the cauda epididymis fluid contains glyceryl phosphorylcholine (GPC) and alkaline phosphatase, the prostate secretion contains large amounts of electrolytes, especially sodium and chloride, the seminal vesicle secretion contains ergothioneine, inositol, citric acid, and fructose as well as the majority of the ejaculate proteins, while the bulbourethral glands secretes large amounts of sialic acid (Einarsson, 1971; Mann and Lutwak-Mann, 1981; Rodriguez-Martinez et al., 2009). Ejaculation is fractionated, i.e., the epididymis and the accessory glands leave their secretions in a certain succession during natural mating (Fig. 3 a) and the same occurs during a semi-automatic collection of the bulk ejaculate (Fig. 3 b). Collecting semen manually (Fig. 3 c) allows the collection of three easily identified fractions, namely the clear pre-sperm-rich fraction (PreSRF) that mainly originates from the prostate and the bulbourethral glands containing some gel and a heavy degree of contamination by cell debris, urine and smegma from the preputium. The following fraction is the most easily recognized, the creamy-white sperm-rich fraction (SRF) mainly originating from the epididymis and the prostate. Finally, the post-sperm-rich fraction (PostSRF, PSRF) that goes from greyish to watery in aspect, with a decreasing sperm content alongside increasing amount of seminal vesicle secretion to mainly register, towards the end, a jelly-rich, tapioca-like floccular secretion from the bulbourethral glands; signaling the end of the ejaculation process (Rodriguez-Martinez et al., 2009). This jelly-like secretion is considered to form a SP-coagulating plug in the cervix and in the corpus uterus after natural mating to limit the backflow from the uterus. If not removed during *ex-corpore* collection, it will eventually coagulate the semen. The components of the different fractions obviously mirror the gland secretions involved, preSRF contains mostly electrolytes, Na and Cl (urethral and prostate-dominated), the SRF increasing amounts of proteins but also of steroid hormones (testosterone and estrogens), GPC, fructose, glucose, inositol, citrate, bicarbonate and zinc (representing the caudal contents and the prostate); whereas the postSRF has increasing amounts of proteins, bicarbonate and Zn (seminal vesicles), and of Na, Cl and sialic acid (bulbo-urethral glands) (Mann and Lutwak-Mann, 1981).

Comparison of the ejaculate fractions provide a clear view that sperm numbers are the greatest in the SRF decreasing steadily thereafter, alongside the PostSRF (Rodriguez-Martinez et al., 2009) (Fig. 3 d, upper panel). Of interest is that the large amounts of proteins of the boar SP, almost 40 mg/mL (Rodriguez-Martinez et al., 2011), where the large spermadhesins follow a contrasting pattern to sperm numbers; proteins dominate the postSRF fraction (Rodriguez-Martinez et al., 2009) (Fig. 3 d, lower panel). Over the past decade, a sperm peak-portion/fraction has been defined in terms of sperm numbers as the first 10 mL of the SRF (P1) where up to a 25% of the spermatozoa in the ejaculate are emitted, and where the amounts of spermadhesins are distinctly lowest (Rodriguez-Martinez et al., 2009) (Fig. 3 d, lower panel). Over the years, it has also become evident that spermatozoa in this particular SRF-portion depict the highest resilience to sperm handling, including cryosurvival (Alkmin et al., 2014; Hossain et al., 2011; Peña et al., 2006; Saravia et al., 2009; Siqueira et al., 2011) and could constitute the vanguard sperm subpopulation reaching the oviductal sperm reservoir *in vivo* (Wallgren et al., 2010). This higher sperm homeostasis might relate to their fortuitous location a sperm peak-fraction with very low levels of spermadhesins (see Fig. 3 d, lower panel) and half of the amount of bicarbonate, a molecule known to destabilize the plasmalemma of the boar sperm (Rodriguez-Martinez, 1990) present in the postSRF (Saravia et al., 2009). A note of caution is to be raised here, the SP is -*in vivo*- in differential contact to spermatozoa, often briefly (for instance the vanguard spermatozoa are exposed mainly to prostate secretion). The SP placed by natural mating is removed from the female uterus and does not necessarily enter the oviduct beyond the sperm reservoir even if specific SP-proteins are attached to the plasmalemma phospholipids (Müller et al., 2023) and follow spermatozoa to the oocyte, participating in fertilization (Caballero et al., 2005; Rodríguez-Martinez et al., 1998). A different situation is present when either specific fractions (as the SRF) are collected and then extended with buffers, or the entire ejaculate is collected and spermatozoa exposed to a mixture of secretions, not necessarily close to the *in vivo* situation. This clarification becomes a point when considering that most analyses of the ejaculate are done *ex-corpore*, after semen is collected, fractionated, or in bulk.

Classical (Mann and Lutwak-Mann, 1981) and more detailed recent studies of proteomic (Pérez-Patiño et al., 2019b, 2018), transcriptomic (Rodriguez-Martinez et al., 2021), genomic and metabolomic (Mateo-Otero et al., 2021) evaluations confirm that the boar SP contains free organic and inorganic components (Barranco et al., 2015a), hormones (Padilla et al., 2021), and large and small proteins and peptides (Pérez-Patiño et al., 2018). Some of these components, including metabolites, have been associated to sperm homeostasis and fertility and could thus be considered valuable biomarkers (see Table 1) for fertility and sperm cryotolerance. Specific components as the nerve growth factor-ß (NGF-ß), can stimulate ovulation (Robertson and Martin, 2022), and SP proteins can maintain the stability of the sperm plasma membrane and participate in the interaction of sperm with female lining epithelium and the oocyte vestments during fertilization (Rodriguez-Martinez et al., 2021). Some enzymes can keep reactive oxygen species (ROS) levels at physiological limits (Barranco et al., 2017, 2015c; Parrilla et al., 2020) or -as the proteins PSP-I and -II- stimulate invasion of polymorphonuclear leukocytes (PMNs) to counteract eventual pathogens and to eliminate defective and surplus spermatozoa and foreign proteins via phagocytosis (Rodriguez-Martinez et al., 2010). Fragile and short-lived cytokines (Barranco et al., 2019a; Padilla et al., 2020a, 2020c) can influence the local female reproductive tract immune system to either tolerate or reject antigen-bearing spermatozoa, embryos and placentae (Alvarez-Rodriguez et al., 2020, 2019b; Martinez et al., 2020). Semen, either by spermatozoa or SP components can dramatically change the expression of genes related to sperm function to warrant fertilization and conceptus development (Alvarez-Rodriguez et al., 2019a; Álvarez-Rodríguez et al., 2020; Martinez et al., 2020; Schjenken and Robertson, 2020), thus signaling the female immune system, the ultimate ruler for fertility. Perhaps the most interesting of these findings is the fact that, both *in vitro* (Barranco et al., 2020) and *in vivo*, gene expression changes appear in the mucosa of the internal genital tract of sows. *In*

vivo changes appear already after insemination in the pre- or peri-ovulatory period, as detected 24 h post mating or infusion with SP (Alvarez-Rodriguez et al., 2019a; Atikuzzaman et al., 2017) and are present along the different segments of the uterus, oviduct and particularly, of the sperm reservoir, a clearly immune-privileged compartment for vanguard spermatozoa (Rodriguez-Martinez, 2007). While expression of miRNAs (Álvarez-Rodríguez et al., 2024) and of crucial oxidative-reductive transcripts (Álvarez-Rodríguez et al., 2023) also occur in the lining epithelium, the overall findings confirm earlier assumptions that semen, or even the SP *per se*, could clearly modulate the genital immunology of the female pig. When exposure to SP is done before a mating (Flowers and Esbenshade, 1993; Robertson, 2007; Rodriguez-Martinez et al., 2009; Rozeboom et al., 2000) it appears to condition the tolerance to paternal antigens which, later on, may shape the fate of maternal receptivity to the hemi-allogeneic embryo (Waberski et al., 2018), promote the survival of the embryos and their placentae (Martinez and Rodriguez-Martinez, 2022). Moreover, SP infusion before AI was shown to enhance the expression of endometrial genes and pathways involved in embryo development (Gil et al., 2024; Martinez et al., 2020).

Some different SP proteins may be related to sperm performance and fertility (Table 1). For instance, fibronectin-1 and N-acetyl-ß-hexosaminidase could be markers of sperm freezability (Vilagran et al., 2015; Wysocki et al., 2015) while particular proteins can be related to fertility (Perez-Patiño et al., 2016; Pérez-Patiño et al., 2018) (Table 1). Lipocalins (related to retinol transport, and prostaglandin synthesis, (Flower, 1996), lipocalin-type prostaglandin D-synthase or epididymal secretory protein-1, present in the sperm-peak portion have been related to boar fertility (Flowers, 2001). Glutathione peroxidase 5 (GPX5), an hydrogen peroxide (H_2O_2)-scavenging enzyme present at the highest levels in the SP-SRF, was positively associated with farrowing rate and litter size (Barranco et al., 2016; Novak et al., 2010). Similarly, paraoxonase type 1, a hydrolytic enzyme with protective capacity against oxidative stress particularly present in the sperm-peak portion was also positively related to farrowing rate (Barranco et al., 2015c) (Table 1). In contrast, the spermadhesin porcine SP-protein -I (PSP-I) was found negatively correlated with litter size (total piglets born) (Novak et al., 2010). Of interest, testing heterospermic breeding with high quality semen from stud boars displaying different concentrations of specific SP-proteins, revealed the 25.9 kDa/5.9 pI SP-protein as explaining 66% of the highly significant variation observed in the proportion of pigs sired within a litter among boars (Flowers et al., 2013, 2016). This particular protein, either possibly being transforming growth factor-β (TGFß active form, with a 26 kDa) or the serine protease 55 (F. Zhu et al., 2021), could promote fertility. Such effect could be effected by ensuring the mated/inseminated spermatozoa would maintain function in boars having large concentrations of this protein compared to sires which do not, or that could have more proteins with deleterious effects, for instance PSP-I (Novak et al., 2010). In any case, it will depend on the fraction of SP considered, as the relative proteins amounts are greater in some fractions than in others (Pérez-Patiño et al., 2018).

The boar SP contains secreted biomolecules enclosed within seminal EVs (sEVs) (Fig. 4) (Foot and Kumar, 2021; Roca et al., 2022) in comparatively higher numbers than in blood (Skalnikova et al., 2019) which, secreted mainly by the epididymal Ms (Fig. 4 a) and

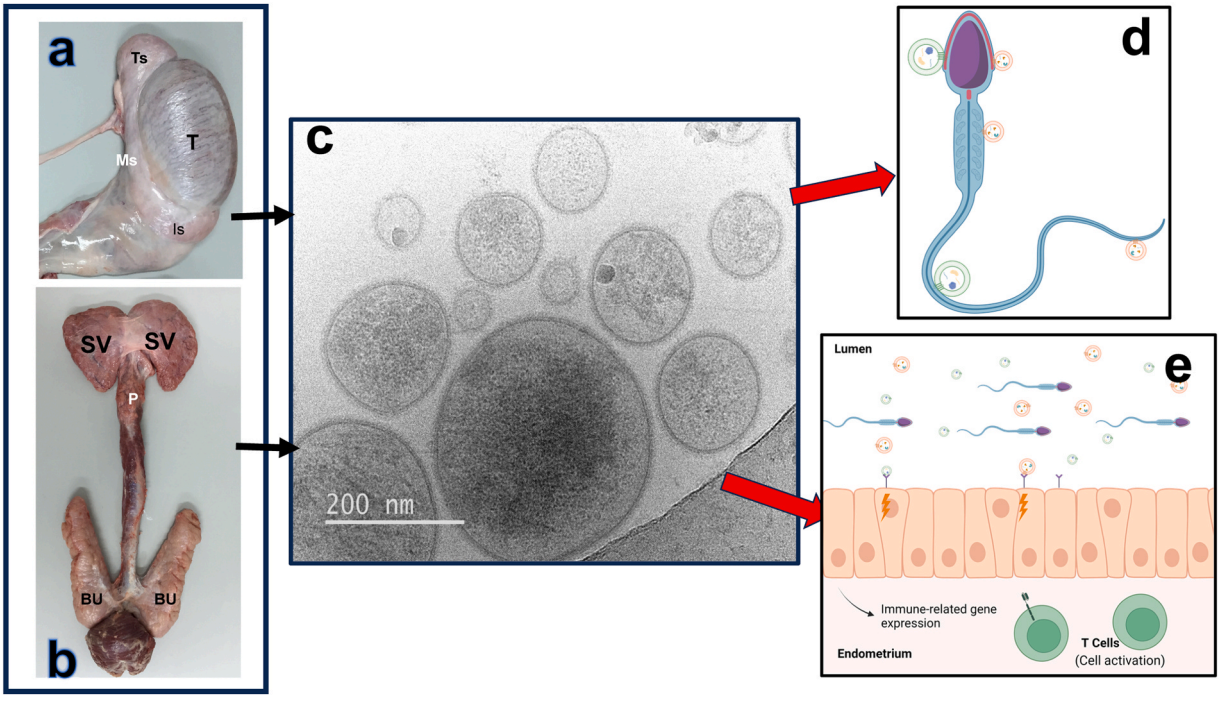

Fig. 4. a-e: The mid segment of the pig epididymis (a, Ms), and the prostate (b, P) secrete nano-sized lipid bilayer pleomorphic (large or small) seminal extracellular vesicles (sEVs, c, Cryo-SEM of ejaculated sEVs isolated using size exclusion chromatography, SEC) holding a molecular cargo that besides affecting sperm maturation in the epididymis, can also influence the maturing/mature spermatozoa (d) and the female genital tract (e) affecting gene expression of immune genes. T: testis, Is: initial segment of epididymis, Ts: terminal segment, SV: seminal vesicles, BU: bulbourethral glands.

the prostate (Fig. 4 b) affect both the spermatozoa and the genital tract of mated female (Rodriguez-Martinez and Roca, 2022)(Fig. 4 d-e). The EVs are defined according to their biogenesis as either exosomes (vesicles 30–100 nm released from cytoplasmic multivesicular bodies) or ectosomes (100–1000 nm size out-budded plasma membrane vesicles) (Fig. 4 c) (Gurunathan et al., 2022; Jeppesen et al., 2023). Either type contains high contents of cholesterol and sphingomyelin (Piehl et al., 2013), lipids involved in the cryosurvival of boar spermatozoa. The EVs encapsulate fragile biological components i.e., lipids, signaling proteins, small non-coding and regulatory RNAs (Barranco et al., 2023). Proteins are also present in SP in free form, as already mentioned, being prone to rapid degradation upon exposure to SP proteases. Other enzymes present in SP, such as nucleases, can easily degrade other molecules, such as free RNAs. Such degradation of SP-free molecules can explain the irregular or even absent response to SP when used as an additive to improve sperm resilience, function, cryosurvival, or even fertility after AI (Rodriguez-Martinez and Roca, 2022). Boar sEVs are, on the other hand, able to survive collection, handling, and storage; preserving their contents (Barranco et al., 2024, 2023). The sEVs are able to interact (add, fuse, internalize) with spermatozoa (Roca et al., 2022) or the female genital tract epithelium (Aleksejeva et al., 2022), releasing to them loaded molecules to exert specific actions on spermatozoa, affecting capacitation and fertilizing capacity (Andrade et al., 2022) or even modifying the immune response of the female genital tract when exposed to paternally derived antigens (Tamessar et al., 2021), (Fig. 4d-e). In this regard, porcine sEVs carry cytokines (Padilla et al., 2023) and small ncRNAs, including miRNAs (nearly 300 identified, representing 9% of total RNA) such as the fertility-related ssc-miR-10b (Xu et al., 2020); (Alvarez-Rodriguez et al., 2020), and the modulation of immune responses by cytokines (Barranco et al., 2020)(Table 1). In fact, miRNAs found in boar spermatozoa (ssc-miR-503) or within small sEVs (ssc-miR-130a, ssc-miR-9) have been related to low sperm cryotolerance in boar semen, thus arising as potential "negative" markers for cryosurvival and hence, for decreased fertility (Pedrosa et al., 2021).

Besides epididymosomes, EVs of other origins (most likely the prostate, i.e. prostasomes; eventually the seminal vesicles) are also present in the SP. This type of distinction seems to be mostly academic. Because semen fractions are *per se* composites of secretions (see Fig. 3 d, upper panel), such distinctions are difficult or even impossible requiring of intensive studies of rather difficult secretion isolates. Thus, it might be wise to define the EVs present in SP as one pleomorphic entity: sEVs (Parra et al., 2023). Due to their pleomorphism (Fig. 4 c), we should focus on concentrating research efforts on their phenotypic and compositional characterization to further elucidate eventual function roles (Roca et al., 2022) (Fig. 6 a). It should be noted, nonetheless, that the population of sEVs in a pig ejaculate, albeit heterogeneous in size and proteins (Barranco et al., 2019a), can be differentiated in subpopulations differing in proteomic load (Barranco et al., 2023) and probably also in RNA-cargo and lipid composition. Methods for isolation and characterization of EVs are evolving (Barranco et al., 2023; Chernyshev et al., 2023).

5. The ejaculate and fertility: what are we (or should be) looking for?

A classical, routine semen analysis, such as those performed in boar studs basically includes determining sperm numbers, motility and in some cases even morphology of some defects (cytoplasmic droplets, tail defects). These variables are not only readily assessed, but they display a clear, basic relation to fertility: a certain number of potentially fertile spermatozoa is needed for fertilization (Alm et al., 2006). Over the years, the industry has moved from inseminating high sperm numbers towards the use of smaller AI-doses, containing down to 1–2 $\times$ 10^9 spermatozoa (Roca et al., 2011), making more evident differences in fertility among boars, differences apparently masked by the previous use of high sperm numbers (Mellagi et al., 2023). Likewise, this decrease in sperm numbers is done by a higher extension of the sperm suspension, i.e. decreasing the amount of SP present in the AI-dose. Considering previous statements, this disclosure of fertility differences among boars might be dual, including spermatozoa and the SP.

Andrological evaluations have in the past identified boars with evident flaws in their spermiogram that would, a priori, yield low fertility. In other words, basic evaluations of aspect, volume, pH, sperm count (concentration, total number), sperm motility (mass motility and individual motility), and sometimes, sperm morphology (unstained/stained, vital dyes (sperm nigrosine) and even survival resistance *in vitro*… have been valuable, and some of these analyses are still routinely used, as they provide a basic view of a "healthy" ejaculate, and thus could help discriminate between sires, and separate those potentially less fertile (Fig. 5). We are, however, aware that despite passing the thresholds of these evaluations, there is still a 5–10% of stud sires displaying sub-fertility, i.e., their fertility is lower than of the breed, batch and even among siblings (Roca et al., 2015). Use of newer methods including particle quantifiers for sperm numbers (Sevilla et al., 2023), Computer-assisted sperm analyses (CASA) for detailed kinematics, Computer-assisted sperm morphology analyses (ASMA) for morphology, use of fluorophores and flow cytometry (FC) for analyses of membrane intactness and for location of specific molecules related to essential events (as capacitation or acrosome exocytosis, (Keller and Kerns, 2023) have increased our capacity for semen evaluation, of value in academic settings and often in relation to intensive diagnosis or research (Hossain et al., 2011; Maside et al., 2023; Peña and Rodriguez-Martinez, 2023; Rodriguez-Martinez, 2014). Transcriptomics have been one of the most advanced techniques, for its association to male fertility (Indriastuti et al., 2022). Likewise, immunological evaluations have been pivotal for the characterization of sperm membrane proteins and the contents of EVs (Barranco et al., 2023; Rodriguez-Martinez, 2019)(Fig. 5). Genomics, transcriptomics, epigenomics, proteomics (and even metabolomics for the metabolic-poor spermatozoon) have revolutionized sperm research, by allowing the determination of gene changes/mutations during spermatogenesis, and of induction of changes in loads of DNA/RNA/ncRNA or different families during the post-testicular transport along the epididymis and ejaculation. In consequence, we now know more on the impact of methylation of DNA during spermatogenesis on fertility, more about the impact of small and large proteins in the ejaculate, and of the relevance of the interchange of the load of various molecules between the EVs of the seminiferous and lining epithelia with spermatozoa, to name a few consequences.

But which are the most relevant biomarkers for sperm resilience and fertility? Biomarkers are per definition molecules of different types that are present in semen and that ought to reflect their relevance for a certain sperm function, acquired during sperm production and maturation, but ultimately having a strong relation to fertility, irrespective if they are lipids, proteins, enzymes, or RNA molecules.

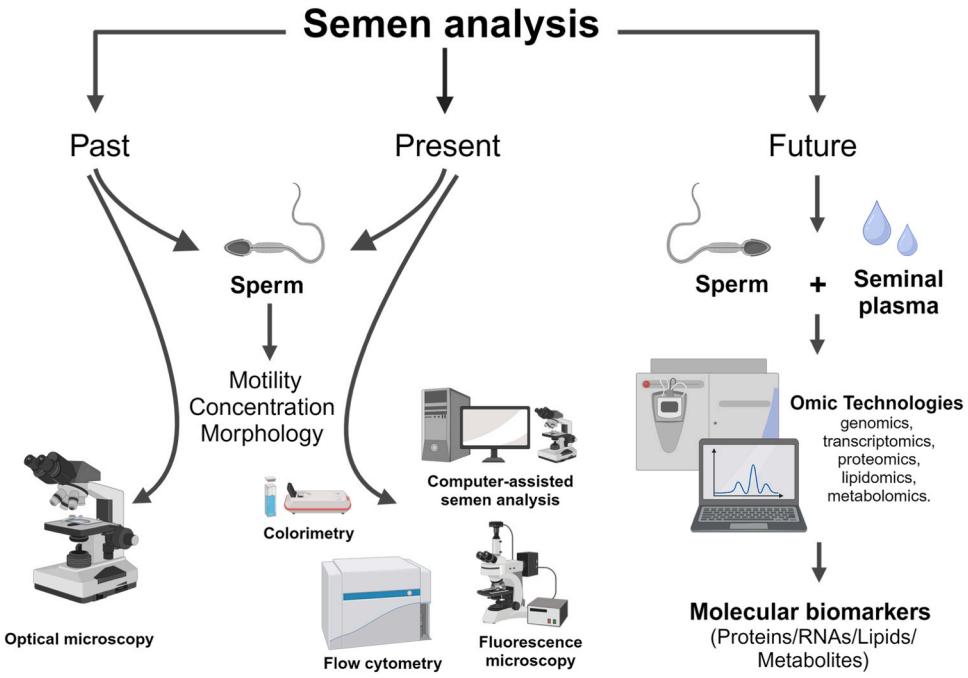

Fig. 5. Overview of past, current and eventual future methods for semen analyses. Most past analyses, often those routine at commercial level have focused on the spermatozoa, and had been mostly operator-dependent. Currently, the increase in computer-aided equipment have made examinations more objective (CASA, FC, ASMA, fluorophores) but still concern spermatozoa. Future methods (some of them already established) shall focus further on the relation sperm-SP intending disclosure of biomarkers for molecules (proteins/enzymes, RNAs or lipids) relevant to explain potential for capacitation and fertilization and with a significant association to fertility (Created with BioRender.com).

A short list of published significant biomarkers for fertility in boar semen is presented (Table 1), a list that is prompt to be revised by future research.

A fact we described at the start of this essay was the large variation among boars in their capacity to withstand the process of cryopreservation. Quite early, it was established that the prevalence of some lipids in the boar plasma membrane contributed to such variation (Buhr et al., 1994; Maldjian et al., 2005). The changes in lipid composition arisen during sperm maturation in the epididymis, with an increase in PUFA contents make boar spermatozoa quite susceptible to ROS-attack, in the eventual absence of SP-antioxidant enzymes, as when the semen is extended in simple buffers. Today, after assaying different ways of modifying the relative contents of lipids (Kasimanickam and Buhr, 2016), it is reported that both lipid metabolism levels and long-chain PUFAs are main contributors to cryotolerance and therefore suggested that oleic acid and some PUFAs can become candidate biomarkers for cryosurvival (X. Zhang et al., 2023). Considering the destabilization of the sperm membrane as one of the relevant signs for impending capacitation, sperm capacitation has been again been put forward as a rightful biomarker for fertility (Keller and Kerns, 2023; Rodriguez-Martinez and Barth, 2007).

Unfortunately, there are not too many proteomic studies that have compared the semen protein set of fertile and sub-fertile boars to identify candidate proteins as fertility biomarkers (Roca et al., 2020). Among the few existing studies, it is worth mentioning that of Perez-Patiño et al. (Pérez-Patiño et al., 2018), comparing the SP-proteome of boars with clear differences in fertility after AI of more than 25,000 sows (>100 sows inseminated per boar). This study identified 11 proteins that were quantitatively different between boars with high and low farrowing rates, and four proteins that were quantitatively different between boars with large and small litter sizes. Notably, some of the proteins identified as differentially abundant have never been associated with reproductive functions. For example, sphingomyelin acid phosphodiesterase 3 A, a protein found to be more abundant in boars with high farrowing rates, that regulates cholesterol, affects cell differentiation, and is involved in immune regulation (Shin and Chung, 2023; Zhang et al., 2022). These findings reinforce the relevance of omics to identify fertility biomarkers in semen, as they highlight novel or unexpected molecules involved in molecular pathways that regulate fertility. Other proteins that were found with potential fertility prediction as biomarkers include triosephosphate isomerase, calcium-binding messenger protein calmodulin, and mitochondrial malate dehydrogenase-2-NAD in non-capacitated spermatozoa (Kwon et al., 2015). Other proteins to be highlighted are those with clear antioxidant activities in SP (Song et al., 2024), defining an easily measurable total antioxidant capacity significantly related to fertility (Table 1) (Barranco et al., 2015d; Nedić et al., 2023).

Apart from interferon-gamma (IFN-γ) which is positively related to increased sperm membrane permeability (Barranco et al., 2019a) (Table 1), the boar SP contains at least other fourteen measurable cytokines (Barranco et al., 2015b), small-size proteins involved in cellular events and the immune system, playing crucial and different roles in modulating reproductive processes. These SP-cytokines are primarily produced in the testis, seminal vesicles, the prostate gland, and the epididymis (Fraczek and Kurpisz, 2015),

in concentrations varying across ejaculate fractions and seasons (Barranco et al., 2015b; Padilla et al., 2020b) and exerting multi-faceted effects on reproductive physiology and fertility, and by inducing either pro-inflammatory or anti-inflammatory responses, contributing to immune modulation within the female reproductive tract and facilitating successful pregnancy (Dai et al., 2023; Parrilla et al., 2020). The transforming growth factor (TGF)-β_1 isoform of the multifunctional cytokine TGF-β (Robertson et al., 2002) is found in high concentrations in boar SP (O'Leary et al., 2011), and has been infused intra-cervically to sows before insemination, without significant effects on either sow pregnancies or prolificacy (Parrilla et al., 2022), confirming previous negative results (Rhodes et al., 2006). This lack of effectiveness may be attributed to the interconnected networks in which seminal plasma cytokines operate, rather than on individual effects (Fraczek and Kurpisz, 2015). On the other hand, cytokine supplementation, either individually or in combination, to IVP media has shown positive effects across various species, increasing maturation rates, embryo quality, and cryotolerance (Javvaji et al., 2023; Oh et al., 2022; Wooldridge et al., 2019). For example, adding leukemia inhibitory factor to pig oocyte maturation medium improved maturation rates and quality of the blastocysts (Dang-Nguyen et al., 2014). A cocktail of fibroblast growth factor 2, leukemia inhibitory factor, and insulin-like growth factor-1 enhanced pig oocyte maturation and blastocyst development, resulting in a fourfold increase in the number of genetically modified piglets (Yuan et al., 2017). Also, the addition of interleukin-6 to the embryo culture medium improved embryo development in pigs (Yin et al., 2020). These findings suggest that cytokine supplementation could be a feasible strategy to enhance IVP outcomes, but their generic use as biomarkers for fertility remains elusive.

We have found a series of miRNAs in boar spermatozoa (ssc-miR-362, ssc-miR-486, and ssc-miR-122–5p) involved in the regulation of sperm motility through a series of different pathways, particularly the mitogen-activated protein kinase (MAPK) signaling (Martinez et al., 2022) (Table 1). More recently, studies of sEV-coupled miRNA profiling have enabled the diagnostics of semen quality in stud boars, aiding more biomarkers to our current arsenal (Dlamini et al., 2023).

Although we have certainly advanced in our capacity to identify molecules with a clear relation to fertility (after AI or *in vitro*) we must remark that none of these biomarkers have relevance unless they can be easily identified/quantified/validated in samples of semen in an easy manner, so that do not remain (as today) a simple academic question. Once again, we need to understand the biology behind it to be able to design a methodology, but in the case of biomarkers we even need to refine the methods to the very end, considering that we still need to preserve spermatozoa to improve industry-based breeding. It is thus logical to consider a major difference between using fertility-related biomarkers in spermatozoa versus those present in SP, even when both can be diagnostic in nature.

How about intending to increase fertility? One could think of using sperm selection for those spermatozoa carrying the biomarker in a general sperm population, and then use only an enriched sperm sample, but this scenario is somewhat distant. Free proteins, enzymes, and cytokines presently identified in SP could, on the other hand be isolated and used as additives. Yet, their routine application in other ARTs as AI, has so far been deceiving (Parrilla et al., 2022). The use of 10–20% of bulk SP from high fertility or good freezer boars (Hernández et al., 2007; Recuero et al., 2019; Rozeboom et al., 2000) has, likewise, provided variable results.

On the other hand, the use of sEVs as additives has been tested *in vitro* (Rodriguez-Martinez and Roca, 2022) and *in vivo* (Bai et al.,

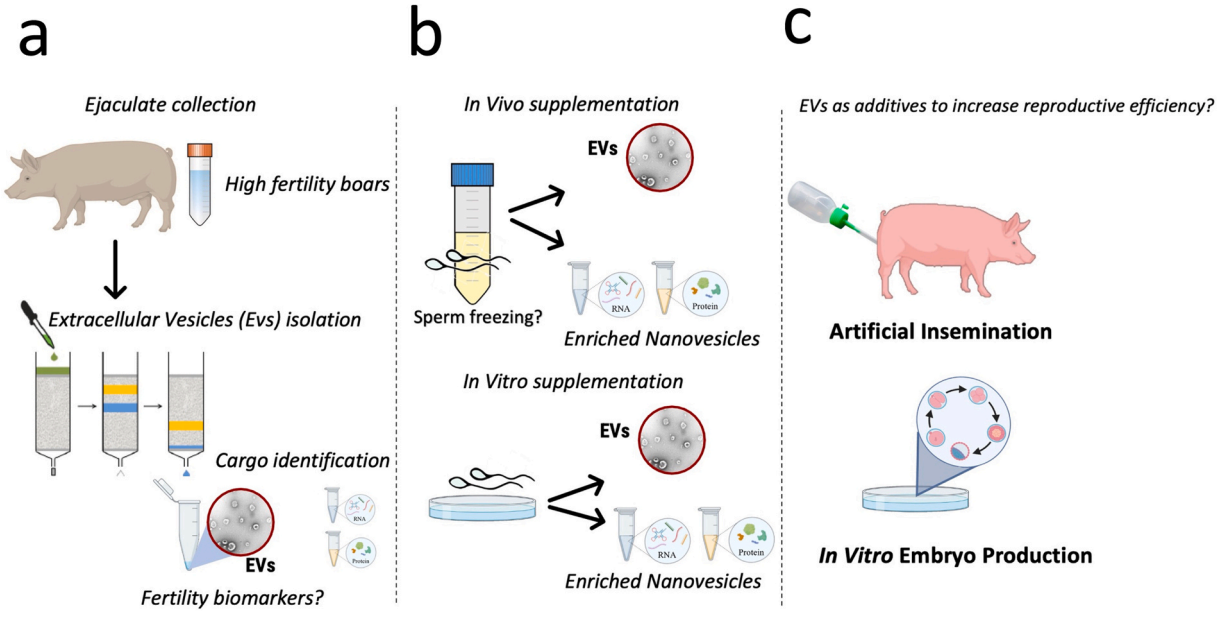

Fig. 6. a-c: Seminal Extracellular Vesicles (sEVs) from the boar ejaculate, can be isolated, their constituent molecules characterized, and used as biomarkers (Panel a). Native sEVs or enriched lipid nanovesicles can be used for *in vivo* or *in vitro* supplementation, including for sperm freezing (Panel b). The EVs can be relevant for use as additives during artificial insemination or *in vitro* embryo production (Panel c). (Created with BioRender.com).

2018; Godakumara et al., 2022), yielding promising results facing the use of harvested and characterized sEVs from high-fertile boars (Fig. 6, panel a) to promote fertility *in vitro* and *in vivo* (by enriching AI-semen doses with sEVs) (Fig. 6, panels b-c). Considering sEVs can modulate gene expression of pig cumulus cells *in vitro* (Mateo-Otero et al., 2022) alternatives are also foreseen, including the enrichment of harvested sEVs with specific biomolecules or the laboratory preparation of lipid nanovesicles filled with specific cargos (Fig. 6, panel b) (Roca et al., 2022; Rodriguez-Martinez and Roca, 2022), including the transfer of CRISPR/Cas9 (Horodecka and Düchler, 2021). The ultimate goal is to employ suitable additives that are to easy to prepare, stored, and use on a routine basis.

6. Future considerations

What has advanced in our understanding of the reproductive function of a breeding boar over the time elapsed during the ICBSP meetings? Plenty, as exposed above, and much more is expected. We are now able to determine, using novel proteomic, (epi)genomic and transcriptomic analyses of the end-product: the semen, not only the status of the testes, the epididymis, or the accessory sexual glands, but even considering prognosis of fertility, by way of eventual biomarkers. Besides diagnostics and potential fertility prognosis, we now have the opportunity of using semen additives, such as specific molecules (proteins, peptides, enzymes, etc.) or of EVs and their valuable cargo, which is protected in nanovesicles that can be harvested, stored, and even manipulated or *de novo* produced *in vitro*. We should nevertheless remember that the first meeting in 1985 faced the problem of suboptimal cryopreservation. There is an increasing trend for the collection of bulk ejaculates using semi-automated methods, arguing for better welfare alongside cost-benefits. Spermatozoa are, under such handling, exposed to higher levels of spermadhesins and, with cholesterol being lost during handling, the cells have been difficult to cryopreserve. Spermatozoa from the SRF or of its sperm-peak fraction have shown a higher cryosurvival and resilience to freezing-thawing. Why? Could it be that those spermatozoa are exposed to a lower amount of spermadhesins? As well as they are exposed to large numbers of sEVs of epididymal and prostatic source? As already described, the sEVs fuse with spermatozoa, and apart from the cargo of specific proteins and nucleic acids, they contribute with their cholesterol-rich lipids, incorporating them to the sperm membrane. Would this be the most relevant future use of harvested sEVs? Undoubtedly, this possibility is to be tested, if the sEVs can be collected from highly fertile, good freezers boars, to start with (Fig. 6, panel b). There is high confidence that this area, evidently under-researched in producing animals, including the pig, is going to become a priority in the immediate future.

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Ethical statement

Animal husbandry and experimental procedures, when applicable to the studies reported here by the authors, were done in accordance with the current European Directive 2010/63/EU, 22/09/20), and Swedish (SJVFS 2017:40) and Spanish (ES300130640127) legislation and approved in advance.

CRediT authorship contribution statement

Heriberto Rodriguez-Martinez: Conceptualization, Writing – original draft, Writing – review & editing, Project administration, Investigation, Funding acquisition. **Cristina A. Martinez-Serrano:** Writing – review & editing, Software, Investigation, Funding acquisition. **Manuel Alvarez-Rodriguez:** Writing – review & editing, Project administration, Investigation, Funding acquisition. **Emilio A. Martinez:** Writing – review & editing, Software, Investigation. **Jordi Roca Aleu:** Writing – review & editing, Visualization, Software, Project administration, Methodology, Investigation, Funding acquisition.

Declaration of Competing Interest

The current submission is an Invited plenary paper for the nextcoming 10th International Conference on Boar Semen Preservation, Vic (Barcelone province) Spain.

On behalf of the Authors

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