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Review article

Genomic, transcriptomic and epigenomic analysis towards the understanding of porcine semen quality traits. Past, current and future trends

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ABSTRACT

The importance of boar reproductive traits, including semen quality, in the sustainability of pig production system is increasingly being acknowledged by academic and industrial sectors. Research is needed to understand the biology and genetic components underlying these traits so that they can be incorporated into selection schemes and managerial decisions. This article reviews our current understanding of genome biology and technologies for genome, transcriptome and epigenome analysis which now facilitate the identification of causal variants affecting phenotypes more than ever before. Genetic and transcriptomic analysis of candidate genes, Genome-Wide Association Studies, expression microarrays, RNA-Seq of coding and noncoding genes and epigenomic evaluations have been conducted to profile the molecular makeups of pig sperm. These studies have provided insightful information for a several semen-related parameters. Nonetheless, this research is still incipient. The spermatozoon harbors a reduced transcriptome and highly modified epigenome, and it is assumed to be transcriptionally silent for nuclear gene expression. For this reason, the extent to which the sperm's RNA and epigenome recapitulate sperm biology and function is unclear. Hence, we anticipate that single-cell level analyses of the testicle and other male reproductive organs, which can reveal active transcription and epigenomic profiles in cells influencing sperm quality, will gain popularity and markedly advance our understanding of sperm-related traits. Future research will delve deeper into sperm fertility, boar resilience to environmental changes or harsh conditions, especially in the context of global warming, and also in transgenerational inheritance and how the environment influences the sperm transcriptome and epigenome.

1. Introduction

The current population growth and increased demand for high-quality food (United Nations, 2017) coupled with crisis in climate, biodiversity and resources, requires urgent and decisive action. This enquires the livestock sector to match the food demand and simultaneously adapt to mitigate its impact on these crises (European Comission, 2020). Livestock must be more productive,

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resource-efficient, resilient to harsh conditions or changing environments, resistant to pathogens, and friendly to the environment, while also maintaining and improving welfare standards. Genomic selection (GS) is one of the front runners in addressing these demanding challenges and making the livestock sector more sustainable at an accelerated pace. To this end, breeders need to increase genetic pressure to the existing breeding goals and to a new set of traits not considered until now. For instance, selection of carcass and meat quality traits has been the focus in paternal lines whilst reproduction, i.e., litter size and survival rate, has traditionally been considered a set of female traits (Harsh and Boler, 2024). In swine, reproduction strategies mostly rely on the semen from genetically elite boars present in artificial insemination (AI) centres. Thus, these boars are largely responsible for the genetic progress achieved in the population. The efficient transmission of the best genetic material to the descendent generations depends on the reproductive performance of the elite boars, mainly assessed by evaluating several semen quality parameters. While fertility remains the ultimate objective, current methods employed at AI farms are limited to the routine evaluation of various semen quality parameters including sperm concentration, motility and morphology. There are additional quality parameters that are rarely assessed in routine practice at AI studs including sperm viability, mitochondrial activity, DNA integrity, plasma membrane integrity and acrosome integrity (Maside et al., 2023). AI centres often operate as independent entities, where economic revenue hinges on semen quality, making the evaluation of these parameters crucial for maximizing economic profit. Noteworthy, none of these routinely evaluated parameters, whether assessed individually or in combination, have demonstrated consistent reliability as indicators of the sperm's fertilising ability (Jung et al., 2015). Although some studies have reported some links (Broekhuijse et al., 2012; Yeste et al., 2010), these findings lack consistency across different research endeavors (Schulze et al., 2021, 2013).

Male reproductive traits should be a primary focus of selection. Recently, few companies have begun incorporating specific semen traits into their selection indexes. Both breeders and researchers are increasingly focusing on boar reproductive traits, including aspects of semen quality. Further research is crucial to unravel the molecular and genetic underpinnings of these traits. With this knowledge, solutions can be developed to select elite boars with optimal semen quality and reproductive performance (Robinson and Buhr, 2005). Moreover, leveraging the vast datasets available on semen quality and male fertility from boar AI centres could provide significant value for other livestock species and for biomedical research.

This review aims to detail the current knowledge on genome activity and gene regulation, as well as the methods and technologies available today to identify genetic and molecular markers linked with porcine semen traits. We begin by providing an overview of the evolution of animal breeding from its inception to current times, culminating in the opportunities offered by the most recent developments in genome biology and state-of-the-art technologies. In this part, we explore tools to evaluate the genome, transcriptome and epigenome using both bulk and cell-based approaches. Subsequent sections focus on genetics, transcriptomics and epigenomics studies carried to date in pig sperm. Finally, we discuss future perspectives, focusing on the needs and opportunities for genomics research in the field of boar semen quality.

2. A brief history of animal breeding

Systematic selective breeding in farm animals traces its roots back to the British agricultural revolution in the 18th century, spearheaded by Robert Bakewell. Bakewell established a strategy that combined the annotation of productive values for desired traits and pedigrees to inform breeding decisions in sheep, cattle and horse. His approach laid the foundation for modern breeding practices. A significant leap forward ocurred in the latter half of the 20th century with the introduction of best linear unbiased prediction (BLUP), a statistical method that revolutionized the evaluation of genetic merit in animals (Henderson, 1975). The use of genetic markers in pig selection schemes emerged in the early 1990s, with the development of a protocol to erradicate the porcine stress syndrome. This protocol employed the contemporary molecular technologies to genotype the C1843T polymorphism of the *Ryanodine 1 Receptor* (*RYR1*) gene (Otsu et al., 1992). The mid-1990s witnessed the advent of the first genome-scale studies that facilitated the mapping of Quantitative Trait Loci (QTL) associated with agronomic traits (Andersson et al., 1994). The limited technological capacity at that time, however, constrained these studies to relatively small sets of markers and animal cohorts.

The emergence of chip microarrays containing nucleic acid probes capable of hybridizing to DNA or RNA molecules marked the onset of the genomics era in the early 21st century. These chips enabled the genotyping of tens or even hundreds of thousands of Single Nucleotide Polymorphisms (SNPs) distributed throughout the genome at an affordable price. SNPs are a ubiquitous type of genetic variation that involves the substitution of a single nucleotide by another at a specific position in the genome. The advent of SNP chips facilitated Genome Wide Association Studies (GWAS) to identify SNPs and genomic regions genetically associated with specific traits with unprecedented precision (Duijvesteijn et al., 2010). These chips replaced BLUP by allowing the implementation of GS schemes, which are now widely used in intensive pig breeding (Cleveland and Hickey, 2013). The GS requires the initial comparison of the genetic profile at a genomic scale, and phenotypic values in a reference set of animals from a population to develop a model capable of predicting the breeding value of animals based on their genotypic profile. Subsequently, this information can be applied to other animals within the same population to determine their breeding values. With GS, breeders can predict genetic merit without the need to record trait values. This approach helps reduce generation intervals and accelerates genetic progress (Goddard and Hayes, 2009). The onset of high throughput sequencing technologies (HTS), also known as next-generation sequencing (NGS), marked a significant milestone in genomics during the first decade of the 21st century. These advancements facilitated the cost-effective sequencing of nucleic acids on a massive scale and catalyzed the development of reference genome sequences for numerous animal species, including swine (Groenen et al., 2012). Notably, some breeding companies soon transitioned from SNP chips to NGS in their GS schemes.

3. Present and future of genomics research applied to animal breeding

3.1. Current knowledge on genome biology and technologies eases the identification of causal variants

Presently, the cost of sequencing whole mammalian genomes has plummeted to less than $1000, with further reductions expected soon (Schroth, 2024). Consequently, the routine sequencing of numerous genomes has become commonplace in both research and applied settings. This surge in sequencing efforts has revealed milions of SNPs in the pig genome. Moreover, the use of more recent long-read sequencing technologies is now accelerating the identification of structural variants, involving segments at least 50 base pairs long, including insertions/deletions (InDels), translocations, inversions, duplications, and copy number variations (Li et al., 2017; Tian et al., 2020). The growing body of research on structural variants at a genomic scale is expected to help uncovering the genetic basis of agronomically significant phenotypes (Blaj et al., 2022; Kwon et al., 2024). While this topic warrants comprehensive examination, its detailed discussion exceeds the scope of the current review.

Currently, GWAS and GS schemes rely on markers selected based on their genotype informativity and other technical criteria, most often overlooking their functional and causative potential. It is likely that most of these variants lack a functional relationship with the traits under study, instead being linked to ungenotyped and unknown causal variants through linkage disequilibrium (LD). Causal variants can influence traits by altering either protein sequence or gene expression, with most expected to regulate gene expression (Georges et al., 2019). Identifying these causal variants would be a big step forward for improving selection schemes, as they capture a larger proportion of trait variance and have consistent additive effects across populations and generations. The identification of causal variants requires deep knowledge on the molecular basis of the traits and the regions of the genome that are involved in these processes. Until recently, such information was inaccessible, but advancements in our understanding in genome biology and the availability of affordable and robust sequencing technologies now facilitate the characterization of the genome's functional landscape and the identification of causal variants in farm animals.

The genome can be conceptualized as a linear puzzle comprised of segments with diverse functions. These segments can be broadly classified as functional and non-functional. The functional segments encompass specific sequences within the genome that can serve various roles. Some act as templates for the transcription of coding RNAs, which are then translated into proteins, or noncoding RNAs (ncRNAs). Others function in the regulation of gene expression. These regulatory elements mainly include enhancers, promoters, silencers, locus control regions (LCRs) and insulators as described below.

3.2. The genome harbors a complex set of noncoding RNAs that can be studied by RNA-Seq

It is now widely acknowledged that a large portion of the genome is transcribed into a complex catalog of different types of ncRNAs with diverse functions (Zhao et al., 2018). These ncRNAs can play crucial roles in modulating gene expression, RNA stability, protein translation, or chromatin organisation. The advent of HTS technologies, such as RNA-Seq, revolutionized the study of RNA profiles, or transcriptomes, revealing a plethora of ncRNAs with regulatory roles. ncRNAs are categorized into short and long types, with the latter defined as exceeding 200 base pairs (bp) in length. The most profoundly studied ncRNAs in livestock are microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), and long non-coding RNAs (lncRNAs), including both linear and circular (circRNA) forms. With the exception of piRNAs, ncRNAs have, as a whole, ubiquotous expression (Chen et al., 2019; Shen et al., 2023) although individually, many show tissue preferential or even specific presence. miRNAs are the most well understood familiy of ncRNAs in liverstock species as they were the first widespread regulatory RNAs to be discovered in the early 2000s (Lau et al., 2001). These are ∼22 nucleotide (nt) long RNAs which function by promoting the degradation or inhibiting protein translation of target messenger RNAs (mRNA). Currently, there are 461 miRNAs annotated in pig (www.mirbase.org; accessed March 23, 2024) but the real number is with all certainty, much larger if we compare it with the miRNA catalog of other mammals (e.g., over 2500 in human; www.mirbase.org). piRNAs are slightly bigger in size (26–32 nt long), are mainly considered germline specific and their presence in non-gonadal tissues remains controversial (Tosar et al., 2018). piRNAs have an important role in gametogenesis, mainly by methylating and silencing transposable elements in primordial germline cells and in late spermatocytes thereby providing genome stability (Aravin et al., 2008; Gòdia et al., 2018b). In late spermatocytes, piRNAs also regulate the levels of mRNA and long non-coding RNA (lncRNA) genes (Watanabe et al., 2015). lncRNAs, exceeding 200 nt in length, can undergo splicing and can be transcribed from intergenic, intronic or, antisense frorm coding regions. Through their interaction with DNA, RNA or proteins, lncRNAs exhibit different, not fully understood, functions including chromatin remodelling, transcriptional regulation, alternative splicing and miRNA sequestering (Mattick et al., 2023). Their discovery and discrimination from other RNAs including protein coding RNAs is complex, which hinders the throughput screening of their biological functions (Mattick et al., 2023). circRNAs have a circular structure, formed through back-splicing of coding or non-coding transcripts. These RNAs modulate gene expression, stability and translation through diverse mechanisms (Huang et al., 2020). Among their various functions, circRNAs have been mostly defined by their role as miRNA sponges. In this capacity, circRNAs harbor binding motifs for miRNAs, sequestering and rendering them unavailable to target mRNAs. Consequently, the captured miRNAs are unable to exert their regulatory influence on their target mRNAs (Hansen et al., 2013). Notably, all these types of ncRNAs have been implicated in diverse phenotypes of interest for the livestock production sector, including boar semen quality, as elaborated below.

3.3. The regulation of RNA expression at a genomic scale can be studied using different epigenomics techniques

Promoters and enhancers both promote gene expresssion but differ in their locations relative to the genes they control. Promoters are typically situated immediately upstream of the genes they regulate, whereas enhancers tend to be at a further distance away.

Table 1
Summary of the studies that evaluated heritability in pig sperm.

Study	Breed	MOT	PMOT	ABN	NCELLS	DD	PD	BT	CT	DMR	CON	VOL
Smital et al.Anim Reprod Sci. 2005 86 (1−2):119–30	several breeds	0.38		0.34	0.42						0.49	0.58
WolfReprod Dom Anim. 2009 44(2):338–44	Large White	0.06		0.04							0.13	0.14
WolfReprod Dom Anim. 2009 44(2):338–44	Landrace	0.16		0.12							0.20	0.24
WolfJ Anim Sci. 2017 88(9):2893–903	Large White	0.08		0.12	0.10						0.18	0.20
WolfJ Anim Sci. 2017 88(9):2893–903	Landrace	0.12		0.10	0.12						0.18	0.25
Marques et al.J Anim Sci.2017 95 (10):4251–9	Pietrain	0.37	0.43	0.42	0.47							
Marques et al.J Anim Sci.2017 95 (10):4251–9	Duroc	0.37	0.46	0.27	0.44							
Marques et al.J Anim Sci.2017 95 (10):4251–9	Large White	0.31	0.4	0.28	0.23							
Marques et al.J Anim Sci.2017 95 (10):4251–9	Landrace	0.28	0.34	0.20	0.34							
Li et al.J Anim Breed Genet. 2019 136:183–9	Duroc	0.42	0.34	0.26							0.34	0.25
Li et al.J Anim Breed Genet. 2019 136:183–9	Landrace	0.11		0.15							0.23	0.21
Li et al.J Anim Breed Genet. 2019 136:183–9	Large White	0.26		0.21							0.27	0.23
Zhao et al.Animals (Basel) 2019 9(10):710	Duroc					0.29	0.24	0.14	0.03	0.27		
Ogawa et al.J Anim Sci. 2022 100(3): skac055	Duroc			0.20	0.23						0.28	0.29
Krupa et al.Genes (Basel) 2023 14(11):2003	Dam lines	0.14		0.24							0.10	0.28
Krupa et al.Genes (Basel) 2023 14(11):2003	Sire lines	0.10		0.22							0.10	0.26
Hong et al.Front Genet. 2022; 13: 805651	Large White	0.11		0.20							0.17	0.23
Hong et al.Front Genet. 2022; 13: 805651	Landrace	0.24		0.15							0.09	0.23
Gruhot et al.Anim Reprod Sci. 2019 206:85–92	Duroc	0.08	0.1		0.16	0.18	0.21	0.13		0.24		
average		0.22	0.34	0.22	0.30	0.23	0.22	0.13	0.03	0.26	0.26	0.27

MOT: percentage of motile spermatozoa; PMOT: percentage of spermatozoa with progressive (in a forward straight line) motility; ABN: percentage of spermatozoa with abnormal morphology; NCELLS: number of sperm cells in the ejaculate; DD: percentage of sperm cells with distal cytoplasmatic droplets; PD: percentage of sperm cells with distal cytoplasmatic droplets; BT: percentage of sperm cells with a bent tail (bending exceeding 20°/μm); CT: percentage of sperm cells with a coiled tail (the tail bends 180° or more over its length); DMR: percentage of sperm cells with distal midpiece reflex (the tail is wrapped around a distal cytoplasmic droplet and returns to the sperm head); CON: concentration of sperm cells in ejaculate; VOL: volume of ejaculate.

Promoters generally regulate gene expression of ubiquotous or cell type or state specific genes in stable manner. On the contrary, enhancers fine-tune gene expression in dynamic ways that depend heavily on physiological or pathological states and environmental cues. Moreover, enhancers tend to be less evolutionary conserved than promoters (Villar et al., 2015). Silencers function by suppressing the expression of the genes they regulate. LCRs are large regulatory segments that regulate the expression of several genes or gene clusters. Lastly, insulators act as barriers or blockers, preventing enhancers from affecting neighbouring genes that should remain unaffected. The activity of all these genomic elements depend upon their chromatin state (Ernst et al., 2011), which can be classified as active, poised, or repressed. These states and their activity are marked by the interplay of several epigenetic marks such as DNA methylation, nucleosome positioning, histone modifications and the transcriptional machinery, namely RNA polymerases and transcription factors (Ernst et al., 2011; Pan et al., 2021; The Encode Project Consortium et al., 2020). This set of epigenetic marks determine the accessibility and signaling of the distinct regions of the genome, and they do so in a cell type specific manner. DNA methylation, mostly characterized in cytosines, is typically associated to the inhibition of transcription. Consistently, the promoters of highly expressed genes tend to show low levels of cytosine methylation (Jones, 2012). The absence of nucleosomes is associated with DNA that is accessible to the transcriptional machinery and is therefore active (Felsenfeld et al., 1996). Different post-translational modifications of histones within nucleosomes are associated to promoters (H3K4me3), enhancers (H3K4me1), active enhancers and promoters (H3K27Ac), or silenced (H3K27me3) elements (Ernst et al., 2011). All these epigenetic marks can now be studied using different approaches coupled with NGS. For example, cytosine methylation can be evaluated by bisulfite conversion of unmethylated cytosines or several of its derivatives (Lister et al., 2009). The genomic location of histones, histone modifications, polymerases and transcription factors can be mapped by chromatin immunoprecipitation (ChIP) techniques (Solomon et al., 1988). Genome accessibility can be profiled by Assay for Transposase-Accessible Chromatin (ATAC) methods (Buenrostro et al., 2013). There is another, higher level of genome organisation that also plays a crucial role in regulating gene expression. This is the tertiary structure in which the genome is packed in the nucleus of eukaryotic cells, creating loopings and physical interactions between elements that are distant in the linear dimension of the genome (Kosak and Groudine, 2004). This 3D structure can be studied by different technologies such as Hi-C (Lieberman-Aiden et al., 2009). These epigenomic elements and approaches to map them in the genome are nicely described in a review published by the ENCODE Consortium whose aim is to characterize the functional elements of the human and mouse genomes (The Encode Project Consortium et al., 2020) and an article published by the NIH Roadmap Epigenomics Consortium (Roadmap Epigenomics Consortium et al., (2015), whose goal is to characterize human epigenomes in relation to gene regulation, development and disease.

Table 2
Summary of the GWAS studies in sperm.

Study	Region	chr:start-end	Gene	Chr	Positional relationship	Phenotype	Breed	SNPs	Approach
Zhao et al.Theriogenology 2020, 141:9–15	1	1:1470,000–2270,000		1		PD	Duroc	21	wssGWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72	2	1:13,501,755–13,501,756		1	less than 3 Mbp	PD	Pietrain	1	GWAS
Gunawan et al.Anim Reprod Sci. 2011 128(1−4):11–21		1:14,217,036–14,493,363	*ESR1*	1		MT, PD	Pietrain		candidate gene
Marques et al.Genet Sel Evol. 2018, 50:40	3	1:55,610,000–56,470,000		1	same region	MOT	Landrace	26	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		1:55,610,000–56,470,000		1		PMOT	Landrace	26	wssGWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72	4	1:82,900,000–83,490,000		1		HABN	Pietrain	8	GWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72	5	1:94,880,000–98,740,000		1		HABN	Pietrain	8	GWAS
Diniz et al.Animal reprod Sci. 2014, 151 (3–4):201–7	6	1:117,230,000–119,560,000		1		MOT	Large White	6	GWAS with DEBV
Gòdia et al.Genet Sel Evol. 2020, 52:72	7	1:126,397,198–126,397,199		1		HABN	Pietrain	1	GWAS
Marques et al.Genet Sel Evol. 2018, 50:40	8	1:135,510,000–136,310,000		1	same region	MOT	Large White	17	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		1:135,510,000–136,310,000		1		PMOT	Large White	17	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		1:135,510,000–136,310,000		1		ABN	Large White	17	wssGWAS
Reyer et al.Genes (Basel) 2024, 15 (3):382	9	1:231,230,000–232,230,000		1		NCELLS	Pietrain	2	GWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72	10	1:243,860,000–246,440,000		1		NABN	Pietrain	11	GWAS
Marques et al.Genet Sel Evol. 2018, 50:40	11	1:255,480,000–256,280,000		1	same region	MOT	Large White	17	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		1:255,480,000–256,280,000		1		ABN	Large White	17	wssGWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72		1:258,540,000–258,550,000		1	less than 3 Mbp	NABN	Pietrain	2	GWAS
Marques et al.Genet Sel Evol. 2018, 50:40	12	1:270,940,000–271,740,000		1		NCELLS	Landrace	10	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	13	1:290,900,000–291,840,000		1	same region	MOT	Large White	25	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		1:290,900,000–291,840,000		1		ABN	Large White	25	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	14	1:305,180,000–305,980,000		1		NCELLS	Large White	23	wssGWAS
Zhang et al.Animals (Basel) 2023, 13 (3):365	15	2:6190,000–6580,000		2		MOT	Duroc	6	wssGWAS
Zhang et al.Animals (Basel) 2023, 13 (3):365	16	2:15,980,000–16,090,000		2	less than 3 Mbp	NCELLS	Duroc	3	wssGWAS
Zhang et al. nimals (Basel) 2023, 13 (3):365		2:17,690,000–18,090,000		2		NCELLS	Duroc	8	wssGWAS
Zhao et al.Theriogenology 2020, 141:9–15	17	2:143,740,000–144,540,000		2	less than 3 Mbp	BT	Duroc	16	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		2:145,690,000–146,490,000		2		NCELLS	Landrace	16	wssGWAS
Gao et al.BMC Genomics 2019, 20:797		2:147,710,000–148,510,000		2		MOT	Duroc	12	wssGWAS

(*continued on next page*)

Table 2 (*continued*)

Study	Region	chr:start-end	Gene	Chr	Positional relationship	Phenotype	Breed	SNPs	Approach
Marques et al.Genet Sel Evol. 2018, 50:40	18	2:154,030,000–154,830,000		2		PMOT	Landrace	23	wssGWAS
Reyer et al.Genes (Basel) 2024, 15 (3):382	19	3:679,027–679,028		3	less than 3 Mbp	NCELLS	Pietrain	1	GWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72		3:2911,413–2911,414		3		HABN	Pietrain	1	GWAS
Lin et al.J Anim Breed Genet. 2006 123 (6):384–8		3:4082,216–4090,356	*ACTB*	3		ABN, MOT	Pietrain		candidate gene
Marques et al.Genet Sel Evol. 2018, 50:40	20	3:28,530,000–29,330,000		3	same region	MOT	Large White	18	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		3:28,530,000–29,330,000		3		ABN	Large White	18	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	21	3:110,290,000–111,090,000		3	less than 3 Mbp	PMOT	Landrace	19	wssGWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72		3:113,750,000–113,840,000		3		NABN	Pietrain	3	GWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72	22	4:2410,000–2420,000		4	less than 3 Mbp	ACRO	Pietrain	2	GWAS
Gao et al.BMC Genomics 2019, 20:797		4:5150,000–5950,000		4		PMOT	Duroc	17	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	23	4:28,250,000–29,050,000		4	same region	MOT	Large White	12	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		4:28,250,000–29,050,000		4		PMOT	Large White	12	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		4:28,250,000–29,050,000		4		ABN	Large White	12	wssGWAS
Reyer et al.Genes (Basel) 2024, 15 (3):382	24	4:64,980,000–65,010,000		4		NCELLS	Pietrain	2	GWAS
Marques et al.Genet Sel Evol. 2018, 50:40	25	4:84,900,000–85,730,000		4	same region	MOT	Large White	21	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		4:84,900,000–85,730,000		4		PMOT	Large White	21	wssGWAS
Reyer et al.Genes (Basel) 2024, 15 (3):382	25	4:109,593,443–109,593,444		4		MOT	Pietrain	1	GWAS
Zhang et al.Animals (Basel) 2023, 13 (3):365	26	4:121,170,000–121,570,000		4	less than 3 Mbp	PMOT	Duroc	10	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		4:123,120,000–124,200,000		4	same region	MOT	Large White	30	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		4:123,120,000–124,200,000		4		PMOT	Large White	30	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		4:123,120,000–124,200,000		4		ABN	Large White	30	wssGWAS
Zhao et al.Theriogenology 2020, 141:9–15	27	5:100,000–900,000		5		DD	Duroc	17	wssGWAS
Reyer et al.Genes (Basel) 2024, 15 (3):382	28	5:5220,000–5290,000		5		VOL	Pietrain	3	GWAS
Mańkowska et al.Theriogenology. 2022 189:199–208	29	5:10,994,215–11,017,950	*IFT27*	5	less than 3 Mbp	Freezability	Large White		candidate gene
Gao et al.BMC Genomics 2019, 20:797		5:11,710,000–12,510,000		5		NCELLS	Duroc	8	wssGWAS
Zhao et al.Anim Biotechnol. 2019 30 (4):311–6	39	5:17,385,390–17,408,511	*NR4A1*	5	less than 3 Mbp	ABN, MT	Duroc, Large White, Landrace		candidate gene
Marques et al.Genet Sel Evol. 2018, 50:40		5:17,610,000–18,470,000		5	same region	MOT	Large White	26	wssGWAS

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Table 2 (*continued*)

Study	Region	chr:start-end	Gene	Chr	Positional relationship	Phenotype	Breed	SNPs	Approach
Marques et al.Genet Sel Evol. 2018, 50:40		5:17,610,000–18,470,000		5		PMOT	Large White	26	wssGWAS
Zhao et al.Theriogenology 2020, 141:9–15	31	5:68,660,000–69,680,000		5		PD	Duroc	6	wssGWAS
Zhao et al.Theriogenology 2020, 141:9–15	32	6:3400,000–4200,000		6	overlap	CT	Duroc	13	wssGWAS
Zhao et al.Theriogenology 2020, 141:9–15		6:3450,000–4250,000		6		BT	Duroc	11	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	33	6:8240,000–9130,000		6	same region	MOT	Large White	20	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		6:8240,000–9130,000		6		PMOT	Large White	20	wssGWAS
Mei et al.J Anim Sci. 2021, 99(7): skab188	34	6:23,477,206–23,477,207		6		MOT	Duroc	1	GWAS with DEBV
Mei et al.J Anim Sci. 2021, 99(7): skab188	35	6:49,350,594–49,350,595		6		CON	Duroc	1	GWAS with DEBV
Gòdia et al.Genet Sel Evol. 2020, 52:72	36	6:65,600,000–66,660,000		6		ACRO	Pietrain	2	GWAS
Marques et al.Genet Sel Evol. 2018, 50:40	37	6:83,320,000–84,120,000		6		ABN	Landrace	12	wssGWAS
Zhao et al.Theriogenology 2020, 141:9–15	38	6:153,640,000–154,440,000		6		DMR	Duroc	13	wssGWAS
Gao et al.BMC Genomics 2019, 20:797	39	7:130,000–930,000		7		PMOT	Duroc	16	wssGWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72	40	7:6200,000–6380,000		7		MOT	Pietrain	2	GWAS
Reyer et al.Genes (Basel) 2024, 15 (3):382	41	7:20,070,000–20,110,000		7	less than 3 Mbp	MOT	Pietrain	2	GWAS
Zhao et al.Theriogenology 2020, 141:9–15		7:21,110,000–21,910,000		7		CT	Duroc	6	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	42	7:82,560,000–83,360,000		7	same region	MOT	Landrace	10	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		7:82,560,000–83,360,000		7		PMOT	Landrace	10	wssGWAS
Reyer et al.Genes (Basel) 2024, 15 (3):382		7:82,580,000–82,750,000		7	overlap	MOT	Pietrain	4	GWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72		7:85,730,000–86,880,000		7	less than 3 Mbp	NABN	Pietrain	2	GWAS
Zhang et al.Animals (Basel) 2023, 13 (3):365	43	7:97,930,000–98,260,000		7		ABN	Duroc	10	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	44	7:116,370,000–117,280,000		7	same region	MOT	Landrace	25	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		7:116,370,000–117,280,000		7		ABN	Landrace	25	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	45	8:16,060,000–16,860,000		8	same region	MOT	Large White	20	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		8:16,060,000–16,860,000		8		PMOT	Large White	20	wssGWAS
Reyer et al.Genes (Basel) 2024, 15 (3):382	46	8:127,722,766–127,722,767		8		NCELLS	Pietrain	1	GWAS
Marques et al.Genet Sel Evol. 2018, 50:40	47	8:133,900,000–134,940,000		8	same region	MOT	Landrace	20	wssGWAS

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Table 2 (*continued*)

Study	Region	chr:start-end	Gene	Chr	Positional relationship	Phenotype	Breed	SNPs	Approach
Marques et al.Genet Sel Evol. 2018, 50:40		8:133,900,000–134,940,000		8		PMOT	Landrace	20	wssGWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72	48	9:5760,000–5780,000		9		HABN	Pietrain	2	GWAS
Marques et al.Genet Sel Evol. 2018, 50:40	49	9:9320,000–10,310,000		9	same region	MOT	Landrace	19	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		9:9320,000–10,310,000		9		PMOT	Landrace	19	wssGWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72	50	9:28,463,580–28,463,581		9		MOT	Pietrain	1	GWAS
Marques et al.Genet Sel Evol. 2018, 50:40	51	9:36,460,000–37,260,000		9		MOT	Landrace	12	wssGWAS
Gao et al.BMC Genomics 2019, 20:797	52	9:121,150,000–121,950,000		9		NCELLS	Duroc	8	wssGWAS
Gao et al.BMC Genomics 2019, 20:797		9:131,550,000–132,350,000		9		MOT	Duroc	13	wssGWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72	53	9:137,959,590–137,959,591		9	less than 3 Mbp	ACRO	Pietrain	1	GWAS
Marques et al.Genet Sel Evol. 2018, 50:40		9:139,530,000–140,630,000		9	same region	PMOT	Large White	23	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		9:139,530,000–140,630,000		9		NCELLS	Large White	23	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		9:139,530,000–140,630,000		9		ABN	Large White	23	wssGWAS
Zhao et al.Theriogenology 2020, 141:9–15	54	10:4450,000–5250,000		10		DMR	Duroc	10	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	55	10:10,580,000–11,450,000		10	same region	MOT	Large White	23	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		10:10,580,000–11,450,000		10		PMOT	Large White	23	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		10:10,580,000–11,450,000		10		NCELLS	Large White	23	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		10:10,580,000–11,450,000		10		ABN	Large White	23	wssGWAS
Brym et al.Theriogenology. 2021 166:112–23	56	10:40,053,355–40,060,319	*BAMBI*	10	less than 3 Mbp	Freezability	Large White		candidate gene
Reyer et al.Genes (Basel) 2024, 15 (3):382		10:40,750,000–41,000,000		10		MIT, ACRO	Pietrain	2	GWAS
Zhang et al.Animals (Basel) 2023, 13 (3):365	57	11:15,980,000–16,370,000		11		NCELLS	Duroc	10	wssGWAS
Reyer et al.Genes (Basel) 2024, 15 (3):382	58	11:19,893,118–19,893,119		11		MOT	Pietrain	1	GWAS
Marques et al.Genet Sel Evol. 2018, 50:40	59	11:41,050,000–41,850,000		11	same region	MOT	Landrace	11	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		11:41,050,000–41,850,000		11		PMOT	Landrace	11	wssGWAS
Zhao et al.Theriogenology 2020, 141:9–15	60	11:68,700,000–69,500,000		11	less than 3 Mbp	CT	Duroc	12	wssGWAS
Brym et al.Theriogenology. 2021 166:112–23		11:70,251,446–70,422,192	*FGF14*	11		Freezability	Large White		candidate gene
Gao et al.BMC Genomics 2019, 20:797	61	12:6200,000–7000,000		12	overlap	ABN	Duroc	9	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		12:6230,000–7030,000		12		MOT	Large White	32	wssGWAS

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Table 2 (*continued*)

Study	Region	chr:start-end	Gene	Chr	Positional relationship	Phenotype	Breed	SNPs	Approach
Zhao et al.Theriogenology 2020, 141:9–15	62	12:17,560,000–18,360,000		12		PD	Duroc	10	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	63	12:40,760,000–41,560,000		12	same region	MOT	Large White	17	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		12:40,760,000–41,560,000		12		PMOT	Large White	17	wssGWAS
Wang et al.Anim Biotechnol. 2023 34 (7):2106–10		12:44,174,948–44,218,146	*NOS2*	12	less than 3 Mbp	ABN, MT, CON	Duroc		candidate gene
Zhang et al.Animals (Basel) 2023, 13 (3):365		12:46,050,000–46,450,000		12	less than 3 Mbp	ABN	Duroc	7	wssGWAS
Zhao et al.Theriogenology 2020, 141:9–15	64	12:51,460,000–52,260,000		12		DMR	Duroc	7	wssGWAS
Zhang et al.Animals (Basel) 2023, 13 (3):365	65	12:58,830,000–59,210,000		12	same region	MOT	Duroc	10	wssGWAS
Zhang et al.Animals (Basel) 2023, 13 (3):365		12:58,830,000–59,210,000		12		ABN	Duroc	10	wssGWAS
Zhang et al.Animals (Basel) 2023, 13 (3):365	66	12:62,160,000–62,440,000		12	less than 3 Mbp	PMOT	Duroc	6	wssGWAS
Gao et al.BMC Genomics 2019, 20:797	67	13:1250,000–2050,000		13	overlap	ABN	Duroc	7	wssGWAS
Gao et al.BMC Genomics 2019, 20:797		13:1290,000–2090,000		13		MOT	Duroc	5	wssGWAS
Reyer et al.Genes (Basel) 2024, 15 (3):382		13:2630,671–2630,672		13	less than 3 Mbp	VOL	Pietrain	1	GWAS
Ma et al.Anim Reprod Sci. 2013 143 (1−4):97–101		13:3764,784–3783,122	*DAZL*	13	less than 3 Mbp	ABN, MT, CON	Duroc		candidate gene
Marques et al.Genet Sel Evol. 2018, 50:40	68	13:11,350,000–12,150,000		13	less than 3 Mbp	ABN	Landrace	19	wssGWAS
Reyer et al.Genes (Basel) 2024, 15 (3):382		13:14,128,868–14,128,869		13		MOT	Pietrain	1	GWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72	69	13:25,360,000–28,470,000		13	less than 3 Mbp	HABN	Pietrain	18	GWAS
Brym et al.Theriogenology. 2021 166:112–23		13:28,524,796–28,692,327	*LARS2*	13		Freezability	Large White		candidate gene
Gòdia et al.Genet Sel Evol. 2020, 52:72	70	13:33,820,000–37,650,000		13		HABN	Pietrain	3	GWAS
Marques et al.Genet Sel Evol. 2018, 50:40	71	13:107,480,000–108,280,000		13		MOT	Landrace	10	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	72	13:143,610,000–144,690,000		13	same region	MOT	Large White	13	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		13:143,610,000–144,690,000		13		PMOT	Large White	13	wssGWAS
Zhao et al.Theriogenology 2020, 141:9–15		13:146,980,000–147,780,000		13	less than 3 Mbp	BT	Duroc	3	wssGWAS
Zhao et al.Theriogenology 2020, 141:9–15	73	13:199,340,000–200,140,000		13		DD	Duroc	10	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	74	14:4130,000–5220,000		14	same region	MOT	Large White	19	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		14:4130,000–5220,000		14		PMOT	Large White	19	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		14:4130,000–5220,000		14		ABN	Large White	19	wssGWAS
Gao et al.BMC Genomics 2019, 20:797	75	14:14,490,000–15,290,000		14		NCELLS	Duroc	16	wssGWAS

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Table 2 (*continued*)

Study	Region	chr:start-end	Gene	Chr	Positional relationship	Phenotype	Breed	SNPs	Approach
Brym et al.Theriogenology. 2021 166:112–23	76	14:71,610,475–71,651,102	*SLC25A16*	14	less than 3 Mbp	Freezability	Large White		candidate gene
Marques et al.Genet Sel Evol. 2018, 50:40		14:72,830,000–73,630,000		14		NCELLS	Large White	16	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	77	14:99,700,000–100,510,000		14	same region	MOT	Large White	25	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40		14:99,700,000–100,510,000		14		PMOT	Large White	25	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	78	15:37,170,000–37,970,000		15		ABN	Landrace	20	wssGWAS
Mei et al.J Anim Sci. 2021, 99(7): skab188	79	15:45,048,041–45,048,042		15		VOL	Duroc	1	GWAS with DEBV
Marques et al.Genet Sel Evol. 2018, 50:40	80	15:61,930,000–62,730,000		15		MOT	Large White	15	wssGWAS
Sironen et al.BMC Genomics 2010, 11:699	81	15:95,680,726–101,722,463		15		Knobbed acrosome	Large White (Finnish Yorkshire)	4	genetic association under a recessive model
Gao et al.BMC Genomics 2019, 20:797	82	15:135,890,000–136,690,000		15		PMOT	Duroc	10	wssGWAS
Gòdia et al.Genet Sel Evol. 2020, 52:72	83	16:6476,358–6476,359		16		MOT	Pietrain	1	GWAS
Gao et al.BMC Genomics 2019, 20:797	84	16:26,760,000–27,560,000		16		ABN	Duroc	7	wssGWAS
Mei et al.J Anim Sci. 2021, 99(7): skab188	85	16:31,722,381–31,722,382		16		VOL	Duroc	1	GWAS with DEBV
Zhao et al.Theriogenology 2020, 141:9–15	86	18:780,000–1580,000		18		DD	Duroc	8	wssGWAS
Zhang et al.Animals (Basel) 2023, 13 (3):365	87	18:14,230,000–14,600,000		18	same region	MOT	Duroc	13	wssGWAS
Zhang et al.Animals (Basel) 2023, 13 (3):365		18:14,230,000–14,600,000		18		PMOT	Duroc	13	wssGWAS
Marques et al.Genet Sel Evol. 2018, 50:40	88	18:42,800,000–43,600,000		18		NCELLS	Landrace	19	wssGWAS

This table includes GWASs and also the candidate gene studies for genes that map less than 3 million base pairs away from a GWAS hit. Other candidate genes are not included in the table.
Theriogenology 2020, 141:9–15 and BMC Genomics 2019, 20:797 used the same animal resource. A proportion of the animals analysed in Genet Sel Evol 2018, 50:40 and Anim Reprod Sci. 2014, 151 (3−4):201–7 is probably common.
The interval mapping study Anim Reprod Sci. 2009; 114(1−3):210–8 performed using microsatellite markers and genomic positions as centiMorgans instead of nucleotide position in the referenc genome is not included.

3.4. Efforts to annotate the functional elements of the genome in swine

A similar worldwide collaborative effort coined as FAANG, which stands for the Functional Annotation of Animal Genomes consortium was established in 2015 with the aim to improve the identification of genetic variants affecting phenotypes to optimize animal breeding (Andersson et al., 2015). FAANG priorities include improving the annotation of animal genomes by: (i) genome re-sequencing and genotyping to catalogue genetic variants associated to target traits; (ii) transcriptome profiling to characterize the gene's biological relevance; and (iii) identify the genome's regulatory elements in different animal populations, tissues, cells and conditions (Andersson et al., 2015). These efforts have already led to the completion of genome characterizations for certain porcine tissues and breeds (Choi et al., 2015; Kern et al., 2021; Pan et al., 2021). The vast majortiy of these studies, however, have not yet been designed to understand agronomical traits. The FarmGTEx, one of the main FAANG projects, aims to discover regulatory variants by characterizing their control over gene expression in farm animals. Within this endeavor, FarmGTEx recently conducted a large pilot study in pigs, utilizing 5457 RNA-Seq from 34 tissues and cell types, as well as 1602 whole-genome sequencing samples. The study demonstrated the widespread presence of genetic regulatory variants throughout the genome, with thousands of associations between DNA variants and a large proportion of protein-coding genes and noncoding RNAs (Teng et al., 2024).

3.5. Single cell -omics enables genome functional annotation at the cell-type level

Until recently, NGS studies were limited to bulk approaches, where the cells in a sample are lysed to release their nucleic acids. Consequently, sequencing results provide a sort of an average representation of all cells processed. Nevertheless, recent advancements in nano-technologies and computational methods have spurred the development of novel technologies enabling the automated characterization of thousands of individual cells within a sample. This breakthrough marks a pivotal stride in molecular characterization, as cells, rather than tissues, are recognized as the functional units of a tissue. Moreover, single cell technologies have further progressed to allow for the examination of cells across various molecular layers, such as transcriptome and chromatin accessibility through RNA-Seq and ATAC-Seq, respectively (Cao et al., 2018). Noteworthy, this multiome analysis of the very same cell is unattainable with bulk tissue approaches. The availability of the two layers of information from the same cells enhances the understanding of regulatory mechanisms, including the linkage of accessible promoters and enhancers to gene expression (Allaway et al., 2021). This significantly improves the accuracy and reliability in identifying genomic regulators specific to these genes within certain cell types.

3.6. Integrative -omics can help finding causal variants affecting pig semen traits

The availability of Whole Genome Sequencing (WGS) now allows obtaining genotypes for virtually all the variants in a genome, including causal variants influencing traits. The integration of GWAS, WGS, transcriptomics and epigenomics, alongside other metabolomics, proteomics, and phenomics approaches, implemented through well-planned experiments using key animals and relevant tissues, holds immense promise. These integrative genomics approaches are poised to yield unprecendented insights into the identification of causal variants affecting traits of agronomic significance. Consequently, it is anticipated to streamline GS protocols by employing a smaller yet more informative set of markers compared to current practices.

The research aimed at characterizing the molecular and genetic basis of semen-related phenotypes is considerably limited compared to other traits that have been the focus of genetic selection for decades, such as traits related to growth or meat production, disease resistance, feed efficiency and female reproduction. In the following section, we will discuss these studies and provide insights considering their findings, addressing current knowledge gaps, available technologies, and socio-economic needs.

4. Genetic basis of pig semen traits

Research across various studies has consistently demonstrated the genetic basis of several phenotypic parameters associated with semen quality (Li et al., 2019; Marques et al., 2017). These studies have estimated the heritability, defined as the proportion of variation in a population for a particular trait that is attributable to inherited genetic factors for parameters such as ejaculate volume, sperm cell count, sperm morphology, and motility in different pig breeds including Duroc, Large White, Landrace or Pietrain (Table 1). The discernible heritability values for these traits suggest their potential for targeted selection in breeding programs across breeds.

Other studies, several of which are described below, directly investigated the association of genetic variation in the pig genome with semen traits and further demonstrated that a portion of this genetic variation is detectable with current methods and can be attributed to specific genetic variants. These studies have predominantly focused on cosmopolitan breeds such as Pietrain, Duroc, Large White and Landrace and mainly used either a candidate gene or a genome-level approach. Candidate gene approaches query the genetic association of DNA variants within genes (typically in the coding sequence) previously selected for known relationship with a related molecular function or phenotype in the same or in other species, most often human. Conversely, genome-level strategies interrogate the whole genome or a significant section of it in an unbiased manner using a set of DNA markers with known locations in the genome. Most studies have primarily focused on standard semen quality traits that are regularly measured in AI centers, including volume, concentration, total sperm count, total motility, progressive motility or abnormal morphology.

Several candidate genes such as *ACTG2*, *ACTN1* (Wimmers et al., 2005), *C7H15orf39*, *NOS2* (Wang et al., 2023), *NR4A1* (Zhao et al., 2019), *SPAG6* (Bai et al., 2023), *LARS2* (Brym et al., 2021), *STK35* and *IFT27* (Mańkowska et al., 2022a) among others have been explored in different experiments. These genes have shown genetic associations with various semen quality parameters including motility, morphology, sperm concentration in ejaculate, ejaculate volume, or cryo-tolerance.

Back in 2002, Thurston and colleagues used the amplified restriction fragment length polymorphisms (AFLP) method to provide the first evidence of genetic variants associated with a semen quality trait (viability of frozen-thawed sperm) using a non-targeted genome-wide approach (Thurston et al., 2002). In spite of this, this method does not identify the genomic location of these markers. The first genome scale study to map the genetic basis of semen traits in pigs was conducted by Xing and colleagues in 2009, employing an approach commonly used before the onset of GWAS. This approach involved identifying quantitative trait loci (QTL) by predicting the QTL genotype using genotyped markers in F_2 populations through a method called interval mapping. This study was carried out on a Duroc x Erhuhalian intercross and identified 4 QTLs, each associated with semen pH on chromosomes 2 and 12, ejaculate volume on chromosome 15, and ejaculation times on chromosome 17 (Xing et al., 2009).

The studies that followed all employed GWAS based methods, mostly weighted single step GWAS (wssGWAS) instead of conventional GWAS because the former allows the inclusion of animals without genotype data but with phenotype records by considering the pedigree relationships. Also, it can potentially estimate the SNP effects with higher accuracy than standard GWAS. As a result, these studies provide improved power and precision to detect genetic associations, especially when the cohort of genotyped and phenotyped animals is small (Wang et al., 2012). Moreover, most studies used phenotypic data recorded at the AI studs within their routine evaluations and included multiple ejaculates within each boar, thereby reducing the within-boar (non-genetic) variation.

Sironen and colleagues evaluated a Finnish Yorkshire population characterized by a relatively high frequency of a knobbed acrosome defect (Sironen et al., 2010). Their GWAS, which included 14 affected and 21 control animals, yielded one single genomic region, in a homozygous state, of a relatively small size (0.7 Mbp). This region harbored a compelling candidate gene, *HECW2*, for its known role in ubiquitin signaling, which had been previously shown to be important in mouse acrosome development (Sironen et al., 2010). This result is characteristic of a single, fully penetrant, variant causing the defect. In such cases, it is relatively straightforward to identify genetic signals with a small number of animals in a GWAS. The variation typically observed in different parameters of semen quality, however, is complex, resulting from a polygenic nature with a significant influence of environmental factors. The GWASs that interrogated these complex traits have identified nearly 100 regions across different traits and breeds or genetic backgrounds (Table 2). Overall, as also happens for other traits, these studies show little concordance that can stem from both technical and biological factors. The most obvious technical limitations inherent to GWAS include the typically low number of animals and genetic variants evaluated, as well as potential inaccuracies in phenotypic and genotypic data. The potential biological factors underlying these differences are several. First, the genetic basis segregating within each population, as well as the LD structure, may vary between the populations analyzed in each study. Furthermore, the limited concordance between studies may suggest that the genetics of semen quality traits is characterized by one or more of the following factors: (i) high polygenicity, with few or no discernible genetic variants of large effect; (ii) high influence of non-additive effects and epistatic interactions; (iii) strong influence from environmental factors that are challenging to control for in these studies. Indeed, the high within-boar variability of semen quality, which refers to differences in semen quality records of different ejaculates from the same boar, also indicates the substantial impact of environmental effects (Marques et al., 2017). Noteworthy, to address this within-boar variability, most GWAS studies for semen quality in swine have adopted approaches that involve the measurement of multiple semen samples within each screened animal. Many of the GWAS regions identified by these studies, however, are represented by a single SNP marker (Table 2). This means that they are not supported by additional SNPs that are supposedly also in LD with the causal variant, rendering these GWAS hits more prone to false and spurious results.

While the study of genetic variants can only capture genetic variation, the RNA levels in sperm for each gene can capture both genetic and environmental influences. Thus, the combination of GWAS and RNA-Seq can improve the influence of genetics and environment in the final phenotype. Two studies used both approaches. Mei et al. carried out a GWAS in a Duroc population for ejaculate volume, sperm cell concentration in ejaculate and percentage of motile spermatozoa (Mei et al., 2021). They identified GWAS hits for the three traits and within these regions determined a set of candidate genes due to their concordant position with the GWAS hits. In addition, they used publicly available RNA-Seq data on the testicle samples from 11 boars, 6 with high and 5 with low DNA Fragmentation Index (DFI), a trait that has been related to sperm concentration, volume and motility. With this data, they undertook a weighted gene co-expression network analysis (WGCNA) and used this network to determine modules of genes according to their co-expression and then associated this with the DFI. The module that showed highest correlation with DFI also included 6 of the GWAS positional candidate genes (*B9D2*, *TMEM145*, *WWC2*, *CDKN2AIP*, *TRAPPC11*, and *PELO*) which were thus considered strong candidates (Mei et al., 2021). The other study was undertaken by Gòdia and co-authors, who performed a standard GWAS on 25 semen quality parameters measured by the researchers using one single ejaculate (Gòdia et al., 2020c). The number of animals included in the GWAS was small when compared with other GWASs for boar semen quality. Yet, the study conducted RNA-Seq on a proportion of the boars evaluated by GWAS and this allowed integrating the results of both analyses. First, combining the GWAS and the correlation between RNA levels of each gene and semen quality parameters, they built a gene interaction network. To try determining whether he GWAS SNP hits were tagging causal variants that alter protein sequence and function or gene expression, they carried two additional analyses. First, they identified genetic variants in the RNA-Seq dataset, in LD with the GWAS hits. Second, they also interrogated the association between the GWAS SNP hits and the RNA abundance of genes which transcript abundance correlated with the same phenotype. They finally designed a panel of 73 SNPs that explained between 5 % and 36 % of the phenotypic variance of the sperm traits in their population (Gòdia et al., 2020c).

Most overlaps are observed between regions identified within the same study, primarily by Marques et al. (Marques et al., 2018) which also reported the most GWAS hits. Marques et al. conducted two independent wssGWAS, one on a Large White and the other on a Landrace, populations targeting identical traits. These traits included the percentage of motile sperm, the percentage of sperm cells moving in a straight line, the percentage of sperm cells with morphological abnormalities, and the log transformed total number of sperm cells in the ejaculate. They identified 20 and 16 genomic regions genetically associated with these traits in the Large White and Landrace populations, respectively. Among these, 16 and 7 regions were associated with more than one phenotypic parameter, notably between the two motility traits and, to a lesser extent, with the morphological abnormality trait, which is somehow expected as these traits yielded the largest proportion of GWAS hits. Interestingly, there were no overlaps between the genetic findings of the two breeds.

Nonetheless, some genomic regions identified in different studies were mapping in either overlapping positions or at least in close vicinity to each other. We assessed LD decay in the pig genome using genotypes obtained from nearly 10,000 randomly selected SNPs genotyped in 276 Pietrain boars (Gòdia et al., 2020c). Taking into account the calculated LD decay in the genome and considering $r^2 < 0.1$ as indicative of low LD, we established a distance threshold of three mega base pairs (Mbp) to determine whether two genomic regions are close enough to represent the same or a different locus (Fig. 1). Of particular interest are these positional similarities involving the same or related traits, regardless of the breed. Although these occurrences could be coincidental, they also provide added robustness to the identified hits, potentially indicating genuine genetic associations with specific genomic regions. Among the 13 instances of genomic co-location across studies, three involved the same trait (Table 2). Specifically, these co-locations were found on chromosomes 1 for morphological abnormalities (Gòdia et al., 2020c; Marques et al., 2018), 4 for motility (Marques et al., 2018; Zhang et al., 2023) and 7 again for motility (Marques et al., 2018; Reyer et al., 2024), within genomic regions 255.5–258.5, 121.2–124.2 and 82.6–86.9 Mbp, respectively. Furthermore, two GWAS regions appeared in three studies. This includes the previous region in chromosome 7, which also involved the percentage of cells with neck morphological abnormalities identified by Gòdia and co-authors (Gòdia et al., 2020c); and a region in chromosome 2 (143–148 Mbp) for the proportion of spermatozoa with bent tails (Zhao et al., 2020), number of cells in ejaculate (Marques et al., 2018), and the proportion of motile spermatozoa (Gao et al., 2019). In the last case, however, it must be noted that the study performed by Gao and colleagues (Gao et al., 2019) used a subset of the animals screened by Zhao et al. (Zhao et al., 2020). Notably, some of these GWAS hits map nearby genes genetically associated with similar phenotypes through candidate gene studies. For instance, the Estrogen Receptor 1 (*ESR1*), associated with the proportion of cells with proximal cytoplasm droplets in Pietrain (Gunawan et al., 2011), lies approximately 0.7 Mbp from a GWAS hit for the same phenotype and breed on chromosome 1 (Gòdia et al., 2020c), (Table 2). Similarly, Beta Actin (*ACTB*) (Lin et al., 2006) maps near a GWAS locus on chromosome 3 for the proportion of morphologically abnormal spermatozoa in Pietrain (Gòdia et al., 2020c) and the Nuclear Receptor Subfamily 4 Group A Member 1 (*NR4A1*) (Zhao et al., 2019) co-locates with a GWAS hit for motility on chromosome 5 in Large White (Marques et al., 2018). Furthermore, the Deleted In Azoospermia Like (*DAZL*) (Ma et al., 2013) aligns with a GWAS region on chromosome 13 for morphological abnormalities and motility in Duroc (Gao et al., 2019). Additionally, some gene-GWAS hit co-locations occurred for the same trait but in different breeds, as seen with the Nitric Oxide Synthase 2 (*NOS2*) for motility in Duroc

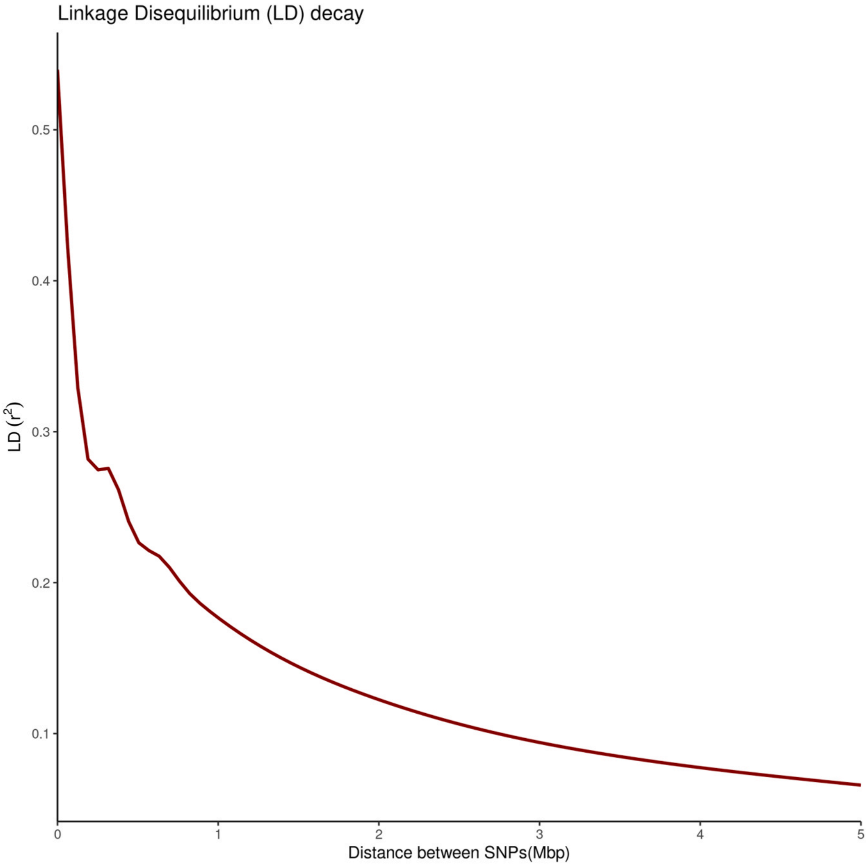

Fig. 1. Linkage disequilibrium decay in the pig genome. Linkage disequilibrium was calculated as r^2. We see that LD is close to 0.1 when the distance between two SNPs is around 3 million base pairs.

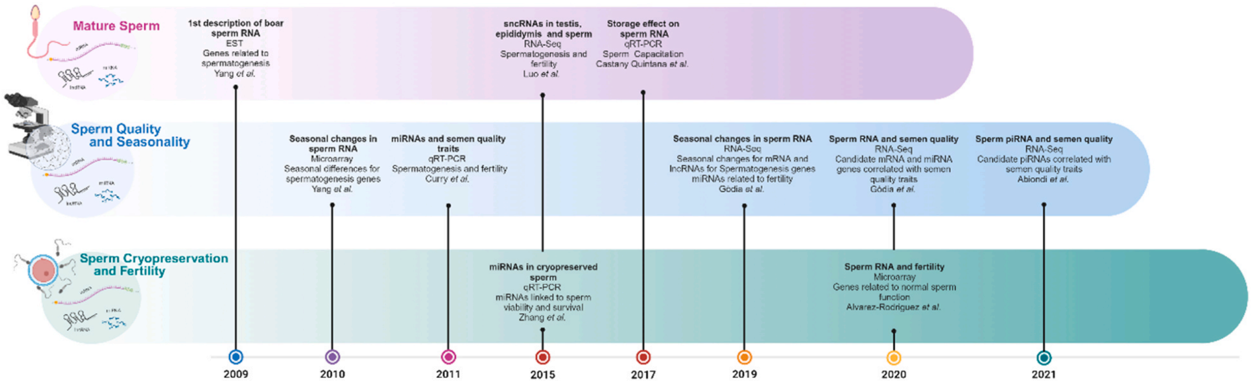

Fig. 2. Timeline of the transcriptomic studies in boar sperm.

(Wang et al., 2023) and a GWAS region for progressive motility on chromosome 12 in Large White (Marques et al., 2018).

Finally, another study explored the genetic basis of spermatogenesis using an original approach different than GWAS. Gòdia and co-authors conducted whole-genome sequencing (WGS) on the genomes from three Pietrain boars, identifying heterozygous sites. Leveraging the haploid nature of sperm cells, they analyzed the proportion of sequencing reads carrying each allele at these sites (Gòdia et al., 2020a) as a proxy of the proportion of haploid cells carrying each allele (Gòdia et al., 2020a). This analysis revealed 378 genes containing coding SNPs with allelic ratio distortion from the expected 0.5 in at least one sample, with minimal overlap between pigs. Many of these genes were directly associated with various stages of spermatogenesis, from meiosis to spermiogenesis (Gòdia et al., 2020a). These findings underscore the complexity of sperm development. Expanding upon this strategy to include a larger number of samples across various breeds may result in an expanded list of genetic variants robustly linked to the efficiency of spermatogenesis and ejaculated sperm cell count.

Overall, these results, which provide relevant information on the genetic basis of semen quality, should be taken with caution as they would require validation for consistency in different and larger cohorts of animals from different genetic backgrounds.

5. The transcriptome of the pig sperm and its relation to semen traits

In mammals, sperm cells are not transcriptionally active. It is widely accepted that the RNA content in sperm is the residual evidence of transcription in the final stages of spermatogenesis (Kramer and Krawetz, 1997). The importance of both protein-coding and regulatory RNAs in livestock has received considerable attention. Over the past 15 years, several contributions have been made regarding their effects on production traits, including their role in pig reproduction and fertility (Fig. 2).

5.1. Protein coding RNAs

In 2009, Yang et al. provided the first characterization of porcine sperm coding RNAs using expressed sequence tags (ESTs). They identified a highly fragmented RNA pool consisting of 514 unique sequences, half of which were not annotated (Yang et al., 2009). Several of these transcripts belonged to genes with known functions in spermatogenesis, including protamine P1 (*PRM1*) and protamine P2 (*PRM2*). Noteworthy, transcripts with significant roles during embryogenesis were also found. These included sperm-specific antigen 2 (*SSFA2*) and Sestrin 1 (*SESN1*). While this effort to characterize boar sperm RNAs proved fruitful, the study focused on samples from a single ejaculate obtained from a Landrace boar with good semen quality. Nearly ten years later, Gòdia et al. provided a technical framework outlining the conditions for the use of RNA-Seq to study the porcine sperm transcriptome (Gòdia et al., 2018a). Here, the authors explored several library preparation kits optimized for different conditions and highlighted the importance of selecting library kits optimized for low RNA input and highly fragmented RNAs. In 2019, Gòdia and co-authors expanded upon their previous work by using RNA-Seq to explore the transcriptome content of pig sperm (Gòdia et al., 2019). As pig sperm is prone to seasonal variation, leading to a general decline in semen quality during the summer and resulting in economic losses (Flowers, 1997), the authors also investigated seasonal changes in the pig sperm transcriptome. They used ejaculates from 10 Pietrain boars, equally distributed between summer and winter. They identified 4436 annotated genes. The genes exhibiting highest RNA abundance were from both mitochondrial (*COX1*, *COX2*, *ATP6* and *ATP8*), and autosomal origin, i.e. *PRM1*, ornithine decarboxylase antizyme (*OAZ3*), and heat shock protein 9 (*HSPB9*). These genes were primarily linked, in other studies, to normal sperm function and embryogenesis. The analysis also revealed 36 transcripts displaying significant differences in RNA abundance between the two seasonal periods. In agreement with previous reports (Zasiadczyk et al., 2015), some of these genes were related to oxidative stress and autophagy. This study marked the first use of RNA-Seq to assess seasonal changes in porcine sperm, but it had a limited sample size (five ejaculates in each seasonal group) and also, each of the ejaculates belonged to a different boar which means that this was not a matched-pair study (Gòdia et al., 2019). Notwithstanding, the seasonal changes on the pig sperm transcriptome had been addressed previously using a sperm-specific oligonucleotide microarray (Yang et al., 2010). In this study, Yang et al. identified 67 genes showing differential RNA abundance between ejaculates collected in summer and winter from six boars. Notably, significantly higher levels of testis-specific serine kinase 6 (*TSSK6*) and testis-specific kinase 1 (*TESK1*) transcripts were observed in winter, both of which play important roles in spermatogenesis. They also detected altered RNA levels of heat shock protein coding genes. These findings highlighted the pivotal role of transcriptomics in clarifying the genetic and environmental factors affecting semen traits. Remarkably, there were no coincidences between these two studies.

In 2012, Kaewmala et al. attempted to associate sperm RNA levels and semen quality (Kaewmala et al., 2012). They used real-time quantitative polymerase chain reaction (RT-qPCR) to quantify the expression of phospholipase C zeta (*PLCZ1*) and cyclooxygenase isoenzyme type 2 (*COX2*) genes, both with important roles in spermatogenesis. The study considered ejaculates from six boars, assigned into two groups based on high or low semen quality. The authors did however not report significant differences in RNA levels of these two genes between groups. Significant efforts were made to link sperm cell transcriptome analysis to semen quality and fertility traits. As previously described in the section on the genetic basis of pig semen traits, Gòdia et al. addressed transcriptome changes associated with semen quality by combining RNA-Seq and GWAS, and link transcript levels with semen quality traits (Gòdia et al., 2020c). The RNA-Seq analysis, performed on 40 Pietrain sperm samples with data for 25 sperm quality parameters, from different boars, found 6128 correlations involving the RNA levels of 3007 genes. Notably, among the most frequently correlated genes were tetratricopeptide repeat domain 28 (*TTC28*), which plays a crucial role during cell division, and ATP-binding cassette (*ABCA3*), correlated with up to nine motility-related parameters. Finally, the results of GWAS and RNA-seq were integrated to highlight the most important genes, which included genes related to calcium influx, DNA repair or chromatin remodeling. The impact of capacitation in the boar transcriptome has also been studied under the hypothesis that nuclear genes are expressed in sperm while in the female reproductive tract until fertilization (Gur and Breitbart, 2006). Studying 5 samples (before and after capacitation *in vitro*) the authors identified 5342 differentially abundant genes, related to sperm apoptosis, mitochondrial membrane potential and spermatogenesis alteration (Li et al., 2018). We, nevertheless, suggest taking results with caution as not enough evidence support the hypothesis that active nuclear transcription can occur in sperm. A different scenario is the contribution of RNAs from different reproductive organs (epididymis, prostate, seminal vesicles, testes) through extracellular vesicles (EV) present in the seminal plasma on sperm maturation and capacitation. These RNAs can enter the sperm cell, thereby altering the spermatozoon RNA content and functional characteristics. To the best of our knowledge, two studies have recently interrogated the miRNA content of seminal-plasma vesicles in swine and also their potential relation with semen quality. Overall, these studies found hundreds of miRNAs, although the miR-10 family was by far the most abundant miRNA (Dlamini et al., 2023). Moreover, Dlamini and co-authors compared the corresponding miRNA profiles of sperm samples classified as having good or bad quality according to several semen quality parameters (Dlamini et al., 2023). The analysis showed significantly different (false discovery rate, FDR correction) miR-9828–3p levels in the two groups (Dlamini et al., 2023). A comparison of the seminal plasma EV in pig sperm samples classified in two groups depending in their motility, also showed some miRNAs (e.g.; miR-486, miR-122–5p) with differential RNA abundance (Zhao et al., 2024). Following, Alvarez-Rodriguez et al. used microarrays to study the link between the sperm transcript content and fertility traits (Alvarez-rodriguez et al., 2020). They analyzed 28 ejaculates from seven Landrace and Large White pigs. To assess fertility outcomes, they used different ejaculates of these boars to inseminate over 1000 sows and recorded farrowing rate and litter size to classify the ejaculate into high and low fertility groups. Differential RNA abundance analysis between these groups revealed 521 genes with significant differences, including pivotal genes associated with normal sperm function such as *CATSPERG* and *GRK4*. The study is however limited by the small sample size and the use of different ejaculates for insemination compared to those collected for RNA analysis. Future efforts should focus on associating RNA content from samples of the same ejaculate to provide a clearer picture of paternal contributions to fertilization and even possible evidence of intergenerational epigenetic inheritance.

Another important aspect in the context of AI is to understand the effect of the conditions in which ejaculate doses are commercialized on the sperm transcriptome. These conditions include the dilution with extenders and subsequent storage, which have been linked to semen quality changes (Rodriguez et al., 2017). A recent study by Castany Quintana et al. addressed this aspect by questioning the implications of dilution and storage on the transcriptome of sperm cells (Castany Quintana et al., 2022). The authors quantified the mRNA abundance of several aquaporin genes (e.g., *AQP3* and *AQP7*) using RT-qPCR in 10 sperm samples obtained from pooled ejaculates of three boars. The study highlighted changes in the mRNA content of aquaporins and emphasized a positive correlation with the capacitation rate, which increased with storage time. It, however, is worth noting that the study had a limited number of observations obtained from pooled ejaculates. Pooling ejaculates may not be the most suitable approach for describing mRNA abundance, as it makes it difficult to discern whether differences observed in mRNA levels are specific to a particular boar from the pool or represent a general pattern.

Cryopreservation of semen is an inefficient procedure for boar reproduction, as it often yields lower fertility outcomes as compared to liquid-stored semen (Knox, 2015). RNAs have also been suggested to regulate spermatozoa under freezing conditions. Ding-Hui and colleagues performed RNA-Seq in 11 individuals with matching fresh and cryopreserved sperm. A total of 567 genes were found differentially abundant, including genes related to the activation of calcium ion pump or AKT signaling pathway (Dai et al., 2019). Nevertheless, we suggest taking the results with caution as the number of mapped reads was very low compared to other RNA-Seq studies. Furthermore, it may be important to emphasize the need for a specific washing step for cryopreserved sperm prior to RNA extraction. The use of several reagents for the cryopreservation protocol, including among others egg yolk, could potentially alter the RNA profile. Cryopreservation was also studied by Fraser et al., who performed RNA-Seq in Polish Large White boars divided between good and poor semen freezers (Fraser et al., 2020). By studying fresh ejaculates from the same individuals as a proxy, the authors identified 52 differentially abundant genes, enriched for functions related to energy metabolism, as *ACADM* and *ND6*. The same group had recently validated using RT-qPCR the differently abundant RNA levels of *TXNRD1* and *HSPA4L* genes for their role in stress and heat shock in 10 boars. In spite of this, the protein abundance of these genes in the pig sperm could not be validated in a follow up study (Mańkowska et al., 2022b). As a general comment, most of these studies used a relatively small sample size and their results should be taken with caution.

5.2. *Regulatory RNAs*

Sperm cells carry an RNA payload that can be considered as debris from the spermatogenesis process but may also include RNAs that will be delivered and used upon fertilization and embryo development (Gòdia et al., 2018b). Aside from the population of protein coding genes discussed in the previous section, ncRNAs also play an important role in regulation of RNA abundance and maintenance of the genome (Gòdia et al., 2018b). In porcine spermatozoa, some studies have aimed at characterizing ncRNA populations and elucidate their potential functions or use as biomarkers (Fig. 2).

In 2011, Curry et al. questioned whether variations in miRNAs abundance were associated with differences in semen quality traits (Curry et al., 2011). Using RT- qPCR, the authors quantified the abundance of 10 miRNAs predicted to have regulatory function over genes involved in spermatogenesis. This quantification was performed on 22 sperm samples, including both normal sperm quality and samples with high abnormality rate or low motility. The authors identified miRNAs more prevalent in the abnormal group as compared to the normal group. Among these miRNAs were miR-22, with regulatory functions during spermatogenesis (Abhari et al., 2014), and let-7a, let-7d, and let-7e miRNAs, which have been associated with embryo development in other species (Liu et al., 2012; Viñas et al., 2013). Zhang et al. also employed RT-qPCR to investigate the role of miRNAs in the context of semen cryopreservation (Zhang et al., 2015). They measured the abundance of 15 miRNAs in epididymal sperm, and sperm from fresh and cryopreserved ejaculates collected

Table 3
Summary of the epigenetic studies in pig sperm.

Epigenetic mechanism	Study	Objective	Technique	Main findings
chomatin compactation	Godia et al.PeerJ. 2023; 11:e15520	Assess nucleosome-retained DNA regions in sperm	MNAse-Seq	Nucleosome-associated DNA is 0.3 % in mature sperm. There is programmatic retention near genes related to fertilization and embryogenesis.
DNA methylation	Congras et al.Biol Reprod. 2014; 91:137	Assess global methylation levels and identify differences between control and infertile boars	LUMA	Mean methylation level of 77 %. No differences found between groups.
	Congras et al.Biol Reprod. 2014; 91:137	Test 38 imprinted loci to find differences between control and infertile boars	MeDIP-qPCR	Genes associated with low sperm quality with hypermethylation (*RTL1, MEG3 DMR, DLK1/MEG3, NESP55, GNASXL ICR, GRB10, RASGRF1, PEG10, WT1, IMPACT B, DAZL*) and hypomethylation (*RASGRF1 DMR, IMPACT*).
	Congras et al.Biol Reprod. 2014; 91:137	Validate previous hyper- hypomethylated loci.	BS-pyrosequencing	Only *NESP55* locus could be validated. Additional extension of the genomic region studies suggested *GNAS* as a candidate locus.
	Perrier et al.BMC Genomics 2018; 19 (1):404	Study overall methylation levels and compare to bull's sperm	RRBS	Average methylation level of 72.6 %, higher than in bull's sperm (45.5 %).
	Khezri et al.BMC Genomics 2019; 20 (1):897	Identify differences across methylation patters in different levels of DFI	RRBS	Similar patterns of sperm methylation across DFI groups (average 33 %). Differentially methylated genes were enriched for membrane function, metabolic cascade and antioxidant defense system.
	Pertille et al.Int. J. Mol. Sci. 2021; 22(5):2679	Identify DMR between fertile and infertile boars and across different season of ejaculates' collection	GBS-MeDIP	No DMRs were identified using statistical correction with FDR. Suggestive DMRs (*P*-value) were identified in the fertility comparison (46 DMRs) and seasonality (40–49 DMRs) groups. Genes within those DMRs were related to sperm quality and capacitation.

BS-pyrosequencing: Bisulfite conversion coupled with pyrosequencing. DFI: DNA Fragmentation Index. DMR: Differentially Methylation Region. FDR: False Discovery Rate. GBS-MeDIP: Genotype-By-Sequencing followed by Methylated DNA Immunoprecipitation. LUMA: Luminometric Methylation Assay. MeDIP-qPCR: Methylated DNA Immunoprecipitation followed by Real-Time PCR. MNAse-Seq: Micrococcal Nuclease followed by sequencing. RRBS: Reduced Representation Bisulfite Sequencing.

from three Landrace boars. They observed higher miRNA abundance in mature fresh and cryopreserved semen as compared to epididymal sperm. More interestingly, they described a decrease in the abundance of let-7c, mir-26a, and miR-186 from fresh to cryopreserved sperm. Notably, mir-26a has recently been linked to sperm viability and survival (Wang et al., 2020), whereas the human orthologue of miR-186 has been associated with fertility outcomes (Zhao et al., 2023a). In 2015, Luo and co-authors addressed the potential role of miRNAs during spermatogenesis by characterizing the testis, epididymis, and ejaculated sperm through RNA-Seq (Luo et al., 2015). The authors identified 4761 potential miRNAs and among the most abundant in all three tissues were mir-10b, miR-26a, and miR-191, all implicated in embryonic development. miR-34c and miR-16 were differentially expressed in sperm compared to both testis and epididymis. The authors highlighted a potential role of miR-34c in sperm development, but more recent evidence also involved this gene in regulating the transcriptome of the zygote after fertilization (Cui et al., 2023). In recent years, Gòdia and co-authors have provided light into the seasonal effects on microRNAs and their correlation with semen quality parameters. In their 2019 study, which described the sperm transcriptome using RNA-Seq on 10 ejaculates collected in both summer and winter, the authors identified 7 miRNAs with significant seasonal differences (Gòdia et al., 2019). Notably, miR-34c, miR-191, miR-30d, miR-10b, and let-7a were among the most abundant miRNAs identified, with miR-34c, miR-1249, and miR-106, all previously associated with fertility, also showing significant seasonal differences. In the subsequent GWAS-RNA study, the authors linked regulatory RNAs with semen quality traits by correlating RNA abundance levels with 25 sperm quality measurements (Gòdia et al., 2020c). They identified 95 small non-coding RNAs (sncRNAs), 87 of which were significantly correlated with multiple semen traits. Examples included miR-23a, miR-122, and miR-27a, previously linked to sperm morphology and fertility in human.

Not only have miRNAs been studied in porcine sperm as sncRNA regulators. Ablondi and colleagues profiled the piRNA population for their role as transcriptional silencers of transposable elements to ensure genome stability (Ablondi et al., 2021). Using a set of 34 small RNA-Seq datasets from mature boars from the study published by Gòdia et al. (Gòdia et al., 2020c), the authors annotated the piRNA landscape identifying over 280,000 piRNAs (know and novel). Of these, 1355 piRNA were found correlated with sperm quality parameters of the same boars, thereby suggesting a potential involvement in these traits. The set of lncRNAs in boar sperm have also been the subject of study. In 2019, Gòdia et al. briefly described the identification of 27 lncRNAs predictive to regulate genes as *ZNF217* and *DYNLRB2* with functions related to spermatogenesis, sperm motility and normal sperm function (Gòdia et al., 2019). This study, however, focused only on lncRNAs annotated in the pig genome, which are still under ongoing improvements. Alongside with the expected improvement on this annotation, we can foresee that the population of lncRNAs identified in in boar sperm will grow rapidly. The same group also used their RNA-Seq dataset from 40 Pietrain samples (Gòdia et al., 2020c) to identify potential circRNAs. The study yielded 1598 putative circRNAs, and the host genes were enriched for epigenetic regulation and spermatogenesis gene ontology functions (Gòdia et al., 2020b). The authors also found that the RNA levels of 148 circRNAs correlated with sperm motility parameters. Two out of a total of 6 circRNAs selected could be validated with Sanger Sequencing and RT-qPCR.

6. The epigenome of the pig sperm and its relation to semen traits

The spermatozoon undergoes an orchestrated condensation of its chromatin by a progressive replacement of histones with protamines for compacting the paternal genome in the sperm head (Ward and Coffey, 1991), thus ensuring genomic integrity for fertilization and normal embryo development (Ward, 2010). Only a minor fraction of sperm DNA retains its structure in histones, with estimates ranging from 4 % to 10 % in humans (Brykczynska et al., 2010; Castillo et al., 2014; Gatewood et al., 1987; Hammoud et al., 2009), 13.5 % in cattle (Samans et al., 2014) and 1–2 % in mice (Balhorn et al., 1977; Carone et al., 2014; Erkek et al., 2013; Johnson et al., 2016). Recent research conducted by Gòdia and colleagues suggested that in porcine mature sperm, there is a retention of only 0.3 % nucleosome-associated DNA (Gòdia et al., 2023). The results pointed towards a systematic retention of nucleosomes in gene promoters, particularly enriched in those genes associated with embryo development and organ morphogenesis (Table 3). Additionally, these retained DNA regions showed enrichment of motifs for transcription factors linked to embryo development and implantation, such as *HOXA1*, *RUNX2* or *Znf263*. Despite the general state of transcriptional silence in mature spermatozoa, genes located within histones-retained DNA presented significantly higher RNA abundance compared to baseline levels, suggesting non-random presence of genes' RNA levels. We also observed an enriched co-location of nucleosome-associated DNA with piRNAs associated with sperm quality parameters, including motility, viability, and morphological abnormalities (Ablondi et al., 2021). Yet, no significant co-location of histone-retained DNA was found with GWAS regions related to sperm quality traits from the same porcine breed population (Gòdia et al., 2020c). Despite the efforts to study histone modifications following chromatin extraction, insufficient DNA was obtained for further analysis (Gòdia et al., 2023). Increasing the initial cell number to a minimum 300 million cells is advisable. Additionally, conducting multiple replicates is also indispensable to mitigate potential limitations due to MNase digestion susceptibility or variation in DNA yield. To date, no studies on histone modifications have been performed in swine sperm, but in humans, mature spermatozoa histone modifications localize preferentially in developmental loci (Hammoud et al., 2009), thus this approach presents a promising avenue for future investigation in mature sperm.

At the onset of spermatogenesis, DNA methylation begins to accumulate in the DNA, ultimately resulting in mature mammalian sperm cells with over 70 % methylation (Lismer and Kimmins, 2023). To date, few studies have investigated global DNA methylation in porcine sperm. The first study was performed by Congras and colleagues in 2014 by comparing 5 Large White and Pietrain boars with normal sperm quality parameters and 8 boars with low parameters, including asthenospermia, teratospermia, oligospermia, or combinations thereof (Congras et al., 2014). The authors identified an average methylation level of 77 % but no differences between the case and the control groups (Congras et al., 2014). Following, methylation levels were specifically quantified for 38 candidate loci involved in imprinting, as alterations during the formation of the epigenetic marks can result in sperm defects. Of these, 17 loci presented significant differences in methylation levels between the studied groups. The authors then validated the results performing bisulfite conversion followed by pyrosequencing. Increased methylation levels of *NESP55* and *GNASXL* genes, belonging to the imprinted *GNAS* locus were found in the three teratospermic boars presented (Table 3) (Congras et al., 2014). Further research increasing sample size and with different sperm etiologies could help discern the involvement of DNA methylation levels of *GNAS* in sperm quality. In 2018, Perrier et al., reported an average of 72.6 % CpG methylation in three porcine sperm samples (Table 3) (Perrier et al., 2018). This study, however, primarily focused on the methylome of bulls' sperm, providing no specific details on the porcine methylation landscape. Shortly after, Khezri and colleagues searched for differences in methylation patterns between boars with divergent DFI, which are known to be associated with fertility (Khezri et al., 2019). In their study, the authors reported surprisingly low (33 %) CpG methylation levels (Table 3). Despite the possibility of this issue being of a technical nature, the study identified differentially methylated cytosines when comparing the extreme groups. Notably, these differences were mainly annotated in promoter regions of genes enriched for acetylation and phosphorylation pathways, as well as antioxidant defense system (Khezri et al., 2019). The role of methylation in porcine fertility and season of ejaculate collection has also been studied by Pértille et al., (Pértille et al., 2021). The authors did not identify significant differences between fertility nor seasonality groups when correction for multiple testing with FDR was set in their analyses. Yet, without an FDR filter, 46 differentially methylated regions (DMRs) were found in relation to fertility, and 40–49 DMRs were found across different seasonal periods compared. Some of the genes annotated within those regions were related to sperm motility, concentration, development or capacitation (Table 3). These results suggest the implication of seasonality on shaping the DNA methylome in sperm. We would notwithstanding suggest increasing the sample size, assessing potential cofounding effects between fertility and seasonality, and to be cautious with the use of GBS-MeDIP with the restriction enzyme *Pst*I in swine. *In-silico* analysis indicates a preferential enzyme cut for repetitive elements and it may bias the results (data not shown). We also encourage using high quality mapped reads (mapping quality > 20) to avoid multi-mapped reads that can hamper further analyses.

7. Single cell RNA-Seq studies on porcine testicle

As previously mentioned, the sperm cell is typically considered transcriptionally silent, lacking active transcription of nuclear genes. Indeed, the RNA content of sperm is significantly lower compared with other cell types, and these RNAs are often highly fragmented. Additionally, sperm chromatin is predominantly ultra-compacted with protamines, rendering it inactive. The majority of studies that have interrogated RNA levels in relation to semen traits have targetted spermatozoa because these cells are highly accessible in the ejaculate and can be collected with non-inavise methods. The sperm cell is the end result of spermatogenesis, which involves germline (spermatogonia, spermatocytes, spermatids) and somatic cells (Sertoli, Leydig, Peritubular myoid) in the testicle. These spermatogenic cells are actively transcribing genes and exhibit dynamic and active epigenetic marks. Thus, semen quality is likely influenced by the transcriptional and epigenetic events occurring throughout spermatogenesis in these cells. Furthermore, our understanding is limited regarding how accurately the mature sperm's transcriptome and epigenome reflect those of their precursor cells.

Single-cell based approaches hold strong promise for studying semen traits and even boar fertility, as they enable the molecular characterization of each cell in a single experiment. To date, six published studies have interrogated the pig testicle's coding transcriptome using single-cell RNA-Seq. The first published article focused on identifying and characterizing the different cell types of the pig testis, along with a list of candidate gene markers for each cell type, with a special focus on spermatogonia (Zhang et al., 2022). In this study, the authors identified 12 candidate markers for spermatogonia, two of which were validated and suggested to allow identifying a subset of undifferentiated spermatogonia (*CD99*) and the global set of differentiating spermatogonia (*PODXL2*). Other studies have focused on various aspects of sexual development or on spermatogenesis. Some of this research centered their efforts on somatic cells, while others investigated spermatogonia or cells involved in spermiogenesis, the latest stage of spermatogenesis whereby the spermatozoon acquires its morphology and molecular characteristics, which mainly involve spermatids. More in detail, Zhang and colleagues evaluated the dynamic changes in cell types, cell number and their gene expression with special focus on somatic cells through sexual maturation in Guanzhong black pigs (Zhang et al., 2022). Voigt et al. extended this research by exploring the microenvironment of spermatogonial stem cells during boar sexual development. Using the scRNA-Seq datasets from the two previous articles, scRNA-Seq from human testicles, phospho-proteomics and lipidomics (Voigt et al., 2023). As Sertoli cells are responsible of providing the niche to spermatogonial development, the authors focused on this cell type. Their analyses revealed associations between development of spermatgonial development, which in pigs starts at the age of 8 weeks, lipid composition in seminiferous tubules, and the onset of Sertoli cell maturation (Voigt et al., 2023). Transitioning to the later stages of spermatogenesis, Zhao et al. delved into spermiogenesis, characterizing dynamic gene expression changes during spermatid to spermatozoon develoopment in pig and humans (Zhao et al., 2023b). Their analysis identified both shared and species-specific patterns and uncovered a novel gene, *SNRPD2*, potentially playing a significant role in regulating gene expression during spermiogenesis (Zhao et al., 2023b). Additionally, Giassetti and colleagues conducted scRNA-Seq on testes from mouse, cattle and pigs, alongside knockout studies in mice, to investigate the expression and function of *ARRDC5* in spermiogenesis and its effect on sperm morphology, motility, capacitation ability and fertility (Giassetti et al., 2023). Continuing the exploration of spermatogenesis and associated cellular dynamics, Liu et al. carried scRNA-Seq on both porcine and murine testicles and epididymis (Liu et al., 2024). In addition to characterizing the testicular cell types throughout spermatogenesis they also investigated epithelial cells in the the caput, corpus, and cauda segments of the epididymis. These sequential segments of the epididymis play vital roles in the maturation of sperm cells as they transit throught this duct system (Liu et al., 2024). Overall, these studies show remarkable similarity albeit also species-specifities between human, mouse and pig gene expression in the different testicular cell types. None of these research efforts have, notwithstanding, been carried in the context of understanding the variation underlying semen quality or boar fertility.

8. Future perspectives and conclusions

Enhanced comprehension of the molecular and genetic underpinnings of semen quality traits in swine holds significant potential for informing husbandry and managerial decisions aimed at improving these traits. Moreover, integrating this knowledge into GS schemes could yield considerable benefits. While currently, only a few companies have begun incorporating certain semen quality parameters into their selection indexes, we anticipate a broader adoption of this trend by other pig genetic companies in the near future. The identification of genetic markers, ideally causal variants directly shaping these traits would help in GS. Research efforts should also focus on several additional aspects of sperm quality. These efforts should prioritize identifying a combination of semen quality parameters to effectively assess fertility indicators such as conception rate litter size. It is likely that the set of regularly evaluated semen quality parameters does not fully explain fertility, as fertility is also influenced by genetic and molecular factors that govern the fertilization of the egg, genome recognition and embryogenesis. Consequently, we need to better understand these molecular loads, whether metabolic, proteomic, transcriptomic or epigenomic. Future studies should also aim to elucidate how variation in sperm RNA and its epigenomic makeup contribute to intergenerational inheritance and how we can leverage this to obtain better offspring. In this regard, understanding to which extent and how environmental factors, including the season of collection, the age of the boar, nutrition and housing conditions, influence the sperm transcriptome and epigenome as well the offspring across generations is essential to assess its potential in animal breeding and health.

Considering the notable intra-individual variation in semen quality, research should also invest on identifying the genetic basis of the robustness across ejaculates from individual boars. Both pig genetics companies and AI farms stand to gain from these genetic markers as they would facilitate the selection of more resilient animals, ensuring more predictable outcomes during servicing. In the context of climate warming, it is also crucial to understand the molecular changes and the genetic basis of resilience associated with decreased semen quality in geographical regions where summers are warm.

Despite the valuable insights we can obtain from evaluating the molecular makeup of ejaculated sperm using non-invasive collection methods, we must also acknowledge that these molecular contents in sperm may not accurately reflect the events that occurred during spermatogenesis. Employing single-cell approaches in the testicle and other male sexual organs can assess this issue by profiling each specific cell type. Coupled with genetic studies such as GWAS and WGS, particularly in animals with extreme phenotype or genetic merit for male reproductive traits, this approach could significantly advance the identification of causal variants affecting these traits.

Ethical approval

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CRediT authorship contribution statement

Yu Lian: Writing – review & editing, Writing – original draft. **Nicole Lewis:** Writing – review & editing, Writing – original draft. **Marta Gòdia:** Writing – review & editing, Writing – original draft. **Pedro Sa:** Writing – review & editing, Writing – original draft. **Alex Clop:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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