



Metabolomics, transcriptomics, and sperm quality analyses to investigate biomarkers in boar semen related to sexual maturity

Asmita Shrestha

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Faculty of Applied Ecology, Agricultural Sciences and Biotechnology

Abstract

Puberty to sexual maturity marks a key transition in boar reproductive development, yet the molecular and functional changes that occur in semen during this transition remain poorly described. In modern pig breeding, artificial insemination is central to breeding and genetic improvement, and the ability to use boars as young as possible can reduce costs and accelerate genetic gains. At present, decisions about when the boars are ready for semen collection rely mainly on age and analysis of ejaculate volume, sperm concentration, motility, and morphology, which do not fully capture the biological processes underlying maturation. This thesis addresses these gaps by following Duroc boars longitudinally from puberty to sexual maturity, with metabolomics, transcriptomics, and sperm quality assessments to uncover the molecular and functional signatures of maturation. Semen samples were collected longitudinally from individual boars through three maturation ages: at approximately 7 months, 6 weeks later, and finally 12 weeks after the initial sample collection. This longitudinal approach eliminated inter-individual genetic variability while capturing the genuine maturational period.

Across four studies, complementary approaches were applied to investigate different biological layers, including targeted metabolomics, untargeted metabolomics, sperm functional assessment, and transcriptomic profiling. Targeted metabolomics was used to quantify amino acids and amines in sperm and seminal plasma, identifying glutamate, alanine, aspartate, choline, and taurine in sperm as key metabolites that changed significantly with age and may serve as potential biomarkers of sexual maturity. Untargeted metabolomics expanded this analysis, capturing thousands of features and highlighting pathways such as porphyrin metabolism, taurine and hypotaurine metabolism, and glycerolipid metabolism, which were significantly enriched during sexual maturation. Functional analyses showed that sperm motility, acrosome integrity and viability, DNA integrity, and tolerance to semen storage all improved significantly as boars matured. Several metabolites, including glutamate, cystine, aspartate, taurine, and choline, were linked directly to these functional gains. Transcriptomic profiling of sperm revealed dynamic changes in both coding and non-coding RNAs. A total of 60 genes and six miRNAs were identified as differentially expressed, which are associated with key reproductive processes such as spermatogenesis and germ cell development. This provides insights into the molecular mechanisms that drive functional improvements. Furthermore, an integrated mRNA-miRNA analysis identified regulatory networks that could influence the

maturation process. Together, these findings provide the first comprehensive longitudinal characterization of sexual maturation in boars.

This thesis contributes both to fundamental knowledge and applied value to the field of animal reproduction. From a practical perspective, it establishes a basis for further research to develop molecular biomarkers that can guide the usage of young boars in semen production and breeding, thereby reducing non-productive days and accelerating genetic gain in breeding programs.

Sammendrag

Overgangen fra pubertet til seksuell modenhet markerer en viktig fase i råners reproduktive utvikling, men de molekylære og funksjonelle endringene som skjer i spermene i denne overgangsfasen er fortsatt begrenset beskrevet. I moderne svineavl er kunstig inseminasjon sentralt for avl og genetisk framgang, og jo tidligere unge rånere kan tas i bruk, desto større er muligheten for å redusere kostnader og øke genetisk gevinst. Per i dag baseres beslutninger om når rånere er klare for sæduttak hovedsakelig på alder og analyse av ejakulatvolum, spermiekonsentrasjon, motilitet og morfologi, som ikke fullt ut fanger opp de biologiske prosessene som ligger til grunn for modningen. Denne avhandlingen tar for seg disse kunnskapshullene ved å følge Duroc-rånere fra pubertet til seksuell modenhet, med bruk av metabolomikk, transkriptomikk og sædkvalitetsanalyser for å avdekke de molekylære og funksjonelle kjennetegnene ved seksuell modning. De samme individene ble fulgt gjennom tre stadier: fra rundt 7 måneders alder, seks uker senere og tolv uker etter første prøvetaking. Denne longitudinelle tilnærmingen eliminerer genetisk variasjon mellom individer og fanger opp den reelle modningsprosessen.

Gjennom fire studier ble komplementære metoder brukt for å undersøke ulike biologiske nivåer: målrettet metabolomikk, global metabolomikk, funksjonelle analyser av spermier og transkriptomisk profilering. Målrettet metabolomikk ble anvendt til å identifisere aminosyrer og aminer som glutamat, alanin, aspartat, kolin og taurin i spermier som ble betydelig endret med alder og kan fungere som biomarkører for seksuell modenhet. Global metabolomikk utvidet dette bildet, med tusenvis av påviste metabolitter og sentrale metabolske veier som porfyrinmetabolisme, taurin og hypotaurinmetabolisme, og glyserolipidmetabolisme. Funksjonelle analyser viste at motilitet, akrosomintegritet og levedyktighet, DNA integritet og lagringstoleranse alle forbedret seg betydelig med alder. Flere metabolitter, inkludert glutamat, cystin, aspartat, taurin og kolin, var direkte knyttet til disse funksjonelle forbedringene. Transkriptomisk profilering av sædprøver fra seks rånere viste dynamiske endringer i både kodende og ikke-kodende RNA, inkludert 60 differensielt uttrykte gener og seks miRNA forbundet med prosesser som spermatogenese og kimeutvikling. En integrert mRNA-miRNA analyse identifiserte dessuten regulatoriske nettverk som kan påvirke modningsprosessen. Samlet gir disse funnene den første omfattende longitudinelle karakteriseringen av seksuell modning hos rånere.

Denne avhandlingen bidrar både med grunnleggende kunnskap og anvendt verdi innen reproduksjonsforskning på gris. Vitenskapelig sett bidrar dette arbeidet til økt forståelse av reproduktiv biologi ved å identifisere sentrale metabolitter, gener og signalveier som styrer modningen. Fra et praktisk perspektiv etablerer resultatene i denne avhandlingen et grunnlag for videre forskning for utvikling av molekulære biomarkører som kan brukes i utvelgelsen av unge råner i avlsprogram, redusere ikke-produktive dager i sædproduksjonen og øke den genetiske framgangen.

Preface

When I look back on these past three years, I see more than experiments and data. I see patience, resilience, a great learning experience, and the people who kept me steady along the way. These pages exist not only because of my persistence but also because of the kindness, wisdom, and encouragement of many people.

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Asmita Shrestha

Hamar, September 2025

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- II Shrestha, A., Gaustad, A. H., Øiaas, J. B., Nordborg, A., Kommisrud, E., van Son, M., Hansen, T., & Alm-Kristiansen, A. H. (2025). Untargeted metabolomics reveals changes in boar sperm and seminal plasma metabolites associated with sexual maturity. *Journal of Animal Science and Biotechnology*, 16, 123. <https://doi.org/10.1186/s40104-025-01258-x>
- III Rouzbehani, S.¹, Shrestha, A.¹, Haque, N., Nushrat, S.A., Zeremicheal, T.T., Nordborg, A., Gaustad, A.H., Alm-Kristiansen, A.H. Influence of sexual maturity on sperm quality in Duroc boar. *Manuscript submitted to Animal Reproduction Science*.
- IV Shrestha, A., van Son, M., Hashim, A., Rouzbehani, S., Gilfillan, G., Bergen, U., Gaustad, A.H., Kommisrud, E., Alm-Kristiansen, A.H. Sperm RNA landscape during sexual maturation in Duroc boars. *Manuscript submitted to BMC Genomics*.

List of abbreviations

<i>ACADM</i>	acyl-CoA dehydrogenase medium chain	<i>DNAJB8</i>	DnaJ heat shock protein family member B8
Adj <i>P</i>	adjusted <i>P</i> -value	<i>EAF2</i>	ELL associated factor 2
AGPC	guanidinium thiocyanate-phenol- chloroform	<i>EVI5</i>	ecotropic viral integration site 5
AI	artificial insemination	<i>FOS</i>	Fos proto-oncogene
<i>AKAP1</i>	A-kinase anchoring protein 1	<i>FOXP4</i>	forkhead box P4
ALH	amplitude of lateral head displacement	FSH	Follicle-stimulating hormone
<i>ALMS1</i>	ALMS1 centrosome and basal body associated protein	<i>FSIP2</i>	fibrous sheath interacting protein 2
ATP	adenosine triphosphate	GABA	gamma-aminobutyric acid
<i>BAMBI</i>	BMP and activin membrane bound inhibitor	gDNA	genomic DNA
BCF	beat cross frequency	GnRH	Gonadotropin-releasing hormone
<i>BSN</i>	bassoon presynaptic cytomatrix protein	GO	Gene Ontology
cAMP	cyclic adenosine monophosphate	HDS	high DNA stainability
CASA	computer-assisted sperm analysis	HILIC	hydrophilic interaction liquid chromatography
<i>CATSPERG</i>	cation channel sperm associated auxiliary subunit gamma	hr	hour
<i>CDC25B</i>	cell division cycle 25B	<i>HSP90AA1</i>	heat shock protein 90 alpha family class A member 1
cDNA	complementary DNA	Hz	Hertz
<i>CIB2</i>	Calcium and integrin binding family member 2	<i>ITGAL</i>	Integrin subunit alpha L
DFI	DNA fragmentation index	KEGG	Kyoto Encyclopedia of Genes and Genomes

LC-MS/MS	liquid chromatography-mass spectrometry/mass spectrometry	PLS-DA	partial least squares discriminant analysis
LH	luteinizing hormone	PNA	peanut agglutinin (fluorescein-labelled)
LIN	linearity	<i>PRM1</i>	protamine 1
lncRNAs	Long noncoding RNAs	<i>PRM2</i>	protamine 2
<i>MED13</i>	Mediator complex subunit 13	<i>PTPN2</i>	protein tyrosine phosphatase non-receptor type 2
miRNAs	micro RNAs	<i>PTPRU</i>	protein tyrosine phosphatase receptor type U
mL	milliliters	RapidNP	rapid non-progressive
MRM	Multiple-reaction monitoring		
mRNAs	messenger RNAs	RapidP	rapid progressive
Mt	million tonnes	<i>RGS12</i>	regulator of G protein signaling 12
MtrRNAs	mitochondrial ribosomal RNAs	<i>RIN</i>	RNA Integrity Number
MttRNAs	mitochondrial transfer RNAs	RNA	Ribonucleic acid
<i>NCLN</i>	nicalin	RNA-seq	RNA sequencing
<i>ND6</i>	NADH dehydrogenase subunit 6	rRNA	ribosomal RNA
<i>NFATC3</i>	Nuclear factor of activated T cells 3	RT	room temperature
ng	nanograms	SCSA	Sperm Chromatin Structure Assay
NIST	National Institute of Standards and Technology	<i>SESN1</i>	sestrin 1
NR56	non-return rate at 56 days	sncRNAs	short noncoding RNAs
<i>OAZ3</i>	ornithine decarboxylase antizyme 3	snoRNAs	small nucleolar RNAs
PBS	phosphate-buffered saline	snRNAs	Small nuclear RNAs
PCA	principal component analysis	<i>SPEM1</i>	spermatid maturation 1
<i>PHC1</i>	polyhomeotic homolog 1	<i>SPEM2</i>	spermatid maturation 2
PI	propidium iodine	<i>SSFA2</i>	sperm specific antigen 2
piRNAs	piwi interacting RNAs	STR	straightness

<i>TAX1BP1</i>	Tax1 binding protein 1
TCEP	tris(2-carboxyethyl)phosphine
<i>TMEM260</i>	transmembrane protein 260
<i>TNP1</i>	transition protein 1
<i>TPP2</i>	tripeptidyl peptidase 2
tRNAs	transfer RNAs
VAP	velocity average path
VCL	velocity curvilinear
<i>VDAC3</i>	voltage dependent anion channel 3
VIP	Variable Importance in Projection (scores)
VSL	velocity straight line
WOB	wobble
<i>ZNF644</i>	zinc finger protein 644
log ₂ FC	absolute log ₂ fold change
μm	micrometers
μm/s	micrometers per second
μL	microliters

1. Introduction

1.1 General introduction

The pig breeding industry relies heavily on artificial insemination (AI) to accelerate genetic gain and improve production efficiency (Knox, 2016). Modern breeding programs increasingly select boars at younger ages to reduce the generation interval and maximize genetic progress (Kasinathan et al., 2015). However, using sexually immature boars can compromise semen quality and field fertility, creating conflict between genetic goals and reproductive performance (Schulze et al., 2014).

Sexual maturity represents a critical transition in boar reproductive development. Unlike puberty, which marks the onset of spermatogenesis, sexual maturity indicates when boars produce semen of adequate quality for commercial use (Flowers, 2022). This distinction has profound implications for AI centers, where non-productive days from immature boars represent significant economic losses (Lopez Rodriguez et al., 2017).

Despite decades of research in reproductive biology, our understanding of the molecular changes during boar sexual maturation is limited. Current industry practices rely on empirical observations and age-based criteria rather than objective biological markers (Dyck et al., 2011; Lopez Rodriguez et al., 2017; Schulze et al., 2014). In addition, most of these studies were done with older boars (Borg et al., 1993; Hensel et al., 2023; Xu et al., 2024). This knowledge gap limits optimal utilization of young boars and hinders efforts to accelerate genetic improvement. This thesis addresses these limitations through a comprehensive molecular characterization of sexual maturation in Duroc boars. By employing metabolomics, transcriptomics, and sperm quality assessments, the aim of this thesis was to understand the biological transition and signatures of boar sexual maturation. These findings could advance fundamental reproductive biology and provide essential knowledge for the pig breeding industry, enabling further research to optimize the use of younger boars.

1.2 The pig breeding industry and artificial insemination

Global pork production reached 125.1 million tonnes (Mt) in 2024, up from 124.5 Mt in 2023 and 122.5 Mt in 2022 (FAO, 2024, 2025). Pig meat accounted for roughly 33% of the global meat consumption of 399.4 Mt in 2024, making it the planet's second most-eaten animal meat (FAO, 2025). This massive industry depends on efficient breeding systems that balance genetic

improvement and sustainability with reproductive performance. Over the past five decades, pig breeding has transformed from small-scale family operations to integrated, technology-driven, sophisticated programs utilizing advanced genetic technologies (Neeteson-van Nieuwenhoven et al., 2013).

Modern swine breeding programs generally employ three-tier pyramidal structures: nucleus herds create genetic progress, multiplier farms generate a large number of replacement females, and commercial herds finish pigs for slaughter (Dekkers et al., 2011). Production can be organized by one-site, two-site, or three-site systems that separate breeding, nursery, and finishing stages, improving health and management (Harris, 1999). Some companies operate nucleus-multiplier farms, such as the Hypor facility in Mexico, which houses both pure line (nucleus) and great-grandparent (multiplier) stock on the same site (Hoggan, 2021). Despite the structural differences, all systems maintain the same directional flow of elite genetics from nucleus to market pigs. Elite genetic flow from nucleus herds through AI, which enables rapid dissemination of superior traits. This system concentrates selection intensity on relatively few elite boars, making reproductive efficiency vital for industry-wide genetic progress (Knox, 2016).

Artificial insemination, the process of breeding females with collected semen, was explored in various species during the early 20th century. Russian scientist Ivanov was a pioneer, initiating experimental AI in pigs in the early 1900s (Foote, 2002). Throughout the 1930s, Soviet researchers, including V. Milovanov, conducted extensive studies on boar semen collection and dilution. The first field trials of AI in pigs were reported in 1931 in Missouri, U.S. Later, AI in pigs started in Japan in 1948 and in Western Europe in the 1950s (Foote, 2002). Similarly, Denmark founded its first boar AI station (Hatting) in 1963 (Jakobsen & Danielsen, 2020). In Norway, Norsvin was founded in 1958 as Norway's farmer-owned pig breeding cooperative. Soon thereafter, Norway began commercial AI for swine, and genomic selection has been implemented since 2012 (Topigs Norsvin, 2025).

Artificial insemination has revolutionized pig breeding by enabling one boar to sire thousands of offspring annually. Use of liquid-stored semen allows a single high-index boar's semen to be collected twice weekly, producing around 20-40 traditional AI doses or 40-60 low semen doses per ejaculation. This means that one boar could inseminate around 2000 sows per year (Knox, 2016; Roca et al., 2016). Artificial insemination not only raises selection intensity but also improves biosecurity, i.e., laboratory processing, health screening, and hygienic distribution, which reduces the probability of transmitting venereal or systemic pathogens compared to natural mating (Knox, 2016). Artificial insemination now dominates sow breeding:

it covers over 97% matings in the United States (USDA, 2025), more than 98% in the Netherlands (Feitsma, 2017), above 90% in Germany (BRS, 2023), almost 100% in Sweden (Stenberg et al., 2023), and the clear majority in Norway (98%), Spain (95%), France (95%) and Denmark (95-98%) (Innoreports, 2007; Riesenbeck, 2011; Vangen & Sehested, 1997). Genomic selection further accelerates genetic gain by enabling accurate ranking of animals at younger ages. Boars can now be genetically evaluated before reaching sexual maturity (Meuwissen et al., 2016). It has been reported that genomic selection shortens the effective generation interval from ~1.9 years to ~1.7 years, which translates to ~30% more genetic gain per year when all nucleus piglets are genotyped (Lopez et al., 2016; Ostersen, 2020).

Each boar in an AI center represents a substantial investment in genetics, housing, management, and daily operations. A report published by Michigan State University in 1996 assumed US \$1500 to purchase an on-farm AI boar, US \$2500 for a higher-index sire for a small commercial stud, and US \$0.50 per day for maintenance and miscellaneous other routine costs for management (Johnson, 1996). Considering the present year 2025 and taking into account inflation costs, the annual costs for maintaining one boar would be much higher. Non-productive days from sexually immature boars directly impact profitability, as these animals consume resources without marketable semen doses. The economic pressures intensify with genomic selection, where rapid generation turnover maximizes genetic gain. Across large commercial AI operations, optimizing boar utilization could yield substantial annual savings. In Norway, costs exceed 15,000 NOK plus maintenance for the pigs from buying the piglets until they reach the test station (E. Nordbye, personal communication, September 2, 2025).

Missing just 1 month of output from a high-index sire can mean hundreds of elite doses lost (Knox, 2016). Therefore, breeding companies want semen from the young high-index boars as soon as possible, as selection indexes are available early and there is a strong economic incentive to reduce “age-at-first collection” while staying within physiological limits. Physiological data, however, show that commercial-grade ejaculates are not obtained consistently until ≥ 8 months of age. Collections taken earlier, average of 4.5 to 8 months of age, are reported to have lower sperm concentration, ejaculate volume, motility, and higher morphological defects, and therefore fail quality-control thresholds for commercial use (Schulze et al., 2014).

1.3 Sexual maturation in boars

Puberty in the boar is defined as the point at which sperm first appear in the seminiferous tubules and the boar becomes capable of impregnating a female (FlorCruz & Lapwood, 1978). Sexual maturity is reached later, once semen volume, sperm output, sperm motility, sperm morphology, and libido stabilize (Knox, 2024). Duroc boars are reported to reach sexual maturity at around 9 months (Ding et al., 2016).

Spermatogenesis drives this transition and is regulated by the hypothalamic-pituitary-gonadal axis. The hypothalamus secretes gonadotropic-releasing hormone (GnRH), which stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland (Rey, 2021). FSH stimulates Sertoli cells in the seminiferous tubules to proliferate and support developing germ cells, partly by producing essential factors and nutrients. LH targets Leydig cells to produce testosterone, which is crucial for maintaining spermatogenesis and enabling the function of Sertoli cells (Rey, 2021). Spermatogenesis begins with spermatogonia on the basement membrane of each seminiferous tubule, progresses through mitosis, meiosis I and II, and ends with elongated spermatids released as spermatozoa into the tubule lumen. A complete cycle of spermatogenesis in a boar takes about 40 days. The process of sperm development begins in the seminiferous tubules but reaches completion within the epididymis. The epididymis serves two important functions: it facilitates sperm maturation and also acts as a sperm storage reservoir. Sperm cells only acquire their ability to swim and fertilize an egg after traveling through the epididymis. The tail of the epididymis contains a particularly dense concentration of sperm and serves as the primary storage location for approximately 80% of fully developed sperm cells (França et al., 2005; Tauson, 2020).

Pre-pubertal stage (birth to 4-5 months)

In the months leading up to puberty, young boars are in a pre-pubertal (juvenile) stage. During this period, testes are growing and differentiating but have not yet started producing sperm. Histologically, seminiferous tubules are developing, they lengthen and widen, and a lumen begins to form as the boar approaches puberty. For example, size of the seminiferous tubules increases from approximately 30% in early piglet life (3 weeks) to roughly 70% at around 5 months, reflecting the growth of sperm-producing tissue (Cameron, 1987). Testis growth rate was recorded highest in boars (Landrace × Duroc) between 100 and 130 days (Allrich et al., 1982). Similarly, it has been reported that in Duroc, the diameter of seminiferous tubules starts to increase from 1 month, continues to grow slowly between 4 weeks and ~16 weeks, and the

growth accelerates from 16 weeks onwards (Zheng et al., 2022). Zheng et al. (2022) have also reported that spermatozoa and seminiferous lumen were histologically visible only after 5 months in Duroc boars. During the pre-pubertal phase, boars have low circulating testosterone levels (Gong et al., 2021). This phase is characterized by rapid testicular growth and cellular maturation without actual sperm production or sexual activity.

Puberty onset (around 5-6 months)

Most domestic boars, including Duroc, reach the pubertal threshold at approximately 5 to 6 months of age, although there is variation with genetics, nutrition, and breed. For instance, Chinese Meishan boars mature at an earlier age. Chinese Meishan boars were reported to show detectable sperm in the seminiferous tubules as early as 2.5 months and attain puberty earlier than Duroc as shown in Figure 1: at 75 (2.5 months) days Chinese Meishan boar tubules contain luminal sperm, while Duroc boar at 75 days only show spermatogonia; by 270 days (9 months) duroc boars display sperm along with primary spermatocytes, secondary spermatocytes, and spermatids (Ding et al., 2016; Zheng et al., 2022). During the pubertal phase, boars undergo a surge of reproductive hormones and testicular activation that triggers spermatogenesis. Testosterone production in the testis rises markedly (~10 to 16 ng/mL) at around 5 to 6.3 months (160-190) days, with an increasing number of Leydig cells (Allrich, 1983; Allrich et al., 1982). This increase in testosterone drives the development of secondary sexual characteristics and mating behavior. Correspondingly, boars around 5-6 months old begin to show increased libido and sexual behavior (Flowers, 2008).

Physiologically, the onset of puberty is marked by the completion of key testicular maturation steps. By about 4 months of age (110-130 days), the testes contain early germ cells (spermatocytes and round spermatids), but spermatozoa are still absent. Then, by approximately 5 months (150 days), detectable spermatozoa appear in the seminiferous tubule lumen (Zheng et al., 2022). Around 5-6 months (160-190 days), the weight of testis also increases (Allrich et al., 1982), and boars are first capable of producing ejaculate containing sperm. However, these initial ejaculates are usually of small volume and variable quality. At this age, when boars first begin producing semen (around 6 months old on average), an average ejaculate volume is only about 220 mL, considerably lower than that of a mature boar. Early ejaculates also have lower sperm counts (~50 billion). These parameters improve rapidly after puberty as boars age (Mottais, 2022).

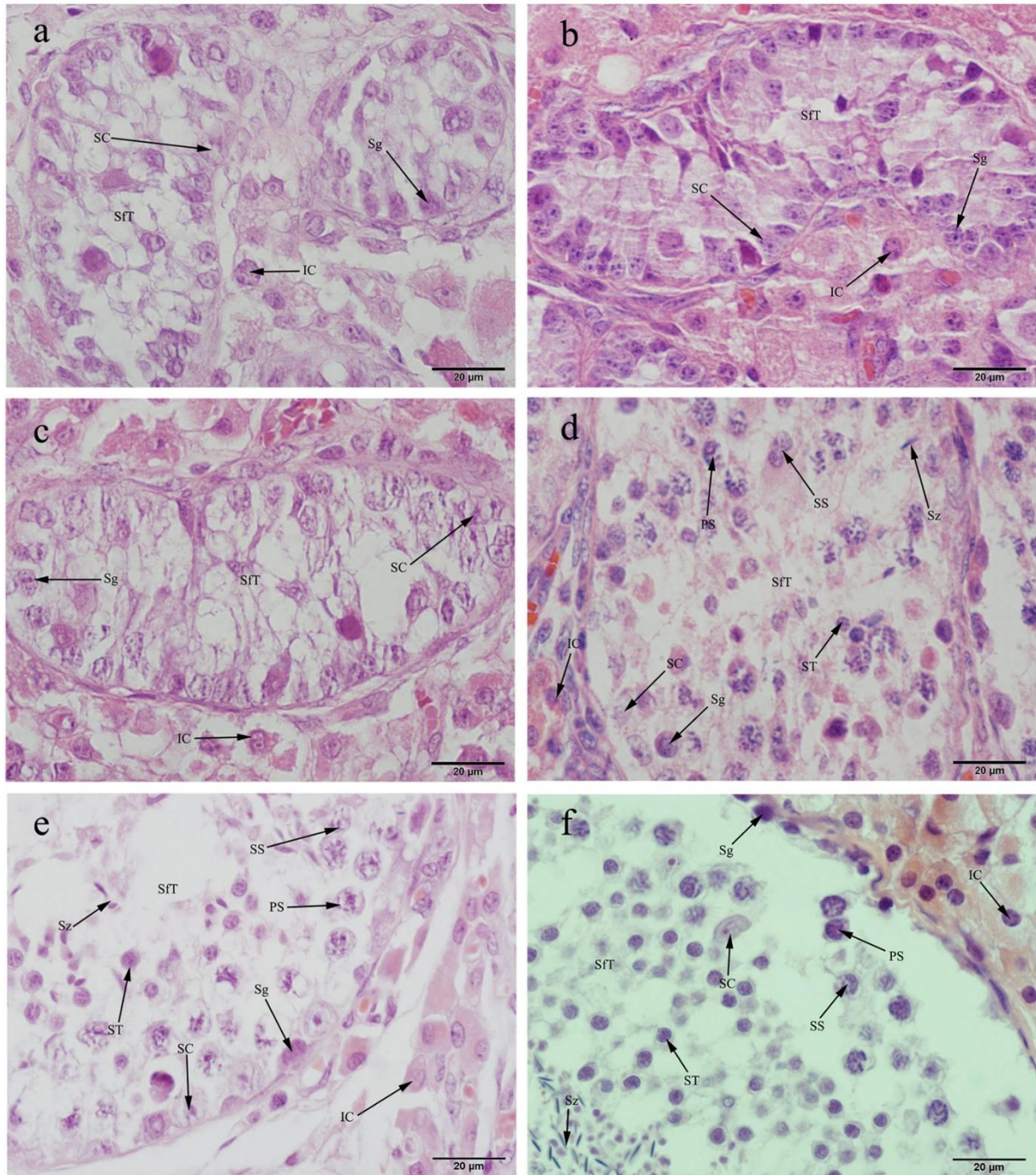


Figure 1: Testis histology of Duroc (a, c, and e) and Meishan boars (b, d, and f) at different developmental stages. Sections are stained with hematoxylin and eosin. Nuclei appear blue to purple, and cytoplasm appears pink. Labels: SFT- seminiferous tubule, SC- Sertoli cell, IC- interstitial Leydig cell, Sg- spermatogonia, PS- primary spermatocyte, SS- secondary spermatocyte, ST- spermatid, Sz- spermatozoon. The picture was reprinted from “Histological and transcriptome analyses of testes from Duroc and Meishan boars,” by Ding, H., Luo, Y., Liu, M., Huang, J., & Xu, D., 2016, *Scientific Reports*, 6, 20758. <https://doi.org/10.1038/srep20758>. CC BY 4.0.

Post-pubertal development and sexual maturity

From 6-7 months onward, the boar is fertile, but fertility continues to improve. Testicular development continues at a rapid rate during this period. After puberty, the seminiferous tubules and testes grow further in size and mass. Testicular and epididymal weight doubles between 6 months and 8 months (FlorCruz & Lapwood, 1978), and the size of the testes increases until boars reach 18 months of age, while daily sperm output rises (Knox, 2024) directly correlating with enlarged testis. The level of testosterone also increases from 130 days onwards, reaches its peak during the period of 190-220 days, and then declines from 250 days. The level of estradiol hormones increases during this period (190-250 days) (Allrich et al., 1982). Ding et al. (2016) reported histological observation of primary spermatocytes, secondary spermatocytes, and sperm in testis of 270 days old Duroc boars, which were absent in 75 days old Duroc boars as illustrated in Figure 1.

Semen volume, total number of sperm, and total motile sperm counts increase from 8-9 months and reach a plateau around 19-24 months (Knecht et al., 2017). As boars mature, ejaculate volume rises from an average of ~220 mL at puberty (6 months) and stabilizes at about 300 mL by 20 months of age. Sperm concentration gradually increases from 280 million/mL (first ejaculate) to about 320 million/mL around the 20th month. Similarly, total sperm output in an ejaculate reaches ~100 billion in fully mature boars (Mottais, 2022). AI studs start weekly collections from 8 to 9 months (Huang et al., 2010; Knox, 2024). The distinction between puberty and sexual maturity matters in AI centers. Even though pubertal boars can produce semen, the quality is generally unsuitable for commercial distribution, and the advisable age of boars for semen collection is ≥ 8 months with $\geq 75\%$ motility (24 hr) and $\leq 20\%$ morphologically abnormal sperm (Schulze et al., 2014).

Duroc boars

Duroc pigs originated in the United States in the mid-19th century from crossing the Red Jersey and New York “Duroc” lines. The modern breed is recognized globally as a red-haired, lop-eared terminal-sire line noted for robustness and meat quality (National Swine Registry, 2023). In Norwegian breeding lines, Norwegian Duroc has been reported to have a lower proportion of motile and progressively motile sperm, as well as higher sperm concentration compared to Norwegian Landrace (Tremoen et al., 2018).

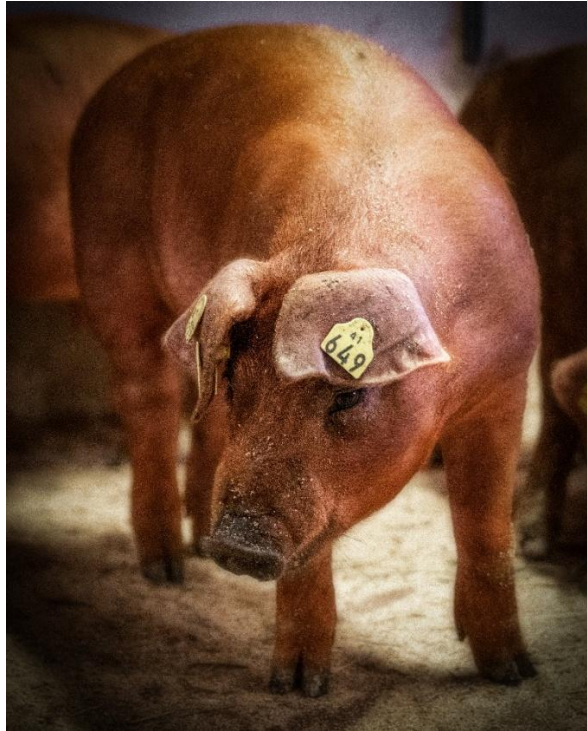


Figure 2: Young Norwegian Duroc boar from the Norsvin breeding nucleus. © 2025 Norsvin SA. Used with permission.

The Norwegian Duroc breed (Figure 2) has been integrated into breeding programs of Norsvin SA since the 1990s. Initially, the Duroc line focused primarily on production and meat quality characteristics, with litter size added to the breeding objectives only recently. Current breeding goals encompass production traits (feed consumption and growth), carcass quality, meat quality, litter size, and animal health and robustness (Jochems, 2022). Today, the line contributes terminal sire genetics to the Topigs Norsvin TN Duroc program, with nucleus testing of around 5000 boars per year at the Delta Norway (Karlsson-Drangsholt, 2024).

1.4 Sperm quality parameters

1.4.1 Traditional evaluation methods

Conventional sperm evaluation includes sperm concentration, motility, and morphology assessments. In addition, many AI stations have started to use objective analysis methods, including computer-assisted sperm analysis (CASA) and flow cytometry, to reduce subjectivity and inter-observer variation (Ayad et al., 2021; Comhaire & Vermeulen, 1995; Dougherty et al., 1975).

Motility is an important predictor of reproductive performance. Immotile sperm are not able to get across cervical mucus, while sperm with non-progressive (circular/rotatory) motility struggle in the journey through the uterotubal junction (Blandau & Rumery, 1964; Tampion & Gibbons, 1963). Fertility tends to decline when total motility falls below 60%, whereas 70-80% motility defines a high-quality ejaculate (Flowers, 1997; Maside et al., 2023).

1.4.2 Computer-assisted sperm analysis (CASA)

CASA systems couple phase-contrast microscopy with video capture and image-analysis algorithms, tracking individual sperm movement patterns, calculating velocity, linearity, and beat frequency. The measured kinematic parameters include velocity straight line (VSL, $\mu\text{m/s}$), velocity curvilinear (VCL, $\mu\text{m/s}$), velocity average path (VAP, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %), linearity (LIN, %), and wobble (WOB, %). These parameters reveal subtle differences in swimming patterns that manual evaluation misses (Dascanio, 2014).

Kinematic parameters for single cells can also be used to define distinct sperm subpopulations within boar ejaculates, each demonstrating unique motility characteristics associated with sperm quality and fertility outcomes (Quintero-Moreno et al., 2007). CASA has enhanced sperm analysis by providing quantitative methods to evaluate sperm movement patterns and their relationship to fertilization capacity (Tanga et al., 2021). However, we also need to remember that CASA faces limitations related to differences in sample preparation, image acquisition, and analytical parameters across different CASA systems (Hackerova et al., 2025; Waberski et al., 2022). Therefore, laboratories must standardize their settings and calibration. In routine practice, CASA is primarily used for motility assessment, whereas viability and acrosome integrity are more robustly measured by flow cytometry. Consistent use of such tools underpins accurate monitoring of boar sperm quality in breeding programs (Bonet et al., 2013).

1.4.3 Flow cytometric analysis

While CASA provides a detailed motility assessment, flow cytometry offers complementary functional analysis, including plasma membrane integrity, acrosome integrity, and DNA integrity (Myromslien et al., 2019; Narud et al., 2020). This technique combines hydrodynamic focusing, laser excitation, and optical detectors to measure light scatter and fluorescence of thousands of sperm in seconds (Mahfouz et al., 2009). Cell size is estimated by forward scatter, side scatter, and granularity, which enables gating the sperm population and excluding debris

even in unstained samples (Petrunkina et al., 2010). Fluorochromes add functional details and can uncover biochemical traits that are often missed by routine microscopy or CASA (Elango et al., 2022). Propidium iodide (PI), a DNA intercalator, is excluded by intact membranes, so only membrane-damaged cells fluoresce red and are counted as non-viable (Graham et al., 1990). Acrosomal status is assessed with lectins such as fluorescein-labelled peanut agglutinin (PNA), which binds to galactose residues exposed when the outer acrosomal membrane is compromised. Intact acrosomes remain non-fluorescent, while damaged ones emit fluorescence upon laser excitation (Mortimer et al., 1987). Flow cytometry enables the collection of multiparametric data on sperm viability and acrosomal integrity, providing a rapid and objective complement to CASA for sperm evaluation (Peña, 2015).

1.4.4 DNA integrity assessment

By applying the Sperm Chromatin Structure Assay (SCSA[®]), sperm DNA integrity and chromatin structure can be assessed, which are the parameters that directly impact embryo development and fertility. Damaged DNA in fertilizing sperm compromises fetal development and offspring health (Evenson & Jost, 2001; Evenson, 2022). SCSA[®] measures chromatin susceptibility to DNA denaturation following low-pH treatment with acridine orange, a DNA-specific probe, combined with flow cytometry (Evenson & Jost, 2001; Evenson, 2022). Acridine orange produces green fluorescence when bound to intact double-stranded DNA and red fluorescence when attached to denatured single-stranded DNA. Flow cytometry analyzes 5000 sperm cells per sample, and this technique generates two clinically significant measurements. The DNA fragmentation index (%DFI) represents the percentage of sperm with detectable DNA strand breaks, while high DNA stainability (%HDS) measures the percentage of sperm with abnormal chromatin structure caused by incomplete replacement of histones with protamines during spermatogenesis. Elevated %HDS indicates faulty chromatin condensation that may prevent proper gene expression during embryonic development (Evenson, 2022).

1.4.5 Applications of CASA and flow cytometry in the analysis of sperm

CASA and flow cytometry have been utilized in the study of motility, acrosome integrity and viability in sperm of various species. In a study with Norwegian Duroc and Norwegian Landrace boars, CASA-derived kinematics at collection (Day 0) and after 96 hr storage (Day 4) at 18°C were linked to field fertility (total number born, TNB). Motility declined with storage in both breeds. Higher LIN at Day 0 and WOB at Day 4 were associated with higher TNB in

Norwegian Landrace. On Duroc, a higher percentage of motile cells and greater ALH, along with lower VCL at Day 0, and higher LIN at Day 4, were associated with higher TNB (Tremoen et al., 2018). Xu et al. (2024) reported significantly lower viability, and compromised acrosome in boars aged 5-6 years compared to boars aged 2-3 years, though progressive motility and survival duration remained similar between these age groups. Tvrdá et al. (2025) showed that Duroc boars aged 8-12 months have significantly lower sperm motility, membrane and acrosome integrity, and mitochondrial activity compared to adult boars aged 24-36 months.

In fertility studies of Norwegian Red bulls, CASA-derived motility parameters showed significant correlations with field fertility (NR56), with high-fertility bulls demonstrating superior hyperactive motility, average path velocity, and curvilinear velocity compared to low-fertility bulls (Narud et al., 2020). Complementary flow cytometric analysis revealed that high-fertility bulls had significantly higher proportions of viable sperm and acrosome-intact live cells (Narud et al., 2020). Age-related maturation studies in young Norwegian Red bulls demonstrated improvements in both motility and viability assessments, with bulls progressing from 14 to 17 months showing enhanced hyperactive motility, increased curvilinear velocity, and improved viability in frozen-thawed samples (Narud et al., 2022).

For SCSA[®], species-specific fertility thresholds have been established through extensive validation studies. In boars, %DFI values above 2.1% correlate with reduced litter size. When this threshold was exceeded, Hampshire, Landrace, and Danish Large White boars produced 0.5, 0.7, and 0.9 fewer piglets per litter, respectively (Boe-Hansen et al., 2008). In bulls, fertility ratings were significantly negatively correlated with both the %DFI (COMP_{at}, $r = -0.40$, $P < 0.01$) and the standard deviation of DNA fragmentation (SD_{at}, $r = -0.58$, $P < 0.01$) (Ballachey et al., 1987; Evenson, 2016). Stallions demonstrate reduced breeding success above 28% DFI, while human male fertility risk increases significantly when %DFI surpasses 25-27% for both natural conception and intrauterine insemination (Evenson, 2016). Human clinical applications demonstrate SCSA's diagnostic value across thousands of internationally analyzed samples. Men with %DFI values exceeding 27% show poor intrauterine insemination success, producing only four pregnancies from 87 inseminations (Evenson, 2016).

While these individual assessment techniques provide valuable information about sperm function, in recent years, analysis of metabolomics and transcriptomics has contributed to increased understanding and knowledge in reproductive biology (Figure 3) (Rodriguez-Martinez et al., 2024).

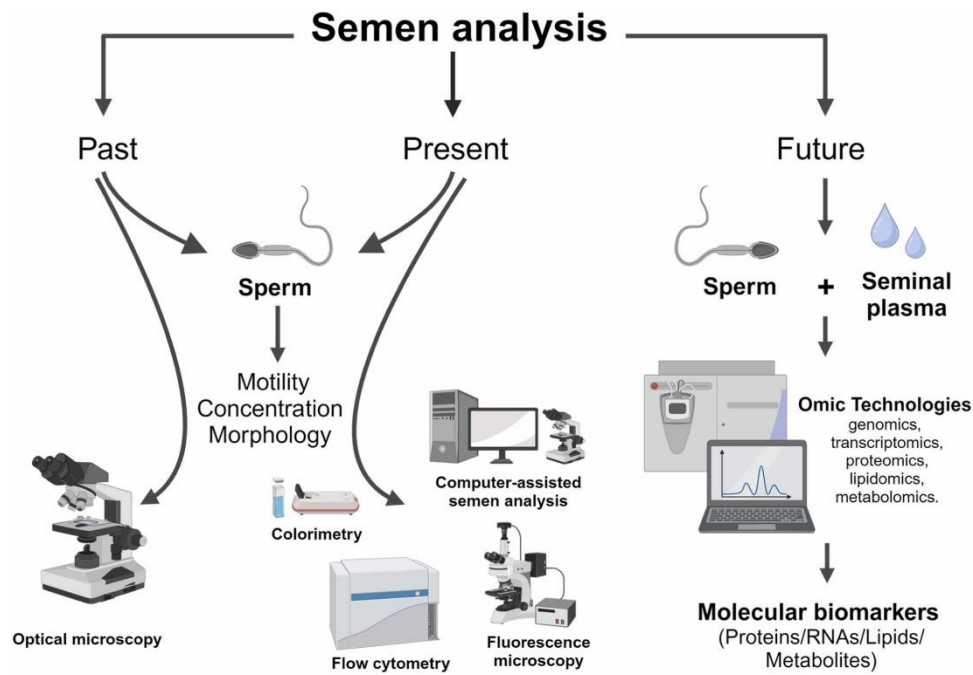


Figure 3: Evolution of semen analysis from traditional microscopy and colorimetry to computer-assisted methods and omics-based biomarker discovery. The image is reprinted from “Reproductive physiology of the boar: What defines the potential fertility of an ejaculate?” by Rodriguez-Martinez, H., Martinez-Serrano, C.A., Alvarez-Rodriguez, M., Martinez, E.A., & Roca, J., 2025, *Animal Reproduction Science*, 257, 107362. <https://doi.org/10.1016/j.anireprosci.2024.107476>. CC BY 4.0.

1.5 Metabolomic changes in semen

Metabolomics represents the systematic study of small molecular compounds present in biological systems. These metabolites, typically weighing less than 1,500 Daltons, serve as final products of cellular processes and provide direct insights into an organism’s physiological state. This approach offers two distinct methodological strategies: targeted and untargeted analysis. Targeted analysis provides absolute quantification of predetermined metabolite groups with higher accuracy for selected compounds. Untargeted analysis employs a comprehensive screening approach that detects measurable compounds within biological samples, capturing both known and unknown metabolites. This makes untargeted metabolomics valuable for discovering novel biomarkers without prior assumptions and understanding complex biological processes (Ashokan et al., 2023).

Recent metabolomic studies in human, bull, stallion, and boar sperm and seminal plasma have primarily focused on fertility and not sexual maturity (Blaurock et al., 2022; Castiglione Morelli et al., 2021; Mateo-Otero, Fernández-López, Delgado-Bermúdez, et al., 2021; Narud et al.,

2022; Narud et al., 2020; Sangeeta et al., 2015; Velho et al., 2018). Research on bull seminal plasma has identified several metabolites that distinguish between high- and low-fertility bulls, including 2-oxoglutaric acid and fructose (Velho et al., 2018). Similarly, Menezes et al. (2019) reported gamma-aminobutyric acid (GABA), carbamate, benzoic acid, lactic acid, and palmitic acid as potential biomarkers of fertility identified in bull sperm. Furthermore, Narud et al. (2020) reported that cysteine and glutamic acid showed inverse correlations with reproductive success, while aspartic acid, leucine, and serine displayed positive associations with fertility as indicated by 56-day non-return rates.

In donkey semen, phenylalanine levels in seminal plasma declined markedly as sperm motility decreased, and it was entirely absent in cases of azoospermia. Multiple metabolites, including alanine, carnitine, choline, citrate, creatine, glutamate, lactate, leucine, myoinositol, and glycerophosphocholine concentrations were significantly reduced in azoospermic stallions. Similarly, alanine, carnitine, choline, and leucine levels showed positive relationships with sperm concentration (Castiglione Morelli et al., 2021). Research in human reproductive biology has explored age-related metabolic alterations by examining seminal plasma composition across different age groups, specifically comparing younger males (26-30 years) with older males (45-54 years). These studies revealed significant disruptions in fundamental metabolic processes, including alterations in the citric acid cycle, oxidative phosphorylation pathways, and glutathione metabolism, illustrating the impact of aging on seminal plasma biochemistry and reproductive capacity (Guo et al., 2023).

Studies focusing on boar reproductive metabolism have established connections between specific metabolites and various aspects of sperm performance. Mateo-Otero, Fernández-López, Delgado-Bermúdez, et al. (2021) investigated the seminal plasma metabolome to find *in vivo* biomarkers of fertility and found that lactate levels showed discriminative capacity for farrowing rates, while carnitine, hypotaurine, sn-glycero-3-phosphocholine, glutamate and glucose distinguished differences in litter size. Additionally, citrate, creatine, phenylalanine, tyrosine, and malonate levels were predictive of stillbirths per litter. The study also found that malonate and fumarate levels could discriminate gestation length differences. Additional research identified glutamate, carnitine, and isoleucine as compounds linked to overall sperm quality parameters, while leucine, hypotaurine, carnitine and isoleucine were found to influence sperm's capacity for liquid preservation (Mateo-Otero, Fernández-López, Ribas-Maynou, et al., 2021). In the metabolomics analysis of seminal plasma from Duroc boars with good and poor preservation ability, Song et al. (2023) reported amino acids/peptides to be the most discriminatory compounds, with enrichment of arginine and proline metabolism as well as other

amino acid pathways. D-proline, arginine, L-citrulline, phenylalanine, leucine, DL-proline, DL-serine, and indole were reported to be candidate biomarkers of liquid preservation ability (Song et al., 2023).

Age-related metabolomic changes have also been documented across species. Argov-Argaman et al. (2013) reported differences in lipid profiles in aging Holstein Friesian bulls between ~2-year-old and ~7-year-old animals. Bulls at around 2 years were reported to have higher levels of polyunsaturated fatty acids, omega-3 fatty acids, and docosahexaenoic acid in sperm and seminal plasma as compared to bulls at 7 years. Zhao et al. (2023) identified L-arginine, L-prolinamide, (R)-4-hydroxymandelate, glutathione, and homovanillic acid as key metabolites in sexual maturation in chickens. Similarly, Yaxiao et al. (2025) have reported carnitine and choline, along with 56 other metabolites, as potential biomarkers of sexual maturity in laying hens (transition from 15 weeks to 22 weeks).

These studies demonstrate that metabolomics is a powerful tool for reproductive assessment. However, comprehensive metabolomic characterization during boar sexual maturation remains unexplored.

1.6 Sperm transcriptomics biomarkers in male reproduction

Transcriptomics involves studying the transcriptome, which is the entire set of RNA molecules generated by the genome within a particular cell, tissue, or organism under specific conditions. This technology examines gene expression patterns in cells and tissues, helping researchers understand biological processes and find molecular markers (Kasimanickam et al., 2024).

Research has shown that spermatozoa contain functional RNA molecules, serving not only as genetic carriers (Marta Gòdia et al., 2018; Gur & Breitbart, 2006). Boar spermatozoa contain multiple RNA classes: messenger RNAs (mRNAs), micro RNAs (miRNAs), long noncoding RNAs (lncRNAs), short noncoding RNAs (sncRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), Piwi interacting RNAs (piRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), mitochondrial ribosomal RNAs (MtrRNAs), and mitochondrial transfer RNAs (MttRNAs) (Gòdia, Castelló, et al., 2020; Gòdia et al., 2019; Gòdia, Reverter, et al., 2020; Sa et al., 2024; Yang et al., 2009). Sperm RNA are reported to be associated with sperm development and function (Jodar et al., 2013), key reproductive milestones such as spermatogenesis, fertilization, post-fertilization oocyte activation, placental development, early embryogenesis (Prakash et al., 2021; Selvaraju et al., 2018; Sandler et al., 2013), and

reproductive success (Gòdia, Castelló, et al., 2020; Gòdia, Reverter, et al., 2020; Yang et al., 2009; Zhang et al., 2021).

Initial analysis of boar sperm identified fragmented RNA transcripts encoding spermatogenesis-related proteins (protamine 1 (*PRM1*), protamine 2 (*PRM2*)) and embryonic development factors (sperm-specific antigen 2 (*SSFA2*), sestrin 1 (*SESNI*)) (Yang et al., 2009). Advanced RNA sequencing technology later detected 4,436 genes in boar sperm, including mitochondrial transcripts and essential spermatogenesis regulatory elements like *PRM1* and ornithine decarboxylase antizyme 3 (*OAZ3*) (Gòdia et al., 2019; M. Gòdia et al., 2018). Comparative transcriptomic analysis identified over 500 differentially expressed genes and over 100 miRNAs between fresh and frozen-thawed boar sperm (Dai et al., 2019). Research comparing high fertile and low fertile boars also revealed over 500 differentially expressed genes (347 upregulated and 174 downregulated) (Alvarez-Rodriguez et al., 2020). High-fertility boars showed enhanced expression of motility-related genes, including cation channel sperm associated auxiliary subunit gamma (*CATSPERG*) (Alvarez-Rodriguez et al., 2020). Fraser et al. (2020) identified *FOS* (Fos proto-oncogene, AP-1 transcription factor subunit), *NFATC3* (nuclear factor of activated T cells 3), *ITGAL* (integrin subunit alpha L), *EAF2* (ELL associated factor 2), *BAMBI* (BMP and activin membrane bound inhibitor), *PTPRU* (protein tyrosine phosphatase receptor type U), *PTPN2* (protein tyrosine phosphatase non-receptor type 2), *ND6* (NADH:Ubiquinone Oxidoreductase Core Subunit 6), and *ACADM* (acyl-CoA dehydrogenase medium chain) to be upregulated in boar sperm with poor freezability. In ram sperm, protein-coding genes such as *SPEM1* (spermatid maturation 1) and *SPEM2* (spermatid maturation 2) are implicated in spermatid to spermatozoa maturation, sperm motility, and the acrosome reaction (Ureña et al., 2022).

Studies on non-coding RNAs have reported differential miRNA expression associated with sperm morphology, function, and quality (Curry et al., 2011). Analysis of morphologically abnormal or poorly motile sperm revealed altered expression of several miRNAs, particularly miR-22 and let-7 family members (let-7a, let-7d, let-7e) (Curry et al., 2011). Maturation from epididymis to ejaculation involves 15 differentially expressed miRNAs in Landrace boars. This maturation process reduces expression of let-7 family members and ssc-miR-26a while increasing ssc-miR-19b and ssc-miR-34c expression. These miRNAs target genes that control cell-cycle regulation and apoptosis (Chang et al., 2016). Tissue-specific analysis reported that 15% of miRNAs are co-expressed across testis, epididymis, and sperm (Luo et al., 2015). Martinez et al. (2022) identified four miRNAs (miR-1285, miR-92a, miR-34c, miR-30) that differ between ejaculated and epididymal sperm, and four other miRNAs (miR-182, miR-1285,

miR-191, miR-96) that showed different abundances between high and low fertility boars. Interestingly, a recent study by Gòdia, Reverter, et al. (2020) has reported links between sperm quality parameters and gene expression, identifying abundant mRNAs (*PRM1*, *OAZ3*, *DNAJB8* (DnaJ heat shock protein family (Hsp40) member B8), *TPP2* (tripeptidyl), *TNP1* (transition protein 1)) and miRNAs (ssc-miR-30d, ssc-miR-34c, ssc-miR-30c-5p, ssc-miR-191, let-7 family, ssc-miR-425-5p) associated with 25 different sperm quality measures.

These studies show that transcriptomics offers valuable insights into the biology and fertility of boar spermatozoa. However, research investigating transcriptomic changes during sexual maturation remains limited. The transition from puberty to sexual maturation represents a critical developmental window, yet the molecular mechanisms underlying this process remain poorly understood.

1.7 Knowledge gaps and research rationale

Despite advances in reproductive biology, knowledge gaps remain in understanding boar sexual maturation. To our knowledge, no studies have comprehensively described the metabolomic and transcriptomic changes that occur during the transition from puberty to sexual maturity. Existing metabolomics research in boar semen is focused on fertility (Mateo-Otero, Fernández-López, Delgado-Bermúdez, et al., 2021) and liquid preservation (Mateo-Otero, Fernández-López, Ribas-Maynou, et al., 2021), leaving the maturation window unexplored. This knowledge gap also extends to experimental design approaches. There is a lack of longitudinal studies tracking individual boars through this maturation timeframe. Most existing studies on sperm functional parameters examined boars only after they attained sexual maturity. Repeated sampling from the same individuals would reveal true developmental trajectories rather than population averages (Sullivan, 2008).

Breed-specific information is also scarce, particularly for commercially important lines like Duroc. Many studies on boar reproduction have studied mixed breeds, i.e., pooled samples from several breeds together, such as Landrace, Large White, Yorkshire, and other cross-breeds (Knecht et al., 2017; Tsakmakidis et al., 2012). Given documented breed differences in puberty and maturation timing (Ding et al., 2016), targeted research on specific breed is essential.

From an applied perspective, the pig breeding industry needs objective biomarkers for assessing sexual maturity to complement conventional assessments of sperm quality and physical characteristics. Currently, breeding centers rely primarily on chronological age and basic semen parameters to determine when young boars are ready for semen collection. Molecular

biomarkers could enable precise management of young boars. Identifying metabolites or genes that indicate sexual maturity would provide a more precise basis for informed management decisions, such as collection timing. This would help to minimize non-productive days while ensuring semen quality meets commercial standards.

In addition, characterizing key molecules and pathways would deepen our understanding of reproductive biology. Such insights could inform strategies to accelerate maturation or enhance sperm quality and optimize the productive use of young boars. Earlier productive use would reduce generation intervals and accelerate genetic gain (Samorè & Fontanesi, 2016). Economic and genetic assessment support this, with Wuensch et al. (2000) reporting that introducing boars to breeding straight after their performance testing increased monetary genetic gain by about 5.5% and profit by roughly 13%.

Our study addresses these knowledge gaps through a detailed longitudinal molecular characterization of boars during puberty to sexual maturity transition. We apply targeted and untargeted metabolomic profiling, alongside transcriptomic analyses and sperm quality assessments in the same cohort of boars followed longitudinally from puberty to sexual maturity. We correlate targeted metabolomic profiles with sperm quality parameters, thereby linking molecular changes to functional outcomes. These findings would advance our understanding of boar sexual maturation and could inform practical application in commercial breeding practices.

2. Aims of the thesis

Sexual maturity represents an important transition in boar reproductive development. The pig breeding industry relies on artificial insemination (AI) in their breeding programs. Optimal utilization of young boars in breeding can enhance genetic gain and reduce costs associated with non-productive days at AI centers. Despite advances in reproductive biology, more knowledge of the molecular landscape during sexual maturation in boars is needed. Understanding metabolomic changes, gene expression changes, and functional sperm quality during the critical window of transition from puberty to sexual maturity represents both fundamental research necessity and a practical requirement. Therefore, the overall aim of this thesis was to characterize molecular and functional signatures of sexual maturation in Duroc boars through longitudinal analysis of semen from puberty to sexual maturity, thereby advancing knowledge of reproductive biology.

The following objectives were outlined:

- To identify amino acid and amine biomarkers of sexual maturity by examining metabolic changes in boar sperm and seminal plasma using targeted LC-MS/MS analysis (Paper I).
- To characterize the global metabolomic landscape of sexual maturation through untargeted metabolomic profiling of boar sperm and seminal plasma, identifying key metabolites and pathways changing during this critical reproductive transition (Paper II).
- To evaluate functional sperm quality parameters during sexual maturation and investigate their correlation with amino acids and amines concentrations (Paper III).
- To examine sperm transcriptomic landscape during sexual maturation through comparative analysis of total RNA and miRNA expression profiles between pubertal and sexually mature boars and explore molecular mechanisms and biological pathways underlying sexual maturation (Paper IV).

2.1 Overview of experimental approach

To address our objectives, we designed an integrated longitudinal study following the same Duroc boars across three maturation time points during sexual maturation (Figure 4). This approach combined metabolomics (targeted and untargeted), transcriptomics (total RNA and miRNA), and conventional sperm quality assessment to investigate potential biomarkers of sexual maturity from complementary molecular and physiological perspectives.

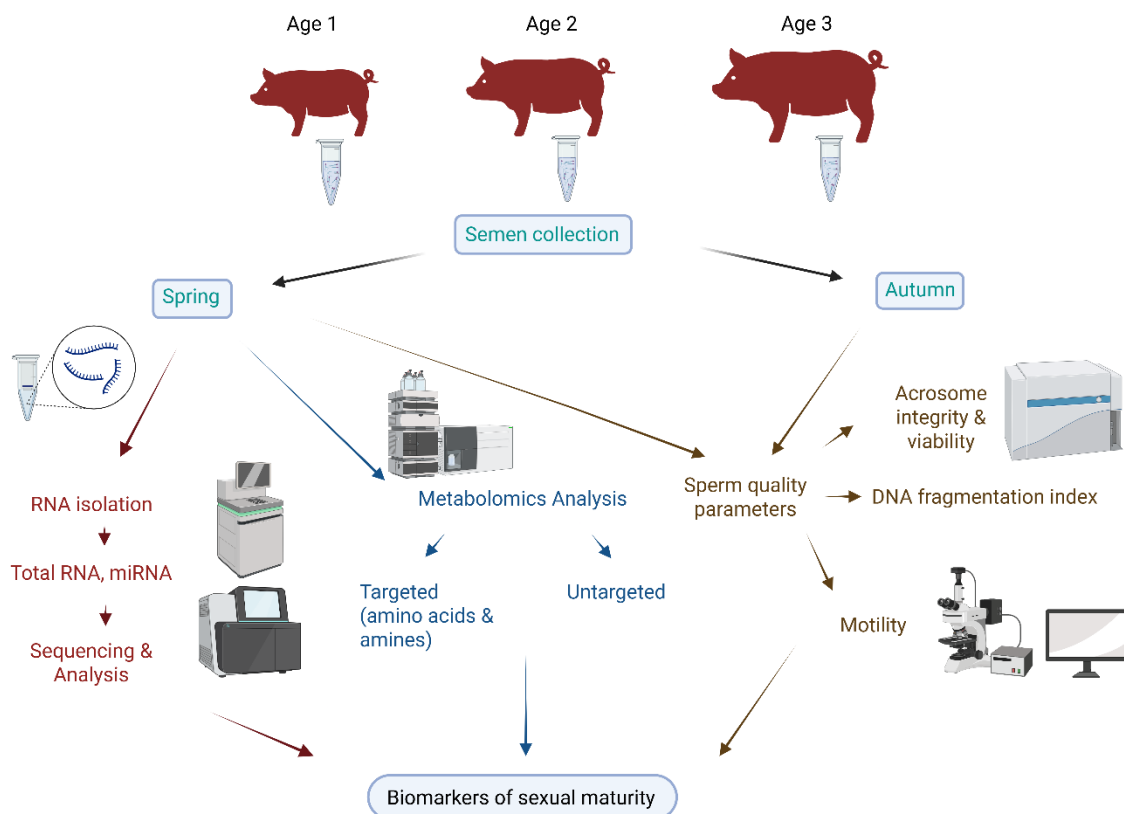


Figure 4: Experimental design for investigation of sexual maturity biomarkers in Duroc boars. Semen samples were collected from the same boars ($n = 15$ in Spring, $n = 13$ in Autumn) at three ages: approximately ~ 7 months (Age 1), then 6 weeks later (Age 2), and again 12 weeks after the first collection (Age 3). Analyses included total RNA isolation for transcriptomics (total RNA and miRNA sequencing), metabolomics analysis (targeted amino acids/amines and untargeted), and conventional sperm quality assessment (motility and kinematic parameters, acrosome integrity and viability, DNA fragmentation index). These complementary approaches aimed to characterize the signatures of sexual maturity from both molecular and physiological perspectives. Created by the author in <https://BioRender.com>

3. Results: Summary of individual papers

Paper I: A metabolomic study uncovering key amino acids and amines in Duroc boar semen as biomarkers of sexual maturity

The aim of this study was to investigate changes in amino acids and amines in Duroc boar sperm and seminal plasma during the transition from puberty to sexual maturity. Semen was collected from 15 Duroc boars longitudinally at three timepoints: approximately 7 months, 8.5 months, and 10 months. Targeted LC-MS/MS analysis was used to quantify amino acids and amines in sperm and seminal plasma separately. Multivariate analysis showed a clear separation between sperm and seminal plasma, indicating significant differences in their metabolic compositions. We observed pronounced age-dependent changes in amino acid and amine concentrations in sperm between samples of 7 months and 10 months, with 8.5 months showing more overlap with 10 months, suggesting it as a transitional phase. Seminal plasma showed more subtle shifts in metabolite concentrations with age compared to sperm. In sperm, glutamate, alanine, aspartate, and choline were identified as top contributors to age group classification (VIP scores > 1.5). Fifteen amino acids showed significant pairwise differences across age groups (adj $P < 0.05$). Glutamate, aspartate, and taurine concentrations decreased significantly with increasing age, particularly between 7 months and 8.5 months and between 7 months and 10 months of age. Alanine and choline levels also decreased significantly with age. In seminal plasma, alanine and glycine were the key contributors to age group classification (VIP scores > 1.5). Only glutamate and taurine showed significant pairwise decreases with age. Glutamate was the most predominant metabolite in both sperm and seminal plasma. Moreover, glutamate exhibited the highest significant positive correlation between boar sperm and seminal plasma ($r = 0.79$), suggesting metabolic interactions. Pathway analysis identified alanine, aspartate and glutamate metabolism, histidine metabolism, tyrosine metabolism, tryptophan metabolism, arginine metabolism, and taurine and hypotaurine metabolism to be strongly associated with age. The study concluded that these robust differences between amino acid and amine concentrations could be potential biomarkers for assessing sexual maturity in boars. These findings are initial steps towards optimizing the use of younger boars in breeding programs, potentially through screening or targeted dietary interventions to improve semen quality and enhance genetic gain.

Paper II: Untargeted metabolomics reveals changes in boar sperm and seminal plasma metabolites associated with sexual maturity

This study aimed to characterize the comprehensive semen metabolome during boar sexual maturation using an untargeted metabolomic approach and to identify key metabolites and pathways associated with sexual maturation. Semen samples were collected from 15 Duroc boars longitudinally at three time points: approximately 7 months, 8.5 months, and 10 months. Sperm and seminal plasma were separated and analyzed using hydrophilic interaction and reversed-phase liquid chromatography coupled with high-resolution quadrupole time-of-flight mass spectrometry to capture a wide range of metabolites. A total of 4,491 features were identified, with 92 metabolites successfully annotated. Amino acids, peptides and analogues constituted the most abundant metabolite class, followed by fatty acid esters. Principal component analysis revealed that younger boars (7 months) displayed a greater metabolomic variability compared to older boars (8.5 and 10 months). Partial least squares discriminant analysis demonstrated effective age group classification with high accuracy (0.76-0.87) and strong predictive capability ($Q^2 = 0.64-0.80$) across both analytical methods. In sperm, eight metabolites showed significant temporal changes ($\text{adj } P < 0.05$), with L-glutamic acid and decanoyl-L-carnitine decreasing, while homoisovanillic acid and N-(1,3-Thiazol-2-yl)benzenesulfonamide increased with age. Seminal plasma analysis revealed 17 metabolites with significant changes in abundance, including increases in 1-formylpyrrolidine-2-carboxylic acid and myo-inositol, and decreases in glycerophosphocholine and glyceric acid. Homoisovanillic acid emerged as a key discriminating metabolite in both sperm and seminal plasma. Random forest analysis ranked L-glutamic acid, homoisovanillic acid, and N-(1,3-Thiazol-2-yl)benzenesulfonamide as top contributors for age classification in sperm, while homoisovanillic acid, 1-formylpyrrolidine-2-carboxylic acid, and glyceric acid were most important for age classification in seminal plasma. Pathway analysis identified porphyrin metabolism, taurine and hypotaurine metabolism, and glycerolipid metabolism as significantly enriched in sperm, while glutathione and nitrogen metabolism were prominent in seminal plasma. The study provides the first comprehensive metabolomic characterization of boar semen during sexual maturation, establishing foundational knowledge for future reproductive biology research and breeding program applications.

Paper III: Influence of sexual maturity on sperm quality in Duroc boar

This study assessed sperm functional parameters during sexual maturation in Duroc boars and investigated their relationships with amino acids and amines concentrations measured by targeted metabolomics analysis. Semen samples were collected from 28 Duroc boars longitudinally at approximately 7 months of age, with follow-up collections 6 weeks and 12 weeks after the first sampling. Sperm motility, kinematic parameters, acrosome integrity and viability were assessed on the day of collection and after five days of storage at 18°C using CASA and flow cytometry. The DNA fragmentation index was measured using the SCSA[®] on five-day stored samples. Amino acids and amines concentrations in sperm and seminal plasma from 15 boars, as in Paper I, were correlated with sperm quality parameters using repeated measures correlation analysis. Overall motility, rapid progressive motility, and acrosome intact live sperm increased significantly ($\text{adj } P < 0.05$) as boars matured from around 7 to 10 months. Mature boar samples displayed superior tolerance for storage, maintaining higher sperm quality after five days compared to younger boars. Cystine, glutamate, aspartate, choline, and taurine showed significant negative correlations with progressive motility and positive correlations with rapid non-progressive motility. Age-related improvements in most of the sperm quality parameters remained consistent across spring and autumn cohorts, validating the robustness of maturation effects. These findings demonstrate that sperm quality continues to improve beyond initial reproductive capability in Duroc boars, with significant enhancements in motility, acrosome integrity, DNA integrity, and storage resilience occurring between ~7 and ~10 months of age. The identified relationships between specific amino acids and sperm functionality provide new insights into the biochemical basis of sperm quality during sexual maturation, offering potential applications for developing precise assessment tools to optimize the utilization of younger boars in breeding programs.

Paper IV: Sperm transcriptome dynamics from puberty to sexual maturity in Duroc boar

This study investigated transcriptomic changes in sperm during sexual maturation through longitudinal RNA sequencing analysis of Duroc boars. Semen samples were collected from six Duroc boars at approximately 7 months (puberty) and 10 months (sexual maturity), enabling paired comparison of the same individuals across the maturation transition. Sperm samples underwent rigorous processing, including somatic cell lysis, confirmation of sperm cell lysis via microscopic examination, and total RNA isolation using the miRNeasy Mini Kit. The sequencing was conducted using the Illumina NovaSeq-6000 for total RNA-seq and the NextSeq-500 for miRNA-seq, followed by bioinformatics analysis that included quality control, alignment to the *Sus scrofa* reference genome (Sscrofa 11.1), and differential expression analysis using DESeq2 with paired statistical modeling to account for repeated measurements. Initial transcriptomic characterization showed 15,637 genes, of which 86.91% were protein-coding genes and 11.56% were long non-coding RNAs. Differential expression analysis identified 60 differentially expressed genes (adj $P < 0.05$, $|\log_2FC| \geq 0.5$). Sexual maturation displayed mostly transcriptional activation, with 39 genes (65%) upregulated and 21 genes (35%) downregulated in mature boars. Hierarchical clustering revealed clear age-dependent expression patterns, with samples clustering by age rather than by individual. Key upregulated genes in mature boars included *NCLN*, *RGS12*, *CIB2*, *FOXP4*, *PHC1*, *CDC25B*, and *AKAP1*. Prominent downregulated genes in mature boars encompassed *HSP90AA1*, *FSIP2*, *VDAC3*, and *ALMS1*. miRNA analysis identified six differentially expressed miRNAs (adj $P < 0.05$, $|\log_2FC| \geq 0.5$) with five upregulated (ssc-miR-193a-5p, ssc-miR-574-3p, ssc-miR-126-3p, ssc-miR-196a, ssc-miR-210) and one downregulated (ssc-miR-338) in sexually mature boars. Integrated miRNA-mRNA analysis identified 17 regulatory pairs, including ssc-miR-193a-5p targeting six downregulated mRNAs (*MED13*, *ZNF644*, *ALMS1*, *TAX1BP1*, *TMEM260*, *EVI5*) and three upregulated targets (*CDC25B*, *FOXP4*, *BSN*). Gene ontology enrichment analysis revealed 11 significantly enriched biological processes (adj $P < 0.05$) directly related to reproductive development, including germ cell development, spermatogenesis, spermatid development, and male gamete generation, alongside four molecular functions emphasizing protein kinase activities. The study establishes the first comprehensive transcriptomic characterization of sperm RNA dynamics during boar sexual maturation, providing molecular insights into reproductive development.

4. Discussion

In this thesis, semen from Duroc boars was examined with different analytical approaches to capture molecular and functional changes during the transition from puberty to sexual maturity. Figure 5 provides a conceptual overview of this perspective, with a spermatozoon shown under magnification to represent the closer investigation of its biology through analysis of metabolites, transcriptomes, and sperm functional traits.

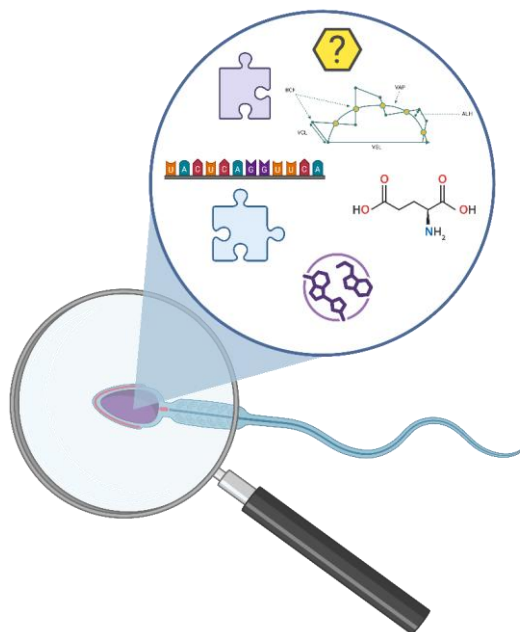


Figure 5: Conceptual illustration of a spermatozoon under magnification, representing the detailed investigation carried out in this thesis, highlighting different analyses such as transcriptomics, metabolomics, and sperm quality analyses to assess sperm biology from various perspectives. Created by the author in <https://BioRender.com>

4.1 Methodological considerations

4.1.1 Experimental Designs

Rationale for Duroc boar selection

Duroc boars were selected for this study following practical discussions with Norsvin SA. For this study, Landrace and Duroc boars were considered, and the latter was chosen since they often stay longer than Landrace in the breeding station, which provided distinct advantages for longitudinal research through extended periods of consistent semen production (Huang et al., 2010; Kondracki & Górski, 2025). In addition, Duroc boars represent a commercial breed

in modern pig breeding (National Swine Registry, 2023), ensuring our findings would have industry relevance, not only for Norsvin SA but also for other breeding companies worldwide. Thus, Duroc boars were selected for this study.

Longitudinal design and age selection

We designed our study to work within Norsvin SA's commercial boar management workflow. The study design had the choice between boars of different ages using different individuals in each group (cross-sectional design) or following the same individuals over time (longitudinal design). The longitudinal approach was more demanding in terms of planning and logistics, as it required careful coordination with breeding schedules and extended periods of sample collection. However, it offered important advantages by eliminating the genetic variability between individuals and capturing genuine differences associated with sexual maturation rather than inter-individual differences (Caruana et al., 2015).

The timeline of semen collection follows three key stages. First, semen was collected from boars that were in the isolation unit under quarantine and training for mounting and ejaculation (~7 months, Age 1). After 6 weeks, when these boars were moved to the AI center (at Age 2), a second sample collection was performed. To maintain consistent intervals, a final collection was performed after another six weeks when the boars were already under regular commercial semen collection (~10 months, Age 3). This design resulted in 3 age groups capturing a window where boars are expected to transition from puberty to sexual maturity.

These six-week intervals between sampling were aligned with Council Directive 90/429/EEC, which requires boars to undergo mandatory quarantine in isolation units for 6 weeks, during which their health status is proven before they are moved to the AI center. During this quarantine period, boars are trained for mounting behaviors, and they undergo health screening through an "all in, all out" system. Semen collected during isolation cannot be used commercially under Council Directive 90/429/EEC. Therefore, we did not have the opportunity to obtain fertility data from boars at the youngest age (Age 1). The fertility data received from boars of Age 2 and Age 3 were incomplete. It would have been interesting to link fertility data to the parameters we analyzed. However, our study was specifically designed with the primary aim of characterizing molecular and functional changes related to sexual maturity during the transition from puberty to sexual maturity, making this constraint less limiting for our research objectives. Nevertheless, it could have provided additional information regarding fertility.

Both the isolation unit and AI center were kept under consistent housing conditions, with controlled ventilation, lighting, and temperature. All animals received a nutritionally balanced

commercial diet for their age. Semen collection procedures were done with the gloved-hand technique, with the same trained personnel conducting all collections. Boars were briefly moved from their pens to a dedicated collection area following identical protocols at each time point. These allowed us to capture genuine physiological changes while minimizing confounding effects. Despite these carefully controlled conditions, the season could influence the results. To address this consideration, samples from two seasons were included in the sperm quality study (Paper III). Interestingly, the effect of age was observed consistently in both seasons, providing evidence that the changes documented related to sexual maturity were robust to seasonal variation. Therefore, we can reasonably assume that the effects observed in the spring season for metabolomics and transcriptomics studies (Paper I, Paper II, and Paper IV) would likely be observed in other seasons as well. However, further studies are needed to confirm this. Ideally, our metabolomic and transcriptomic studies would have benefited from including samples from both seasons, but practical considerations, including cost limitations, constrained our sampling design.

Sample size considerations

Paper I and Paper II followed the same 15 Duroc boars in metabolomics study, providing repeated-measured analyses while remaining manageable for intensive laboratory work (Billoir et al., 2015). Paper III required a broader approach, incorporating 28 boars across two seasons to capture comprehensive data on sperm quality. We began sampling with 18 boars in the spring cohort; however, three boars were culled for various reasons, as often happens in longitudinal research with live animals (Pardon et al., 2012), resulting in a sample size of 15. The autumn cohort started with 15 boars, and 13 boars completed all sampling. We accounted for expected commercial culling practices in our initial sample size calculations. Paper IV's transcriptomic analysis focused on a subset of six boars from our original 15, recognizing that RNA sequencing depth often compensates for smaller sample sizes (Conesa et al., 2016; Schurch et al., 2016). Ideally, we could have had larger sample sizes here as well, but cost considerations influenced our experimental design, particularly for comprehensive metabolomic and transcriptomic analysis. Despite these sample size limitations, we observed statistically significant differences across multiple parameters between samples from different age groups in all studies, including the transcriptomic study, suggesting that our sample sizes were adequate to detect the biological effects that we were investigating.

4.1.2 Sample processing strategies

Several analyses had to be performed on the same day of the semen collection. For some analyses (SCSA, metabolomics, transcriptomics), semen samples had to be stored for later analysis. Thus, it required careful sample processing procedures for each method to ensure a smooth workflow. All procedures were thoroughly tested in pilot experiments to optimize the protocols and determine the number of samples that could be handled efficiently per day. Each analytical platform required specialized handling: metabolomics needed immediate processing and preservation of biochemical states (Smith et al., 2020), transcriptomics required pristine RNA maintenance (Pang et al., 2020) and viable cells were necessary for sperm functional assessments. Collected semen samples were transported from Norsvin SA's facilities to our laboratory in Hamar within 30 minutes in styrofoam containers to maintain ambient temperature. Upon arrival at the laboratory, samples were immediately dedicated to the several distinct sampling processes.

We received raw semen samples prediluted in an equal volume of extender from Age 1, as those samples were not for commercial purposes. They were further extended in our lab immediately upon arrival by diluting to a standardized concentration of 25×10^6 sperm per mL using Androstar® Plus extender (Minitube, 84184, Tiefenbach, Germany), following the same protocol as the breeding company (Norsvin SA). For Age 2 and Age 3, we received both raw and diluted semen samples.

Sample processing for metabolomics

For metabolomics, fresh samples were handled immediately to separate sperm and seminal plasma. We chose to work with raw semen rather than extended semen samples for metabolomic analysis because extenders contain additives that could interfere with metabolite detection and quantification. While washing with phosphate-buffered solution (PBS) might have provided cleaner samples, we opted for direct processing without PBS washing to avoid potential interference and metabolite loss during washing steps and to capture the natural metabolomic state. The separation protocol used centrifugation at $600\times g$ for 10 minutes at room temperature (RT) for sperm. For seminal plasma, it was centrifuged at $600\times g$ for 10 minutes at RT, followed by high-speed clarification at $10,000\times g$ at 4°C to ensure clean compartment separation. A second centrifugation was performed on the seminal plasma as an additional step to ensure the complete removal of sperm. Furthermore, to ensure the complete absence of sperm in the seminal plasma sample, each seminal plasma sample was examined microscopically, as

even minimal contamination with sperm could affect the metabolomic analyses. Both sample types were snap-frozen in liquid nitrogen and stored at -80°C until analysis to preserve their integrity throughout the study. This made it possible to analyze all the samples together at a later stage when sampling was completed.

Sample processing for sperm functional assessments

For sperm quality assessments, extended semen samples were stored at 18°C, which is the temperature the breeding company (Norsvin) uses for semen shipment. Motility analysis, acrosome integrity and viability analysis were conducted using CASA and flow cytometry, respectively, on the day of collection (Day 0) and after 5 days of storage (Day 5). We selected a five-day storage period because Norsvin routinely uses samples stored for up to 4 days for commercial purposes, and we wanted to test one additional storage day. The five-day timepoint also aligned with our practical laboratory scheduling, allowing for processing during the week while providing commercially relevant data. For DNA integrity analysis with SCSA, aliquots of extended samples stored at 18°C were frozen on Day 5 at -80°C until analysis. We chose to assess motility and viability on both Day 0 and Day 5 because these parameters are important parameters associated with sperm quality (Fernández-López et al., 2022; Sutkeviciene et al., 2009). DNA fragmentation index (DFI) was measured only on Day 5, because Norsvin had analyzed DFI in boar sperm from Day 0 to Day 4 in previous studies on many samples (Myromslien et al., 2019). Day 5 measurement provided new information beyond their existing dataset. This approach was also more economical and practical when processing samples for multiple analyses.

We selected these specific parameters (motility, acrosome integrity, viability, and DNA integrity) for our study from among the numerous available sperm quality assessments based on their practicality and established relevance to commercial breeding success. This choice enabled us to efficiently process samples alongside metabolomic and transcriptomic analysis.

Sample processing for transcriptomics

Samples for transcriptomics analysis required the most careful handling due to RNA fragility and contamination concerns (Goodrich et al., 2013). Raw samples were first washed with PBS, then treated with somatic cell lysis buffer to eliminate somatic cells. This step was necessary because sperm cells contain approximately 600 times less RNA than somatic cells (Zhao et al., 2006), meaning that even minimal somatic cell contamination could overwhelm sperm-specific RNA. After somatic cell lysis, we confirmed the absence of somatic cells in each sample through microscopic examination. Only samples showing a complete absence of somatic cells

proceeded to snap-freezing in liquid nitrogen. This validation step ensured the integrity of our transcriptomic results.

Storage and preservation protocols

The standardized freezing and storage protocols across all samples ensured consistency throughout our longitudinal study. Snap-freezing in liquid nitrogen provided instantaneous preservation of biological states (Smith et al., 2020), while -80°C storage maintained long-term stability (Haid et al., 2018; Smith et al., 2020). It allowed samples collected months apart to be analyzed under identical conditions, which was essential for detecting genuine age-related changes rather than storage artifacts.

4.1.3 Analytical Methodologies

In this study, different analytical platforms were employed to elucidate different aspects of the same story: transition from puberty to sexual maturity, across multiple biological layers. It began with targeted metabolomics to quantify amino acids and amines, considering their importance in reproductive function (Endo et al., 2019; Hanley, 2023; Hosseini et al., 2022; Lewis & Aitken, 2001; Li et al., 2023), then expanded to untargeted metabolomics to discover novel metabolites. Sperm quality assessments provided biological functional changes, and we investigated whether amino acids and amines have any correlation with changes in sperm performance. Finally, transcriptomics revealed gene expression changes that potentially may have driven these alterations. Each paper is focused on a single analytical method, rather than attempting full integration across all platforms, because each platform generated extensive datasets that required in-depth analysis. However, integrating sperm quality parameters with targeted metabolomics was performed in Paper III to examine functional relationships. In future studies, it would be interesting to combine transcriptomics and metabolomics data as well.

Targeted metabolomics

Our targeted approach investigated 26 amino acids and three amines in both sperm and seminal plasma using LC-MS/MS. This focused analysis offered high sensitivity for these specific compounds, enabled absolute quantification, and the detection of subtle changes in low-concentration metabolites (Roberts et al., 2012). The analytical workflow involved methanol protein precipitation, AccQ-Tag derivatization for amino acids, and analysis on an Agilent 1290 Infinity II LC system coupled to an Agilent 6495 QqQ mass spectrometer operating in dynamic

multiple-reaction monitoring (MRM) mode. This approach provided reliable quantification of amino acids and amines for their comparison across samples of three ages.

Untargeted metabolomics

While targeted metabolomics examined expected compounds, untargeted metabolomics explored the discovery of novel compounds (Di Minno et al., 2021). We cast a wider net with dual complementary chromatographic approaches: reverse-phase chromatography for hydrophobic metabolites and hydrophilic interaction liquid chromatography (HILIC) for polar compounds (Haggarty et al., 2015). This approach ensured broad coverage of the metabolome. The SIMPLEX extraction protocol efficiently extracted and preserved both lipophilic and hydrophilic metabolites (Coman et al., 2016). Data-dependent acquisition with 0.5-second cycle times captured both high- and low-abundance ions, while Bruker MetaboScape software handled feature extraction, quality control, and NIST database annotation. This approach revealed other novel metabolites, expanding our understanding of metabolic changes during sexual maturation. However, there was a challenge in making sense of the thousands of features. Untargeted metabolomics identified relative differences in metabolite abundance between samples across age groups rather than providing absolute concentrations of metabolites in the sample. This approach is ideal for discovering novel metabolic signatures; however, it poses challenges for performing direct correlations with sperm functional traits, as it reveals differences between groups but not the absolute concentration of each metabolite. Targeted metabolomics is a logical follow-up to untargeted metabolomics to verify absolute changes in metabolites due to age.

Sperm functional assessments

Computer-assisted sperm analysis (CASA) using the Sperm Class Analyzer® (Microptic SL, Spain) provided detailed kinematic profiles, including velocity, linearity, straightness, wobble, amplitude of lateral head displacement, and beat cross frequency (Dearing et al., 2014). Beyond total motility percentages, CASA analysis allowed us to identify distinct sperm subpopulations based on movement patterns (rapid progressive, rapid non-progressive, medium, and slow motile sperm). This subpopulation analysis significantly increased the value of the functional assessments by revealing how the swimming pattern was different between the ages and after storage for five days. The identification of rapid progressive (RapidP) subpopulations was particularly interesting, as these sperm might represent elite swimmers with superior fertilizing potential, similar to what is observed in swim-up selected sperm and fresh bull sperm (Alm-

Kristiansen, 2023). This was not just about whether sperm moved, but how they moved, with different movement patterns.

Flow cytometry with triple staining (SYTOTM 60 Red Fluorescent Nucleic Acid Stain, propidium iodide, PNA-Alexa Fluor 488) simultaneously assessed acrosome membrane integrity and viability on the Beckman Coulter CytoFLEX platform (Beckman Coulter, Fullerton, CA, USA). The SYTOTM 60 staining was included to ensure that only DNA-positive cells were identified as sperm, preventing contamination from debris or other cell types that could confound our viability and acrosome integrity measurements.

DNA integrity analysis using the Sperm Chromatin Structure Assay (SCSA[®]) with acridine orange staining revealed both fragmentation and chromatin condensation status (Boe-Hansen et al., 2005; Evenson & Jost, 2001). The SCSA[®] technique provided insights into chromatin structural changes that occur during sexual maturation, measuring both DNA fragmentation index (DFI) and high DNA stainability (HDS), which together provide an assessment of sperm nuclear integrity and chromatin maturation status.

Transcriptomics

Working with sperm RNA presents unique challenges, primarily due to minimal RNA content and the exceptional resistance of sperm to standard lysis protocols. During spermatogenesis, spermatids lose most of their cytoplasm and hence have far less RNA content than somatic cells (Hecht, 1998; Zhao et al., 2006). Sperm cells are notably resistant to standard guanidinium thiocyanate-phenol-chloroform (AGPC) lysis mixtures such as Trizol or Qiazol (phenol and guanidine thiocyanate) (QIAGEN, 2021), largely because disulfide-rich structures reinforce their plasma membrane and nuclear chromatin (Roszkowski & Mansuy, 2021).

Mammalian sperm pose particular challenges due to their chromatin structure, characterized by a high degree of DNA condensation, which makes *in vitro* studies difficult (Ribas-Maynou et al., 2021). During spermatogenesis, histones are replaced with protamines, a process that involves the oxidation of thiol (-SH) groups to disulfide (-SS-) bonds within and between protamine molecules (Gosálvez et al., 2011). This forms a three-dimensional network of disulfide bridges in the sperm chromatin, conferring high stability upon the sperm head that creates extensively disulfide-crosslinked chromatin (Gosálvez et al., 2011). Notably, boar sperm contain exclusively protamine 1, unlike species such as humans, mice, and horses that possess both protamine 1 and members of the protamine 2 family (Corzett et al., 2002). Boar protamine 1 is particularly distinct, as it possesses 10 cysteine residues per molecule, which is the highest number among the mammalian species examined, including donkey (8), horse (7),

human (6), goat (7), sheep (7), bull (7), white-tailed deer (7), and elephant (5) as reported by Gosálvez et al. (2011). Ribas-Maynou et al. (2021) reported that porcine and bovine sperm required longer lysis treatment for complete sperm DNA decondensation compared to human, horse, and donkey sperm. The challenge of sperm lysis is also compounded by membrane modifications that occur during epididymal maturation, during which sperm acquire additional cholesterol and maintain an asymmetric lipid distribution to stabilize the membrane for ejaculation (Gautier & Aurich, 2022; Nolan & Hammerstedt, 1997).

To overcome the challenge of lysis, we tested different combinations of several lysis reagents, including Qiazol (Qiagen), buffer RLT (Qiagen), beta-mercaptoethanol, and tris(2-carboxyethyl)phosphine (TCEP). We finalized the lysis protocol with the incorporation of TCEP into the Qiazol lysis solution from the miRNeasy mini kit, followed by incubation at 56°C for 60 minutes to achieve complete lysis of sperm cells. TCEP maintains its disulfide reducing capacity across a wide pH range (1.5-8.5), which enables complete disruption of the covalent bonds that stabilize boar sperm structures, thereby facilitating efficient RNA release (Roszkowski & Mansuy, 2021). Complete lysis was confirmed by microscopic examination for each sample. Additional RNA quality validation included TapeStation analysis to assess RNA integrity and confirming the absence of somatic cell contamination through non-detection of 18S and 28S rRNA peaks (Card et al., 2013; Yang et al., 2009). Furthermore, RNA quality was assessed by *PRMI* TaqMan™ Gene Expression Assay and RT-qPCR to verify absence of genomic DNA contamination (Haug et al., 2024). These multiple validation steps were essential as RNA-seq results depend on the quality of the input RNA.

Our transcriptomic approach involved both total RNA sequencing and miRNA sequencing to capture both coding and non-coding elements of the sperm transcriptome. Library preparation used rRNA depletion rather than poly-A selection to capture the diverse sperm transcripts. QiaSeq FastSelect HMR reagents blocked rRNA during cDNA synthesis, improving sequencing efficiency by reducing dominance of ribosomal reads. NEBNext Ultra II Directional Library preparation accommodated partially degraded RNA (RIN 2-6). NovaSeq-6000 sequencing with 150 bp paired-end reads provided the necessary depth and coverage for low-abundance transcripts characteristic of sperm cells. To complement total RNA profiling, miRNA libraries were built with QiaSeq miRNA library kit (Qiagen, Germany) and sequenced on a NextSeq-500 instrument to ensure detection of small RNAs.

4.1.4 Data Analysis

Our diverse analytical platforms created distinct challenges in data analysis that required tailored approaches while preserving the repeated measures' structure, treating each boar as its own control, increasing power to detect subtle maturational changes (Sullivan, 2008). Paper I (targeted metabolomics) yielded absolute concentration values for amino acids and amines, while Paper II (untargeted metabolomics) generated relative intensity values for thousands of detected features. Paper III had both percentage and count data with different distributions, while Paper IV generated FASTA files and count matrices of genes.

Metabolomics data (Papers I and II) were handled in MetaboAnalyst 6.0 (Pang et al., 2024) and R 4.4.2 (R Core Team, 2022). For Paper I, we evaluated normality and homogeneity using the Shapiro-Wilk test and Levene's test, respectively. We implemented repeated measures ANOVA when data met normality assumptions and homogeneity of variance, while the Friedman test was used for non-parametric situations to identify metabolites with significant change across age groups. Paper II had thousands of relative intensity-based data, which required careful normalization strategies based on the platform used to generate data (HILIC, RP-Pos). We employed multivariate analyses including principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to examine the distribution of the data. For annotated metabolites, we used the *limma* package in R (Ritchie et al., 2015) with age as a fixed effect and boar identity as a block. This analysis identified metabolites that showed significant age-related differences while accounting for repeated measures within the same boar. MetaboAnalyst 6.0 provided metabolomics pathway analysis, integrating enrichment analysis and pathway topology using *Sus scrofa* KEGG libraries (Pang et al., 2024). Paper III's sperm quality parameters posed distribution challenges. Percentage data required beta regression with logit links, while count data required Gamma models with log links. The *glmmTMB* package in R provided flexibility to handle these diverse distributions within a mixed-effects framework (McGillcuddy et al., 2025).

The RNA-Seq analysis pipeline began with FastQC quality control (Andrews, 2010) and the Trim Galore adapter trimming (Krueger, 2023), followed by HISAT2 alignment against the *Sus scrofa* genome (Kim et al., 2019). FeatureCounts transformed aligned reads into count matrices (Liao et al., 2013), while sRNAbench handled small RNA annotation against multiple databases (Aparicio-Puerta et al., 2019). Gene expression was conducted with the *DESeq2* package in R (Love et al., 2014) using a paired design with a 10-month vs a 7-month contrast. Gene Ontology (GO) analysis was performed to provide insight into biological processes associated with age-

related differences (Ashburner et al., 2000). With thousands of molecular features, the Benjamini-Hochberg method controlled false discovery rates while maintaining reasonable sensitivity (Ferreira & Zwinderman, 2006).

4.2 Transformations during sexual maturation

Our journey to understand the sexual maturity in Duroc boars began with a targeted investigation into amino acid and amine concentrations, later expanded into untargeted metabolomics and transcriptomics. It revealed how biological changes occur in boar semen during the transition from puberty to sexual maturity.

Amino acids, amines, and their relationship with sperm functional traits

Glutamate emerged as the predominant metabolite driving age-related changes during sexual maturity as identified in both targeted (Paper I) and untargeted (Paper II) metabolomics analyses. This is consistent with earlier findings by Brown-Woodman and White (1974), who found that glutamate was the dominant metabolite in both sperm and seminal plasma metabolomes. In our data, glutamate concentrations in sperm and seminal plasma declined progressively from 7 months to 10 months, showing the highest variable importance in projection (VIP) scores and significant correlations between sperm and seminal plasma ($r = 0.79$, adj $P < 0.05$). L-Glutamic acid also ranked highest in mean decrease accuracy for sperm by random forest analysis (Paper II). Glutamate influences sperm motility through N-methyl-D-aspartate receptor-mediated calcium signaling (Endo et al., 2019), guides sperm chemotaxis (Frolikova et al., 2020), and regulates acrosome reactions, which are important for fertilization (Momeni et al., 2021). However, excessive glutamate levels can compromise sperm quality and mitochondrial integrity, as reported in mice and *Drosophila* (Kayode et al., 2020; Vedelek et al., 2023). Consistent with this, Paper III showed that the glutamate concentrations in boar sperm and seminal plasma were negatively correlated with both progressive motility and rapid progressive motility. These findings suggest that the age-related decline in glutamate represents a form of metabolic optimization rather than deficiency. This hypothesis could be tested in future studies by supplementing glutamate in controlled experiments to assess its impact on sperm function or by comparing glutamate levels across individual boars to see how they relate to fertility outcomes.

Beyond glutamate, our analyses identified alanine, aspartate, choline, and taurine as metabolites that declined significantly in sperm between 7 months and 10 months. Choline has been reported as one of the potential biomarkers of sexual maturity in laying hens (Yaxiao et al.,

2025). Alanine, aspartate and glutamate metabolism, histidine metabolism, and tyrosine metabolism were identified as significantly enriched pathways in boar sperm during sexual maturation. These pathways collectively support energy metabolism, antioxidant defense, and protein phosphorylation cascades essential for sperm function (Brosnan & Brosnan, 2020; Ijiri et al., 2012; Massucci et al., 2013). In seminal plasma, the concentrations of glutamate and taurine decreased significantly with maturity in boars, highlighting their importance in both sperm and seminal plasma. Taurine protects sperm cells through antioxidant mechanisms (Li et al., 2023), choline maintains sperm membrane integrity and supports motility (Hanley, 2023), aspartate has been reported to enhance semen quality in rabbits and promotes testosterone production in Leydig cells in mice (Macchia et al., 2010; Nagata et al., 1999) and tyrosine supports sperm maturation in the epididymis by elevating cyclic adenosine monophosphate (cAMP) (Lewis & Aitken, 2001).

Results from Paper III complemented these findings: in both sperm and seminal plasma, aspartate, choline, and taurine concentrations were negatively correlated with progressive motility and rapid progressive motility, while alanine correlated positively with %DFI. These findings suggest that, rather than acquiring higher concentrations of these metabolites, mature boars achieve superior sperm performance through optimized metabolic profiles.

Several other studies have also reported amino acids to be important metabolites in reproductive metabolomics, often linked to fertility, liquid preservation ability, or sperm quality across boars and bulls (Narud et al., 2022; Narud et al., 2020; Sangeeta et al., 2015; Song et al., 2023), reflecting their central role in many biological processes. They function as energy substrates, signaling molecules, antioxidants, and precursors for hormone synthesis. They also regulate gene expression and metabolic pathways (Wu, 2009). In male reproduction, these compounds have been linked to key functions, including sperm motility, the acrosome reaction, and the modulation of antioxidant defenses.

Broader metabolome transformation

Paper II (untargeted metabolomics) revealed more than 4,000 metabolites in boar semen, the most predominant being amino acids, followed by fatty acid esters. Untargeted metabolomics expanded our understanding beyond amino acids, revealing complex changes across lipids, antioxidant systems, and energy pathways. Notable findings included homoisovanillic acid, which is involved in antioxidative functions (Tai et al., 2012), being the most important metabolite that separates age classification in both sperm and seminal plasma. Interestingly, the structurally related homovanillic acid has been reported as one of the key biomarkers of sexual

maturity in chickens (Zhao et al., 2023). Other findings included a decline in decanoyl-L-carnitine in sperm, linked to energy and fatty acid metabolism (Jeulin & Lewin, 1996), an increase in myo-inositol, which supports motility and DNA integrity (Ghaemi et al., 2024), and elevated levels of antioxidant compounds like 1-formylpyrroline-2-carboxylic acid and 7-hydroxychromanone (Jia & Farrow, 2012; Matsugo & Nakamura, 2023) in the seminal plasma of mature boars. Together, these metabolites reflect that there are changes in the antioxidant defenses, energy metabolism, and fatty acid metabolism during sexual maturation.

Pathway analysis in sperm identified porphyrin metabolism, involved in heme biosynthesis, and supporting cellular respiration (Yien & Perfetto, 2022), taurine and hypotaurine metabolism, which contribute to antioxidant protection (Partyka et al., 2017), and glycerolipid metabolism, which is essential for maintaining membrane integrity (Am-in et al., 2011), as key processes underlying sexual maturation. In seminal plasma, glutathione metabolism and nitrogen metabolism pathways were significantly enriched, supporting a protective environment, such as antioxidant defense (Li, 1975; Song et al., 2023), which is required for mature sperm function. These pathway enrichments provide additional knowledge beyond the metabolite-level findings, highlighting how multiple biochemical systems interact during maturation.

Multivariate assessment in both Paper I and Paper II showed that samples from younger boars (Age 1) exhibited greater metabolomic variability than those from mature boars (Age 2 and Age 3), with metabolites clustering by age. These findings may suggest that young boars are at different phases of achieving maturation, and it stabilizes once they reach Age 2. This stabilization suggests that metabolomic profiles can serve as potential markers of metabolic maturity, providing additional insight into the transition from puberty to sexual maturity.

Sperm quality improvements

Sperm quality assessments revealed maturation-associated changes in functional parameters between young and mature boars, with most changes stabilizing by 8.2 months. These shifts included increases in acrosome integrity, viability, motility, progressive motility, and rapid progressive motility, alongside decreases in medium motility, slow motility, and rapid non-progressive motility in mature boars (Age 2 and Age 3). The sperm swimming pattern also improves as boars age, as shown by our results, which include an increase in kinematic parameters (linearity, velocity, and straightness). Collectively, these changes reflected improvements in sperm quality and functional maturation in mature boars. Young boars produced sperm with higher ALH and more erratic swimming patterns, while mature boars

achieved more efficient linear trajectories. This shift suggests that sexual maturation involves fine-tuning of swimming efficiency rather than simply increasing the proportion of motile cells. While there are studies on comparing sperm quality traits such as motility, acrosome integrity and viability across boar ages, they were mostly done on older males, pooled samples across wide age groups, or with cross-sectional designs (Hensel et al., 2023; Xu et al., 2024), our study is the first to report longitudinal changes in sperm quality traits at six weeks interval during sexual maturation.

Enhanced storage tolerance with maturation

These maturational improvements were not only evident in fresh ejaculates but also in the ability of sperm to withstand storage. While all semen samples showed a decline in sperm quality after five days of storage at 18°C, the magnitude was most pronounced in the youngest boars. Samples from Age 2 and Age 3 maintained superior acrosome integrity, viability, motility, and kinematic parameters compared to Age 1 samples, suggesting that maturation enhances the cellular mechanisms supporting sperm survival under stress conditions.

Differential expression of genes and enrichment of pathways during sexual maturation

Paper IV (Transcriptomic analysis) revealed that sexual maturation involves mostly upregulation of genes, with 65% of differentially expressed genes showing upregulation in mature boars. This activation suggests that sexual maturation represents an active process. Key upregulated genes included *NCLN* (involved in membrane protein organization into lipid bilayers) (McGilvray et al., 2020), *RGS12* (regulates calcium oscillation for oocyte activation) (Ma et al., 2024), *CIB2* (important for calcium homeostasis and spermatogenesis) (Patel et al., 2015; Xu et al., 2020), *FOXP4* (linked to spermatogonial stem cell development) (Luo et al., 2023), *PHC1* (associated with spermatogenesis) (Sisakhtnezhad & Heshmati, 2018), *CDC25B* (linked to non-cycling function in sperm) (Teng et al., 2007), and *AKAP1* (involved in mitochondrial energy production and spermatogenesis) (Reinton et al., 2000; Sang et al., 2021). These genes collectively support membrane organization, calcium signaling, chromatin remodeling, and energy metabolism, processes that are directly aligned with the functional improvements documented in our sperm quality analyses (Paper III).

Downregulation of genes provided equally important insights. *HSP90AA1*, previously associated with poor fertility in bulls (D'Amours et al., 2019), was significantly downregulated in mature boars, suggesting optimization of protein folding mechanisms (Forsythe et al., 2001). The *FSIP2*, *VDAC3*, and *ALMS1* genes, which are involved in sperm fibrous sheath, mitochondrial function, and spermatogenesis (Arsov et al., 2006; Fang et al., 2021; Kwon et

al., 2013), were also downregulated in mature boars. Overexpression of *FSIP2* is linked to abnormal tail and acrosome structure in mouse spermatozoa (Fang et al., 2021), supporting the idea that downregulation prevents such defects. These patterns suggest that sexual maturation not only activates genes but also suppresses genes associated with abnormal morphology or impaired fertility, thereby fine-tuning sperm function during maturation.

Gene Ontology (GO) enrichment analysis revealed reproduction-related biological processes, protein kinase activity, and membrane protein organization as key GO terms. This showed the convergence of developmental processes, signaling pathways, and structural organization during sexual maturation.

miRNA regulatory networks

miRNA analysis revealed coordinated regulatory mechanisms controlling sexual maturation. Five of six differentially expressed miRNAs were upregulated in mature boars, including ssc-miR-193a-5p (linked to sperm motility across species) (Kasimanickam et al., 2022; Li & Chen, 2021), ssc-miR-196a (evolutionarily conserved for spermatogenesis) (Madison-Villar & Michalak, 2011), ssc-miR-126-3p (Sertoli cell function) (Tang et al., 2021), ssc-miR-574-3p (linked to mitochondrial function and ATP generation) (Ma et al., 2020) and miR-210 (linked to spermatogenesis) (Tang et al., 2016). This upregulation pattern may reflect increased regulatory needs as boars mature. Larger, sexually mature animals likely require more complex molecular control over both sperm development and the supporting functions of Sertoli cells.

We found that ssc-miR-196a is upregulated in mature sperm, which contrasts with a previous study that reported ssc-miR-196a expression only in immature porcine testis of 1 month (Lian et al., 2012). This suggests that miR-196a may have different roles during testicular development versus sperm maturation. The upregulation of miR-126-3p in mature boars in our study suggests promoting Sertoli cell proliferation (Tang et al., 2021), while miR-574-3p controls mitochondrial energy production needed for sperm function (Ma et al., 2020).

Only ssc-miR-338 was downregulated in sperm samples of mature boars, which is linked to spermatogenesis (Madison-Villar & Michalak, 2011). The integrated miRNA-mRNA analysis identified 17 regulatory pairs, with three miRNAs (ssc-miR-193a-5p, ssc-miR-126-3p, and ssc-miR-574-3p) targeting 2,459 unique genes. Many genes were targeted by multiple miRNAs, potentially creating overlapping control systems that likely suggest redundant control mechanisms for sexual maturation.

4.3 Integrated understanding of sexual maturation

Sexual maturation in Duroc boars involves coordinated changes across metabolic, functional, and transcriptional levels. The decline in amino acid and amines concentration paralleled improvements in sperm function, while transcriptional changes supported enhanced membrane organization, energy metabolism, and calcium signaling. This may suggest that sexual maturation represents a systemic biological transition rather than isolated developmental changes, as illustrated in Figure 6. Most sperm functional parameters stabilized in the second age group, which is approximately 8 months (mean 8.2 months); some improvements continued through 10 months. This suggests that sexual maturity extends beyond initial semen collection readiness (Age 1). However, it is important to note that sperm quality at Age 1 was already quite good, as these young boars produced motile, viable sperm that could potentially be used commercially with adjusted protocols, such as increasing the sperm concentration per insemination dose to compensate for any quality differences.

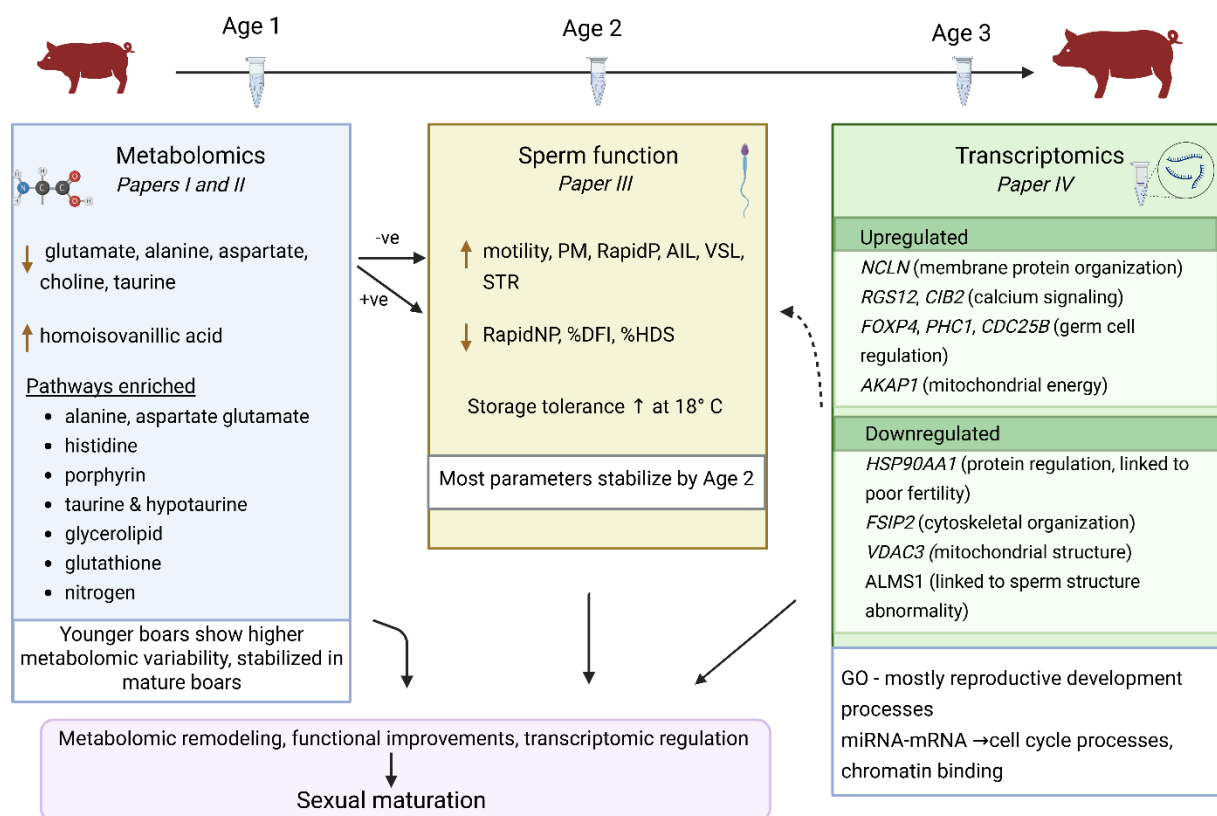


Figure 6: Proposed integrated overview of sexual maturation in Duroc boars based on analyses of semen. Metabolomic profiling (Papers I and II) showed a decline in key amino acids and amines in semen from mature boars, such as glutamate, alanine, aspartate, choline, and taurine, alongside an increase in homoisovanillic acid, with age-related enrichment of several pathways. Functional evaluation (Paper

III) revealed significant improvements in sperm motility, progressive motility (PM), rapid progressive motility (RapidP), acrosome intact live (AIL), velocity straight line (VSL), and straightness (STR), while DNA fragmentation index (%DFI), high DNA stainability (%HDS), and rapid non-progressive motility (RapidNP) decreased significantly in mature boars. Paper III also showed a significant correlation between key metabolites identified in Paper I and sperm quality parameters, linking metabolic shifts with functional outcomes. Transcriptomic analyses (Paper IV) showed upregulation of genes supporting membrane organization (*NCLN*), calcium signaling (*RGS12*, *CIB2*), germ cell regulation (*FOXP4*, *PHC1*, *CDC25B*), and mitochondrial energy regulation (*AKAP1*), while genes linked to poor fertility or abnormal spermatozo structure (*HSP90AA1*, *FSIP2*, *VDAC3*, *ALMS1*) were downregulated in samples from 10 months. Together, these findings highlight that sexual maturation is a biological transition, driven by metabolomic remodeling, functional improvements, and transcriptomic regulation, extending beyond the onset of puberty. Created by the author in <https://BioRender.com>

4.4 Implications, limitations, and future perspectives

4.4.1 Implications

This thesis provides the first longitudinal molecular and functional characterization of sexual maturation in boars. It shows that maturation is not a simple switch but a coordinated process involving the refinement of metabolite usage and pathways, the improvement of sperm quality parameters, and the regulation of gene expression. From an applied perspective, the identification of metabolites such as glutamate, alanine, aspartate, and choline, together with their correlations to sperm function, provides a foundation for developing further research on practical biomarkers of sexual maturity. Such biomarkers could support AI centers in determining or optimizing collection timing, potentially shortening generation intervals and accelerating genetic gain. The transcriptomic insights expand this view by identifying several genes associated with membrane organization, calcium signaling, and energy metabolism, as well as miRNA-mRNA interactions during sexual maturation. Collectively, these contributions strengthen the connection between fundamental molecular biology and applied pig production, offering value both to science and the livestock industry.

4.4.2 Study limitations

Our study had relatively small cohorts of boars. These small cohorts enabled us to perform detailed molecular analyses but may have limited our ability to detect subtle individual variations. We studied only Duroc boars from Norsvin SA's commercial breeding line. This

ensured genetic consistency and practical relevance but restricts broader applicability across different breeds and commercial AI operations.

An untargeted metabolomics approach revealed novel metabolites and pathways but left over 4,300 features unidentified due to current database limitations. This annotation challenge potentially concealed valuable biological insights. Many of these unidentified and identified features could represent novel biomarkers of sexual maturation that could be further validated with a targeted metabolomics approach. Future studies could prioritize validating identified metabolites through targeted approaches and correlating them with sperm functional parameters and fertility. The integration of untargeted discovery with targeted validation, combined with functional assessments, could reveal significant biological insights currently hidden within our datasets.

In our transcriptomics study, we employed fold-change thresholds ($|\log_2FC| \geq 0.5$), which we considered appropriate given the inherently low RNA abundance (600-fold less) in sperm cells as compared to somatic cells (Zhao et al., 2006). Even modest expression changes could be biologically meaningful in this context. However, this approach may have excluded biologically relevant changes with smaller effect sizes. Reliance on human miRNA databases for target prediction, necessitated by the limited pig-specific annotations, may have introduced some limitations in our miRNA-mRNA regulatory network analysis.

Moreover, the absence of field fertility data for Age 1 boars and the incomplete data for Age 2 and Age 3 boars prevented a direct correlation of whether the molecular changes we documented translate to improved reproductive performance in the field. Nevertheless, our comprehensive analyses consistently revealed improvements in motility, rapid progressive motility, and acrosome integrity and viability, as well as their correlation with metabolite optimization (declining glutamate, alanine, aspartate, choline, and taurine) in mature boars, and supportive transcriptional changes (membrane organization, energy metabolism, and signaling pathways). All are pointing towards genuine sexual maturation that likely translates to reproductive advantages.

4.4.2 Future perspectives

Validation of our metabolomics and transcriptomics findings, along with sperm quality parameters across various pig breeds and seasons, would help establish the generalizability of the findings. Collaboration among multiple AI centers across different geographical regions would help assess environmental influences on sexual maturation and broaden the practical

relevance of our results. While six-week sampling intervals aligned with commercial practical relevance, they might have missed developmental events between time points. Since sexual maturation likely involves continuous rather than discrete changes, future studies could employ continuous sampling to capture the full complexity of this biological process. However, such intensive sampling protocols would require careful consideration of the costs and practical challenges in commercial settings.

The lack of complete field fertility data in our study highlights a key area for future research. Large-scale, longitudinal studies with increased sample sizes that account for commercial culling practices would provide sufficient fertility data and would validate the significance of metabolomics and transcriptomics signatures, as well as sperm quality parameters. Such studies could follow boars from initial semen collection through seasons, correlating molecular profiles with conception rates, litter sizes, and offspring viability. This fertility validation would enable the development of practical screening protocols for identifying sexually mature boars ready for commercial use, potentially reducing generation intervals and accelerating genetic progress, with positive economic implications.

Our identification of glutamate, alanine, aspartate, and choline as key metabolic biomarkers, along with their correlation with sperm function, suggests opportunities for developing rapid and cost-effective assays to assess boar readiness. Practical implementation could involve developing rapid assays for key metabolites like glutamate, enabling quick assessment of boar readiness at AI stations. They also indicate research opportunities for targeted nutritional interventions, such as amino acid optimization to potentially enhance sperm quality or accelerate sexual maturation in younger boars. Similarly, the improved storage tolerance documented in mature boars (Age 2 and Age 3) opens up research possibilities for exploring age-specific semen preservation protocols that could better maintain semen quality from younger animals. This might bridge the gap between early genetic selection goals and optimal reproductive performance.

Other advanced molecular approaches, such as proteomics, genetics, and epigenetics, could reveal additional biological layers of sexual maturity, enhancing our understanding at a molecular level. An integrated analysis combining metabolomics, transcriptomics, and sperm quality data could identify novel regulatory networks and biomarker combinations. As a foundational first approach, our current study focused on individual analytical methods to establish a baseline understanding of each biological layer, providing the necessary groundwork for future integrated analysis.

5. Conclusions

This thesis achieved its aim of characterizing the molecular signatures of sexual maturation in Duroc boars through longitudinal analysis of metabolites, transcriptomes, and sperm functional parameters, thereby advancing knowledge of reproductive biology.

Paper I identified key amino acid and amine biomarkers of sexual maturity through targeted LC-MS/MS analysis. We discovered glutamate, alanine, aspartate, and choline as biomarkers whose decline from 7 to 10 months characterizes the reproductive maturation. Pathway analysis revealed alanine, aspartate and glutamate metabolism as key processes underlying sexual maturation. These findings establish that sexual maturation involves metabolic refinement rather than simple quantitative increases.

Paper II comprehensively characterized the global metabolomic landscape through untargeted metabolomic profiling, establishing distinct metabolomic fingerprints. L-glutamic acid and decanoyl-L-carnitine decreased while homoisovanillic acid increased with age. Porphyrin metabolism, taurine and hypotaurine metabolism, glycerolipid metabolism, nitrogen metabolism, and glutathione metabolism were significantly enriched pathways underlying sexual maturation.

Paper III evaluated functional sperm quality parameters and their relationships with amino acids and amines concentrations. We documented increased overall motility, progressive motility, changed swimming patterns, improved DNA integrity, and increased storage tolerance, with most parameters stabilizing after 8 months of age. The correlations between declining amino acid concentrations and improved sperm performance validated the biological significance of our metabolomic discoveries.

Paper IV profiled the sperm transcriptome landscape through a comparative analysis of total RNA and miRNA expression profiles. We revealed upregulation of genes linked to membrane organization, calcium signaling, and energy metabolism (*NCLN*, *RGS12*, *CIB2*, *FOXP4*, *AKAP1*), alongside downregulation of genes involved in protein folding, sperm structure, and mitochondrial function (*HSP90AA1*, *FSIP2*, *VDAC3*, *ALMS1*). The identification of miRNA regulatory networks adds complexity to our understanding of sexual maturation.

Together, these studies show that sexual maturation involves coordination across metabolic, functional, and transcriptional levels, rather than discrete shifts. Beyond providing fundamental insights into reproductive biology, this thesis also identifies molecular and functional biomarkers with potential value in pig breeding programs that require further research. This

dual contribution of fundamental insights and applied value defines the broader significance of the research and establishes a framework for future integrated studies in reproductive development.

6. References

- Allrich, R. (1983). Pubertal development of the boar: Age-related changes in testicular morphology and in vitro production of testosterone and estradiol-17 β . *Biology of Reproduction*, 28(4), 902–909. <https://doi.org/10.1095/biolreprod28.4.902>
- Allrich, R. D., Christenson, R. K., Ford, J. J., & Zimmerman, D. R. (1982). Pubertal development of the boar: Testosterone, estradiol-17 β , cortisol and LH concentrations before and after castration at various ages. *Journal of Animal Science*, 55(5), 1139–1146. <https://doi.org/10.2527/jas1982.5551139x>
- Alm-Kristiansen, A. H. (2023). Motility subpopulations with distinct motility characteristics using swim-up-selected sperm cells from Norwegian Red bulls: Effects of freezing–thawing and between-bull variation. *Biology*, 12(8).
- Alvarez-Rodriguez, M., Martinez, C., Wright, D., Barranco, I., Roca, J., & Rodriguez-Martinez, H. (2020). The transcriptome of pig spermatozoa, and its role in fertility. *International Journal of Molecular Sciences*, 21(5). <https://doi.org/10.3390/ijms21051572>
- Am-in, N., Kirkwood, R. N., Techakumphu, M., & Tantasuparuk, W. (2011). Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility. *Theriogenology*, 75(5), 897–903. <https://doi.org/10.1016/j.theriogenology.2010.10.032>
- Andrews, S. (2010). *FastQC: A quality control tool for high throughput sequence data*. Babraham Bioinformatics. <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>
- Aparicio-Puerta, E., Lebrón, R., Rueda, A., Gómez-Martín, C., Giannoukakos, S., Jaspez, D., Medina, J. M., Zubkovic, A., Jurak, I., Fromm, B., Marchal, J. A., Oliver, J., & Hackenberg, M. (2019). sRNAbench and sRNAtoolbox 2019: Intuitive fast small RNA profiling and differential expression. *Nucleic Acids Research*, 47(W1), W530–W535. <https://doi.org/10.1093/nar/gkz415>
- Argov-Argaman, N., Mahgrefthe, K., Zeron, Y., & Roth, Z. (2013). Variation in lipid profiles within semen compartments—the bovine model of aging. *Theriogenology*, 80(7), 712–721. <https://doi.org/10.1016/j.theriogenology.2013.05.024>
- Arsov, T., Silva, D. G., O'Bryan, M. K., Sainsbury, A., Lee, N. J., Kennedy, C., Manji, S. S., Nelms, K., Liu, C., Vinuesa, C. G., de Kretser, D. M., Goodnow, C. C., & Petrovsky, N. (2006). Fat aussie--a new Alström syndrome mouse showing a critical role for ALMS1 in obesity, diabetes, and spermatogenesis. *Molecular Endocrinology*, 20(7), 1610–1622. <https://doi.org/10.1210/me.2005-0494>

- Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T., Harris, M. A., Hill, D. P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J. C., Richardson, J. E., Ringwald, M., Rubin, G. M., & Sherlock, G. (2000). Gene Ontology: Tool for the unification of biology. *Nature Genetics*, 25(1), 25–29. <https://doi.org/10.1038/75556>
- Ashokan, M., Rana, E., Sneha, K., Namith, C., Naveen Kumar, G. S., Azharuddin, N., Elango, K., Jeyakumar, S., & Ramesha, K. P. (2023). Metabolomics—a powerful tool in livestock research. *Animal Biotechnology*, 34(7), 3237–3249. <https://doi.org/10.1080/10495398.2022.2128814>
- Ayad, B., Oyeyipo, I., Van Der Horst, G., & Du Plessis, S. (2021). Cementing the relationship between conventional and advanced semen parameters. *Middle East Fertility Society Journal*, 26. <https://doi.org/10.1186/s43043-021-00086-z>
- Ballachey, B. E., Hohenboken, W. D., & Evenson, D. P. (1987). Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. *Biology of Reproduction*, 36(4), 915–925. <https://doi.org/10.1095/biolreprod36.4.915>
- Billoir, E., Navratil, V., & Blaise, B. J. (2015). Sample size calculation in metabolic phenotyping studies. *Briefings in Bioinformatics*, 16(5), 813–819. <https://doi.org/10.1093/bib/bbu052>
- Blandau, R. J., & Rumery, R. E. (1964). The relationship of swimming movements of epididymal spermatozoa to their fertilizing capacity. *Fertility and Sterility*, 15(6), 571–580. [https://doi.org/10.1016/S0015-0282\(16\)35401-2](https://doi.org/10.1016/S0015-0282(16)35401-2)
- Blaurock, J., Baumann, S., Grunewald, S., Schiller, J., & Engel, K. M. (2022). Metabolomics of human semen: A review of different analytical methods to unravel biomarkers for male fertility disorders. *International Journal of Molecular Sciences*, 23(16), 9031.
- Boe-Hansen, G. B., Christensen, P., Vibjerg, D., Nielsen, M. B., & Hedeboe, A. M. (2008). Sperm chromatin structure integrity in liquid stored boar semen and its relationships with field fertility. *Theriogenology*, 69(6), 728–736. <https://doi.org/10.1016/j.theriogenology.2007.12.004>
- Boe-Hansen, G. B., Morris, I. D., Ersbøll, A. K., Greve, T., & Christensen, P. (2005). DNA integrity in sexed bull sperm assessed by neutral Comet assay and sperm chromatin structure assay. *Theriogenology*, 63(6), 1789–1802. <https://doi.org/10.1016/j.theriogenology.2004.08.004>
- Bonet, S., Garcia, E., & Sepúlveda, L. (2013). The boar reproductive system. In S. Bonet, I. Casas, W. V. Holt, & M. Yeste (Eds.), *Boar Reproduction: Fundamentals and new*

- biotechnological trends* (pp. 65–107). Springer Berlin Heidelberg.
https://doi.org/10.1007/978-3-642-35049-8_3
- Borg, K. E., Lunstra, D. D., & Christenson, R. K. (1993). Semen characteristics, testicular size, and reproductive hormone concentrations in mature Duroc, Meishan, Fengjing, and Minzhu boars. *Biology of Reproduction*, 49(3), 515–521.
<https://doi.org/10.1095/biolreprod49.3.515>
- Brosnan, M. E., & Brosnan, J. T. (2020). Histidine metabolism and function. *The Journal of Nutrition*, 150, 2570S–2575S. <https://doi.org/10.1093/jn/nxaa079>
- Brown-Woodman, P. D., & White, I. G. (1974). Amino acid composition of semen and the secretions of the male reproductive tract. *Australian Journal of Biological Sciences*, 27(4), 415–422. <https://doi.org/10.1071/bi9740415>
- BRS. (2023). *A quality offensive in pig reproduction*. Bundesverband Rind und Schwein e. V. (BRS). Retrieved July 22, 2025 from <https://www.rind-schwein.de/brs-schwein/a-quality-offensive-in-pig-reproduction.html>
- Cameron, R. D. A. (1987). Sexual development and semen production in boars. *Pig News and Information*, 8(4), 389–396.
- Card, C. J., Anderson, E. J., Zamberlan, S., Krieger, K. E., Kaproth, M., & Sartini, B. L. (2013). Cryopreserved bovine spermatozoal transcript profile as revealed by high-throughput ribonucleic acid sequencing. *Biology of Reproduction*, 88(2), 49.
<https://doi.org/10.1095/biolreprod.112.103788>
- Caruana, E. J., Roman, M., Hernández-Sánchez, J., & Solli, P. (2015). Longitudinal studies. *Journal of Thoracic Disease*, 7(11), E537–540. <https://doi.org/10.3978/j.issn.2072-1439.2015.10.63>
- Castiglione Morelli, M. A., Ostuni, A., Giangaspero, B., Cecchini, S., Carluccio, A., & Boni, R. (2021). Relationships between seminal plasma metabolites, semen characteristics and sperm kinetics in donkey (*Equus asinus*). *Animals*, 11(1).
<https://doi.org/10.3390/ani11010201>
- Chang, Y., Dai, D.-h., Li, Y., Zhang, Y., Zhang, M., Zhou, G.-b., & Zeng, C.-j. (2016). Differences in the expression of microRNAs and their predicted gene targets between cauda epididymal and ejaculated boar sperm. *Theriogenology*, 86(9), 2162–2171.
<https://doi.org/10.1016/j.theriogenology.2016.07.012>
- Coman, C., Solari, F. A., Hentschel, A., Sickmann, A., Zahedi, R. P., & Ahrends, R. (2016). Simultaneous metabolite, protein, lipid extraction (SIMPLEX): A combinatorial

- multimolecular omics approach for systems biology. *Molecular & Cellular Proteomics*, 15(4), 1453–1466. <https://doi.org/10.1074/mcp.M115.053702>
- Comhaire, F., & Vermeulen, L. (1995). Human semen analysis. *Human Reproduction Update*, 1(4), 343–362. <https://doi.org/10.1093/HUMUPD/1.4.343>
- Conesa, A., Madrigal, P., Tarazona, S., Gomez-Cabrero, D., Cervera, A., McPherson, A., Szczeniak, M. W., Gaffney, D. J., Elo, L. L., Zhang, X., & Mortazavi, A. (2016). A survey of best practices for RNA-seq data analysis. *Genome Biology*, 17(1), 13. <https://doi.org/10.1186/s13059-016-0881-8>
- Corzett, M., Mazrimas, J., & Balhorn, R. (2002). Protamine 1: Protamine 2 stoichiometry in the sperm of eutherian mammals. *Molecular Reproduction and Development*, 61(4), 519–527. <https://doi.org/10.1002/mrd.10105>
- Curry, E., Safranski, T. J., & Pratt, S. L. (2011). Differential expression of porcine sperm microRNAs and their association with sperm morphology and motility. *Theriogenology*, 76(8), 1532–1539. <https://doi.org/10.1016/j.theriogenology.2011.06.025>
- D'Amours, O., Calvo, É., Bourassa, S., Vincent, P., Blondin, P., & Sullivan, R. (2019). Proteomic markers of low and high fertility bovine spermatozoa separated by Percoll gradient. *Molecular Reproduction and Development*, 86(8), 999–1012. <https://doi.org/10.1002/mrd.23174>
- Dai, D. H., Qazi, I. H., Ran, M. X., Liang, K., Zhang, Y., Zhang, M., Zhou, G. B., Angel, C., & Zeng, C. J. (2019). Exploration of miRNA and mRNA profiles in fresh and frozen-thawed boar sperm by transcriptome and small RNA sequencing. *International Journal of Molecular Sciences*, 20(4). <https://doi.org/10.3390/ijms20040802>
- Dascanio, J. J. (2014). Computer-assisted sperm analysis. In *Equine reproductive procedures* (pp. 368–372). <https://doi.org/10.1002/9781118904398.ch113>
- Dearing, C. G., Kilburn, S., & Lindsay, K. S. (2014). Validation of the sperm class analyser CASA system for sperm counting in a busy diagnostic semen analysis laboratory. *Human Fertility*, 17(1), 37–44. <https://doi.org/10.3109/14647273.2013.865843>
- Dekkers, J., Mathur, P., & Knol, E. (2011). Genetic improvement of the pig. *CABI*, 390–425. <https://doi.org/10.1079/9781845937560.0390>
- Di Minno, A., Gelzo, M., Stornaiuolo, M., Ruoppolo, M., & Castaldo, G. (2021). The evolving landscape of untargeted metabolomics. *Nutrition, Metabolism and Cardiovascular Diseases*, 31(6), 1645–1652. <https://doi.org/10.1016/j.numecd.2021.01.008>

- Ding, H., Luo, Y., Liu, M., Huang, J., & Xu, D. (2016). Histological and transcriptome analyses of testes from Duroc and Meishan boars. *Scientific Reports*, 6(1), 20758. <https://doi.org/10.1038/srep20758>
- Dougherty, K. A., Emilson, L. B., Cockett, A. T., & Urry, R. L. (1975). A comparison of subjective measurements of human sperm motility and viability with two live-dead staining techniques. *Fertility and Sterility*, 26(7), 700–703. [https://doi.org/10.1016/s0015-0282\(16\)41238-0](https://doi.org/10.1016/s0015-0282(16)41238-0)
- Dyck, M. K., Foxcroft, G. R., Novak, S., Ruiz-Sanchez, A., Patterson, J., & Dixon, W. T. (2011). Biological markers of boar fertility. *Reproduction in Domestic Animals*, 46 Suppl 2, 55–58. <https://doi.org/10.1111/j.1439-0531.2011.01837.x>
- Elango, K., Layek, S. S., & Kumaresan, A. (2022). Advances in bovine sperm quality assessment: from motility to fertility. In A. Kumaresan & A. K. Srivastava (Eds.), *Current Concepts in Bovine Reproduction* (pp. 263–291). Springer Nature Singapore. https://doi.org/10.1007/978-981-19-0116-4_14
- Endo, D., Kon, S., Sato, T., Toyama, F., Katsura, Y., Nakauchi, Y., Takayama-Watanabe, E., & Watanabe, A. (2019). NMDA-type glutamate receptors mediate the acrosome reaction and motility initiation in newt sperm. *Molecular Reproduction and Development*, 86(9), 1106–1115. <https://doi.org/10.1002/mrd.23225>
- Evenson, D., & Jost, L. (2001). Sperm chromatin structure assay for fertility assessment. *Current Protocols in Cytometry*, Chapter 7, Unit 7.13. <https://doi.org/10.1002/0471142956.cy0713s13>
- Evenson, D. P. (2016). The sperm chromatin structure assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. *Animal Reproduction Science*, 169, 56–75. <https://doi.org/10.1016/j.anireprosci.2016.01.017>
- Evenson, D. P. (2022). Sperm Chromatin Structure Assay (SCSA®) for fertility assessment. *Current Protocols*, 2(8), e508. <https://doi.org/10.1002/cpz1.508>
- Fang, X., Gamallat, Y., Chen, Z., Mai, H., Zhou, P., Sun, C., Li, X., Li, H., Zheng, S., Liao, C., Yang, M., Li, Y., Yang, Z., Ma, C., Han, D., Zuo, L., Xu, W., Hu, H., Sun, L., & Li, N. (2021). Hypomorphic and hypermorphic mouse models of *Fsip2* indicate its dosage-dependent roles in sperm tail and acrosome formation. *Development*, 148(11). <https://doi.org/10.1242/dev.199216>
- FAO. (2024). *Meat Market Review: Overview of global market developments in 2023*. <https://openknowledge.fao.org/handle/20.500.14283/cd0465en>

- FAO. (2025). *Meat market review. Overview of global market developments in 2024*. FAO. <https://openknowledge.fao.org/items/b553c447-f6b2-4ff8-825e-024767b27760>
- Feitsma, H. (2017). *Artificial insemination in pigs, research and developments in the Netherlands, a review*. Engormix. https://en.engormix.com/pig-industry/swine-management/artificial-insemination-pigs-research_a40422/
- Fernández-López, P., Garriga, J., Casas, I., Yeste, M., & Bartumeus, F. (2022). Predicting fertility from sperm motility landscapes. *Communications Biology*, 5(1), 1027. <https://doi.org/10.1038/s42003-022-03954-0>
- Ferreira, J. A., & Zwinderman, A. H. (2006). On the Benjamini-Hochberg method. *Annals of Statistics*, 34, 1827–1849.
- FlorCruz, S. V., & Lapwood, K. R. (1978). A longitudinal study of pubertal development in boars investigation of the relationships between gonadal and epididymal development and plasma luteinizing hormone and testosterone profiles. *International Journal of Andrology*, 1(1–6), 317–330. <https://doi.org/10.1111/j.1365-2605.1978.tb00603.x>
- Flowers, W. L. (1997). Management of boars for efficient semen production. *Journal of Reproduction and Fertility. Supplement*, 52, 67–78.
- Flowers, W. L. (2008). Genetic and phenotypic variation in reproductive traits of AI boars. *Theriogenology*, 70(8), 1297–1303. <https://doi.org/10.1016/j.theriogenology.2008.06.016>
- Flowers, W. L. (2022). Factors affecting the production of quality ejaculates from boars. *Animal Reproduction Science*, 246, 106840. <https://doi.org/10.1016/j.anireprosci.2021.106840>
- Foote, R. H. (2002). The history of artificial insemination: Selected notes and notables. *Journal of Animal Science*, 80, 1–10.
- Forsythe, H. L., Jarvis, J. L., Turner, J. W., Elmore, L. W., & Holt, S. E. (2001). Stable association of hsp90 and p23, but Not Hsp70, with active human telomerase. *Journal of Biological Chemistry*, 276(19), 15571–15574. <https://doi.org/10.1074/jbc.C100055200>
- França, L. R., Avelar, G. F., & Almeida, F. F. L. (2005). Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigs. *Theriogenology*, 63(2), 300–318. <https://doi.org/10.1016/j.theriogenology.2004.09.014>
- Fraser, L., Brym, P., Pareek, C. S., Mogielnicka-Brzozowska, M., Paukszto, Ł., Jastrzębski, J. P., Wasilewska-Sakowska, K., Mańkowska, A., Sobiech, P., & Żukowski, K. (2020). Transcriptome analysis of boar spermatozoa with different freezability using RNA-Seq. *Theriogenology*, 142, 400–413. <https://doi.org/10.1016/j.theriogenology.2019.11.001>

- Frolikova, M., Otcenaskova, T., Valasková, E., Postlerova, P., Stopkova, R., Stopka, P., & Komrskova, K. (2020). The role of taste receptor mTAS1R3 in chemical communication of gametes. *International Journal of Molecular Sciences*, 21(7), 2651.
- Gautier, C., & Aurich, C. (2022). “Fine feathers make fine birds” – The mammalian sperm plasma membrane lipid composition and effects on assisted reproduction. *Animal Reproduction Science*, 246, 106884. <https://doi.org/10.1016/j.anireprosci.2021.106884>
- Ghaemi, M., Seighali, N., Shafiee, A., Beiky, M., Kohandel Gargari, O., Azarboo, A., Shafiti, V., Jafarabady, K., Eshraghi, N., Haddadi, M., Akbari, R., Panahi, Z., & Hantoushzadeh, S. (2024). The effect of Myo-inositol on improving sperm quality and IVF outcomes: A systematic review and meta-analysis. *Food Science & Nutrition*, 12(11), 8515–8524. <https://doi.org/10.1002/fsn3.4427>
- Gòdia, M., Castelló, A., Rocco, M., Cabrera, B., Rodríguez-Gil, J. E., Balasch, S., Lewis, C., Sánchez, A., & Clop, A. (2020). Identification of circular RNAs in porcine sperm and evaluation of their relation to sperm motility. *Scientific Reports*, 10(1). <https://doi.org/10.1038/s41598-020-64711-z>
- Gòdia, M., Estill, M., Castelló, A., Balasch, S., Rodríguez-Gil, J. E., Krawetz, S. A., Sánchez, A., & Clop, A. (2019). A RNA-seq analysis to describe the boar sperm transcriptome and its seasonal changes. *Frontiers in Genetics*, 10, 299. <https://doi.org/10.3389/fgene.2019.00299>
- Gòdia, M., Mayer, F. Q., Nafissi, J., Castelló, A., Rodríguez-Gil, J. E., Sánchez, A., & Clop, A. (2018). A technical assessment of the porcine ejaculated spermatozoa for a sperm-specific RNA-seq analysis. *Systems Biology in Reproductive Medicine*, 64(4), 291–303. <https://doi.org/10.1080/19396368.2018.1464610>
- Gòdia, M., Reverter, A., González-Prendes, R., Ramayo-Caldas, Y., Castelló, A., Rodríguez-Gil, J.-E., Sánchez, A., & Clop, A. (2020). A systems biology framework integrating GWAS and RNA-seq to shed light on the molecular basis of sperm quality in swine. *Genetics Selection Evolution*, 52(1), 72. <https://doi.org/10.1186/s12711-020-00592-0>
- Gòdia, M., Swanson, G., & Krawetz, S. A. (2018). A history of why fathers’ RNA matters. *Biology of Reproduction*, 99(1), 147–159. <https://doi.org/10.1093/biolre/iox007>
- Gong, T., Wang, W., Xu, H., Yang, Y., Chen, X., Meng, L., Xu, Y., Li, Z., Wan, S., & Mu, Q. (2021). Longitudinal expression of testicular TAS1R3 from prepuberty to sexual maturity in Congjiang Xiang pigs. *Animals*, 11(2), 437.

- Goodrich, R. J., Anton, E., & Krawetz, S. A. (2013). Isolating mRNA and small noncoding RNAs from human sperm. In *Methods in Molecular Biology* (Vol. 927, pp. 385–396). https://doi.org/10.1007/978-1-62703-038-0_33
- Gosálvez, J., López-Fernández, C., Fernández, J. L., Gouraud, A., & Holt, W. V. (2011). Relationships between the dynamics of iatrogenic DNA damage and genomic design in mammalian spermatozoa from eleven species. *Molecular Reproduction and Development*, 78(12), 951–961. <https://doi.org/10.1002/mrd.21394>
- Graham, J. K., Kunze, E., & Hammerstedt, R. H. (1990). Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. *Biology of Reproduction*, 43(1), 55–64. <https://doi.org/10.1095/biolreprod43.1.55>
- Guo, Y., Li, J., Hao, F., Yang, Y., Yang, H., Chang, Q., Kong, P., Liu, W., Jiao, X., & Teng, X. (2023). A new perspective on semen quality of aged male: The characteristics of metabolomics and proteomics. *Frontiers in Endocrinology*, 13. <https://doi.org/10.3389/fendo.2022.1058250>
- Gur, Y., & Breitbart, H. (2006). Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes. *Genes & Development*, 20(4), 411–416. <https://doi.org/10.1101/gad.367606>
- Hackerova, L., Pilsova, A., Pilsova, Z., Zelenkova, N., Tymich Hegrova, P., Klusackova, B., Chmelikova, E., Sedmikova, M., Simonik, O., & Postlerova, P. (2025). Boar sperm motility assessment using computer-assisted sperm analysis: Current practices, Limitations, and Methodological Challenges. *Animals*, 15(3).
- Haggarty, J., Oppermann, M., Dalby, M. J., Burchmore, R. J. S., Cook, K., Weidt, S., & Burgess, K. E. V. (2015). Serially coupling hydrophobic interaction and reversed-phase chromatography with simultaneous gradients provides greater coverage of the metabolome. *Metabolomics*, 11, 1465–1470.
- Haid, M., Muschet, C., Wahl, S., Römisch-Margl, W., Prehn, C., Möller, G., & Adamski, J. (2018). Long-term stability of human plasma metabolites during storage at -80 °C. *Journal of Proteome Research*, 17(1), 203–211. <https://doi.org/10.1021/acs.jproteome.7b00518>
- Hanley, P. J. (2023). Elusive physiological role of prostatic acid phosphatase (PAP): Generation of choline for sperm motility via auto-and paracrine cholinergic signaling. *Frontiers in Physiology*, 14. <https://doi.org/10.3389/fphys.2023.1327769>

- Harris, D. L. (1999). *One-site and multi-site swine rearing systems*. Retrieved September 6, 2025 from https://www.pig333.com/articles/one-site-and-multi-site-swine-rearing-systems_19565/
- Haug, L. M., Wilson, R. C., & Alm-Kristiansen, A. H. (2024). Epigenetic-related transcriptional reprogramming elucidated by identification and validation of a novel reference gene combination for RT-qPCR studies in porcine oocytes of contrasting quality. *Molecular Biology Reports*, 51(1), 368. <https://doi.org/10.1007/s11033-024-09319-6>
- Hecht, N. B. (1998). Molecular mechanisms of male germ cell differentiation. *BioEssays*, 20(7), 555–561. [https://doi.org/10.1002/\(sici\)1521-1878\(199807\)20:7%3C555::aid-bies6%3E3.0.co;2-j](https://doi.org/10.1002/(sici)1521-1878(199807)20:7%3C555::aid-bies6%3E3.0.co;2-j)
- Hensel, B., Pieper, L., Jung, M., & Schulze, M. (2023). Influence of age, breed, and season on the quality of boar semen stored at low-temperature. *Theriogenology*, 208, 102–108. <https://doi.org/10.1016/j.theriogenology.2023.06.010>
- Hoggan, N. Á. (2021). *New multiplier nucleus farm supports investment in Mexico*. Hypor (Hendrix Genetics). Retrieved August 26, 2025 from <https://www.hypor.com/en/news/new-multiplier-nucleus-farm-supports-investment-in-mexico/>
- Hosseini, E., Amirjannati, N., Bazrafkan, M., Moghaddasfar, H., & Gilany, K. (2022). P-052 Targeted metabolomics (amino acids) profiling of seminal plasma distinguished teratozoospermia from normozoospermic samples. *Human Reproduction*, 37(Supplement_1). <https://doi.org/10.1093/humrep/deac107.048>
- Huang, Y. H., Lo, L. L., Liu, S. H., & Yang, T. S. (2010). Age-related changes in semen quality characteristics and expectations of reproductive longevity in Duroc boars. *Animal Science Journal*, 81(4), 432-437. <https://doi.org/10.1111/j.1740-0929.2010.00753.x>
- Ijiri, T. W., Mahbub Hasan, A. K., & Sato, K. (2012). Protein-tyrosine kinase signaling in the biological functions associated with sperm. *Journal of Signal Transduction*, 2012, 181560. <https://doi.org/10.1155/2012/181560>
- Innoreports. (2007). *The inside scoop on semen*. Innoscripta AG. Retrieved September 6, 2025 from <https://www.innovations-report.com/agriculture-environment/agricultural-and-forestry-science/report-80474/>
- Jakobsen, K., & Danielsen, V. (2020). A short history of the development of pig production and pig nutrition. In *Lærebog i fysiologi*. Landbrug & Fødevarer Sektor for Gris.

- Jeulin, C., & Lewin, L. (1996). Role of free L-carnitine and acetyl-L-carnitine in post-gonadal maturation of mammalian spermatozoa. *Human Reproduction Update*, 2(2), 87–102. <https://doi.org/10.1093/humupd/2.2.87>
- Jia, Q., & Farrow, T. M. (2012). 7-hydroxy chromones as potent antioxidants (U.S. Patent No. US8110555B2). U. S. Patent. <https://patents.google.com/patent/US8110555B2/en>
- Jochems, R. (2022). *In vitro embryo production in Norwegian Duroc and Landrace pigs* (Publication Number 2022:22) [Doctoral thesis, Norwegian University of Life Sciences]. Ås, Norway.
- Jodar, M., Selvaraju, S., Sendler, E., Diamond, M. P., & Krawetz, S. A. (2013). The presence, role and clinical use of spermatozoal RNAs. *Human Reproduction Update*, 19(6), 604–624. <https://doi.org/10.1093/humupd/dmt031>
- Johnson, T. (1996). *Artificial insemination ... A revised comparison*. MSU Pork Quarterly. <https://www.canr.msu.edu/pork/uploads/files/pq%20vol%201.3.pdf>
- Karlsson-Drangsholt, T. M. (2024). *Delta Norway's central role in breeding*. Topigs Norsvin. Retrieved August 6, 2025 from <https://topignorsvin.com/news/delta-norways-central-role-in-breeding/>
- Kasimanickam, V., Kastelic, J., & Kasimanickam, R. (2024). Transcriptomics of bovine sperm and oocytes. *Animal Reproduction Science*, 271, 107630. <https://doi.org/10.1016/j.anireprosci.2024.107630>
- Kasimanickam, V., Kumar, N., & Kasimanickam, R. (2022). Investigation of sperm and seminal plasma candidate microRNAs of bulls with differing fertility and in silico prediction of miRNA-mRNA interaction network of reproductive function. *Animals (Basel)*, 12(18). <https://doi.org/10.3390/ani12182360>
- Kasinathan, P., Wei, H., Xiang, T., Molina, J. A., Metzger, J., Broek, D., Kasinathan, S., Faber, D. C., & Allan, M. F. (2015). Acceleration of genetic gain in cattle by reduction of generation interval. *Scientific Reports*, 5(1), 8674. <https://doi.org/10.1038/srep08674>
- Kayode, O. T., Rotimi, D. E., Kayode, A. A. A., Olaolu, T. D., & Adeyemi, O. S. (2020). Monosodium glutamate (MSG)-induced male reproductive dysfunction: A mini review. *Toxics*, 8(1). <https://doi.org/10.3390/toxics8010007>
- Kim, D., Paggi, J. M., Park, C., Bennett, C., & Salzberg, S. L. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature Biotechnology*, 37(8), 907–915. <https://doi.org/10.1038/s41587-019-0201-4>
- Knecht, D., Jankowska-Mąkosa, A., & Duziński, K. (2017). The effect of age, interval collection and season on selected semen parameters and prediction of AI boars

- productivity. *Livestock Science*, 201, 13–21.
<https://doi.org/10.1016/j.livsci.2017.04.013>
- Knox, R. V. (2016). Artificial insemination in pigs today. *Theriogenology*, 85(1), 83–93.
<https://doi.org/10.1016/j.theriogenology.2015.07.009>
- Knox, R. V. (2024). *Boar Management*. Merck & Co., Inc. Retrieved September 1, 2025 from
<https://www.merckvetmanual.com/management-and-nutrition/management-of-reproduction-pigs/boar-management>
- Kondracki, S., & Górski, K. (2025). Analysis of factors of variation in characteristics of boar ejaculates. *Animals*, 15(14).
- Krueger, F., James, F., Ewels, P., Afyounian, E., Weinstein, M., Schuster-Boeckler, B., Hulselmans, G., Sclamons. (2023). FelixKrueger/TrimGalore: v0.6.10 - add default decompression path. In: Zenodo.
- Kwon, W. S., Park, Y. J., Mohamed el, S. A., & Pang, M. G. (2013). Voltage-dependent anion channels are a key factor of male fertility. *Fertility and Sterility*, 99(2), 354–361.
<https://doi.org/10.1016/j.fertnstert.2012.09.021>
- Lewis, B., & Aitken, R. J. (2001). Impact of epididymal maturation on the tyrosine phosphorylation patterns exhibited by rat spermatozoa. *Biology of Reproduction*, 64(5), 1545–1556. <https://doi.org/10.1095/biolreprod64.5.1545>
- Li, L., & Chen, S. (2021). Screening, identification and interaction analysis of key microRNAs and genes in asthenozoospermia. *International Journal of Medical Sciences*, 18(7), 1670–1679. <https://doi.org/10.7150/ijms.54460>
- Li, T.-K. (1975). The glutathione and thiol content of mammalian spermatozoa and seminal plasma. *Biology of Reproduction*, 12(5), 641–646.
<https://doi.org/10.1095/BIOLREPROD12.5.641>
- Li, Y., Peng, Q., Shang, J., Dong, W., Wu, S., Guo, X., Xie, Z., & Chen, C. (2023). The role of taurine in male reproduction: Physiology, pathology and toxicology. *Frontiers in Endocrinology*, 14, 1017886. <https://doi.org/10.3389/fendo.2023.1017886>
- Lian, C., Sun, B., Niu, S., Yang, R., Liu, B., Lu, C., Meng, J., Qiu, Z., Zhang, L., & Zhao, Z. (2012). A comparative profile of the microRNA transcriptome in immature and mature porcine testes using Solexa deep sequencing. *The FEBS Journal*, 279(6), 964–975.
<https://doi.org/10.1111/j.1742-4658.2012.08480.x>
- Liao, Y., Smyth, G. K., & Shi, W. (2013). featureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*, 30(7), 923–930.
<https://doi.org/10.1093/bioinformatics/btt656>

- Lopez, B. M., Kang, H. S., Kim, T. H., Viterbo, V. S., Kim, H. S., Na, C. S., & Seo, K. S. (2016). Optimization of swine breeding programs using genomic selection with ZPLAN+. *Asian-Australasian Journal of Animal Sciences*, 29(5), 640–645. <https://doi.org/10.5713/ajas.15.0842>
- Lopez Rodriguez, A., Van Soom, A., Arsenakis, I., & Maes, D. (2017). Boar management and semen handling factors affect the quality of boar extended semen. *Porcine Health Management*, 3(1), 15. <https://doi.org/10.1186/s40813-017-0062-5>
- Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with *DESeq2*. *Genome Biology*, 15(12), 550. <https://doi.org/10.1186/s13059-014-0550-8>
- Luo, S. W., Tang, L., Zhou, D., Bo, H., & Fan, L. Q. (2023). FOXP4 promotes proliferation of human spermatogonial stem cells. *Asian Journal of Andrology*, 25(3), 322–330. <https://doi.org/10.4103/aja202254>
- Luo, Z., Liu, Y., Chen, L., Ellis, M., Li, M., Wang, J., Zhang, Y., Fu, P., Wang, K., Li, X., & Wang, L. (2015). microRNA profiling in three main stages during porcine spermatogenesis. *Journal of Assisted Reproduction and Genetics*, 32(3), 451–460. <https://doi.org/10.1007/s10815-014-0406-x>
- Ma, J., Chen, Q., Wang, S., Ma, R., Jing, J., Yang, Y., Feng, Y., Zou, Z., Zhang, Y., Ge, X., Xue, T., Liang, K., Cao, S., Wang, D., Chen, L., & Yao, B. (2020). Mitochondria-related miR-574 reduces sperm ATP by targeting ND5 in aging males. *Aging*, 12(9), 8321–8338. <https://doi.org/10.18632/aging.103141>
- Ma, T., Zhou, S., Xie, X., Chen, J., Wang, J., & Zhang, G. (2024). A case report of a family with developmental arrest of human prokaryotic stage zygote. *Frontiers in Cell and Developmental Biology*, 12, 1280797. <https://doi.org/10.3389/fcell.2024.1280797>
- Macchia, G., Topo, E., Mangano, N., D’Aniello, E., & Boni, R. (2010). DL-aspartic acid administration improves semen quality in rabbit bucks. *Animal Reproduction Science*, 118(2), 337–343. <https://doi.org/10.1016/j.anireprosci.2009.07.009>
- Madison-Villar, M. J., & Michalak, P. (2011). Misexpression of testicular microRNA in sterile *Xenopus* hybrids points to tetrapod-specific microRNAs associated with male fertility. *Journal of Molecular Evolution*, 73(5-6), 316–324. <https://doi.org/10.1007/s00239-011-9478-8>
- Mahfouz, R. Z., Said, T. M., & Agarwal, A. (2009). The diagnostic and therapeutic applications of flow cytometry in male infertility. *Archives of Medical Science Special Issues*, S99–S108.

- Martinez, C. A., Roca, J., Alvarez-Rodriguez, M., & Rodriguez-Martinez, H. (2022). miRNA-profiling in ejaculated and epididymal pig spermatozoa and their relation to fertility after artificial insemination. *Biology*, 11(2). <https://doi.org/10.3390/biology11020236>
- Maside, C., Recuero, S., Salas-Huetos, A., Ribas-Maynou, J., & Yeste, M. (2023). Animal board invited review: An update on the methods for semen quality evaluation in swine - from farm to the lab. *Animal*, 17(3), 100720. <https://doi.org/10.1016/j.animal.2023.100720>
- Massucci, F. A., DiNuzzo, M., Giove, F., Maraviglia, B., Castillo, I. P., Marinari, E., & Martino, A. D. (2013). Energy metabolism and glutamate-glutamine cycle in the brain: a stoichiometric modeling perspective. *BMC Systems Biology*, 7(1), 103. <https://doi.org/10.1186/1752-0509-7-103>
- Mateo-Otero, Y., Fernández-López, P., Delgado-Bermúdez, A., Nolis, P., Roca, J., Miró, J., Barranco, I., & Yeste, M. (2021). Metabolomic fingerprinting of pig seminal plasma identifies in vivo fertility biomarkers. *Journal of Animal Science and Biotechnology*, 12(1), 113. <https://doi.org/10.1186/s40104-021-00636-5>
- Mateo-Otero, Y., Fernández-López, P., Ribas-Maynou, J., Roca, J., Miró, J., Yeste, M., & Barranco, I. (2021). Metabolite profiling of pig seminal plasma identifies potential biomarkers for sperm resilience to liquid preservation. *Frontiers in Cell and Developmental Biology*, 9, 669974. <https://doi.org/10.3389/fcell.2021.669974>
- Matsugo, S., & Nakamura, Y. (2023). Pyrrole-2-carboxaldehydes: Origins and physiological activities. *Molecules*, 28(6). <https://doi.org/10.3390/molecules28062599>
- McGillcuddy, M., Popovic, G., Bolker, B. M., & Warton, D. I. (2025). Parsimoniously fitting large multivariate random effects in *glmmTMB*. *Journal of Statistical Software*, 112(1), 1–19. <https://doi.org/10.18637/jss.v112.i01>
- McGilvray, P. T., Anghel, S. A., Sundaram, A., Zhong, F., Trnka, M. J., Fuller, J. R., Hu, H., Burlingame, A. L., & Keenan, R. J. (2020). An ER translocon for multi-pass membrane protein biogenesis. *eLife*, 9. <https://doi.org/10.7554/eLife.56889>
- Menezes, E. B., Velho, A. L. C., Santos, F., Dinh, T., Kaya, A., Topper, E., Moura, A. A., & Memili, E. (2019). Uncovering sperm metabolome to discover biomarkers for bull fertility. *BMC Genomics*, 20(1), 714. <https://doi.org/10.1186/s12864-019-6074-6>
- Meuwissen, T., Hayes, B., & Goddard, M. (2016). Genomic selection: A paradigm shift in animal breeding. *Animal Frontiers*, 6(1), 6–14. <https://doi.org/10.2527/af.2016-0002>

- Momeni, H. R., Etemadi, T., Alyasin, A., & Eskandari, N. (2021). A novel role for involvement of N-methyl-D-aspartate (NMDA) glutamate receptors in sperm acrosome reaction. *Andrologia*, 53(10), e14203. <https://doi.org/10.1111/and.14203>
- Mortimer, D., Curtis, E. F., & Miller, R. G. (1987). Specific labelling by peanut agglutinin of the outer acrosomal membrane of the human spermatozoon. *Journal of Reproduction and Fertility*, 81(1), 127–135. <https://doi.org/10.1530/jrf.0.0810127>
- Mottais, D. (2022). *Boar performance: How does it change over the course of the boar's life?* GENEPRO. <https://www.boars-and-semen.com/articles/boar-performance-over-his-life/>
- Myromslien, F. D., Tremoen, N. H., Andersen-Ranberg, I., Fransplass, R., Stenseth, E. B., Zeremichael, T. T., van Son, M., Grindflek, E., & Gaustad, A. H. (2019). Sperm DNA integrity in Landrace and Duroc boar semen and its relationship to litter size. *Reproduction in Domestic Animals*, 54(2), 160–166. <https://doi.org/10.1111/rda.13322>
- Nagata, Y., Homma, H., Lee, J. A., & Imai, K. (1999). D-aspartate stimulation of testosterone synthesis in rat Leydig cells. *FEBS Letters*, 444(2-3), 160–164. [https://doi.org/10.1016/s0014-5793\(99\)00045-9](https://doi.org/10.1016/s0014-5793(99)00045-9)
- Narud, B., Khezri, A., Nordborg, A., Klinkenberg, G., Zeremichael, T. T., Stenseth, E.-B., Heringstad, B., Kommisrud, E., & Myromslien, F. D. (2022). Semen quality parameters including metabolites, sperm production traits and fertility in young Norwegian Red AI bulls. *Livestock Science*, 255. <https://doi.org/10.1016/j.livsci.2021.104803>
- Narud, B., Klinkenberg, G., Khezri, A., Zeremichael, T. T., Stenseth, E.-B., Nordborg, A., Haukaas, T. H., Morrell, J., Heringstad, B., Myromslien, F. D., & Kommisrud, E. (2020). Differences in sperm functionality and intracellular metabolites in Norwegian Red bulls of contrasting fertility. *Theriogenology*, 157, 24–32. <https://doi.org/10.1016/j.theriogenology.2020.07.005>
- National Swine Registry. (2023). *Duroc The world's terminal sire*. National Swine Registry. <https://nationalswine.com/about/breeds/about-duroc.php>
- Neeteson-van Nieuwenhoven, A.-M., Knap, P., & Avendaño, S. (2013). The role of sustainable commercial pig and poultry breeding for food security. *Animal Frontiers*, 3(1), 52–57. <https://doi.org/10.2527/af.2013-0008>
- Nolan, J. P., & Hammerstedt, R. H. (1997). Regulation of membrane stability and the acrosome reaction in mammalian sperm. *The FASEB Journal*, 11(8), 670–682. <https://doi.org/10.1096/fasebj.11.8.9240968>

- Ostersen, T. (2020). *10 years of genomic selection: A quantum leap for pig breeding*. DANBRED. Retrieved July 22 from <https://danbred.com/10-years-genomic-selection/>
- Pang, W.-K., Kang, S., Ryu, D.-Y., Rahman, M. S., Park, Y.-J., & Pang, M.-G. (2020). Optimization of sperm RNA processing for developmental research. *Scientific Reports*, 10(1), 11606. <https://doi.org/10.1038/s41598-020-68486-1>
- Pang, Z., Lu, Y., Zhou, G., Hui, F., Xu, L., Viau, C., Spigelman, Aliya F., MacDonald, Patrick E., Wishart, David S., Li, S., & Xia, J. (2024). MetaboAnalyst 6.0: Towards a unified platform for metabolomics data processing, analysis and interpretation. *Nucleic Acids Research*, 52(W1), W398–W406. <https://doi.org/10.1093/nar/gkae253>
- Pardon, B., De Bleecker, K., Hostens, M., Callens, J., Dewulf, J., & Deprez, P. (2012). Longitudinal study on morbidity and mortality in white veal calves in Belgium. *BMC Veterinary Research*, 8(1), 26. <https://doi.org/10.1186/1746-6148-8-26>
- Partyka, A., Rodak, O., Bajzert, J., Kochan, J., & Niżański, W. (2017). The effect of L-carnitine, hypotaurine, and taurine supplementation on the quality of cryopreserved chicken semen. *BioMed Research International*, 2017(1), 7279341. <https://doi.org/10.1155/2017/7279341>
- Patel, K., Giese, A. P., Grossheim, J. M., Hegde, R. S., Delio, M., Samanich, J., Riazuddin, S., Frolenkov, G. I., Cai, J., Ahmed, Z. M., & Morrow, B. E. (2015). A novel C-terminal CIB2 (Calcium and Integrin Binding Protein 2) mutation associated with non-syndromic hearing loss in a Hispanic family. *PLOS ONE*, 10(10), e0133082. <https://doi.org/10.1371/journal.pone.0133082>
- Peña, F. J. (2015). Multiparametric flow cytometry: A relevant tool for sperm function evaluation. *Animal Reproduction*, 12(3), 351–355.
- Petrunkina, A. M., Waberski, D., Bollwein, H., & Sieme, H. (2010). Identifying non-sperm particles during flow cytometric physiological assessment: a simple approach. *Theriogenology*, 73(7), 995–1000. <https://doi.org/10.1016/j.theriogenology.2009.12.006>
- Prakash, M. A., Kumaresan, A., Ebenezer Samuel King, J. P., Nag, P., Sharma, A., Sinha, M. K., Kamaraj, E., & Datta, T. K. (2021). Comparative transcriptomic analysis of spermatozoa from high- and low-fertile crossbred bulls: Implications for fertility prediction. *Frontiers in Cell and Developmental Biology*, 9.
- QIAGEN. (2021). *Qiazol Handbook*. QIAGEN.
- Quintero-Moreno, A., Rigau, T., & Rodríguez-Gil, J. E. (2007). Multivariate cluster analysis regression procedures as tools to identify motile sperm subpopulations in rabbit semen

- and to predict semen fertility and litter size. *Reproduction in Domestic Animals*, 42(3), 312–319. <https://doi.org/10.1111/j.1439-0531.2006.00785.x>
- R Core Team. (2022). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. <https://www.R-project.org/>
- Reinton, N., Collas, P., Haugen, T. B., Skålhegg, B. S., Hansson, V., Jahnsen, T., & Taskén, K. (2000). Localization of a novel human A-kinase-anchoring protein, hAKAP220, during spermatogenesis. *Developmental Biology*, 223(1), 194–204. <https://doi.org/10.1006/dbio.2000.9725>
- Rey, R. A. (2021). The role of androgen signaling in male sexual development at puberty. *Endocrinology*, 162(2). <https://doi.org/10.1210/endo/bqaa215>
- Ribas-Maynou, J., Garcia-Bonavila, E., Hidalgo, C. O., Catalán, J., Miró, J., & Yeste, M. (2021). Species-specific differences in sperm chromatin decondensation between eutherian mammals underlie distinct lysis requirements. *Frontiers in Cell and Developmental Biology*, 9. <https://doi.org/10.3389/fcell.2021.669182>
- Riesenbeck, A. (2011). Review on international trade with boar semen. *Reproduction in Domestic Animals*, 46(s2), 1–3. <https://doi.org/10.1111/j.1439-0531.2011.01869.x>
- Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., & Smyth, G. K. (2015). *limma* powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research*, 43(7), e47–e47. <https://doi.org/10.1093/nar/gkv007>
- Roberts, L. D., Souza, A. L., Gerszten, R. E., & Clish, C. B. (2012). Targeted metabolomics. *Current Protocols in Molecular Biology*, 98(1), 30.32.1–30.32.24. <https://doi.org/10.1002/0471142727.mb3002s98>
- Roca, J., Parrilla, I., Bolarin, A., Martinez, E. A., & Rodriguez-Martinez, H. (2016). Will AI in pigs become more efficient? *Theriogenology*, 86(1), 187–193. <https://doi.org/10.1016/j.theriogenology.2015.11.026>
- Rodriguez-Martinez, H., Martinez-Serrano, C. A., Alvarez-Rodriguez, M., Martinez, E. A., & Roca, J. (2024). Reproductive physiology of the boar: What defines the potential fertility of an ejaculate? *Animal Reproduction Science*, 269, 107476. <https://doi.org/10.1016/j.anireprosci.2024.107476>
- Roszkowski, M., & Mansuy, I. M. (2021). High efficiency RNA extraction from sperm cells using guanidinium thiocyanate supplemented with tris(2-carboxyethyl)phosphine. *Frontiers in Cell and Developmental Biology*, 9, 648274. <https://doi.org/10.3389/fcell.2021.648274>

- Sa, P., Gòdia, M., Lewis, N., Lian, Y., & Clop, A. (2024). Genomic, transcriptomic and epigenomic analysis towards the understanding of porcine semen quality traits. Past, current and future trends. *Animal Reproduction Science*, 269, 107543. <https://doi.org/10.1016/j.anireprosci.2024.107543>
- Samorè, A. B., & Fontanesi, L. (2016). Genomic selection in pigs: State of the art and perspectives. *Italian Journal of Animal Science*, 15(2), 211–232. <https://doi.org/10.1080/1828051X.2016.1172034>
- Sang, Y., Liu, J., Li, X., Zhou, G., Zhang, Y., Gao, L., Zhao, Y., & Zhou, X. (2021). The effect of SiNPs on DNA methylation of genome in mouse spermatocytes. *Environmental Science and Pollution Research*, 28(32), 43684–43697. <https://doi.org/10.1007/s11356-021-13459-8>
- Sangeeta, S., Arangasamy, A., Kulkarni, S., & Selvaraju, S. (2015). Role of amino acids as additives on sperm motility, plasma membrane integrity and lipid peroxidation levels at pre-freeze and post-thawed ram semen. *Animal Reproduction Science*, 161, 82–88.
- Schulze, M., Buder, S., Rüdiger, K., Beyerbach, M., & Waberski, D. (2014). Influences on semen traits used for selection of young AI boars. *Animal Reproduction Science*, 148(3), 164–170. <https://doi.org/10.1016/j.anireprosci.2014.06.008>
- Schurch, N. J., Schofield, P., Gierliński, M., Cole, C., Sherstnev, A., Singh, V., Wrobel, N., Gharbi, K., Simpson, G. G., Owen-Hughes, T., Blaxter, M., & Barton, G. J. (2016). How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use? *RNA*, 22(6), 839–851. <https://doi.org/10.1261/rna.053959.115>
- Selvaraju, S., Parthipan, S., Somashekar, L., Binsila, B. K., Kolte, A. P., Arangasamy, A., Ravindra, J. P., & Krawetz, S. A. (2018). Current status of sperm functional genomics and its diagnostic potential of fertility in bovine (*Bos taurus*). *Systems Biology in Reproductive Medicine*, 64(6), 484–501. <https://doi.org/10.1080/19396368.2018.1444816>
- Sendler, E., Johnson, G. D., Mao, S., Goodrich, R. J., Diamond, M. P., Hauser, R., & Krawetz, S. A. (2013). Stability, delivery and functions of human sperm RNAs at fertilization. *Nucleic Acids Research*, 41(7), 4104–4117. <https://doi.org/10.1093/nar/gkt132>
- Sisakhtnezhad, S., & Heshmati, P. (2018). Comparative analysis of single-cell RNA sequencing data from mouse spermatogonial and mesenchymal stem cells to identify differentially expressed genes and transcriptional regulators of germline cells. *Journal of Cellular Physiology*, 233(7), 5231–5242. <https://doi.org/10.1002/jcp.26303>

- Smith, L., Villaret-Cazadamont, J., Claus, S. P., Canlet, C., Guillou, H., Cabaton, N. J., & Ellero-Simatos, S. (2020). Important considerations for sample collection in metabolomics studies with a special focus on applications to liver functions. *Metabolites*, 10(3). <https://doi.org/10.3390/metabo10030104>
- Song, C., Chang, L., Wang, B., Zhang, Z., Wei, Y., Dou, Y., Qi, K., Yang, F., Li, X., Li, X., Wang, K., Qiao, R., & Han, X. (2023). Seminal plasma metabolomics analysis of differences in liquid preservation ability of boar sperm. *Journal of Animal Science*, 101. <https://doi.org/10.1093/jas/skad392>
- Stenberg, H., Malmberg, M., & Hayer, J. (2023). Screening for atypical porcine pestivirus in Swedish boar semen used for artificial insemination and a characterisation of the seminal RNA microbiome including the virome. *BMC Veterinary Research*, 19(1), 219. <https://doi.org/10.1186/s12917-023-03762-6>
- Sullivan, L. M. (2008). Repeated measures. *Circulation*, 177, 1238–1243.
- Sutkeviciene, N., Riskeviciene, V., Januskauskas, A., Zilinskas, H., & Andersson, M. (2009). Assessment of sperm quality traits in relation to fertility in boar semen. *Acta Veterinaria Scandinavica*, 51(1), 53. <https://doi.org/10.1186/1751-0147-51-53>
- Tai, A., Sawano, T., & Ito, H. (2012). Antioxidative properties of vanillic acid esters in multiple antioxidant assays. *Bioscience, Biotechnology, and Biochemistry*, 76(2), 314–318. <https://doi.org/10.1271/bbb.110700>
- Tampion, D., & Gibbons, R. A. (1963). Swimming rate of bull spermatozoa in various media and the effect of dilution. *Journal of Reproduction and Fertility*, 5, 259–275. <https://doi.org/10.1530/jrf.0.0050259>
- Tang, D., Huang, Y., Liu, W., & Zhang, X. (2016). Up-regulation of microRNA-210 is associated with spermatogenesis by targeting IGF2 in male infertility. *Medical Science Monitor*, 22, 2905–2910. <https://doi.org/10.12659/MSM.897340>
- Tang, X., Chen, Y., Luo, H., Bian, Q., Weng, B., Yang, A., Chu, D., Ran, M., & Chen, B. (2021). miR-126 controls the apoptosis and proliferation of immature porcine Sertoli cells by targeting the PIK3R2 gene through the PI3K/AKT signaling pathway. *Animals*, 11(8). <https://doi.org/10.3390/ani11082260>
- Tanga, B. M., Qamar, A. Y., Raza, S., Bang, S., Fang, X., Yoon, K., & Cho, J. (2021). Semen evaluation: Methodological advancements in sperm quality-specific fertility assessment — A review. *Animal Bioscience*, 34(8), 1253–1270. <https://doi.org/10.5713/ab.21.0072>
- Tauson, A. H. (2020). Reproduction. In *Lærebog i fysiologi*. Landbrug & Fødevarer Sektor for Gris.

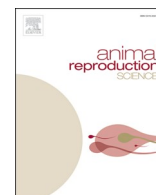
- Teng, Y. N., Chung, C. L., Lin, Y. M., Pan, H. A., Liao, R. W., & Kuo, P. L. (2007). Expression of various CDC25B isoforms in human spermatozoa. *Fertility and Sterility*, 88(2), 379–382. <https://doi.org/10.1016/j.fertnstert.2006.11.186>
- Topigs Norsvin. (2025). *More than 100 years of progress*. Topigs Norsvin. Retrieved July 23, 2025 from <https://topignorsvin.us/about/more-than-100-years-of-progress/>
- Tremoen, N. H., Gaustad, A. H., Andersen-Ranberg, I., van Son, M., Zeremichael, T. T., Frydenlund, K., Grindflek, E., Våge, D. I., & Myromslien, F. D. (2018). Relationship between sperm motility characteristics and ATP concentrations, and association with fertility in two different pig breeds. *Animal Reproduction Science*, 193, 226–234. <https://doi.org/10.1016/j.anireprosci.2018.04.075>
- Tsakmakidis, I. A., Khalifa, T. A., & Boscós, C. M. (2012). Age-related changes in quality and fertility of porcine semen. *Biological Research*, 45(4), 381–386. <https://doi.org/10.4067/s0716-97602012000400009>
- Tvrđá, E., Bučko, O., Ďuračka, M., Kováčik, A., Benko, F., & Kačániová, M. (2025). Age-related dynamics in the conventional, non-conventional, and bacteriological characteristics of fresh and liquid-stored porcine semen. *Animals*, 15(3). <https://doi.org/10.3390/ani15030377>
- Ureña, I., González, C., Ramón, M., Gòdia, M., Clop, A., Calvo, J. H., Carabaño, M. J., & Serrano, M. (2022). Exploring the ovine sperm transcriptome by RNAseq techniques. I Effect of seasonal conditions on transcripts abundance. *PLOS ONE*, 17(3), e0264978. <https://doi.org/10.1371/journal.pone.0264978>
- USDA. (2025). *Hogs & Pork - Sector at a Glance*. United States Department of Agriculture. Economic Research, Service. Retrieved July 22, 2025 from <https://www.ers.usda.gov/topics/animal-products/hogs-pork/sector-at-a-glance/>
- Vangen, O., & Sehested, E. (1997). Swine production and research in Norway. *Pig News and Information*, 18, 29N–34N.
- Vedelek, V., Vedelek, B., Lőrincz, P., Juhász, G., & Sinka, R. (2023). A comparative analysis of fruit fly and human glutamate dehydrogenases in *Drosophila melanogaster* sperm development. *Frontiers in Cell and Developmental Biology*, 11. <https://doi.org/10.3389/fcell.2023.1281487>
- Velho, A. L. C., Menezes, E., Dinh, T., Kaya, A., Topper, E., Moura, A. A., & Memili, E. (2018). Metabolomic markers of fertility in bull seminal plasma. *PLOS ONE*, 13(4), e0195279. <https://doi.org/10.1371/journal.pone.0195279>

- Waberski, D., Suarez, S. S., & Henning, H. (2022). Assessment of sperm motility in livestock: Perspectives based on sperm swimming conditions in vivo. *Animal Reproduction Science*, 246, 106849. <https://doi.org/10.1016/j.anireprosci.2021.106849>
- Wu, G. (2009). Amino acids: Metabolism, functions, and nutrition. *Amino Acids*, 37(1), 1–17. <https://doi.org/10.1007/s00726-009-0269-0>
- Wuensch, U., Nitter, G., Bergfeld, U., & Schueler, L. (2000). Genetic and economic evaluation of genetic improvement schemes in pigs. II. Comparison of selection strategies in a three-way crossbreeding scheme. *Archives Animal Breeding*, 43(2), 139–150. <https://doi.org/10.5194/aab-43-139-2000>
- Xu, C., Yang, X., Sui, H., Tong, X., Zhang, D., Zheng, X., Jiao, J., Wang, C., Cao, Z., & Zhang, Y. (2024). Effects of different ages on frozen semen quality and in vitro fertilization efficiency in Wannan Black pigs. *Frontiers in Veterinary Science*, 11. <https://doi.org/10.3389/fvets.2024.1395718>
- Xu, Z., Miyata, H., Kaneda, Y., Castaneda, J. M., Lu, Y., Morohoshi, A., Yu, Z., Matzuk, M. M., & Ikawa, M. (2020). CIB4 is essential for the haploid phase of spermatogenesis in mice. *Biology of Reproduction*, 103(2), 235–243. <https://doi.org/10.1093/biolre/ioaa059>
- Yang, C. C., Lin, Y. S., Hsu, C. C., Wu, S. C., Lin, E. C., & Cheng, W. T. K. (2009). Identification and sequencing of remnant messenger RNAs found in domestic swine (*Sus scrofa*) fresh ejaculated spermatozoa. *Animal Reproduction Science*, 113(1), 143–155. <https://doi.org/10.1016/j.anireprosci.2008.08.012>
- Yaxiao, H., Ran, Z., Rongyan, Z., Zhenhong, Z., Yinliang, Z., Shulong, Y., & Lanhui, L. (2025). Identification of biomarkers associated with sexual maturity in laying hens based on metabolomics and gut microbiology. *Poultry Science*, 104(9), 105469. <https://doi.org/10.1016/j.psj.2025.105469>
- Yien, Y. Y., & Perfetto, M. (2022). Regulation of heme synthesis by mitochondrial homeostasis proteins. *Frontiers in Cell and Developmental Biology*, 10, 895521. <https://doi.org/10.3389/fcell.2022.895521>
- Zhang, B., Yan, Z., Wang, P., Yang, Q., Huang, X., Shi, H., Tang, Y., Ji, Y., Zhang, J., & Gun, S. (2021). Identification and characterization of lncRNA and mRNA in testes of Landrace and Hezuo boars. *Animals*, 11(8). <https://doi.org/10.3390/ani11082263>
- Zhao, J., Pan, H., Zhao, W., Li, W., Li, H., Tian, Z., Meng, D., Teng, Y., Li, X., He, Y., Shi, H., Ge, C., & Wang, K. (2023). Untargeted metabolomics revealed potential biomarkers of small yellow follicles of chickens during sexual maturation. *Metabolites*, 13(2), 176.

- Zhao, Y., Li, Q., Yao, C., Wang, Z., Zhou, Y., Wang, Y., Liu, L., Wang, Y., Wang, L., & Qiao, Z. (2006). Characterization and quantification of mRNA transcripts in ejaculated spermatozoa of fertile men by serial analysis of gene expression. *Human Reproduction*, 21(6), 1583–1590. <https://doi.org/10.1093/humrep/del027>
- Zheng, Y., Gao, Q., Li, T., Liu, R., Cheng, Z., Guo, M., Xiao, J., Wu, D., & Zeng, W. (2022). Sertoli cell and spermatogonial development in pigs. *Journal of Animal Science and Biotechnology*, 13(1), 45. <https://doi.org/10.1186/s40104-022-00687-2>

Dissertation articles

1



A metabolomic study uncovering key amino acids and amines in Duroc boar semen as biomarkers of sexual maturity

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ABSTRACT

Metabolomic analysis of boar semen associated with sexual maturation is essential for improving fertility management and breeding, with amino acids and amines playing key roles in the reproductive process. This study aimed to explore changes in amino acids and amines in boar spermatozoa and seminal plasma during puberty to sexual maturity and identify potential biomarkers of sexual maturity. Semen was collected from the same 15 Duroc boars over time at approximately 7 months (Age 1), 8.5 months (Age 2), and 10 months (Age 3). Liquid chromatography-mass spectrometry was used to analyse amino acids and amines in spermatozoa and seminal plasma separately. Multivariate analysis (PLS-DA) revealed pronounced age-dependent changes in amino acids and amines in spermatozoa between Age 1 and Age 3, and more subtle shifts in seminal plasma. Univariate analysis (Repeated measure ANOVA/Friedman) revealed that glutamate and taurine had significant pairwise differences in seminal plasma ($P < 0.05$). In sperm, 15 amino acids (glutamate, alanine, aspartate, choline, taurine, histidine, methionine, tryptophan, leucine, cystine, tyrosine, arginine, lysine, valine and glycine) exhibited significant pairwise differences ($P < 0.05$). VIP scoring (>1.5) prioritised glutamate, alanine, aspartate, and choline as key contributors to the variations and pathway analysis implicated alanine, aspartate and glutamate metabolism, and histidine metabolism linked to sexual maturity. Our study highlights metabolic changes during sexual maturation, identifying potential biomarkers for assessing reproductive maturity. These findings are initial steps toward optimising younger boars' usage in breeding, enhancing genetic gain, and reducing costs associated with their non-productive days at AI centres.

1. Introduction

Boar fertility is a critical factor in the swine industry, as it significantly impacts pork production's productivity, efficiency, and profitability. Age is one of the key factors that influence the reproductive performance of a boar, where sexual maturity marks an important milestone in their reproductive development (Sonderman and Luebke, 2008). Domestic boars begin puberty around 5 months of age (Caires et al., 2008); however, they typically don't reach full sexual maturity until about 7 months, with fertility

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generally improving as boars age from about 9 to 20 months (Singleton and Flowers, 2006). Specifically, Duroc boars reach sexual maturity with a large number of spermatocytes in the seminiferous tubules at around 9 months of age (Ding et al., 2016). They are used in breeding as semen donors from around the same age, i.e., 9 months following appropriate training and qualification (Huang et al., 2010). Important changes occur in the reproductive system in boars from puberty to sexual maturity, with an increase in sperm production, ejaculate volume, sperm motility, testosterone, and testicular size (Bonet et al., 2013; Maistrelli et al., 2022; Shostya et al., 2023).

Semen comprises spermatozoa and seminal plasma, the latter consisting of fluids secreted by the rete testis, epididymis, and accessory glands (Kasimanickam et al., 2019). Particularly in boars, the vesicular gland and bulbourethral glands contribute largely to seminal plasma volume (Buckman et al., 2009). Seminal plasma plays a major role in sperm survival, transport in the female reproductive tract, capacitation, establishment of the oviductal sperm reservoir, modulation of female immune response, and fertilisation (Rodríguez-Martínez et al., 2011). The chemical composition of seminal plasma is distinct from other body fluids, characterised by high concentrations of free amino acids, especially glutamic acid, and small peptides (Mann and Lutwak-Mann, 2012) in addition to ions, carbohydrates, organic compounds, nitrogenous compounds, reducing substances, and reactive oxygen species scavenging enzymes (Chabory et al., 2010; Mann and Lutwak-Mann, 2012). The presence of free amino acids in seminal plasma is reported to be crucial for sperm protection and progression in the female genital tract in humans (Silvestroni et al., 1979).

Metabolomics, the comprehensive analysis of molecular metabolites, has emerged as a powerful tool for investigating the metabolites from biological samples and their alterations, elucidating pathways associated with physiological processes (Ashokan et al., 2023). In recent years, metabolomics has been increasingly applied to study the metabolites in sperm and seminal plasma concerning male fertility in various species, including bulls (Velho et al., 2018; Menezes et al., 2019; Memili et al., 2020; Narud et al., 2020), humans (Blaurock et al., 2022) and boars (Mateo-Otero et al., 2021a; Zhang et al., 2021; Mateo-Otero et al., 2023). These studies have provided valuable insights into the metabolic processes underlying male fertility and identified potential fertility biomarkers in seminal plasma and sperm. Similarly, the metabolomics approach was employed to study age-related changes in different cell types for bulls (Argov-Argaman et al., 2013) and pigs (Rabassa et al., 2013). Argov-Argaman et al. (2013) reported that Holstein Friesian mature bulls exhibited lower proportions of polyunsaturated fatty acids, omega-3 fatty acids, and docosahexaenoic acid in intact sperm, seminal fluid, and sperm head as compared to younger bulls (Argov-Argaman et al., 2013). Rabassa et al. (2013) tracked metabolic parameters in blood from male pigs from three classes: pre-puberty (1–5 months), puberty (6–9 months), and post-puberty (10–12 months) finding glucose levels to decrease with increasing age, while non-esterified fatty acid levels were highest at pre-pubertal stage (Rabassa et al., 2013). While metabolomics research has provided valuable information into male reproductive metabolites across species, we lack a clear picture of how these metabolites evolve in boar spermatozoa and seminal plasma as they progress from puberty to sexual maturity. This gap is critical to address, as young boars are increasingly used in artificial insemination (AI) programs before full sexual maturity and could contribute towards refining breeding strategies in using young boars for AI.

Amino acids and amines are integral to numerous biological functions, including energy metabolism, cell signalling, oxidative processes, precursors for hormones, regulation of gene expression and metabolic pathways (Wu, 2009). In male reproduction, they are linked to a range of functions from sperm motility and the acrosome reaction to modulation of antioxidant defences. For instance, tyrosine is involved in the epididymal sperm maturation through interaction with cyclic adenosine monophosphate (cAMP) elevation (Lewis and Aitken, 2001), while taurine contributes to the protection of spermatozoa through antioxidant defence (Li et al., 2023). Similarly, choline has been reported to be essential for sperm membrane and motility (Hanley, 2023), and glutamate helps in the regulation of sperm chemotaxis, acrosome reaction, and mitochondrial homeostasis through interactions with N-methyl-D-aspartate (NMDA) receptors (Endo et al., 2019; Vedelek et al., 2023). Moreover, alterations in the level of amino acid derivatives have been associated with male fertility. For example, levels of 17 amino acid derivatives showed significant differences between teratozoospermic and fertile semen in humans (Hosseini et al., 2022). Arginine and ornithine were found to be negatively associated with various types of pathospermia (abnormal semen), and their concentrations in seminal plasma can influence fertility outcomes (Papp et al., 1983). For bull semen, sperm concentration of cysteine and glutamic acid was reported to be linked to higher pregnancy success, while aspartic acid, leucine and serine were associated with lower pregnancy success (Narud et al., 2020). It is reported that phenylalanine in the seminal plasma of bulls was correlated with post-thaw viability of sperm (Ugur et al., 2020). Likewise, phenylalanine in seminal plasma of stallions showed a significant decrease in concentration with decreasing motility and complete absence in azoospermia, while carnitine and choline showed a significant decrease in concentrations in azoospermic stallions, and alanine and carnitine showed significant positive correlation with sperm concentration in this species (Castiglione Morelli et al., 2021). Furthermore, Mateo-Otero et al. (2021b) found that glutamate, carnitine, and isoleucine are related to sperm quality, while leucine, hypotaurine, carnitine and isoleucine are associated with spermatozoa's ability to tolerate liquid storage (Mateo-Otero et al., 2021b). While these studies highlight the roles of amino acids and amines for overall semen health, their association with fertility in various species, and liquid storage in boars, to our knowledge, it has not yet been studied how amino acids and amines concentration shift during sexual maturity in boar spermatozoa and seminal plasma.

We hypothesise that the metabolome composition of boar semen undergoes dynamic changes during the transition from puberty to full sexual maturity, reflecting early physiological adaptations critical to reproductive development. In this study, we, therefore, aimed to examine subtle changes in the concentrations of amino acids and amines in spermatozoa and seminal plasma of Duroc boars during the critical transition window from puberty to sexual maturity. By collecting semen samples from the same boars at around 7 months, 8.5 months and 10 months, and using liquid chromatography-mass spectrometry (LC-MS/MS) analysis in sperm and seminal plasma, we sought to identify potential metabolic biomarkers of sexual maturity. Samples were analysed separately for spermatozoa and seminal plasma. This approach provides additional knowledge to better understand metabolic changes during boar sexual maturation, which pig breeding companies can evaluate and utilise to optimise the use of the younger boars in breeding programmes.

2. Materials and methods

2.1. Ethical approval

Ethical approval was not necessary for this study, since the boars were part of regular semen production and regulated by laws in Norway (The Animal Protection Act of 20 December 1974, the Animal Welfare Act of 19 June 2009 and the Regulations for Management of Pigs in Norway of 18 February 2003), and European Union legislation on semen production for pigs (Council Directive 90/429/EEC).

2.2. Experimental design and animal material

The study followed 15 *Sus scrofa* boars of the Duroc breed over three months (March – June 2023). These boars were from a commercial breeding line used in Norsvin SA, a pig breeding company in Norway. Semen was collected from the same individuals at three distinct time points to capture the critical metabolic transitions associated with sexual maturity, as even a small age difference can be significant during critical developmental windows. These time points were: 7.2 ± 0.4 months (Age 1), 8.6 ± 0.5 months (Age 2, six weeks after Age 1), and 9.9 ± 0.5 months (Age 3, 12 weeks after Age 1). Detailed data is shown in Table 1. During Age 1, boars were quarantined and trained for semen collection in an isolation unit, each housed in 4 m² pens, an arrangement approved by the Norwegian Food Safety Authority for young animals of this size. Semen was collected from Age 1 boars as soon as they were interested in mounting the dummy and semen collection was possible. The same boars were relocated to the Artificial Insemination (AI) centre before semen collection at Age 2. There, each boar was kept in a 6 m² pen. Norsvin distributes semen samples for artificial insemination from Age 2, which they consider ready for use in breeding (personal communication).

Standard housing conditions (ventilation, lighting, temperature) were maintained in both the isolation unit and the AI centre. All animals received a nutritionally balanced commercial diet. The semen collection was done using the gloved-hand technique by the same trained personnel for all boars, with boars briefly led from their pens to a separate collection pen. General husbandry practices were kept consistent across both locations to minimise any potential confounding effects on metabolic parameters. By sampling the same 15 boars across three successive ages, we reduced random inter-individual variability and captured longitudinal changes related to sexual maturation rather than random differences among unrelated animals. The combination of uniform management, repeated sampling, and standardised semen collection methods allowed us to capture true physiological shifts while minimising random or management-related effects.

2.3. Handling of semen samples

The semen samples were transported from the AI station to the laboratory within 30 minutes, packed in a Styrofoam box ensuring a stable temperature. The concentration of spermatozoa in the ejaculates was determined by NucleoCounter® SP-100™ (Chemometec, Allerød, Denmark). For the isolation of spermatozoa, for each raw semen sample, 200 million spermatozoa were centrifuged at $600 \times g$ for 10 minutes at room temperature. The supernatant was carefully removed, and the sperm pellets were then snap-frozen in liquid nitrogen and stored at -80°C until further analysis. To prepare seminal plasma samples, 1 ml of raw semen was initially centrifuged at $600 \times g$ for 10 minutes at room temperature. The supernatant was then transferred to a new Eppendorf tube and centrifuged again at $10,000 \times g$ for 10 minutes at 4°C . The resulting supernatant was then transferred to a new tube and a microscopic examination was

Table 1

Age of boars in months and days across three age groups. The ages “Age 1”, “Age 2” and “Age 3” represent different measurement time points for the same group of boars.

Boar ID	Age 1		Age 2		Age 3	
	Months	Days	Months	Days	Months	Days
B1	6.8	207	8.2	249	9.6	291
B2	7.3	223	8.7	265	10.1	307
B3	7.5	228	8.9	270	10.2	311
B4	6.1	185	7.5	227	8.8	269
B5	7.3	223	8.7	265	10.1	307
B6	7.3	223	8.7	265	10.1	307
B7	7.6	230	9.2	279	10.6	322
B8	6.9	208	8.2	250	9.6	291
B9	7.0	210	8.3	252	9.6	293
B10	6.7	204	8.1	246	9.4	287
B11	7.8	237	9.2	279	10.5	320
B12	7.4	226	8.8	268	10.2	311
B13	7.3	221	8.6	263	10.1	306
B14	7.0	214	8.4	256	9.8	299
B15	7.6	232	9.0	274	10.4	317
Mean	7.2	218	8.6	261	9.9	303

Note: Each boar is assigned a unique identifier from B1 to B15.

conducted to confirm the absence of spermatozoa in the seminal plasma sample. Then the samples were snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

2.4. Metabolite analysis

Targeted metabolomics analysis of amino acids and amines in both seminal plasma and spermatozoa was conducted using Liquid Chromatography-Mass Spectrometry (LC-MS/MS). Sample preparation prior to analysis consisted of protein precipitation and dilution as appropriate. For the sperm pellet, 500 μL phosphate-buffered saline (PBS) was employed for reconstituting the sperm pellets, maintaining the physiological pH and osmotic balance, and a smaller (100 μL) portion of the reconstituted spermatozoa were collected for further sample preparation and analysis. For seminal plasma, 100 μL was used for analysis without prior dilution directly. Protein precipitation was done with methanol (MeOH) by adding 400 μL of cold MeOH to 100 μL of the sperm pellet mixture or seminal plasma. This step was followed by vortexing for 60 seconds and incubation on ice to enhance protein precipitation. The samples were then centrifuged at $14,000 \times g$ for 12 minutes, and the supernatant was collected for analysis and dried by vacuum centrifugation (speed-vac). For amino acids, the samples were mixed with the amino acid internal standard mixture and derivatised using an AccQ-Tag Derivatization Kit (Waters, Milford, MA, United States) prior to analysis. Quantitation was done against standards of 26 amino acids included in the amino acid cell culture standard kit (Catalog No. 186009300, Waters). For amine analysis, the dried samples were redissolved in water containing an internal standard mixture (Choline-D13, Creatine-D3, and L-Carnitine-D3). Quantitation was against mixed amine standards.

The quantitative analysis was performed on an Agilent 1290 Infinity II LC system (Agilent, Santa Clara, USA) coupled to an Agilent 6495 QqQ mass spectrometer, using one method for amino acids and one for amines. The mass spectrometer was equipped with a jet-stream ESI source operated in positive mode and for both methods the instrument was operated in dynamic multiple-reaction monitoring (MRM) mode ($\Delta R_t = 1 \text{ min}$) with a unit mass resolution for both mass filters. For the amino acids, the analysis was done using an Ascentis Express C18 column (150 $\times$ 2.1 mm, 2.7 μm , Agilent, Santa Clara, USA). The mobile phases were composed of 25 mM formic acid and acetonitrile, and eluent flow was 0.3 ml/min. The column thermostat was maintained at 20°C and the autosampler at 6°C . For the amines (choline, creatine, and L-carnitine), the chromatographic separation was performed in HILIC mode using a BEH Amide column (2.1 $\times$ 150 mm, 2.7 μm , Waters, Milford, USA) and gradient elution using an 80:20 mixture of 25 mM Formic acid and 50 mM ammonium acetate as eluent A, and acetonitrile as eluent B, at a flow rate of 0.3 ml/min. The column thermostat was maintained at 35°C and the autosampler at 6°C . Data analysis and quantitation were performed in Agilent MassHunter Quant.

2.5. Statistical analysis

Statistical analysis was performed separately for spermatozoa and seminal plasma. Metabolites with no variance in quantitative amounts across all samples were excluded from the data sets. All statistical analyses were performed using R version 4.3.2 (R Core Team, 2022) and MetaboAnalyst 6.0 (Pang et al., 2024). For multivariate analysis, data was normalised by median, log-transformation and auto-scaling. Partial least squares discriminant analysis (PLS-DA) was conducted using MetaboAnalyst 6.0 (Pang et al., 2024). Variable importance in projection (VIP) scores were calculated to identify metabolites contributing the most to the separation between age groups (Chong et al., 2019). In our study, a VIP score > 1.5 was set as the threshold (Menezes et al., 2019).

Descriptive statistics, including mean, median, standard deviation, minimum, and maximum range, were calculated for each age group (Age 1, Age 2, and Age 3) and for all boars combined to provide a comprehensive overview of the data distribution. Data normality was assessed using the Shapiro-Wilk test and visualised using density plots and Q-Q plots. Log10 transformation was applied to achieve a normal distribution of data. The homogeneity of variance was assessed using Levene's test. For metabolites that met the assumptions of normality and homogeneity, repeated measures ANOVA was performed to assess the effect of age group, considering boar as a random effect. Post-hoc pairwise comparisons were conducted using paired t-tests with Benjamini-Hochberg (BH) correction for multiple comparisons. For metabolites that did not meet normality and homogeneity assumptions, the Friedman test was conducted to assess differences across age groups. Post hoc Wilcoxon signed-rank tests with BH correction were performed for pairwise comparisons. Additionally, Pearson correlation analysis was performed to assess the relationships among metabolite concentrations and between spermatozoa and seminal plasma. Repeated measures correlation analysis was conducted to assess relationships between age and metabolite concentrations. For both sperm and seminal plasma data, *P*-values were adjusted using the Benjamini-Hochberg (BH) method to control the false discovery rate (FDR). Statistical significance was set at $P < 0.05$ after adjustment.

2.6. Pathway analysis

Pathway analysis was performed using MetaboAnalyst 6.0 (Pang et al., 2024), a web-based platform to identify the metabolic pathways in which the quantified metabolites are included and their possible association with the observed age-related changes in boar spermatozoa and seminal plasma. The pathway analysis module in MetaboAnalyst 6.0 integrates results from pathway enrichment analysis and pathway topology analysis. The Sus scrofa (pig) KEGG pathway library (abbreviation: ssc) was selected as the reference metabolome for the analysis. The GlobalTest method was chosen for pathway enrichment analysis, which tests whether any of the metabolites in a pathway are associated with the age groups studied. This method is particularly suitable for analysing high-dimensional metabolomics data with a relatively small sample size (Goeman et al., 2004). For the pathway topology analysis, the "relative betweenness centrality" was selected as the node importance measure. This measure considers a metabolite's centrality and

connectivity within a pathway (Xia and Wishart, 2010), giving a better understanding of its importance. The reference metabolome was set to include all compounds in the selected *Sus scrofa* KEGG pathway library. The pathway analysis results were visualised using pathway impact plots, which display the most significant pathways based on their *P*-values from the enrichment analysis and their pathway impact values from the topology analysis. Pathways with a false discovery rate (FDR) adjusted *P*-value < 0.05 were considered significantly enriched.

3. Results

The metabolic profiling of amino acids and amines revealed distinct clusters between spermatozoa and seminal plasma, indicating significant differences in their metabolic compositions (Fig. 1). Principal Component Analysis (PCA) demonstrated that the first principal component (PC1), which accounted for 40.72 % of the variance, along with the second principal component (PC2) capturing an additional 24.44 %, effectively separated the two sample types.

3.1. Multivariate analysis of metabolite concentrations across age groups

Partial Least Squares Discriminant Analysis (PLS-DA) identified the metabolites contributing the most to the classification into three age groups. For spermatozoa, the PLS-DA model demonstrated a cross-validation accuracy of 0.76, indicating a reliable classification rate. The model achieved an R^2 (goodness of fit) value of 0.75, suggesting that age group differences could explain 75 % of the variance in the targeted metabolite data. The Q^2 value (predictive ability) was 0.52, which reflected moderate predictive relevance. Whereas the PLS-DA model for seminal plasma metabolites showed an accuracy of 0.47, indicating a modest classification rate. The latter indicated that the model was not able to capture the complex relationship between metabolites in seminal plasma and age, indicating there could be a potential overlap in the metabolic profiles among the three different age groups, resulting in the correct classification of less than half of the time. The model R^2 and Q^2 were 0.20 and 0.07 respectively, which further indicated a relatively weak model fit and limited predictive capability. Compared to spermatozoa, seminal plasma showed lower performance metrics, suggesting that age-related differences in metabolite concentrations were either more complex or less pronounced.

The PLS-DA scores plot (Fig. 2) indicated a clear differentiation for sperm between Age 1 and Age 3, while Age 2 displayed more overlap with Age 3 than with Age 1. The VIP scores from the PLS-DA model reflect the importance and contribution of each metabolite for class separation, where a VIP score greater than 1 indicates their significant contribution to the classification of age groups [31]. In spermatozoa, glutamate, alanine, aspartate, and choline were identified as the top contributors in the classification of age groups (Fig. 3). Similarly, VIP scores in seminal plasma revealed that alanine (VIP = 1.5514) and glycine (VIP = 1.5424) were the key contributors.

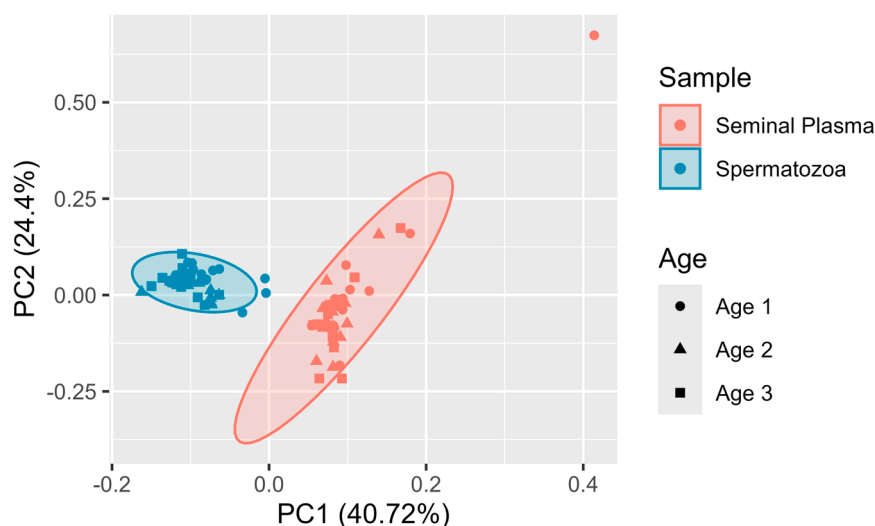


Fig. 1. Principal Component Analysis (PCA) score plot of amino acid and amine concentration in boar spermatozoa and seminal plasma across three ages. Semen samples were collected from 15 Duroc boars at approximately 7 months (Age 1) and again six (Age 2) and twelve (Age 3) weeks later. Spermatozoa were separated from seminal plasma, and metabolite levels were quantified by Liquid Chromatography-Mass Spectrometry (LC-MS). The plot displays the first two principal components (PC1 and PC2), which capture the major variance in metabolite distribution. Each point represents an individual sample, coded by both sample type and age: Seminal Plasma (red) and Spermatozoa (green) indicate sample type, while Age 1 (circle), Age 2 (triangle) and Age 3 (square) denote ages. The ellipses represent 95 % confidence intervals around the mean for each group.

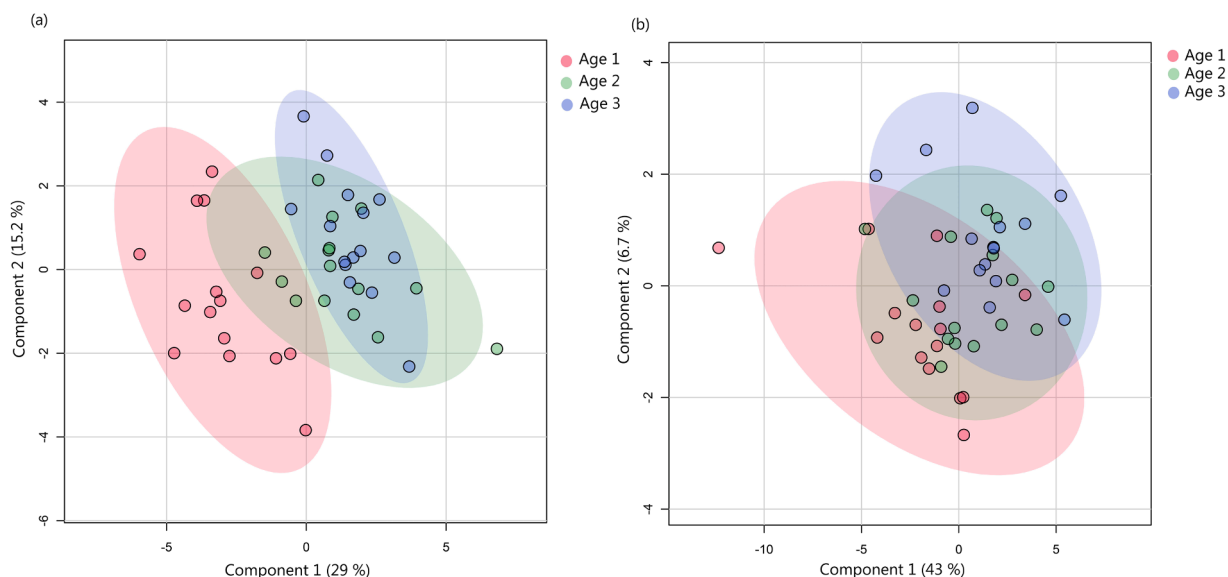


Fig. 2. Partial Least Squares Discriminant Analysis (PLS-DA) score plot of amino acid and amine concentrations in boar spermatozoa (a) and seminal plasma (b) across three ages. Semen samples were collected from 15 Duroc boars at approximately 7 months (Age 1), and again six weeks (Age 2) and twelve weeks (Age 3) later. Spermatozoa were separated from seminal plasma, and metabolite levels were quantified using Liquid Chromatography-Mass Spectrometry (LC-MS). The PLS-DA plot displays component 1 and component 2 showing the distribution of metabolite concentration of samples from boars. Age 1, Age 2, and Age 3 are depicted by red, green, and blue respectively.

3.2. Targeted metabolomic analysis of spermatozoa

The descriptive statistics of all metabolites analysed for spermatozoa are given in [Table S1](#), with concentrations visualised as boxplots in [Figure S1](#). Glutamate (582.67 ± 466.29) showed the highest average concentration while tryptophan (0.38 ± 0.25) exhibited the lowest average concentration of all amino acids analysed. The five metabolites with the highest mean concentration for each age group were glutamate (582.67 ± 466.29), glutamine (355.71 ± 151.11), L-carnitine (211.02 ± 93.24), glycine (152.13 ± 88.57), and creatine (79.82 ± 40.44). Conversely, the five metabolites with the lowest concentrations were tryptophan (0.38 ± 0.25), phenylalanine (0.88 ± 0.26), α -aminobutyric acid (0.93 ± 0.36), cystine (1.07 ± 0.74), and ornithine (3.12 ± 2.36).

Of all the metabolites analysed from spermatozoa, 18 of them, glutamate, histidine, aspartate, alanine, tyrosine, methionine, tryptophan, leucine, choline, taurine, valine, cystine, arginine, lysine, glycine, proline, glutamine and α -aminobutyric acid (AABA) showed overall significant differences across age groups ($P < 0.05$). Among these 18 metabolites, 15 of them except glutamine, AABA and proline demonstrated significance between at least two age groups ([Fig. 4](#), [Table 2](#)). Glutamate, aspartate and taurine concentration decreased significantly with increasing age ($P < 0.001$) and the differences were significant between Age 1- Age 2 and Age 1 - Age 3 ($P < 0.001$) ([Fig. 4](#), [Table 2](#)). Histidine, methionine, tryptophan, leucine, and cystine were also significantly different ($P < 0.05$) between Age 1 and the two older groups ([Fig. 4](#), [Table 2](#)), exhibiting patterns of fluctuation or increase in concentration. The concentration of alanine decreased significantly between Age 3 and each of the earlier age groups ($P < 0.001$). Tyrosine showed a significant difference between each age group ($P < 0.05$), with its concentration increasing at Age 2 and again declining at Age 3. Choline showed significant decrease in concentration, while arginine and lysine showed significant differences between Age 1 and Age 3 ($P < 0.05$). Valine and glycine showed a significant decrease in concentration between Age 2 and Age 3 ($P < 0.05$).

3.3. Targeted metabolomic analysis of seminal plasma

The descriptive statistics of targeted metabolites of seminal plasma are given in [Table S2](#) with concentrations visualised as boxplots in [Figure S2](#). Across age groups, glutamate (3603.87 ± 1751.69), glycine (906.69 ± 410.80), L-carnitine (674.80 ± 266.95), creatine (398.03 ± 149.35), and taurine (227.31 ± 115.19) were the top five metabolites in seminal plasma with the highest mean concentrations of glutamate showing a wide range from $1337.00 \mu\text{M}$ to $7500.00 \mu\text{M}$. Conversely, hydroxylysine (1.17 ± 0.40), α -aminobutyric acid (5.08 ± 3.24), asparagine (5.66 ± 2.20), lysine (6.52 ± 5.77), and cystine (7.77 ± 2.35) were the five metabolites with the lowest mean concentration. Ten metabolites, glutamate, taurine, glutamine, isoleucine, leucine, proline, valine, glycine, phenylalanine, and hydroxyproline showed overall significant differences across age groups, however, post-hoc comparisons revealed significant differences between specific age groups for only taurine and glutamate. Taurine showed a significant decrease in concentration between boars of first age with two older ages (adjusted $P < 0.05$), and glutamate revealed a significant decrease between boars of first and third age groups (adjusted $P < 0.05$) ([Figure S2](#)). The remaining metabolites did not achieve statistical significance in pairwise comparisons, suggesting that while general age-related trends existed, specific group differences were not pronounced for

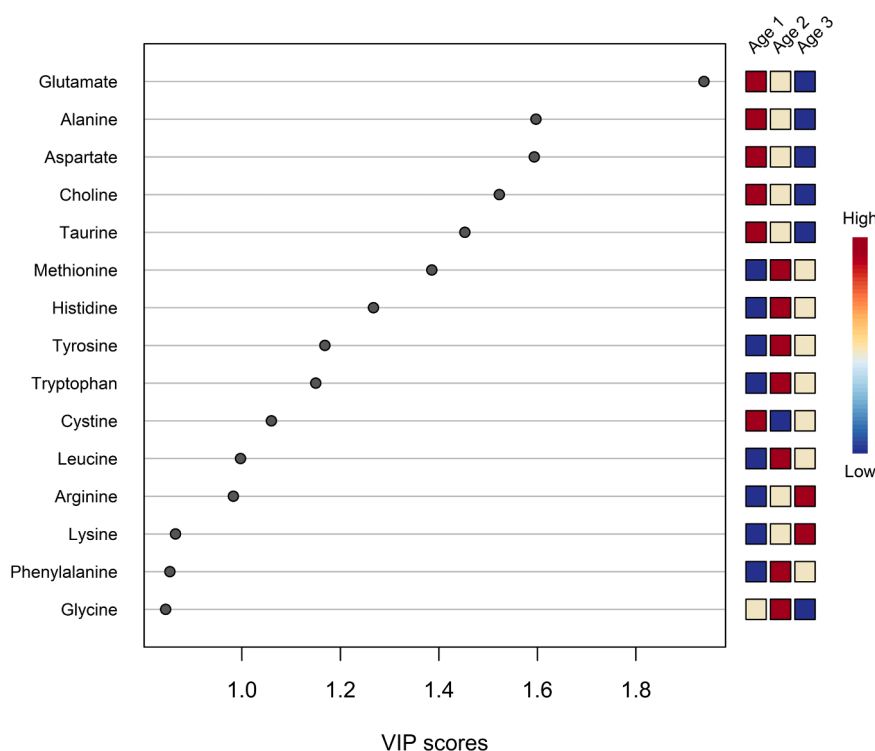


Fig. 3. Variable Importance in Projection (VIP) scores of amino acids and amines in boar spermatozoa across three age groups. Semen samples were collected from 15 Duroc boars at approximately 7 months (Age 1), and again six weeks (Age 2) and twelve weeks (Age 3) later. Following the separation of spermatozoa from seminal plasma, amino acid and amine levels were quantified using Liquid Chromatography-Mass Spectrometry (LC-MS). The plot shows VIP scores on the x-axis, indicating the importance of each metabolite in the Partial Least Squares-Discriminant Analysis (PLS-DA) model used to differentiate age groups. Metabolites are listed on the y-axis in descending order of their importance. The color gradient represents the relative concentration of each metabolite across the age groups where blue indicates a low concentration and red indicates a high concentration.

most metabolites.

3.4. Correlation analysis

3.4.1. Correlation of metabolite concentrations between spermatozoa and seminal plasma

Glutamate exhibited the highest significant correlation between boar spermatozoa and seminal plasma ($r = 0.79$, $P = 2.01 \times 10^{-9}$) followed by hydroxyproline ($r = 0.73$, $P = 1.75 \times 10^{-7}$), taurine ($r = 0.72$, $P = 1.85 \times 10^{-7}$), glycine ($r = 0.67$, $P = 2.61 \times 10^{-6}$), choline ($r = 0.66$, $P = 5.03 \times 10^{-6}$), creatine ($r = 0.61$, $P = 4.48 \times 10^{-5}$), cystine ($r = 0.59$, $P = 1.20 \times 10^{-4}$), aspartate ($r = 0.55$, $P = 3.41 \times 10^{-4}$), L-carnitine ($r = 0.52$, $P = 8.61 \times 10^{-4}$), and α -aminobutyric acid ($r = 0.44$, $P = 5.95 \times 10^{-3}$) (Figure S3).

3.4.2. Correlation among metabolite concentrations

In spermatozoa, strong positive correlations were observed between the concentrations of lysine and arginine (Figure S4, $r = 0.96$, $P < 1 \times 10^{-12}$), hydroxyproline and creatine ($r = 0.91$, $P < 1 \times 10^{-12}$), tyrosine and histidine ($r = 0.85$, $P = 1.22 \times 10^{-11}$), glycine and creatine ($r = 0.82$, $P = 2.51 \times 10^{-10}$), and choline and taurine ($r = 0.80$, $P = 1.48 \times 10^{-9}$). Conversely, notable negative correlations were detected between hydroxyproline and lysine ($r = -0.84$, $P = 3.46 \times 10^{-11}$), and creatine and lysine ($r = -0.82$, $P = 4.25 \times 10^{-10}$).

In seminal plasma (Figure S5), the concentrations of glutamate and L-carnitine were highly correlated ($r = 0.97$, $P < 1 \times 10^{-12}$). Likewise, positive correlations were found between glutamate and choline ($r = 0.96$, $P < 1 \times 10^{-12}$), L-carnitine and choline ($r = 0.96$, $P < 1 \times 10^{-12}$), taurine and asparagine ($r = 0.95$, $P < 1 \times 10^{-12}$), and glutamate and aspartate ($r = 0.95$, $P < 1 \times 10^{-12}$). On the contrary, high negative correlations were observed between tyrosine and hydroxyproline ($r = -0.65$, $P = 7.65 \times 10^{-6}$), proline and isoleucine ($r = -0.61$, $P = 3.92 \times 10^{-5}$), and valine and glutamate ($r = -0.61$, $P = 3.34 \times 10^{-5}$).

3.4.3. Correlation between age and metabolite concentrations in spermatozoa

In spermatozoa, glutamate had a highly significant negative correlation with increasing age ($r_{\text{rm}} = -0.78$, $P = 6.57 \times 10^{-6}$). Likewise, the same was found for the level of alanine ($r_{\text{rm}} = -0.73$, $P = 3.51 \times 10^{-5}$), aspartate ($r_{\text{rm}} = -0.71$, $P = 6.11 \times 10^{-5}$), choline ($r_{\text{rm}} = -0.60$, $P = 2.40 \times 10^{-3}$), taurine ($r_{\text{rm}} = -0.55$, $P = 5.67 \times 10^{-3}$), cystine ($r_{\text{rm}} = -0.46$, $P = 2.00 \times 10^{-2}$) and

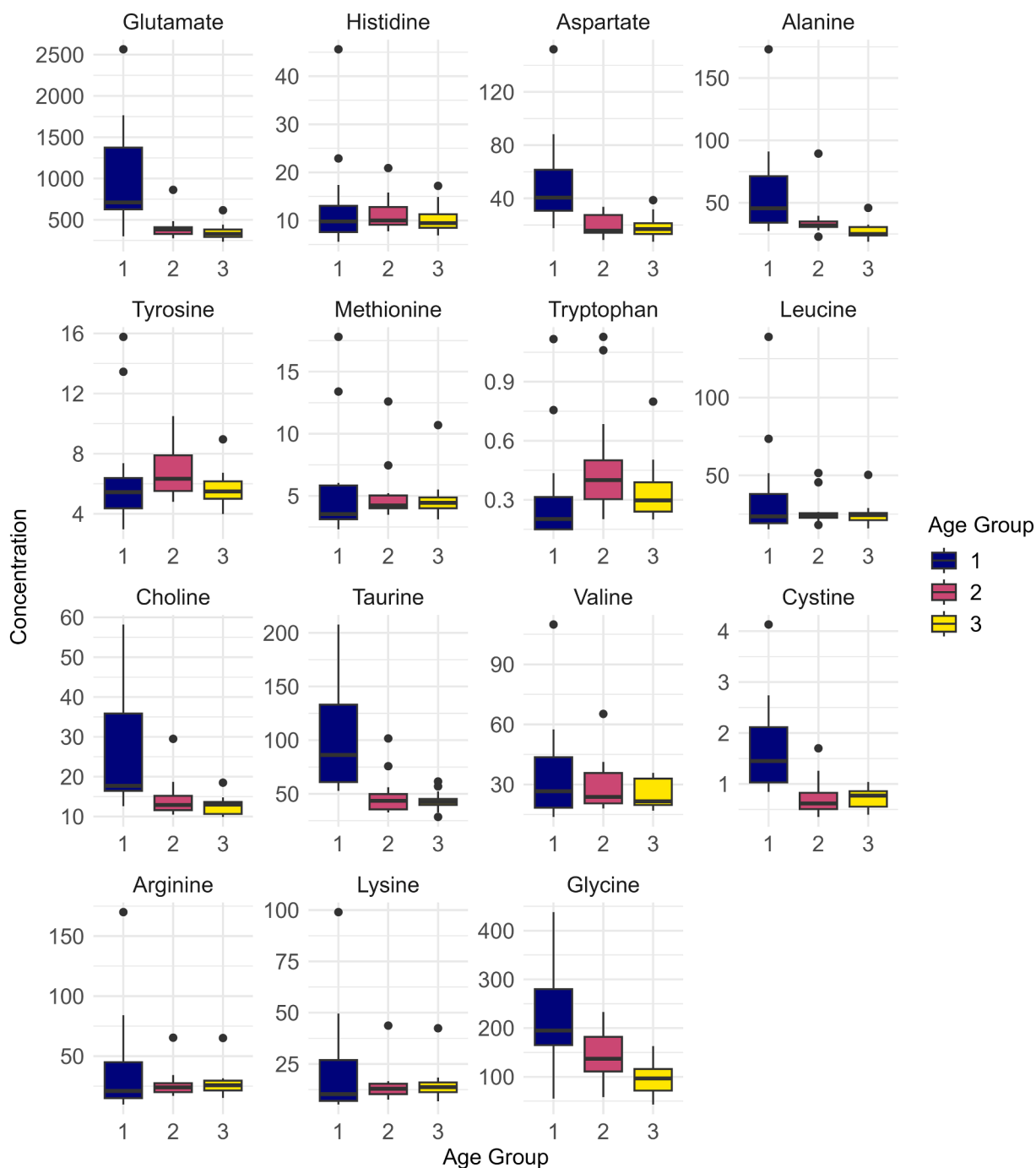


Fig. 4. Boxplot of significant amino acid concentrations in boar spermatozoa across three ages. Semen samples were collected from 15 Duroc boars at approximately 7 months (Age 1, shown in dark blue), and then six weeks (Age 2, pink) and twelve weeks (Age 3, yellow) later. Spermatozoa were separated from seminal plasma, and amino acid and amine levels were quantified using Liquid Chromatography-Mass Spectrometry (LC-MS). Each boxplot represents the concentration (μM) of individual amino acids and amines, highlighting significant age-related variations in metabolites.

glycine ($r_{\text{rm}} = -0.42$, $P = 3.44 \times 10^{-2}$) (Table S3). On the other hand, a significant positive correlation with increasing age was found for histidine ($r_{\text{rm}} = 0.57$, $P = 4.54 \times 10^{-3}$) followed by methionine ($r_{\text{rm}} = 0.55$, $P = 5.67 \times 10^{-3}$), arginine ($r_{\text{rm}} = 0.50$, $P = 1.51 \times 10^{-2}$), tyrosine ($r_{\text{rm}} = 0.49$, $P = 1.55 \times 10^{-2}$), lysine ($r_{\text{rm}} = 0.46$, $P = 2.00 \times 10^{-2}$), tryptophan ($r_{\text{rm}} = 0.46$, $P = 2.00 \times 10^{-2}$) and leucine ($r_{\text{rm}} = 0.45$, $P = 2.17 \times 10^{-2}$).

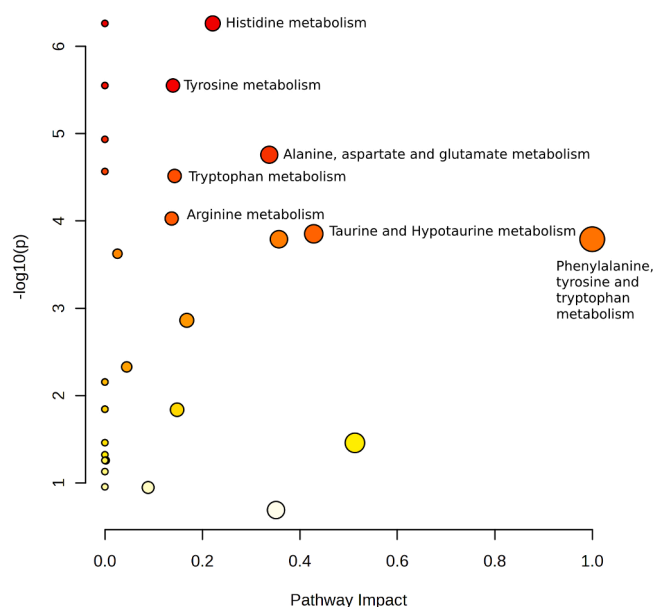


Fig. 5. Pathway analysis of amino acids and amines in boar spermatozoa. Semen samples from 15 Duroc boars were collected at three different ages starting at approximately 7 months (Age 1) and again six and twelve weeks later (Age 2 and Age 3 respectively). Spermatozoa were separated from seminal plasma, and Liquid Chromatography-Mass Spectrometry analysed the amount of amino acids and amines in spermatozoa. The plot shows pathway analysis results for targeted metabolites, with the x-axis representing the pathway impact score and the y-axis showing the negative logarithm of the P -value ($-\log_{10}(p)$), indicating the significance of each pathway's involvement. Points are color-coded from yellow to red, with red indicating the highest significance levels. Only the most important pathways are labeled.

Table 2

Repeated measures ANOVA and Friedman test P -values (P -adjusted) and post hoc P -values for the metabolites across three age groups. P -values are shown for pairwise comparisons between age groups. Age 1 – Age 2: Comparison between boars of 7.2 ± 0.4 and 8.6 ± 0.5 months; Age 1 – Age 3: Comparison between boars of 7.2 ± 0.4 and 9.9 ± 0.5 months; Age 2- Age 3: Comparison between boars of 7.2 ± 0.4 and 9.9 ± 0.5 . NS indicates statistically non-significant differences ($P > 0.05$).

Metabolite	P -adjusted	Age 1 – Age 2	Age 1 – Age 3	Age 2 – Age 3
Glutamate	2.51×10^{-6}	7.51×10^{-4}	7.51×10^{-4}	NS
Histidine	7.40×10^{-6}	7.51×10^{-4}	2.52×10^{-3}	NS
Aspartate	1.01×10^{-5}	2.52×10^{-3}	2.52×10^{-3}	NS
Methionine	5.49×10^{-4}	1.29×10^{-2}	1.29×10^{-2}	NS
Tryptophan	5.49×10^{-4}	9.77×10^{-3}	1.55×10^{-2}	NS
Leucine	3.17×10^{-3}	2.10×10^{-2}	4.94×10^{-2}	NS
Taurine	4.76×10^{-3}	4.94×10^{-2}	1.73×10^{-2}	NS
Cystine	7.62×10^{-3}	3.97×10^{-2}	3.97×10^{-2}	NS
Tyrosine	1.90×10^{-5}	1.26×10^{-3}	1.81×10^{-2}	1.81×10^{-2}
Alanine	1.90×10^{-5}	NS	7.53×10^{-4}	7.51×10^{-4}
Choline	3.32×10^{-3}	NS	6.57×10^{-3}	NS
Arginine	1.13×10^{-2}	NS	4.94×10^{-2}	NS
Lysine	2.58×10^{-2}	NS	3.98×10^{-2}	NS
Valine	4.76×10^{-3}	2.79×10^{-2}	NS	NS
Glycine	2.79×10^{-2}	NS	NS	2.04×10^{-2}

3.5. Pathway analysis

The results of pathway analysis for sperm are shown in Fig. 5. In spermatozoa, alanine, aspartate and glutamate metabolism showed a strong association with age ($FDR = 8.73 \times 10^{-5}$, pathway impact = 0.34). Other highly significant pathways identified were histidine metabolism ($FDR = 8.20 \times 10^{-6}$, pathway impact = 0.22), tyrosine metabolism ($FDR = 2.11 \times 10^{-5}$, pathway impact = 0.14), tryptophan metabolism ($FDR = 1.15 \times 10^{-4}$, pathway impact = 0.14), arginine metabolism ($FDR = 3.13 \times 10^{-4}$, pathway impact = 0.14), and taurine and hypotaurine metabolism ($FDR = 4.05 \times 10^{-4}$, pathway impact = 0.43). Phenylalanine, tyrosine, and tryptophan biosynthesis pathway had the highest impact score of 1 with an FDR of 0.003. The details of all pathways identified along with FDR and pathway impact are given in Table S4.

Similarly, in seminal plasma, phenylalanine, tyrosine, and tryptophan biosynthesis, glycine, serine and threonine metabolism, taurine and hypotaurine metabolism, phenylalanine metabolism, alanine, aspartate, and glutamate metabolism, showed a notable

impact factor of 1, 0.51, 0.43, 0.36 and 0.34 respectively, although they were not significantly enriched ($FDR > 0.05$).

4. Discussion

Our findings revealed significant differences in concentrations of amino acids and amines in spermatozoa among boars during sexual maturation, implying several of these are important for the sexual maturation of Duroc boars. The multivariate analysis suggests that the metabolic profiles of the youngest (Age 1) and oldest groups (Age 3) are distinctly different, with the samples from the boars at Age 2 sharing more similarities with the oldest group, indicating Age 2 as a transition between Age 1 and Age 3. It could also indicate that the amino acids concentration during sexual maturation may reach a plateau or stabilise after boars reach Age 2 of 8.5 months.

4.1. Metabolic crosstalk between spermatozoa and seminal plasma

Glutamate was identified as the predominant metabolite in both spermatozoa and seminal plasma, exhibiting the highest concentration relative to other metabolites. Univariate analysis also showed that glutamate concentrations are significantly different between at least two age groups, with the highest concentration in the youngest boars and progressively decreasing with age. Furthermore, in sperm, glutamate distinguished itself not only through its concentration but also by having the highest VIP score. This indicates a significant contribution to the separation observed between different age groups.

The presence of glutamate in the highest concentration in boar semen in this study, consistent with findings from the previous study (Brown-Woodman and White, 1974), could indicate its vital role in the developmental stages of sperm function. Glutamate plays a role in various aspects of sperm function, including sperm motility, sperm chemotaxis, acrosome reaction, sperm development, and mitochondrial homeostasis (Endo et al., 2019; Frolikova et al., 2020; Kayode et al., 2020; Momeni et al., 2021; Breitbart and Grinshtein, 2023; Vedelek et al., 2023). Glutamate is reported to influence sperm motility by modulating Ca^{2+} signals through interaction with N-methyl-D-aspartate (NMDA) receptors. These receptors facilitate depolarization and repolarization processes in spermatozoa by causing Ca^{2+} influx upon binding glutamate and releasing Mg^{2+} under depolarised conditions (Endo et al., 2019). Similarly, the influence of L-glutamate on sperm chemotaxis, the directed movement of sperm towards chemical attractants, evidenced by varying chemotactic responses of epididymal sperm to different concentrations of L-glutamate, highlight its significance in sperm development and guidance (Frolikova et al., 2020). Glutamate receptors, specifically NMDA receptors, play a role in the acrosome reaction of spermatozoa, a critical step for sperm penetration of the oocyte, by regulating the influx of calcium ions. L-glutamate, an NMDA receptor agonist, has been shown to trigger this acrosome reaction in mouse spermatozoa, suggesting a conserved mechanism across species that increases intracellular calcium levels to initiate the acrosome reaction (AR) (Momeni et al., 2021; Breitbart and Grinshtein, 2023).

Glutamate has a role in boar sperm development by enhancing antioxidant capacity, regulating inflammatory responses and stimulating spermatogonial cell proliferation. Studies show that glutamate supplementation improves testicular morphology, antioxidant enzyme activities, and inflammatory cytokine secretion in boars under oxidative stress (Ni et al., 2016; Tang et al., 2020). Moreover, glutamate and its derivatives, such as NMDA, actively stimulate the expression of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors in spermatogonial cells, enhancing the cell's proliferation mechanisms, evidenced by increased levels of cellular markers like proliferating cell nuclear antigen and Aurora B, which are crucial for cell division and the overall process of sperm production (Santillo et al., 2019).

The decline in glutamate concentrations with age in boar semen observed in our study could reflect a shift in metabolic and cellular priorities as boars mature. Older boars may require less glutamate-mediated signalling as the frequency of spermatogenesis stabilises, or as the efficiency of receptor-mediated processes increases. This hypothesis is supported by studies of male reproductive organs in mice indicating that elevated levels of monosodium glutamate resulted in abnormal sperm, reduced sperm count, smaller testis, smaller epididymis, smaller diameters of seminiferous tubules, oxidative damage, histological modifications, alterations in the anatomy, ultimately affecting sperm quality and reproductive function (Kayode et al., 2020). Likewise, elevated glutamate levels in *Drosophila* spermatids were associated with the formation of abnormal mitochondrial structures (Vedelek et al., 2023).

Taurine is another metabolite showing significant decrease between boars from the first age and the other two older ages in both sperm and seminal plasma. Taurine is a nonprotein amino acid that is synthesised in abundance in male reproductive organs and plays a role in maintaining the structural integrity of sperm membranes, improving sperm viability and motility, and promoting spermatogenesis by acting on the hypothalamus-pituitary-testis axis. It acts as an antioxidant and protects spermatozoa from oxidative stress, preventing DNA damage (Li et al., 2023). The addition of taurine during storage enhances motility, viability and membrane integrity (Jang et al., 2006). One could speculate that an increased level of taurine in younger boars could be beneficial for improving their sperm quality. A practical application of this idea might involve adding taurine to semen extenders to further improve the sperm quality of the youngest boars.

Besides, we observed significant correlations between concentrations of several metabolites between spermatozoa and seminal plasma, including glutamate, hydroxyproline, taurine, choline, creatine, cystine, aspartate, L-carnitine and α -aminobutyric acid. These findings suggest potential metabolic interactions and exchange mechanisms between spermatozoa and seminal plasma (Huacuja et al., 1981). This metabolic crosstalk may play a crucial role in supporting sperm function, maturation, and development. Free amino acids in seminal plasma are produced in the testes, epididymis, and accessory sex glands, as well as by the action of endogenous proteolytic enzymes on seminal proteins after ejaculation (Roussel and Stallcup, 1967).

4.2. Changes in sperm metabolites as boar mature

Our analysis identified variations in the concentrations of key metabolites reflecting significant changes in the metabolome as boars mature. Besides glutamate and taurine, our study also showed a clear decline in alanine concentrations in boar spermatozoa with increasing age. The observed second-highest VIP score underlines the importance of alanine in the metabolic integrity of spermatozoa. Alanine in boar semen is important for sperm function, but studies in rams have shown that high levels may impair both total motility, and progressive motility, and reduce post-thaw sperm motility in rams (Sangeeta et al., 2015). On the other hand, too-low levels are associated with azoospermia in stallions (Castiglione Morelli et al., 2021), indicating that adequate levels of alanine are required.

Aspartate and choline showed similar trends as their concentrations in spermatozoa were reduced significantly when boars reached Age 2, suggesting these amino acids play an important role in the metabolic changes during sexual maturation of the boar. The high VIP scores of aspartate and choline further emphasise their relevance in metabolism during sexual maturation in boars. D-aspartate is an endogenous amino acid in animals, synthesised in testis (D'Aniello et al., 1996; Topo et al., 2009). A study has shown that D-aspartate supplementation significantly increases sperm concentration and motility in oligoasthenozoospermic and asthenozoospermic patients (D'Aniello et al., 2012), stimulates testosterone synthesis in rat Leydig cells (Nagata et al., 1999) and improves the quality of semen in rabbits (Macchia et al., 2010). D-aspartate, however, at higher concentrations was found to suppress germ cell differentiation and reduce the number of meiotic cells in cultured mouse testis tissue (Tomita et al., 2016), indicating a potential negative impact when administered excessively. Choline is integral to the formation and maintenance of sperm membrane structure and fluidity, which are essential for the maturation and fertilizing capacity of spermatozoa. Alterations in enzymes involved in choline metabolism, such as phosphatidylethanolamine N-methyltransferase (PEMT), and choline dehydrogenase (CHDH), due to gene sequence variations can affect spermatogenesis, sperm quality, sperm motility patterns, sperm mitochondrial morphology and sperm ATP concentrations (Johnson et al., 2012; Lazaros et al., 2012; Everett, 2022). Choline promotes sperm motility via cholinergic signalling as it is taken up by sperm and converted to acetylcholine by choline acetyltransferase, and acetylcholine is then released to stimulate sperm motility (Bray et al., 2006; Hanley, 2023). Our results showed the highest level of choline in the youngest group. This may serve as a mechanism to increase motility in spermatozoa from young, not fully matured boars; however, a delicate balance is required, since excess choline can transiently impair spermatogenesis, as observed in rats (Vachhrajani et al., 1993).

Taken together, the observed patterns in this study of the concentration of glutamate, alanine, aspartate, choline, and taurine, and their decline with age in boar sperm may be closely related to the functional demands placed on spermatozoa at different stages of sexual maturity. These findings highlight the dynamic role of these amino acids in initiating critical reproductive processes and adapting to the boar's changing physiological landscape as it matures. Moreover, the pathway analysis highlighted the significance of various metabolic pathways in the sperm maturation process, also identifying alanine, aspartate and glutamate metabolism, histidine metabolism, and tyrosine metabolism as important. Histidine is an essential amino acid that is crucial for protein synthesis, proton buffering, metal ion chelation, scavenging of reactive oxygen and nitrogen species, buffering intracellular pH and is a precursor to critical metabolites like carnosine and histamine (Brosnan and Brosnan, 2020; Holeček, 2020), thus could play vital functions in regulating pH levels, ionic balances, protecting spermatozoa from oxidative stress and other cellular processes and function during sperm development process. Tyrosine phosphorylation, a key aspect of the tyrosine metabolism pathway, is linked to various aspects of sperm development such as spermatogenesis and epididymal maturation of sperm. It promotes the acquisition of motility and fertilization ability through interaction with secretory proteins and biochemical modifications like cyclic adenosine monophosphate (cAMP) elevation (Ijiri et al., 2012). A study by Lewis and Aitken shows that cAMP elevation results in intense tyrosine phosphorylation in sperm proteins of caput and cauda epididymis, thus influencing sperm maturation (Lewis and Aitken, 2001).

4.3. Role of seminal plasma

In this study, 10 amino acids demonstrated overall significant differences between age groups in the boars' seminal plasma, with only glutamate and taurine showing significant pairwise differences between at least two age groups. In comparison, we observed a higher number of amino acids (15) showing significant pairwise age-related changes in the sperm cell metabolome. A less pronounced change in the amino acid composition of seminal plasma suggests that its amino acids may not undergo extensive remodelling during sexual maturation as compared to sperm amino acids. Seminal plasma plays an important role in maintaining an optimal environment for sperm by providing a protective and nutritive medium that supports their maturation. Abundant amino acids in seminal plasma, such as glutamate, glycine, L-carnitine, creatine, and taurine, are known to play key roles in energy metabolism, and antioxidant activity, and serve as essential precursors for various sperm-related processes such as spermatogenesis, sperm maturation, normal sperm morphology, motility, capacitation, fertilization and protection of sperm from oxidative damage (Cheon and Kim, 2015; Umehara et al., 2018; Li et al., 2023; Mateus et al., 2023; Vedelek et al., 2023).

The variable importance projection (VIP) score analysis identified alanine and glycine as the most important metabolites contributing to the seminal plasma amino acids profile variation. Alanine and glycine are abundant amino acids in seminal plasma (Ugur et al., 2020) which provide metabolic support to spermatozoa. The presence of glycine in seminal plasma suggests its potential role as an antioxidant, aiding in the quality of spermatozoa (Nazif et al., 2022).

Overall, distinct metabolite profiles and age-related dynamics observed in spermatozoa and seminal plasma highlight the specialised roles of these compartments in supporting sperm maturation and function.

4.4. Study limitations and practical implications

While our targeted LC-MS analysis in boar sperm and seminal plasma provides insights into metabolic changes during sexual maturation, a few considerations warrant mention. Our standardised housing and feeding conditions minimised confounding factors, and the results are representative of the Duroc population at Norsvin AI station but may not fully reflect the variability of different AI stations around the world. Our study included 15 randomly selected Duroc boars allowing detailed analyses but expanding this sample size to include more animals and other breeds would strengthen the broader context of these findings and add knowledge about breed-related differences. Additionally, employing an untargeted metabolomics approach could help identify novel and various classes of metabolites that differ with age. Establishing relationships between metabolites and sexual maturity could pave the way for screening protocols during the training period to identify sexually matured boars or targeted dietary interventions (e.g., amino acid supplementation) to accelerate maturation or sustain semen quality in young boars. Such strategies would allow breeding programs to integrate young boars earlier without compromising semen quality, optimizing both efficiency and economic returns for the pig industry.

5. Conclusion

This study revealed significant changes in amino acid and amine concentrations in boar spermatozoa and seminal plasma during sexual maturation even within the small age differences. Spermatozoa exhibited pronounced alterations in 15 amino acids, with glutamate, alanine, aspartate, and choline emerging as critical markers, while for seminal plasma glutamate and taurine were identified as pivotal amino acids. These robust differences highlight their utility as potential biomarkers for the assessment of sexual maturity in boars.

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CRediT authorship contribution statement

Asmita Shrestha: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Ann Helen Gaustad:** Conceptualization, Resources, Writing – review & editing. **Janne Beate Øiaas:** Methodology, Investigation. **Elisabeth Kommisrud:** Funding acquisition, Writing – review & editing. **Maren van Son:** Writing – review & editing. **Anna Nordborg:** Methodology, Investigation, Data curation, Writing – review & editing. **Alm-Kristiansen Anne Hege:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors have no conflict of interests to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.anireprosci.2025.107800](https://doi.org/10.1016/j.anireprosci.2025.107800).

References

- Argov-Argaman, N., Mahgrefthe, K., Zeron, Y., Roth, Z., 2013. Variation in lipid profiles within semen compartments—the bovine model of aging. *Theriogenology* 80, 712–721.
- Ashokan, M., Rana, E., Sneha, K., Namith, C., Naveen Kumar, G.S., Azharuddin, N., Elango, K., Jeyakumar, S., Ramesha, K.P., 2023. Metabolomics—a powerful tool in livestock research. *Anim. Biotechnol.* 34, 3237–3249.
- Blaurock, J., Baumann, S., Grunewald, S., Schiller, J., Engel, K.M., 2022. Metabolomics of human semen: a review of different analytical methods to unravel biomarkers for male fertility disorders. *Int. J. Mol. Sci.* 23, 9031.
- Bonet, S., Garcia, E., Sepúlveda, L., 2013. The boar reproductive system. In: Bonet, S., Casas, I., Holt, W.V., Yeste, M. (Eds.), *Boar Reproduction: Fundamentals and New Biotechnological Trends*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 65–107.
- Bray, C., Son, J.-H., Meizel, S., 2006. Acetylcholine causes an increase of intracellular calcium in human sperm. *Mol. Hum. Reprod.* 11, 881–889.
- Breitbart, H., Grinshtein, E., 2023. Mechanisms that protect mammalian sperm from the spontaneous acrosome reaction. *Int. J. Mol. Sci.* 24, 17005.
- Brosnan, M.E., Brosnan, J.T., 2020. Histidine metabolism and function. *J. Nutr.* 150, 2570S–2575S.
- Brown-Woodman, P.D., White, I.G., 1974. Amino acid composition of semen and the secretions of the male reproductive tract. *Aust. J. Biol. Sci.* 27, 415–422.
- Buckman, C., George, T.C., Friend, S., Sutovsky, M., Miranda-Vizuete, A., Ozanon, C., Morrissey, P., Sutovsky, P., 2009. High throughput, parallel imaging and biomarker quantification of human spermatozoa by ImageStream flow cytometry. *Syst. Biol. Reprod. Med* 55, 244–251.
- Caires, K.C., Schmidt, J.A., Oliver, A.P., de Avila, J., McLean, D.J., 2008. Endocrine regulation of the establishment of spermatogenesis in pigs. *Reprod. Domest. Anim.* 43 (2), 280–287.
- Castiglione Morelli, M.A., Ostuni, A., Giangaspero, B., Cecchini, S., Carluccio, A., Boni, R., 2021. Relationships between seminal plasma metabolites, semen characteristics and sperm kinetics in donkey (*Equus asinus*). *Animals (Basel)* 11.

- Chabory, E., Damon, C., Lenoir, A., Henry-Berger, J., Vernet, P., Cadet, R., Saez, F., Drevet, J.R., 2010. Mammalian glutathione peroxidases control acquisition and maintenance of spermatozoa integrity. *J. Anim. Sci.* 88, 1321–1331.
- Cheon, Y.P., Kim, C.H., 2015. Impact of glycosylation on the unimpaired functions of the sperm. *Clin. Exp. Reprod. Med.* 42, 77–85.
- Chong, J., Wishart, D.S., Xia, J., 2019. Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. *Curr. Protoc. Bioinforma.* 68, e86.
- D'Aniello, G., Ronsini, S., Notari, T., Grieco, N., Infante, V., D'Angel, N., Mascia, F., Fiore, M.M.D., Fisher, G., D'Aniello, A., 2012. D-aspartate, a key element for the improvement of sperm quality. *Adv. Sex. Med.* 02, 45–53.
- D'Aniello, A., Cosmo, A.D., Cristo, C.D., Annunziato, L., Petrucci, L., Fisher, G., 1996. Involvement of D-Aspartic acid in the synthesis of testosterone in rat testes. *Life Sci.* 59, 97–104.
- Ding, H., Luo, Y., Liu, M., Huang, J., Xu, D., 2016. Histological and transcriptome analyses of testes from Duroc and Meishan boars. *Sci. Rep.* 6, 20758.
- Endo, D., Kon, S., Sato, T., Toyama, F., Katsura, Y., Nakauchi, Y., Takayama-Watanabe, E., Watanabe, A., 2019. NMDA-type glutamate receptors mediate the acrosome reaction and motility initiation in newt sperm. *Mol. Reprod. Dev.* 86, 1106–1115.
- Everett, C., 2022. Identifying and Treating Poor Male Sperm Function with Gene-Guided Precision Nutrition | SNP Ther.
- Frolíkova, M., Otčenásková, T., Valasková, E., Postlerová, P., Stopkova, R., Stopka, P., Komrskova, K., 2020. The role of taste receptor mTAS1R3 in chemical communication of gametes. *Int. J. Mol. Sci.* 21, 2651.
- Goeman, J.J., van de Geer, S.A., de Kort, F., van Houwelingen, H.C., 2004. A global test for groups of genes: testing association with a clinical outcome. *Bioinformatics* 20, 93–99.
- Hanley, P.J., 2023. Elusive physiological role of prostatic acid phosphatase (PAP): generation of choline for sperm motility via auto-and paracrine cholinergic signaling. *Front. Physiol.* 14.
- Holeček, M., 2020. Histidine in health and disease: metabolism, physiological importance, and use as a supplement. *Nutrients* 12, 848.
- Hosseini, E., Amirjannati, N., Bazrafkan, M., Moghaddasfar, H., Gilany, K., 2022. P-052 Targeted metabolomics (amino acids) profiling of seminal plasma distinguished teratozoospermia from normozoospermic samples. *Hum. Reprod.* 37.
- Huacuja, L., Delgado, N.M., Calzada, L., Wens, A., Reyes, R., Pedrón, N., Rosado, A., 1981. Exchange of lipids between spermatozoa and seminal plasma in normal and pathological human semen. *Arch. Androl.* 7, 343–349.
- Huang, Y.H., Lo, L.L., Liu, S.H., Yang, T.S., 2010. Age-related changes in semen quality characteristics and expectations of reproductive longevity in Duroc boars. *Anim. Sci. J.* 81, 432–437.
- Ijiri, T.W., Mahbub Hasan, A.K., Sato, K., 2012. Protein-tyrosine kinase signaling in the biological functions associated with sperm. *J. Signal Transduct.* 2012, 181560.
- Jang, H., Kong, H., Park, C., Oh, J., Lee, S.-G., Cheong, H., Kim, J., Lee, S., Yang, B., Lee, H.-J., 2006. Effects of taurine on sperm characteristics during in vitro storage of Boar Semen. *Asian-Australas. J. Anim. Sci.* 19, 1561–1565.
- Johnson, A.R., Lao, S., Wang, T., Galanko, J.A., Zeisel, S.H., 2012. Choline dehydrogenase polymorphism rs12676 is a functional variation and is associated with changes in human sperm cell function. *PLoS One* 7, e36047.
- Kasimanickam, V., Buhr, M., Kasimanickam, R., 2019. Patterns of expression of sperm and seminal plasma microRNAs in boar semen. *Theriogenology* 125, 87–92.
- Kayode, O.T., Rotimi, D.E., Kayode, A.A.A., Olaolu, T.D., Adeyemi, O.S., 2020. Monosodium Glutamate (MSG)-Induced Male Reproductive Dysfunction: A Mini Review. *Toxics* 8.
- Lazaros, L., Xita, N., Hatzis, E., Kaponis, A., Makrydimas, G., Takenaka, A., Sofikitis, N., Stefanos, T., Zikopoulos, K., Georgiou, I., 2012. Phosphatidylethanolamine N-methyltransferase and choline dehydrogenase gene polymorphisms are associated with human sperm concentration. *Asian J. Androl.* 14, 778–783.
- Lewis, B., Aitken, R.J., 2001. Impact of epididymal maturation on the tyrosine phosphorylation patterns exhibited by rat spermatozoa. *Biol. Reprod.* 64, 1545–1556.
- Li, Y., Peng, Q., Shang, J., Dong, W., Wu, S., Guo, X., Xie, Z., Chen, C., 2023. The role of taurine in male reproduction: Physiology, pathology and toxicology. *Front. Endocrinol. (Lausanne)* 14, 1017886.
- Macchia, G., Topo, E., Mangano, N., D'Aniello, E., Boni, R., 2010. dl-Aspartic acid administration improves semen quality in rabbit bucks. *Anim. Reprod. Sci.* 118, 337–343.
- Maistrelli, C., Schmicke, M., Hoedemaker, M., Siebert, U., 2022. An Approach for Investigating Sexual Maturity in Wild Boar Males: Testosterone and 17 β -Estradiol Analysis. *Animals* 12, 2295.
- Mann, T., Lutwak-Mann, C., 2012. Male Reproductive Function and Semen: Themes and Trends in Physiology, Biochemistry and Investigative Andrology. Springer Science & Business Media.
- Mateo-Otero, Y., Fernández-López, P., Ribas-Maynou, J., Roca, J., Miró, J., Yeste, M., Barranco, I., 2021b. Metabolite profiling of pig seminal plasma identifies potential biomarkers for sperm resilience to liquid preservation. *Front. Cell Dev. Biol.* 9, 669974.
- Mateo-Otero, Y., Fernández-López, P., Delgado-Bermúdez, A., Nolis, P., Roca, J., Miró, J., Barranco, I., Yeste, M., 2021a. Metabolomic fingerprinting of pig seminal plasma identifies in vivo fertility biomarkers. *J. Anim. Sci. Biotechnol.* 12, 113.
- Mateo-Otero, Y., Madrid-Gambin, F., Llanera, M., Gomez-Gomez, A., Haro, N., Pozo, O.J., Yeste, M., 2023. Sperm physiology and in vitro fertilising ability rely on basal metabolic activity: insights from the pig model. *Commun. Biol.* 6, 344.
- Mateus, F.G., Moreira, S., Martins, A.D., Oliveira, P.F., Alves, M.G., Pereira, M.L., 2023. L-Carnitine and male fertility: is supplementation beneficial? *J. Clin. Med.* 12.
- Memili, E., Moura, A.A., Kaya, A., 2020. Metabolomes of sperm and seminal plasma associated with bull fertility. *Anim. Reprod. Sci.* 220, 106355.
- Menezes, E.B., Velho, A.L.C., Santos, F., Dinh, T., Kaya, A., Topper, E., Moura, A.A., Memili, E., 2019. Uncovering sperm metabolome to discover biomarkers for bull fertility. *BMC Genom.* 20, 714.
- Momeni, H.R., Etemadi, T., Alyasin, A., Eskandari, N., 2021. A novel role for involvement of N-methyl-D-aspartate (NMDA) glutamate receptors in sperm acrosome reaction. *Andrologia* 53, e14203.
- Nagata, Y., Homma, H., Lee, J.A., Imai, K., 1999. D-Aspartate stimulation of testosterone synthesis in rat Leydig cells. *FEBS Lett.* 444, 160–164.
- Narud, B., Klinkenberg, G., Khezri, A., Zeremichael, T.T., Stenseth, E.-B., Nordborg, A., Haukaas, T.H., Morrell, J., Heringstad, B., Myromslien, F.D., Kommisrud, E., 2020. Differences in sperm functionality and intracellular metabolites in Norwegian Red bulls of contrasting fertility. *Theriogenology* 157, 24–32.
- Nazif, M.S., Rehman, Z., Khan, H., Khan, F.A., Hussain, T., Ahmad, A., Farmanullah, Husnain, A., Muhammad, S., Murtaza, G., Gang, L., 2022. Glycine improved cryopreserved spermatozoa quality in Achai Bull. *BioMed. Res. Int.* 2022, 8282387.
- Ni, H., Lu, L., Deng, J., Fan, W., Li, T., Yao, J., 2016. Effects of glutamate and aspartate on serum antioxidative enzyme, sex hormones, and genital inflammation in boars challenged with hydrogen peroxide. *Mediat. Inflamm.* 2016, 4394695.
- Pang, Z., Lu, Y., Zhou, G., Hui, F., Xu, L., Viau, C., Spigelman, Aliya F., MacDonald, Patrick E., Wishart, David S., Li, S., Xia, J., 2024. MetaboAnalyst 6.0: towards a unified platform for metabolomics data processing, analysis and interpretation. *Nucleic Acids Res.* 52, W398–W406.
- Papp, G., Grof, J., Menyhart, J., 1983. The role of basic amino acids of the seminal plasma in fertility. *Int. Urol. Nephrol.* 15, 195–203.
- R Core Team, 2022. R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria.
- Rabassa, V.R., Feijó, J.O., Pereira, R.A., Acosta, D.A.V., Hax, L.T., Silva, A.D.C.J., Oliveira, A.M., Schmitt, E., Del Pino, F.A.B., Bianchi, I., Corrêa, M.N., 2013. Metabolic and endocrine changes of hybrid boars (Landrace x Large White) during the first year of life and its relation to the establishment of puberty. *Brazilian J. Vet. Res. Anim. Sci.* 50, 323–330.
- Rodríguez-Martínez, H., Kvist, U., Emerudh, J., Sanz, L., Calvete, J.J., 2011. Seminal Plasma Proteins: What Role Do They Play? *Am. J. Reprod. Immunol.* 66, 11–22.
- Roussel, J., Stallcup, O., 1967. Some amino acid aspects of bovine semen.
- Sangeeta, S., Arangasamy, A., Kulkarni, S., Selvaraju, S., 2015. Role of amino acids as additives on sperm motility, plasma membrane integrity and lipid peroxidation levels at pre-freeze and post-thawed ram semen. *Anim. Reprod. Sci.* 161, 82–88.
- Santillo, A., Falvo, S., Fiore, M.M.D., Russo, F.D.G., Chieffi, P., Usiello, A., Pinelli, C., Baccari, G.C., 2019. Journal of Cellular Biochemistry. Wiley Online Library.
- Shostya, A., Zamazi, A., Usenko, S., Shpyrna, I., 2023. Peculiarities of the formation of sperm production and the course of peroxide oxidation in boars. *Sci. Messenger LNU Vet. Med. Biotechnol. Ser.: Agric. Sci.* 25, 11–18.
- Silvestroni, L., Morisi, G., Malandrino, F., Frajese, G.V., 1979. Free amino acids in semen: measurement and significance in normal and oligozoospermic men. *Arch. Androl.* 2 (3), 257–261.

- Singleton, W.L., Flowers, W.L., 2006. Management of the boar for natural service, Pork Information Gateway, U.S. Pork Center of Excellence.
- Sonderman, J.P., Luebke, J.J., 2008. Semen production and fertility issues related to differences in genetic lines of boars. *Theriogenology* 70, 1380–1383.
- Tang, W., Wu, J., Jin, S., He, L., Lin, Q., Luo, F., He, X., Feng, Y., He, B., Bing, P., Li, T., Yin, Y., 2020. Glutamate and aspartate alleviate testicular/epididymal oxidative stress by supporting antioxidant enzymes and immune defense systems in boars. *Sci. China Life Sci.* 63, 116–124.
- Tomita, K., Tanaka, H., Kageyama, S., Nagasawa, M., Wada, A., Murai, R., Kobayashi, K., Hanada, E., Agata, Y., Kawauchi, A., 2016. The effect of d-aspartate on spermatogenesis in mouse Testis1. *Biol. Reprod.* 94.
- Topo, E., Soricelli, A., D'Aniello, A., Ronsini, S., D'Aniello, G., 2009. The role and molecular mechanism of D-aspartic acid in the release and synthesis of LH and testosterone in humans and rats. *Reprod. Biol. Endocrinol.* 7, 120.
- Ugur, M.R., Dinh, T., Hitit, M., Kaya, A., Topper, E., Didion, B., Memili, E., 2020. Amino acids of seminal plasma associated with freezability of bull sperm. *Front. Cell Dev. Biol.* 7.
- Umehara, T., Kawai, T., Goto, M., Richards, J.S., Shimada, M., 2018. Creatine enhances the duration of sperm capacitation: a novel factor for improving in vitro fertilization with small numbers of sperm. *Hum. Reprod.* 33, 1117–1129.
- Vachhrajani, K.D., Sahu, A.P., Dutta, K.K., 1993. Excess choline availability: a transient effect on spermatogenesis in the rat. *Reprod. Toxicol.* 7, 477–481.
- Vedelek, V., Vedelek, B., Lőrincz, P., Juhász, G., Sinka, R., 2023. A comparative analysis of fruit fly and human glutamate dehydrogenases in *Drosophila melanogaster* sperm development. *Front. Cell Dev. Biol.* 11.
- Velho, A.L.C., Menezes, E., Dinh, T., Kaya, A., Topper, E., Moura, A.A., Memili, E., 2018. Metabolomic markers of fertility in bull seminal plasma. *PLoS One* 13, e0195279.
- Wu, G., 2009. Amino acids: metabolism, functions, and nutrition. *Amino Acids* 37, 1–17.
- Xia, J., Wishart, D.S., 2010. MetPA: a web-based metabolomics tool for pathway analysis and visualization. *Bioinformatics* 26, 2342–2344.
- Zhang, Y.-t., Liu, Y., Liang, H.-l., Xu, Q.-q., Liu, Z.-h., Weng, X.-g., 2021. Metabolomic differences of seminal plasma between boars with high and low average conception rates after artificial insemination. *Reprod. Domest. Anim.* 56, 161–171.

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Untargeted metabolomics reveals changes in boar sperm and seminal plasma metabolites associated with sexual maturity

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Abstract

Background Boars undergo physiological and biochemical changes in semen composition as they grow from puberty to sexual maturity. However, comprehensive metabolomic profiles of boar semen remain uncharacterised. Understanding metabolic alterations in semen during this period is important for optimising reproductive performance in breeding programs. The aim of this study was to characterise the semen metabolome as boars mature, utilising an untargeted metabolomic approach. Semen samples were collected from 15 Duroc boars at three developmental ages: ~7 months, 8.5 months, and 10 months. Sperm and seminal plasma were separated and analysed by hydrophilic interaction and reversed-phase liquid chromatography coupled with mass spectrometry to capture a wide range of metabolites.

Results We identified a total of 4,491 features in boar semen, annotating 92 distinct metabolites. Amino acids, peptides and analogues constituted the most abundant components, followed by fatty acid esters. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) showed a clear separation between metabolomic profiles by age groups. PERMANOVA analysis of PCA scores confirmed statistically significant differences ($P < 0.05$) between younger (7 months) and more mature boars (8.5 months and 10 months). Pathway analysis identified porphyrin metabolism, taurine and hypotaurine metabolism, and glycerolipid metabolism as significantly enriched pathways in sperm, while glutathione and nitrogen metabolism were prominently enriched in seminal plasma. Using linear modelling, partial Spearman correlation and random forest analyses, we identified homoisovanillic acid as a key metabolite discriminating age groups in both sperm and seminal plasma. Additionally, L-glutamic acid, decanoyl-L-carnitine and N-(1,3-Thiazol-2-yl)benzenesulfonamide emerged as important sperm metabolites, while glyceric acid, myo-inositol, glycerophosphocholine, and several other compounds were identified as critical seminal plasma metabolites.

Conclusion This study provides a detailed characterisation of metabolic changes in Duroc boar semen during the transition from puberty to sexual maturity. Our findings enhance the understanding of reproductive development and could inform strategies to assess sexual maturity in breeding programs.

Keywords Global metabolomics, Seminal plasma, Sexual maturity, Spermatozoa, *Sus scrofa domestica*

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Introduction

Sexual maturity influences reproductive performance, genetic improvement, and overall productivity in swine production. Boars reach sexual maturity between three and seven months, with substantial breed variation influenced by genetic and environmental factors [1–3]. Early maturing breeds like Chinese Meishan and Portuguese Bisaro boars attain spermatogenesis by 2.5–3 months of age [4], with sexual capacity evident between 3.5 and 4.4 months of age [1, 3]. Modern commercial breeds do not reach full sexual maturity until approximately 7 months of age, and their fertility continues to increase substantially with age, reaching its peak between 9 and 20 months of age [4]. Typically, Duroc boars are reported to reach puberty at about 5 months [5], and sexual maturity by 9 months [3], with the highest sperm production at around 18 months [6, 7]. Early maturation enables the use of younger males for breeding, thereby shortening generation intervals and accelerating genetic progress [8].

As boars mature sexually, there is a marked increase in ejaculate volume and total sperm number per ejaculate, which are important for artificial insemination (AI) practices [9, 10]. It is reported that between 8 and 10 months of age, there is an increase in the sperm length, width and head area, along with a reduction in the percentage of sperm with minor abnormalities [11]. This maturation period also features increased testicular weight [12] and hormonal shifts [12, 13] driving the development of secondary sexual characteristics and libido [14].

Determining sexual maturity onset in boars presents several challenges. Traditional methods of assessment, such as physical examinations, hormonal assays, and assessment of semen quality, often have limitations. Physical examinations assess only external characteristics, which may not accurately reflect internal physiological readiness for reproduction. For example, Hezuo boars show libido at around 1.5 months, but develop abundant spermatogonia cells after 4 months [15]. Hormonal assays are influenced by various external factors such as stress and nutrition [16–19], leading to fluctuations in hormone levels, which may not reliably indicate sexual maturity. Parameters such as motility, morphology, and acrosome integrity are reported to vary significantly between boars and within ejaculates from the same boar [20], and their relationship to sexual maturity remains poorly characterised. There is no single parameter that defines the animals' sexual maturity, as progression is gradual rather than an abrupt occurrence [21]. This highlights the need for more comprehensive approaches that could provide complementary information for effective sexual maturity assessment.

Although genetic companies are increasingly using younger males to accelerate genetic progress [22], selecting animals that have not yet achieved true reproductive maturity creates potential conflicts. Untargeted metabolomics is a powerful analytical method that offers thorough profiling of metabolites, which are end products of cellular processes in biological samples, without needing prior knowledge of which specific metabolites are present. This approach provides insights into physiological states, biochemical pathways and metabolic processes occurring within an organism [23, 24]. Zhao et al. utilised untargeted metabolomics to identify potential biomarkers in the small yellow follicle of chickens during sexual maturation, highlighting L-arginine, L-prolinamide, (R)-4-hydroxymandelate, glutathione and homovanillic acid as important metabolites in this process [25].

The sperm metabolite profile can provide insights into their functional capacity, including fertility [26, 27], motility and viability [28]. Studies have identified specific metabolites in sperm associated with fertility outcomes; for example, gamma-aminobutyric acid (GABA), carbamate, benzoic acid, lactic acid, and palmitic acid were reported to be significantly different between high-fertility and low-fertility bulls [26]. Similarly, cysteine and glutamic acid in sperm were reported to have a negative association with fertility, while aspartic acid, leucine and serine were reported to have a positive association with fertility in bulls [27]. Seminal plasma contains a rich array of metabolites that provide nutrients and protective factors to enhance sperm survival and help them navigate through the female reproductive tract [29]. Velho et al. reported an association of 2-oxoglutaric acid and fructose in seminal plasma with the fertility of bulls [30]. A study in humans comparing seminal plasma metabolites in young men aged 26 to 30 and older men aged 45 to 54 identified that age affects citrate cycle, oxidative phosphorylation, and glutathione metabolism [31]. This shows valuable insights into how age impacts seminal plasma composition and consequently male fertility.

Our recent targeted metabolomic analysis of amino acids and amines in boar semen identified key sexual maturity-related changes, particularly in glutamate, alanine, aspartate and choline [32]. However, this targeted approach captured only a fraction of the metabolic landscape, leaving the broader range of lipids, energy metabolites, antioxidants, and signalling molecules unexplored, highlighting the necessity of untargeted metabolomics research in this area.

The present study aimed to provide a comprehensive metabolomic characterisation of boar semen during sexual maturation, identifying key metabolites and pathways that change during this critical reproductive transition. This was performed through a longitudinal study design

collecting semen samples from Duroc boars across three maturation time points (7, 8.5, and 10 months), and analysing sperm and seminal plasma separately by untargeted metabolomic analysis (LC–MS/MS platform). This holistic discovery profiling approach enabled the identification of metabolic fingerprints of reproductive development and major metabolic pathways involved in sexual maturation. Our work represents the first in-depth metabolomic exploration of boar sexual maturation, establishing a foundational knowledge base for future research and breeding strategies.

Methods

Study design and sample collection

Fifteen male Duroc swine (*Sus scrofa domestica*) selected randomly from the commercial breeding line of Norsvin SA (Norway) were followed longitudinally over three months. Semen was collected repeatedly from these boars from March to June 2023, encompassing three growth phases: 7.16 ± 0.44 months (designated as 7 months), 8.56 ± 0.46 months (designated as 8.5 months), and 9.94 ± 0.47 months (designated as 10 months).

During the first semen collection at 7 months of age, the boars were kept in individual pens measuring 4 m^2 within an isolation unit, as approved by the Norwegian Food Safety Authority, where they were trained for semen collection. Semen collection began as soon as the boars showed interest in mounting the dummy and were physically able to produce an ejaculate. The same boars were transferred to an Artificial Insemination (AI) centre at 8.5 months of age, where they were housed in larger pens of 6 m^2 . In compliance with Council Directive 90/429/EEC, Norsvin SA commences commercial semen distribution from boars from approximately 8.5 months of age.

Throughout both the isolation unit and the AI centre, consistent housing conditions, including ventilation, lighting and temperature, were maintained. All the boars received the nutritionally balanced commercial diet. The semen collection was performed using the gloved-hand technique by the same trained personnel for all boars to maintain consistency. The boars were briefly moved from their home pens to a separate area for collection. This longitudinal design with repeated sampling from the same individuals allowed us to capture true developmental changes while minimising random inter-individual variability.

Semen samples

Sperm concentration of the collected samples was assessed at the isolation unit and AI station using NucleoCounter® SP-100™ (Chemometec, Allerød, Denmark). Semen samples were diluted in Androstar® Plus

extender (Minitube, Germany) to 25 million sperm/ml and incubated at 38°C for 20 min before assessment of motility by Sperm Class Analyzer (Microptic SL, Spain). Spermatozoa were identified by head area $20\text{--}80 \mu\text{m}^2$ and the defined threshold for motility was velocity average path $>10 \mu\text{m/s}$. The samples were transported in Styrofoam boxes to the laboratory within 30 min. For spermatozoa isolation, 200 million sperm cells from each raw semen sample were centrifuged at $600 \times g$ for 10 min at room temperature, and the supernatant was discarded. Seminal plasma was separated from the raw semen sample and prepared through a two-step centrifugation protocol ($600 \times g$ at room temperature and $10,000 \times g$ at 4°C) for 10 min. Thereafter, each seminal plasma sample was carefully examined under a microscope across multiple fields of view to confirm the complete absence of spermatozoa. Both separated sperm pellet and seminal plasma were snap-frozen in liquid nitrogen and stored at -80°C until metabolomic analysis.

Untargeted metabolomics profiling

Untargeted metabolomics profiling was conducted by high-performance liquid chromatography (Agilent 1290, Agilent, Santa Clara, CA, USA) coupled to a high-resolution quadrupole time-of-flight mass spectrometer (Bruker Impact II QTOF, Bruker, Bremen, Germany).

Sample extraction was conducted by an in-house modified SIMPLEX extraction [33] for the removal of lipophilic components and isolation of a hydrophilic phase for metabolome analysis. For the seminal plasma, a sample volume of $100 \mu\text{L}$ was utilised. For the spermatozoa samples, the isolated spermatozoa pellet was redissolved in $500 \mu\text{L}$ of PBS buffer prior to sample extraction, and a volume of $100 \mu\text{L}$ was utilised in the sample work-up. To isolate the metabolites, the lipophilic components were first removed by extraction with a combination of methanol ($225 \mu\text{L}$ ice-cold methanol) and $750 \mu\text{L}$ methyl *tert*-butyl ether, added sequentially with the sample vortexed between additions. Finally, $400 \mu\text{L}$ of water was added, followed by another vortex for 20 s. After centrifugation at $14,000 \times g$ for 2 min at 10°C , the major portion of the $400 \mu\text{L}$ of the upper lipophilic phase was removed before the addition of $50 \mu\text{L}$ chloroform, followed by a 10-s vortex, causing an inversion of the lipophilic and hydrophilic phases. $100 \mu\text{L}$ of the upper hydrophilic phase was collected for analysis, evaporated to dryness, and resolubilised in $50 \mu\text{L}$ methanol prior to analysis.

Mass spectrometric data were obtained from a Bruker Impact II mass spectrometer coupled to an Agilent 1290 HPLC. All data was acquired utilising a data-dependent acquisition method with a cycle time of 0.5 s. Two chromatographic methods were utilised, one reverse-phase positive ion mode (RP-Pos) method using a Waters BEH

C18 (2.1 mm×50 mm) column and one separation in hydrophilic interaction liquid chromatography (HILIC) mode employing a Waters BEH amide (2.1 mm×50 mm) column. Data for reversed-phase mode chromatographic separation of metabolites was acquired in positive ionisation mode, while negative ionisation mode was employed for the HILIC separation. Feature extraction, initial data quality control and metabolite annotation were conducted utilising Bruker MetaboScape software. Annotation is based on a match of observed mass and fragments against the NIST metabolite database integrated in the MetaboScape software.

Annotated metabolites were grouped into chemical classes (superclass) by ClassyFire [34].

Statistical analysis

All statistical analyses of metabolites were performed using MetaboAnalyst 6.0 [35], MetaboAnalystR 4.0 [36] and R 4.4.2 [37]. Metabolite data were analysed separately for spermatozoa and seminal plasma to account for the distinct sample types.

For both the identified metabolites in spermatozoa and seminal plasma, data preprocessing before statistical analysis included normalisation, transformation, and scaling steps. The preprocessing steps were chosen based on a systematic evaluation using MetaboAnalyst 6.0, with different methods selected for each dataset to achieve optimal normalisation results. Normalisation effectiveness was evaluated using MetaboAnalyst's integrated diagnostic tools, combining visual assessment of distribution density plots and variance box plots with quantitative metrics including skewness and variance stabilisation. For the spermatozoa RP-Pos data and HILIC dataset, all features were normalised by sum, log₁₀ transformation, and autoscaling. Similarly, annotated spermatozoa metabolites from RP-Pos were normalised by log₁₀ transformation and autoscaling, whereas those from HILIC were normalised by the sum, log₁₀ transformation and mean-centering.

Likewise, all features in seminal plasma from RP-Pos were normalised by sum, log₁₀ transformed, and autoscaled, whereas HILIC data were normalised by sum, log₁₀ transformed, and Pareto-scaled. For annotated metabolites in the seminal plasma, data from both RP-Pos and HILIC were normalised to the sample median, log₁₀ transformed, and autoscaled before statistical analysis. For all discovered features, principal component analysis (PCA) was performed to assess clustering patterns, and a pairwise PERMANOVA test was conducted on PCA scores to evaluate the statistical significance of group differences. The clustering pattern of metabolites was assessed using partial least

squares discriminant analysis (PLS-DA) models. The model performance was evaluated using R^2 and Q^2 values obtained through cross-validation. Model validity was further confirmed using a permutation test (2000 permutations) based on the separation distance.

Annotated metabolites were analysed using the *limma* package in R [38]. Age groups were modelled as a fixed effect, and each boar's three measurements were treated as a block via *limma*'s *duplicateCorrelation* to estimate within-animal correlation. Family relationships were included as a fixed covariate in the design matrix. ANOVA-style contrasts (8.5 months vs. 7 months, 10 months vs. 7 months, 10 months vs. 8.5 months) were tested for overall differences across time points. Additionally, partial Spearman rank correlations were computed on annotated metabolites using the *Correlation and Partial Correlation Analysis module*. The analysis assessed monotonic associations between metabolite abundance levels and longitudinal time points (age groups), independent of boars' influence. The Benjamini–Hochberg method was applied to adjust for multiple testing, with adjusted *P*-values < 0.05 considered statistically significant. Random forest analysis was conducted with 5,000 trees and six predictors, and the model accuracy was evaluated using the out-of-bag (OOB) error rate. Annotated metabolites were ranked for their importance based on the mean decrease accuracy (MDA) value.

Sperm quality traits were handled separately in R 4.4.2 [37]. Concentration was fitted with a Gamma mixed-effects model and a log link, and motility with a binomial logit mixed-effects model, both run in *lme4* 1.1–36 [39]. Age was used as a fixed effect, individual boar and parents were used as random effects, capturing shared genetics and repeated measures. Pairwise age contrasts were extracted with *emmeans* 1.10.7 [40], and their *P*-values were adjusted with Benjamini–Hochberg method. Model fit and residual patterns were checked with *DHARMa* 0.4.7 [41] simulated residuals.

Pathway analysis

Pathway analysis was performed on all identified features in MetaboAnalyst 6.0 [35] using Mummichog version 2 with a *P*-value cutoff of 0.05. The *Sus scrofa* KEGG database was used as a reference for the pathway mapping. Pathway enrichment and topology analyses were conducted to identify significantly altered metabolic pathways, with significance determined based on pathway impact scores and pathway *P*-values, aligning with the significance threshold set at an adjusted *P*-value < 0.05.

Results

Sperm quality was slightly different between the age groups. Sperm concentration declined significantly from 7 months to 8.5 months and 10 months of age, while motility increased in the same time interval (Table 1, adjusted $P < 0.05$).

Global metabolomic profiling

A total of 4,491 features were detected across all samples in both sample types, spermatozoa and seminal plasma. Specifically, 2,410 and 2,251 features were identified using RP-Pos and HILIC methods, respectively. Of these, 92 metabolites were successfully annotated using the NIST metabolite database integrated in the MetaboScape software, of which 65 were from RP-Pos and 27 were from HILIC.

The annotated metabolites were grouped into various chemical superclasses, including organic acids and derivatives, lipids and lipid-like molecules, organic oxygen compounds, benzenoids, organoheterocyclic compounds, organic nitrogen compounds, nucleosides, nucleotides and analogues, phenylpropanoids and polyketides, alkaloids and derivatives, organometallic compounds and organosulfur compounds (Fig. 1). Amino acids, peptides and analogues ($n = 13$) were the predominant compounds in organic acids and derivatives. Likewise, fatty acid esters ($n = 10$) were the most abundant, followed by glycerophosphocholines ($n = 6$) in the lipids and lipid-like molecules superclass. Detailed information can be found in Table S1 (Additional file 1).

Multivariate assessment of semen metabolome

In both spermatozoa and seminal plasma, PCA score plots (Figs. 2a, b and 3a, b) showed intriguing metabolomic profile patterns across age groups in both the RP-Pos and HILIC datasets. The first two principal components (PC1 and PC2) explained a total of 21.3% and 17.5% of the variance in the HILIC and RP-Pos datasets for spermatozoa, respectively (Fig. 2a and b). Seminal

plasma showed comparable variance explanation, with PC1 and PC2 accounting for 16.9% and 19.3% of the variance in the HILIC dataset and RP-Pos dataset, respectively (Fig. 3a and b).

Across both sample types, 7 months displayed higher metabolomic variability compared to those from 8.5 and 10 months. PERMANOVA analysis of the PCA scores revealed significant differences in metabolomic dispersion between younger boars (7 months) and both older age groups (adjusted $P < 0.01$) for all datasets examined. The greater variability in the youngest cohort was particularly pronounced, while samples from 8.5-month and 10-month-old boars clustered more tightly.

The PLS-DA model score plot (Fig. 2c and d) showed effective separation of the three ages in spermatozoa. The RP-Pos dataset showed strong predictive capability (accuracy = 0.76, $R^2 = 0.99$, $Q^2 = 0.64$), while the HILIC dataset showed even better classification capability (accuracy = 0.84, $R^2 = 0.99$, $Q^2 = 0.80$). Seminal plasma analysis yielded similar results. The score plots from PLS-DA (Fig. 3c and d) demonstrated a distinct separation between the three ages for both LC-MS methods, with model performance indicating a strong predictive capability for the HILIC dataset (accuracy = 0.87, $R^2 = 0.99$, $Q^2 = 0.77$). For the RP-Pos dataset, it showed good predictive ability with a Q^2 of 0.62, higher classification accuracy (84%) and goodness of fit ($R^2 = 0.98$).

While PCA revealed variability differences between age groups, supervised PLS-DA modelling could effectively distinguish age-related metabolomic patterns.

Pathway analysis of metabolites in semen

Pathway analysis with all features of spermatozoa identified several pathways that differed among age groups, of which three were significantly enriched in sperm ($P < 0.05$). These included porphyrin metabolism ($P = 0.002$, enrichment factor = 6.25), taurine and hypotaurine metabolism ($P = 0.006$, enrichment factor = 12.5), and glycerolipid metabolism ($P = 0.006$, enrichment

Table 1 Sperm concentration and percentage motility in boar spermatozoa

Parameter	Age	Mean $\pm$ SD	Median	Range
Concentration, $10^6/\text{mL}$	7 months	466.80 $\pm$ 194.50 ^a	508.30	156.70–757.10
	8.5 months	316.90 $\pm$ 117.50 ^b	311.00	125.00–511.90
	10 months	352.40 $\pm$ 138.10 ^b	315.30	107.10–651.40
Motility, %	7 months	85.87 $\pm$ 9.38 ^a	89.43	55.23–92.76
	8.5 months	91.97 $\pm$ 1.59 ^b	91.98	89.87–95.31
	10 months	90.73 $\pm$ 3.22 ^b	91.37	83.92–94.63

Semen samples were collected from 15 Duroc boars at three time points: initially when the animals were approximately 7 months old, followed by subsequent collections at 8.5 and 10 months. Values represent mean $\pm$ standard deviation, with median and range provided for each parameter. Within age, values sharing a letter are not significantly different (GLMM post hoc, adjusted $P \geq 0.05$). Values with different letters differ significantly (GLMM post hoc, adjusted $P < 0.05$)

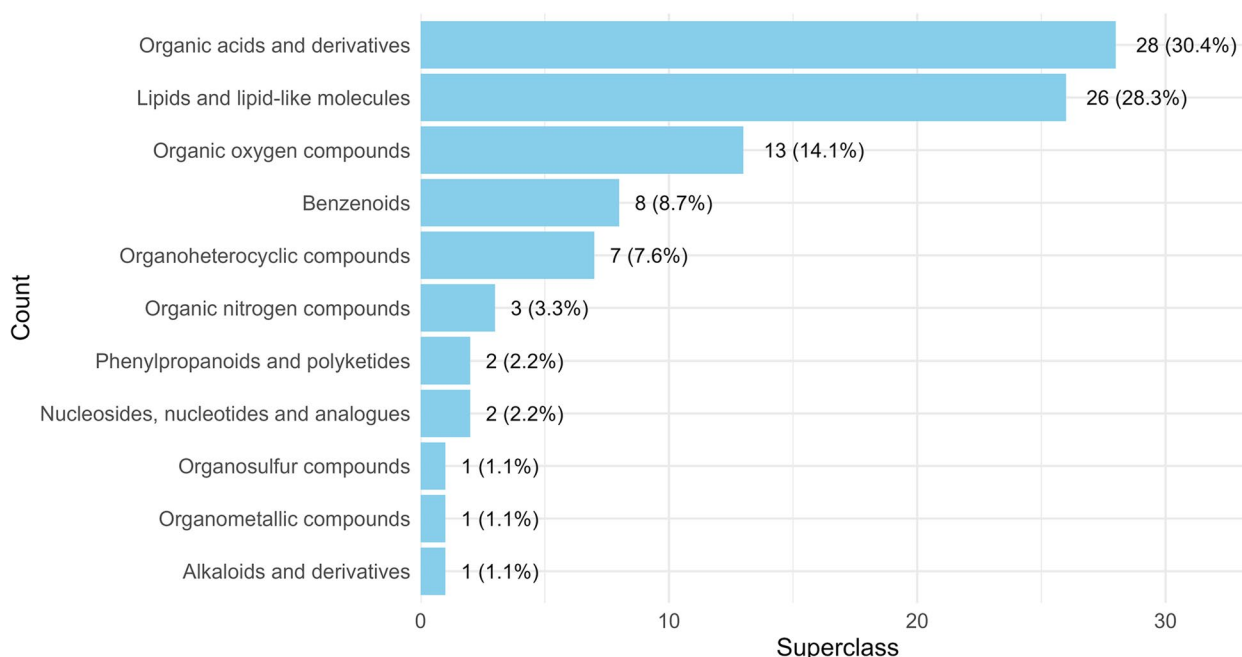


Fig. 1 Chemical superclasses of annotated metabolites in boar semen with percentage distribution. Annotated metabolites were classified into chemical superclasses based on their structural properties. The bar plot displays the percentage and absolute number of metabolites identified in each superclass. Semen samples were collected from 15 Duroc boars, and spermatozoa were isolated from the seminal plasma for metabolomic analysis. Metabolite abundances were measured using liquid chromatography-mass spectrometry (LC-MS) with HILIC and RP-Pos chromatographic methods. Data from both HILIC and RP-Pos were merged for this analysis. The same annotated metabolites were observed in both spermatozoa and seminal plasma

factor = 3.0). In seminal plasma, two pathways were significantly enriched ($P < 0.05$). These included glutathione metabolism ($P = 0.03$, enrichment factor = 2.57) and nitrogen metabolism ($P = 0.03$, enrichment factor = 2.57). Beyond these significant pathways, numerous additional pathways were also identified, showing age-related variations. The complete pathway analysis of spermatozoa and seminal plasma is detailed in Table S2 (Additional file 2) and Table S3 (Additional file 3), respectively.

Identification of key metabolites

In spermatozoa, the longitudinal analysis of metabolite intensity levels across boar sexual development revealed eight annotated metabolites with significant changes across ages (Fig. 4 and Table 2). L-Glutamic acid and decanoyl-L-carnitine showed the pronounced statistical significance, with consistent decreases at both 8.5 months and 10 months compared to 7 months (adjusted $P < 0.05$). Octanoylcarnitine also showed a decrease at 10 months (adjusted $P < 0.05$). Among the phospholipid species, both PC(O-16:0/22:6) and N-(octadecanoyl)sphing-4-enine-1-phosphocholine showed significant decreases at 10 months compared to 7 months (adjusted $P < 0.05$).

Several metabolites showed an abundance increase with age. Homoisovanillic acid and N-(1,3-Thiazol-2-yl) benzenesulfonamide abundance increased at 10 months, while succinic acid displayed increased abundance at 8.5 months compared to 7 months (adjusted $P < 0.05$).

Similarly, the longitudinal analysis of boar seminal plasma identified 17 annotated metabolites with significant temporal abundance changes across three ages ($P < 0.05$, Fig. 5, Table 3). 1-Formylpyrrolidine-2-carboxylic acid and myo-inositol exhibited the most statistically significant changes (adjusted $P = 1.81 \times 10^{-4}$), showing increased abundance at 10 months compared to 7 months. The abundance of 1-formylpyrrolidine-2-carboxylic acid also increased at 8.5 months (adjusted $P < 0.05$). Homoisovanillic acid likewise displayed elevated abundance at 10 months in comparison to both 8.5 months and 7 months (adjusted $P < 0.05$). Several metabolites showed an increase in abundance at 10 months relative to 7 months (adjusted $P < 0.05$), including 7-hydroxychromanone, oleamide, isobutyrylphloroglucinol, 4-O- β -galactopyranosyl-D-mannopyranose, and oleoyl ethylamide.

Several metabolites exhibited a decrease in abundance at 10 months compared to 7 months (adjusted $P < 0.05$) in seminal plasma, including glycerophosphocholine,

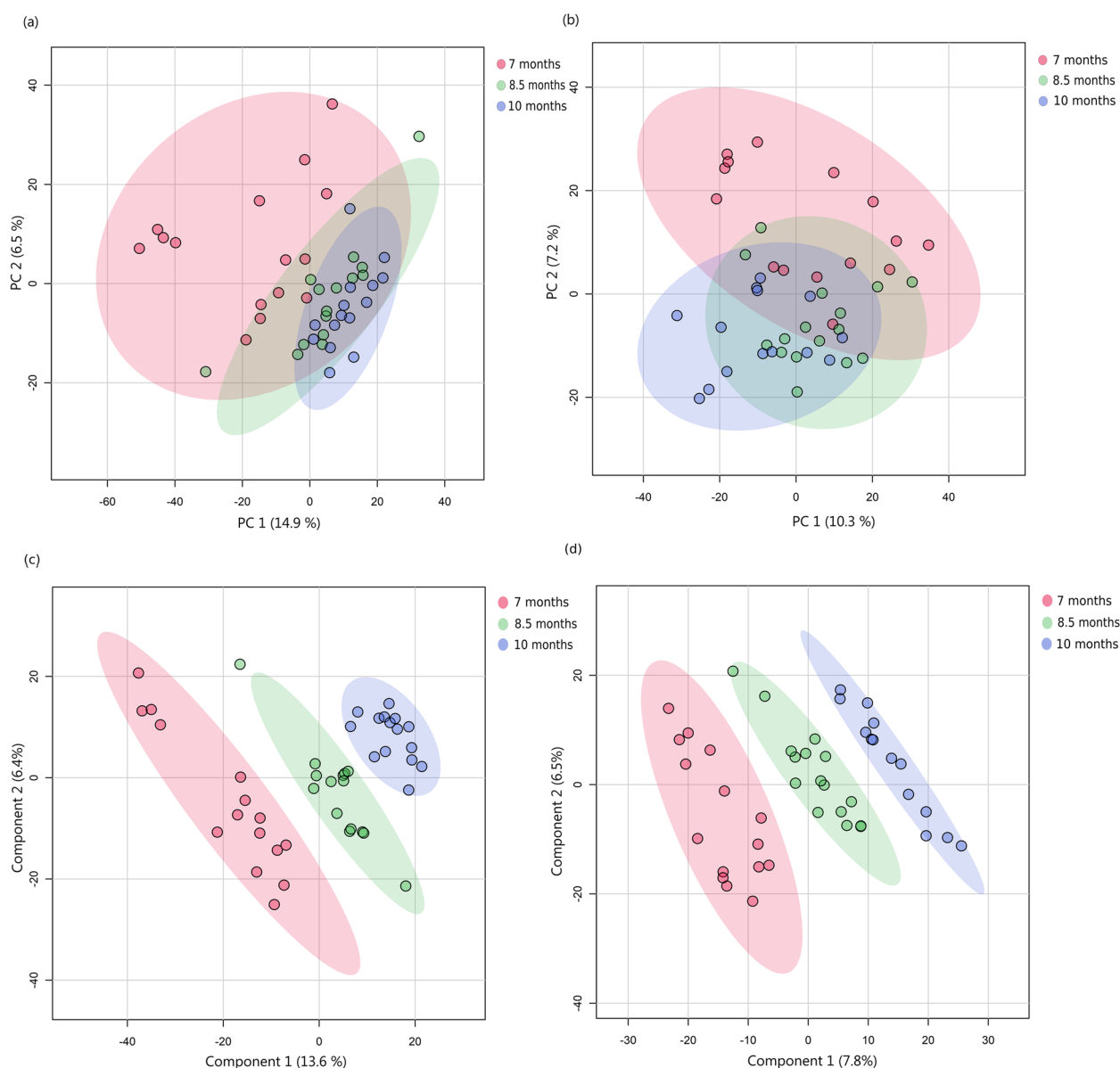


Fig. 2 Score plots from global metabolomic profiling of boar sperm across three ages. Principal Component Analysis (PCA) score plots: **(a)** based on HILIC method and **(b)** based on RP-Pos method, showed metabolomic profiles with greater variability in 7-month-old boars, while Partial Least Squares Discriminant Analysis (PLS-DA) score plots: **(c)** based on HILIC method and **(d)** based on RP-Pos method showed effective classification of age groups. Semen samples were collected from 15 Duroc boars at three time points: initially when the animals were approximately 7 months old, followed by subsequent collections at 8.5 and 10 months. Spermatozoa were isolated from the seminal plasma, and metabolite abundances were measured using liquid chromatography-mass spectrometry (LC-MS) with HILIC and RP-Pos methods

glyceric acid, and 3-indoleacetic acid. Glycerophosphocholine and 3-indoleacetic acid also demonstrated reduced abundance at 8.5 months relative to 7 months. Similarly, N-(octadecanoyl)sphing-4-enine-1-phosphocholine showed a decrease at 8.5 months in comparison to 7 months.

Correlation of semen metabolites with age

Partial Spearman correlation analysis, adjusting for boar variability, confirmed strong relationships between age and four annotated metabolites in spermatozoa (Additional file 4: Table S4): L-glutamic acid ($\rho = -0.58$, FDR = 0.0009), decanoyl-L-carnitine ($\rho = -0.45$, FDR = 0.035),

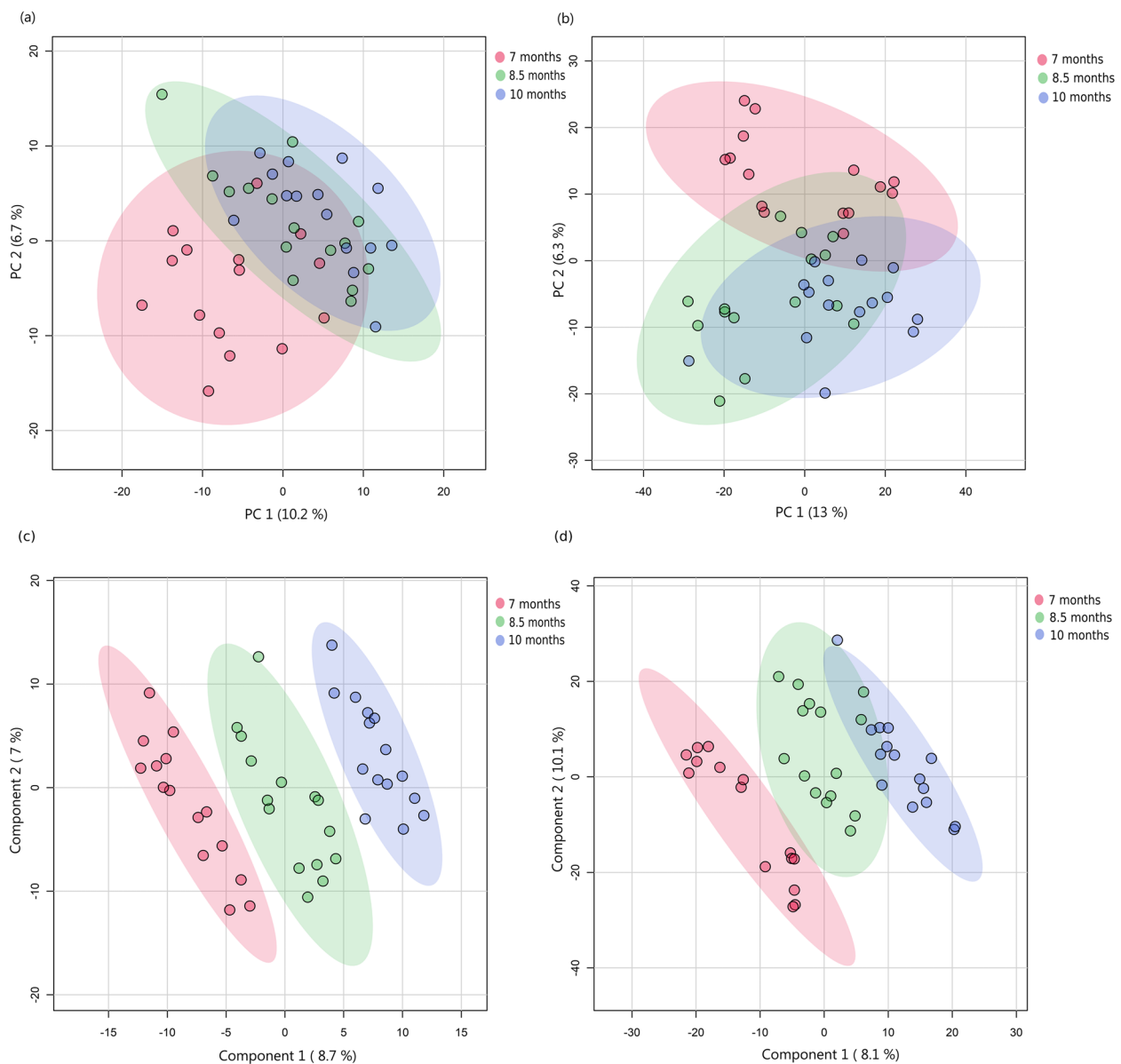


Fig. 3 Score plots from global metabolomic profiling of boar seminal plasma across three ages. Principal Component Analysis (PCA) score plots: **(a)** based on HILIC method and **(b)** based on RP-Pos method, showed metabolomic profiles with greater variability in 7-month-old boars, while Partial Least Squares Discriminant Analysis (PLS-DA) score plots: **(c)** based on HILIC method and **(d)** based on RP-Pos method showed effective classification of age groups. Semen samples were collected from 15 Duroc boars at three time points: initially when the animals were approximately 7 months old, followed by subsequent collections at 8.5 and 10 months. Seminal plasma was separated from spermatozoa, and metabolite abundances were measured using liquid chromatography-mass spectrometry (LC-MS) with HILIC and RP-Pos methods

PC(O-16:0/22:6) ($\rho = -0.41$, $\text{FDR} = 0.044$) and N-(1,3-Thiazol-2-yl)benzenesulfonamide ($\rho = 0.57$, $\text{FDR} = 0.0009$). These aligned with linear model trends (e.g., L-glutamic acid's abundance decreases in older ages of boars, whereas N-(1,3-Thiazol-2-yl)benzenesulfonamide's abundance increases with age). Glycerophosphocholine and octanoylcarnitine showed negative correlation with age ($\rho = -0.43$, $\text{FDR} = 0.035$), and

DL-indole-3-lactic acid showed positive correlation with age ($\rho = 0.43$, $\text{FDR} = 0.035$).

Likewise, in seminal plasma, the partial correlation analysis confirmed temporal associations for 12 annotated metabolites in seminal plasma ($\text{FDR} < 0.05$, Additional file 5: Table S5). Among these, homoisovanillic acid, 1-formylpyrrolidine-2-carboxylic acid, myo-inositol, 7-hydroxychromanone, oleamide, isobutyrylphloroglucinol,

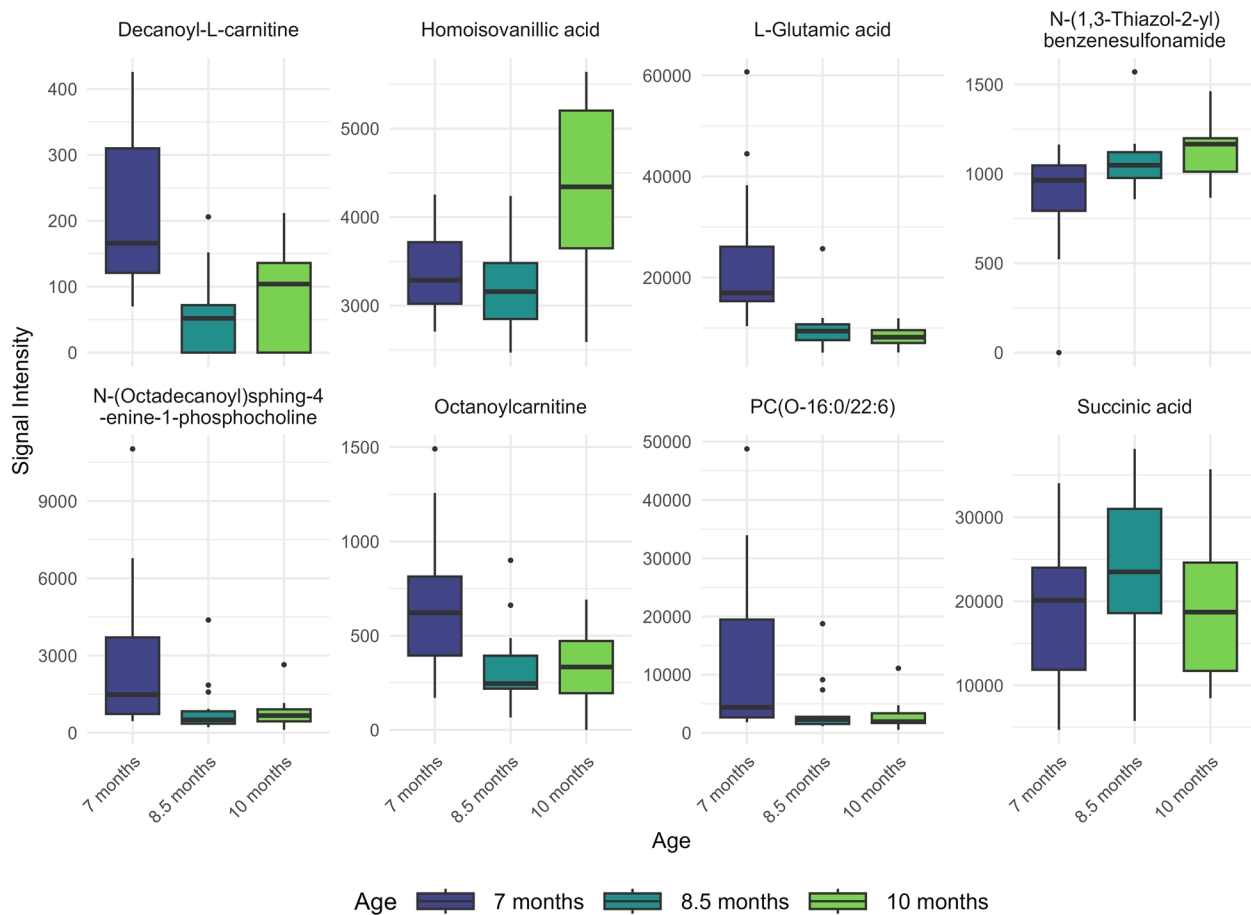


Fig. 4 Longitudinal changes in boar sperm metabolite abundance during sexual maturation. Box plots display the distribution of eight significant metabolites across three ages in boar spermatozoa. The x-axis denotes age groups as 7 months, 8.5 months and 10 months, while the y-axis indicates the abundance of each metabolite. Semen samples were collected from 15 Duroc boars at three time points: initially when the animals were approximately 7 months old, followed by subsequent collections at 8.5 and 10 months. Spermatozoa were isolated from the seminal plasma, and metabolite abundances were measured using liquid chromatography-mass spectrometry (LC-MS) with HILIC and RP-Pos methods

4-O- β -galactopyranosyl-D-mannopyranose and oleoyl ethylamide showed significant positive correlation with age ($\rho=0.42$ – 0.58 , $FDR<0.05$), aligning with their progressive accumulation in linear models. Glycerophosphocholine, glyceric acid and 3-indoleacetic acid showed significant negative correlation ($\rho=-0.40$ to -0.53 , $FDR<0.05$), consistent with sustained suppression in linear models, suggesting a decrease in abundance with increasing age. DL-indole-3-lactic acid emerged exclusively in correlation analysis with gradual reduction in abundance ($\rho=-0.39$, $FDR<0.05$) with age.

Ranking of semen metabolites

In spermatozoa, random forest analysis with an average OOB error of 0.43 ranked L-glutamic acid ($MDA=0.048$), homoisovanillic acid ($MDA=0.025$), N-(1,3-Thiazol-2-yl)-benzenesulfonamide ($MDA=0.019$), DL-indole-3-lactic acid ($MDA=0.014$) and decanoyl-L-carnitine

($MDA=0.013$) as the top five contributors (Fig. 6, Additional file 6: Table S6) to the predictive accuracy of the age group categories.

Similarly, in seminal plasma, random forest analysis with an average OOB error of 0.47 ranked homoisovanillic acid ($MDA=0.064$), 1-formylpyrrolidine-2-carboxylic acid ($MDA=0.025$), glyceric acid ($MDA=0.025$), myo-inositol ($MDA=0.020$) and N-(octadecanoyl)sphing-4-enine-1-phosphocholine ($MDA=0.017$) as the top five contributors (Fig. 7) to the predictive accuracy of the age group categories (Additional file 7: Table S7).

Discussion

This study represents, to our knowledge, the first comprehensive untargeted metabolomic investigation of boar spermatozoa and seminal plasma across three distinct maturation timepoints. Following the boars longitudinally from puberty through sexual maturity, we have

Table 2 List of annotated metabolites with significant abundance changes in boar spermatozoa across ages

Metabolite	Contrast	logFC	Fold change	SE	t-statistic	Adjusted P value
L-Glutamic acid	10 M vs. 7 M	-0.24	1.18	0.04	-5.75	4.11×10^{-5}
L-Glutamic acid	8.5 M vs. 7 M	-0.25	1.19	0.04	-5.89	2.66×10^{-5}
Decanoyl-L-carnitine	10 M vs. 7 M	-0.97	1.96	0.34	-2.86	3.33×10^{-2}
Decanoyl-L-carnitine	8.5 M vs. 7 M	-1.31	2.48	0.34	-3.84	4.96×10^{-3}
PC(O-16:0/22:6)	10 M vs. 7 M	-1.10	2.14	0.34	-3.22	2.84×10^{-2}
Octanoylcarnitine	10 M vs. 7 M	-0.98	1.97	0.34	-2.89	3.33×10^{-2}
N-(Octadecanoyl)sphing-4-enine-1-phosphocholine	10 M vs. 7 M	-1.02	2.03	0.34	-3.01	3.33×10^{-2}
Succinic acid	8.5 M vs. 7 M	0.19	1.14	0.06	3.27	3.23×10^{-2}
N-(1,3-Thiazol-2-yl)-benzenesulfonamide	10 M vs. 7 M	0.30	1.23	0.07	4.13	2.78×10^{-3}
Homoisovanillic acid	10 M vs. 7 M	1.08	2.11	0.34	3.19	2.84×10^{-2}
Homoisovanillic acid	10 M vs. 8.5 M	1.33	2.52	0.34	3.91	3.56×10^{-3}

Fold changes are calculated as 2^{logFC} . Ages correspond to 7 months (7 M), 8.5 months (8.5 M), and 10 months (10 M). SE stands for standard error. Semen samples were obtained from 15 Duroc boars at three time points: initially when the animals were approximately 7 months, followed by subsequent collections at 8.5 and 10 months. Spermatozoa were separated from seminal plasma, and liquid chromatography-mass spectrometry (LC-MS) with HILIC and RP-Pos methods was used to measure metabolite abundances in spermatozoa. Metabolite differences were assessed using linear modelling with individual boar blocking and family relationship covariates

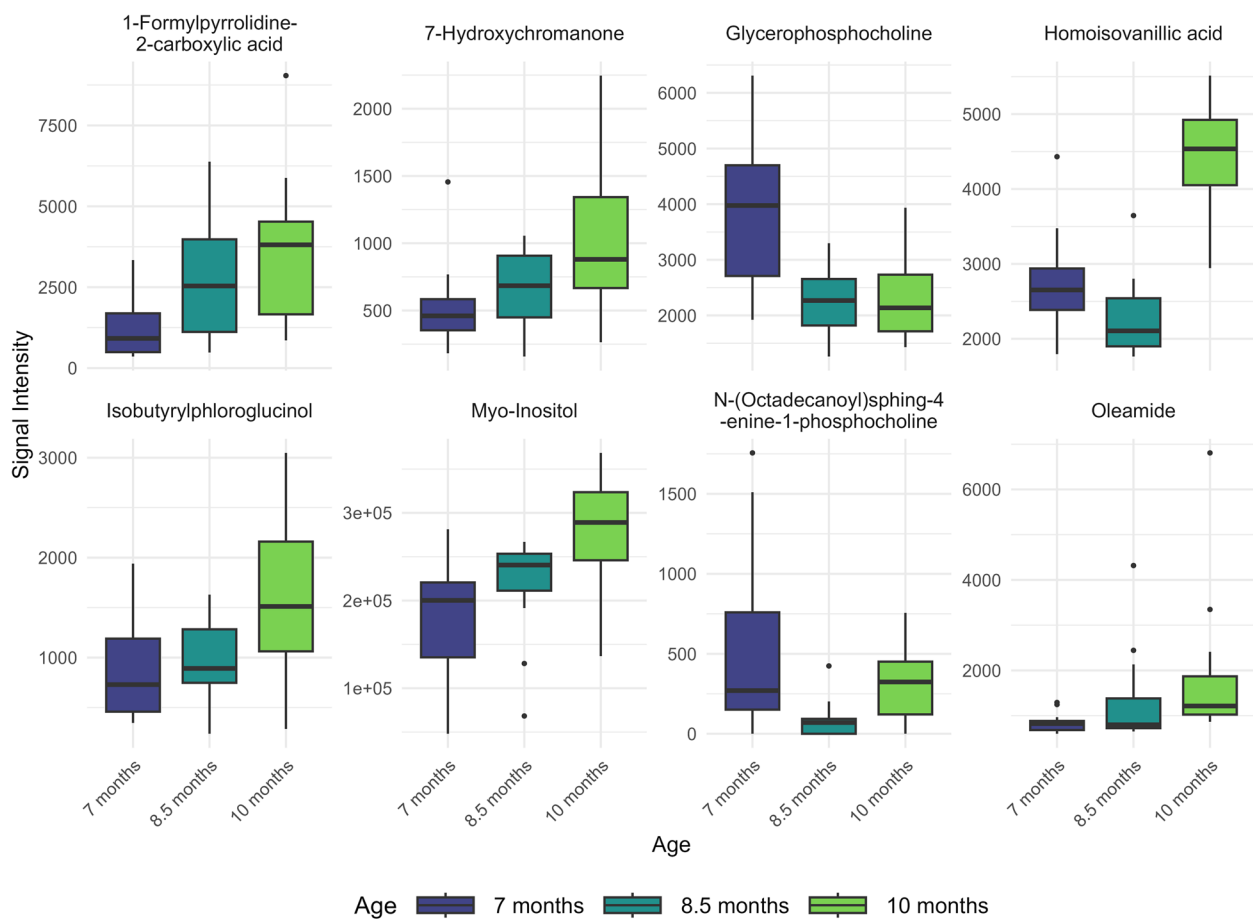


Fig. 5 Longitudinal changes in boar seminal plasma metabolite abundance during sexual maturation. Box plots display the distribution of key metabolites across three ages in boar seminal plasma. The x-axis denotes age groups as 7 months, 8.5 months and 10 months, while the y-axis indicates the abundance of each metabolite. Semen samples were collected from 15 Duroc boars at three time points: initially when the animals were approximately 7 months old, followed by subsequent collections at 8.5 and 10 months. Seminal plasma was separated from spermatozoa, and metabolite abundances were measured using liquid chromatography-mass spectrometry (LC-MS) with HILIC and RP-Pos methods

expanded our understanding of the metabolic landscape that highlights the physiological transformations underlying sexual maturity.

The annotated metabolite profile in boar semen was predominantly comprised of organic acids and derivatives, followed by lipids and lipid-like molecules, with amino acids and fatty acids dominating the composition. This compositional distribution aligns with earlier studies in bull sperm and seminal plasma [26, 30] and extends our previous findings on the amino acid and amine composition in boar semen [32]. These results support the established understanding of mammalian semen composition and its essential roles in sperm physiology as described by Brown-Woodman et al. [42]. For bulls, it has been reported that variations in fatty acid profiles, including myristic acid, polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA), omega-3 fatty acids, and cholesterol, correlate with

differences in sperm motility, morphology, and concentration evident in comparative studies of young versus mature bulls [43].

Our multivariate analyses encompassing all detected features revealed maturation trajectories in the metabolomic landscape. The increased metabolomic variability observed in 7-month-old boars likely reflects the transitional metabolic state during maturation, where individual boars may be at different stages of sexual maturation. The ability of PLS-DA to distinguish between the youngest cohort and the two mature groups in both spermatozoa and seminal plasma suggests that there is a shift in metabolic profile during the transition from puberty to sexual maturity, even across the relatively modest six-week intervals examined in our study. These findings complement and extend our previous observations of age-dependent clustering in amino acid and amine profiles [32].

Table 3 List of annotated metabolites with significant abundance changes in boar seminal plasma across ages

Metabolite	Contrast	logFC	Fold change	SE	t-statistic	Adjusted P value
Glycerophosphocholine	10 M vs. 7 M	-1.31	2.48	0.30	-4.29	4.83×10^{-4}
Glycerophosphocholine	8.5 M vs. 7 M	-0.96	1.94	0.30	-3.14	2.42×10^{-2}
N-(Octadecanoyl)sphing-4-enine-1-phosphocholine	8.5 M vs. 7 M	-1.13	2.19	0.31	-3.65	1.23×10^{-2}
Glyceric acid	10 M vs. 7 M	-1.06	2.09	0.32	-3.34	5.91×10^{-3}
3-Indoleacetic acid	10 M vs. 7 M	-0.95	1.93	0.31	-3.05	1.42×10^{-2}
3-Indoleacetic acid	8.5 M vs. 7 M	-0.93	1.91	0.31	-3.01	2.81×10^{-2}
3'-Galactosyllactose	10 M vs. 7 M	-0.88	1.84	0.32	-2.76	3.17×10^{-2}
4,4,7a-Trimethyl-3a,5,6,7-tetrahydro-3H-indene-1-carboxylic acid	10 M vs. 7 M	-0.88	1.84	0.31	-2.80	2.69×10^{-2}
1-Formylpyrrolidine-2-carboxylic acid	10 M vs. 7 M	1.41	2.66	0.32	4.44	1.81×10^{-4}
Myo-inositol	10 M vs. 7 M	1.39	2.62	0.32	4.38	1.81×10^{-4}
Homoisovanillic acid	10 M vs. 7 M	1.38	2.60	0.30	4.58	2.8×10^{-4}
Homoisovanillic acid	10 M vs. 8.5 M	1.35	2.55	0.30	4.48	4.28×10^{-4}
7-Hydroxychromanone	10 M vs. 7 M	1.20	2.30	0.31	3.87	1.78×10^{-3}
Oleamide	10 M vs. 7 M	1.15	2.21	0.31	3.71	2.41×10^{-3}
Isobutyrylphloroglucinol	10 M vs. 7 M	1.12	2.17	0.31	3.59	3.07×10^{-3}
4-O-β-Galactopyranosyl-D-mannopyranose	10 M vs. 7 M	1.11	2.16	0.32	3.50	4.41×10^{-3}
1-Formylpyrrolidine-2-carboxylic acid	8.5 M vs. 7 M	1.10	2.14	0.32	3.45	1.57×10^{-2}
Oleoyl ethylamide	10 M vs. 7 M	1.04	2.05	0.32	3.28	7.75×10^{-3}
5'-S-Methyl-5'-thioadenosine	8.5 M vs. 7 M	0.99	1.98	0.31	3.19	2.42×10^{-2}
Methanesulfonic acid	10 M vs. 7 M	0.82	1.76	0.31	2.61	4.1×10^{-2}

Fold changes are calculated as $2^{\logFC}$. Ages correspond to 7 months (7 M), 8.5 months (8.5 M), and 10 months (10 M). SE stands for standard error. Semen samples were obtained from 15 Duroc boars at three time points: initially when the animals were approximately 7 months, followed by subsequent collections at 8.5 and 10 months. Seminal plasma was separated from spermatozoa, and liquid chromatography-mass spectrometry (LC-MS) with HILIC and RP-Pos methods was used to measure metabolite abundances in seminal plasma. Metabolite differences were assessed using linear modelling with individual boar blocking and family relationship covariates

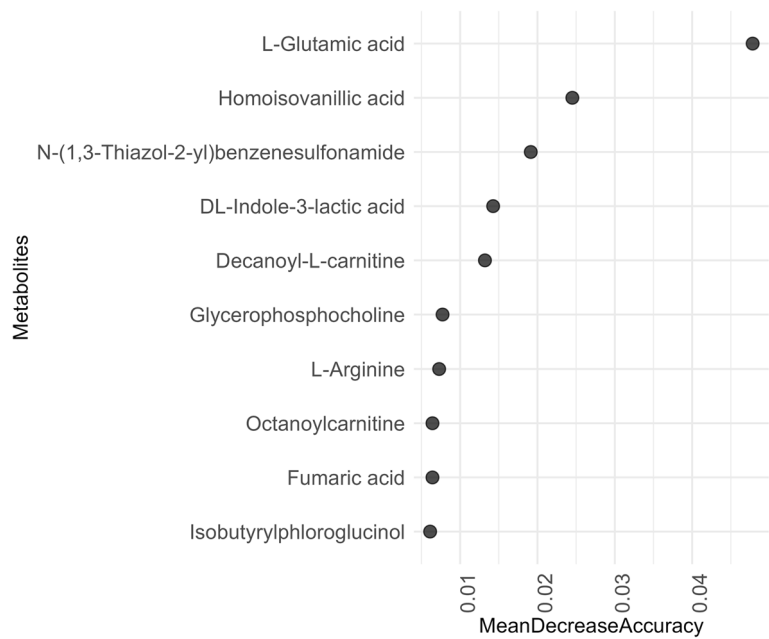


Fig. 6 Top 10 metabolites distinguishing age groups in boar spermatozoa. Metabolites are ranked by mean decrease accuracy (x-axis) from random forest analysis modelling. The plot shows metabolites on the y-axis listed in descending order of their importance for age group classification. Semen samples were collected from 15 Duroc boars at three time points: initially when the animals were approximately 7 months old, followed by subsequent collections at 8.5 and 10 months. Spermatozoa were isolated from the seminal plasma, and metabolite abundances were measured using liquid chromatography-mass spectrometry (LC–MS) with HILIC and RP-Pos methods

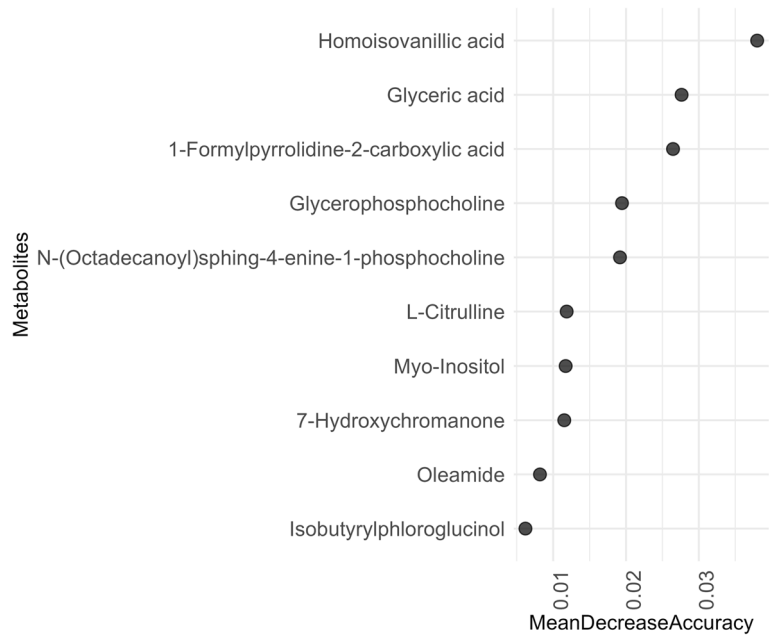


Fig. 7 Top 10 metabolites distinguishing age groups in boar seminal plasma. Metabolites are ranked by mean decrease accuracy (x-axis) from random forest analysis modelling. The plot shows metabolites on the y-axis listed in descending order of their importance for age group classification. Semen samples were collected from 15 Duroc boars at three time points: initially when the animals were approximately 7 months old, followed by subsequent collections at 8.5 and 10 months. Seminal plasma was separated from spermatozoa, and metabolite abundances were measured using liquid chromatography-mass spectrometry (LC–MS) with HILIC and RP-Pos methods

Pathway analysis in this study revealed three key metabolic pathways in spermatozoa underlying sexual maturation: porphyrin metabolism, taurine and hypotaurine metabolism, and glycerolipid metabolism. Porphyrin metabolism is primarily associated with heme biosynthesis, supporting cellular respiration and redox regulation, which are vital during sperm maturation due to high energy demands associated with motility [44].

The enrichment of taurine and hypotaurine metabolism pathways in our dataset aligns with their known abundance in male reproductive tissues, including testis and epididymis [45]. These compounds are particularly concentrated in the epididymis, where sperm maturation occurs [46]. Hypotaurine effectively scavenges reactive oxygen species (ROS), thus preventing lipid peroxidation and preserving membrane integrity, as shown in rabbit spermatozoa [47] and chicken sperm during cryopreservation [48]. It can also maintain mitochondrial integrity, inhibit DNA fragmentation, and promote spermatogenesis through effects on the hypothalamus-pituitary-testis axis [45, 48]. In taurine-supplemented boar semen during storage, it is reported that taurine improves motility and viability [49].

The glycerolipid metabolism pathways are involved in maintaining membrane integrity. Polyunsaturated fatty acids (PUFAs), particularly DHA, maintain membrane fluidity and support motility in boar sperm [50]. In mice, lipid remodelling in the epididymis, characterised by shifts in phosphatidylcholine (PC) acyl groups to DHA and docosapentaenoic acid, significantly enhances sperm motility and fertility [51], processes important for sperm maturation.

Likewise, pathway analysis of our seminal plasma data revealed significant enrichment of glutathione metabolism and nitrogen metabolism pathways during maturation. Glutathione and its associated enzymes, such as glutathione peroxidase (GPx), provide dual protection by scavenging H_2O_2 and regenerating oxidised molecules, thereby preserving membrane integrity and sperm motility [52, 53]. Moreover, the enrichment of nitrogen metabolism pathways in our seminal plasma analysis highlights the importance of nitrogen-containing metabolites such as arginine, proline, and citrulline, which contribute to nitric oxide production, ammonia detoxification, osmotic regulation, and antioxidant defence. These functions are critical for sperm survival under stress conditions [54]. Although direct evidence linking nitrogen metabolism to sexual maturation remains limited, the involvement of these metabolites provides an interesting basis for further exploration.

Homoisovanillic acid, a breakdown product of dopamine, emerged as a significant metabolite in both

spermatozoa and seminal plasma in our study, suggesting fundamental roles in sexual maturity. It has been reported that dopamine activates male sexual behaviour, even in the absence of estrogen receptor α [55], governing both anticipatory (motivation) and consummatory (erection and copulation) phases of sexual behaviour [56]. A structurally related phenolic compound, vanillic acid, has been well documented for its antioxidative properties, such as scavenging free radicals, inhibiting lipid peroxidation, and activation of endogenous antioxidant enzymes, superoxide dismutase and catalase [57, 58]. This protective effect could be especially relevant in the epididymal environment, maintained by V-ATPase and other acid/base transporters, where maintaining the acidic environment is integral for sperm maturation [59, 60].

L-Glutamic acid declined with maturation in this study, consistent with our previous findings [32] and its reported roles in modulating sperm motility, chemotaxis, acrosome reaction, sperm development, and mitochondrial regulation [61–65]. Mechanistically, glutamate binds to N-methyl-D-aspartate receptors, causing calcium influx and magnesium release, which are vital for sperm motility [63] and the acrosome reaction required for oocyte penetration [64, 65]. Glutamate also enhances antioxidant defences, regulates inflammatory responses, and promotes spermatogonial cell proliferation [66, 67].

Decanoyl-L-carnitine, formed through esterification of L-carnitine with decanoic acid [68], was another important metabolite identified in boar sperm by this study. We observed declining decanoyl-L-carnitine abundance with age, suggesting regulatory adjustments in fatty acid metabolism during this critical maturation window. It has been reported that L-carnitine improves progressive and total sperm motility in Duroc boars [69] and Simmental bulls [70], while protecting maturing sperm from oxidative stress by boosting antioxidant enzyme activities. In addition, it supports energy metabolism by generating ATP via transport of long-chain fatty acids into mitochondria for β -oxidation [71, 72]. N-(1,3-Thiazol-2-yl) benzenesulfonamide, a sulfathiazole derivative belonging to the sulfonamide class [73], showed increasing abundance with age in our study. The involvement of sulfonamide-based metabolites in diverse biological activities, including modulation of oxidative stress and cellular signalling pathways [74, 75], highlights their possible influence on spermatogenesis and sperm maturation, which warrants further investigation.

Our seminal plasma metabolome analyses revealed distinctive age-dependent signatures. 1-Formylpyrroline-2-carboxylic acid showed a significant elevation in mature boars (10 months) in our study, aligning with its known antioxidant properties and effects on cellular redox states [76, 77], suggesting a protective role during sperm

maturation. Similarly, 7-hydroxychromanone accumulated steadily with advancing age, bringing potent ROS scavenging and lipid peroxidation inhibition that likely preserve sperm viability, motility and DNA integrity [78, 79]. Oleamide showed a similar increasing abundance in our study, most likely contributing to anti-inflammatory and antioxidant functions [80, 81]. This aligns with the broader role of fatty acid derivatives in spermatogenesis, sperm maturation and improving sperm quality, as highlighted in previous studies [82].

Myo-inositol, a naturally occurring cyclohexane hexol belonging to the vitamin B complex (B8), had an increasing abundance as boars mature in our study. It is interesting to note that previous studies have reported an association of myo-inositol with increased sperm motility, acrosome integrity, testosterone levels, maintenance of DNA integrity of sperm and fertilisation ability [83, 84]. It is noteworthy that motility also increased with age in our study, which implies role of myo-inositol in motility regulation. Its regulatory effect, earlier reported on intracellular calcium levels, further supports mitochondrial function and ATP production, essential for sperm capacitation and maturation [85, 86]. Glycerophosphocholine is predominantly synthesised in the epididymis under androgenic control, and glyceric acid displayed a decline with advancing age in our analysis. Glycerophosphocholine's roles in osmoregulation and membrane composition [87, 88] combined with glyceric acid's involvement in glycolysis [89] and mitochondrial metabolism activation and reduction of systemic inflammation in humans [90] suggest their decline represents optimisation of conditions for maturing spermatozoa. N-(Octadecanoyl) sphing-4-enine-1-phosphocholine, a sphingolipid component of sphingomyelin, showed a significant decline at 8.5 months compared to 7 months. Sphingomyelins contribute to membrane stability and fluidity, supporting critical functions including lipid raft formation and signal transduction essential for capacitation and the acrosome reaction [91, 92]. Their metabolism generates bioactive compounds like ceramides and sphingosine-1-phosphate that regulate apoptosis, lipid remodelling, and gametogenesis, helping maintain membrane integrity and enabling the structural changes necessary for fertilisation [91, 93]. Additionally, sphingolipids facilitate cholesterol efflux, enhancing membrane fluidity and protein interactions critical for maturation [92], while contributing antioxidant protection against oxidative damage [91].

In our study, we selected three age timepoints: 7 months (puberty with onset of mounting behaviour and first ejaculates), 8.5 months (the industry standard for commercial semen collection), and 10 months (sexual maturity with consistent sperm production). This

6-week interval design effectively captured the metabolic changes during this critical puberty to sexual maturation window. The longitudinal design following 15 Duroc boars eliminated genetic variability and captured true metabolic changes with sexual maturation rather than inter-individual differences. However, our modest sample size and single-breed focus limit broader applicability to other genetic lines. In addition, the challenge of metabolite annotation persisted, leaving a considerable number of features (over 4,300) unidentified, potentially concealing valuable biological insights that could prove useful in future studies. Improved metabolite annotation would help unlock this hidden information, providing a deeper understanding of the underlying biological processes.

Our comprehensive metabolomics investigation has revealed complex metabolic changes in boar spermatozoa and seminal plasma during the transition from puberty to sexual maturity. The identified metabolites and pathways contributing to membrane integrity, oxidative protection, and energy metabolism provide fundamental insights into reproductive development. This foundational characterisation enables future development of biomarker-based screening protocols for the early identification of younger boars ready for breeding, reducing generation intervals and accelerating genetic progress in commercial operations. Our findings also provide a knowledge base for developing targeted nutritional interventions to potentially shift metabolite concentrations in young males toward a mature metabolic profile. Furthermore, these metabolites can serve as candidates for semen extender supplementation for young males.

However, validation in larger, more diverse populations will be essential, along with targeted analyses including focused lipidomics and functional assays to clarify the biological roles of these metabolites. Additionally, investigating how these metabolic signatures correlate with fertility outcomes will strengthen their practical relevance to reproductive biotechnology and commercial pig breeding programs.

Conclusions

Our results reveal subtle yet important shifts in the metabolomic composition in boar sperm and seminal plasma as they grow from puberty to sexual maturity. We identified numerous metabolites and pathways important in distinguishing these age groups during sexual maturation. This insight offers a valuable understanding of the evolving metabolome, which can serve as the foundation for future research and potential applications in the field of reproductive biology, especially for pig breeding companies interested in incorporating younger animals in the breeding program.

Abbreviations

AI	Artificial insemination
DHA	Docosahexanoic acid
FDR	False discovery rate
HILIC	Hydrophilic interaction liquid chromatography
LC–MS	Liquid chromatography–mass spectrometry
MDA	Mean decrease accuracy
OOB	Out-of-bag
PC	Principal component
PCA	Principal component analysis
PERMANOVA	Permutational multivariate analysis of variance
PLS-DA	Partial least squares discriminant analysis
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
RP-Pos	Reverse-phase (positive ionisation mode)

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40104-025-01258-x>.

Additional file 1: Table S1: Overview of annotated metabolites in boar semen, including their respective numbers with their chemical classes and superclasses. Note: The metabolites are extracted using global metabolomics with LC–MS platform, including HILIC and RP-Pos methods.

Additional file 2: Table S2: Pathway enrichment analysis results of all features in boar spermatozoa. Note: The table includes pathway's name, total hits, significant hits, expected values, Fisher's exact test *P*-values, and enrichment factors.

Additional file 3: Table S3: Pathway enrichment analysis results of all features in boar seminal plasma. Note: The table includes pathway's name, total hits, significant hits, expected values, Fisher's exact test *P*-values, and enrichment factors.

Additional file 4: Table S4: Partial Spearman correlation analysis of annotated metabolites with age in boar spermatozoa. Note: The table shows partial Spearman correlation coefficients, *t*-statistics, and false discovery rate adjustments.

Additional file 5: Table S5: Partial Spearman correlation analysis of annotated metabolites with age in boar seminal plasma. Note: The table shows Spearman correlation coefficients, *t*-statistics, and false discovery rate adjustments.

Additional file 6: Table S6: Mean decrease accuracy values of annotated metabolites in boar spermatozoa. Note: It is generated from a random forest analysis.

Additional file 7: Table S7: Mean decrease accuracy values of annotated metabolites in boar seminal plasma. Note: It is generated from a random forest analysis.

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Authors' contributions

AS conceptualised the study, designed the methodology, collected the samples, pre-processed samples for LC–MS analysis, performed analysis and the interpretation of data, created visualisation, and was a major contributor in writing the original draft and reviewing and editing the manuscript. AHG contributed to the conceptualisation, provided samples, and was involved in reviewing and editing the manuscript. JBØ was responsible for the LC–MS analysis. AN and TH contributed to the LC–MS analysis, data curation, and manuscript review and editing. EK contributed to the conceptualisation and acquisition of funding for the project, as well as reviewing and editing the manuscript. MS contributed to reviewing and editing the manuscript. AHAK conceptualised the study, provided resources, supervised the project, and contributed to writing, reviewing, and editing the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study did not require ethical approval, as the boars were part of standard semen production practices, conducted per Norwegian legislation, including the Animal Protection Act (December 20, 1974), the Animal Welfare Act (June 19, 2009), the Regulations for Management of Pigs in Norway (February 18, 2003), along with the European Union legislation on semen production for pigs (Council Directive 90/429/EEC).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest.

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References

- Paixão G, Esteves A, Carolino N, Dos Anjos PM, Payan-Carreira R. Evaluation of gonadal macroscopic and microscopic morphometry reveals precocious puberty in Bísaro pig. *Reprod Domest Anim*. 2020;55(12):1706–13.
- Andersson HK, Hullberg A, Malmgren L, Lundström K, Rydhmer L, Squires J. Sexual maturity in entire male pigs: environmental effects, relations to skatole level and female puberty. *Acta Agric Scand A Anim Sci*. 1999;49(2):103–12.
- Ding H, Luo Y, Liu M, Huang J, Xu D. Histological and transcriptome analyses of testes from Duroc and Meishan boars. *Sci Rep*. 2016;6:20758.
- Singleton WL, Flowers WL. Management of the boar for natural service [PIG 08–02–02]. Ames (IA): U.S. Pork Center of Excellence; 2006. Available from: <https://porkgateway.org/wp-content/uploads/2015/07/management-of-the-boar1.pdf>.
- Lervik S, Oskam I, Krogenæs A, Andresen Ø, Dahl E, Haga HA, et al. Androstenedione and testosterone levels and testicular morphology of Duroc boars related to estimated breeding value for androstenedione. *Theriogenology*. 2013;79(6):986–94.
- Wang C, Li JL, Wei HK, Zhou YF, Tan JJ, Sun HQ, et al. Linear growth model analysis of factors affecting boar semen characteristics in Southern China. *J Anim Sci*. 2017;95(12):5339–46.
- Knecht D, Jankowska-Mąkosa A, Duziński K. The effect of age, interval collection and season on selected semen parameters and prediction of AI boars productivity. *Livest Sci*. 2017;201:13–21.
- Zhang B, Yan Z, Gao Y, Li J, Wang Z, Wang P, et al. Integrated analysis of miRNA and mRNA expression profiles in testes of Landrace and Hezuo boars. *Front Vet Sci*. 2022;9:942669.
- Kondracki S, Iwanina M, Wysokińska A, Banaszewska D, Kordan W, Fraser L, et al. The usefulness of sexual behaviour assessment at the beginning of service to predict the suitability of boars for artificial insemination. *Animals (Basel)*. 2021;11(12):3341.
- Banaszewska D, Kondracki S. An assessment of the breeding maturity of insemination boars based on ejaculate quality changes. *Folia Biol*. 2012;60(3–4):151–62.

11. Banaszewska D, Kondracki S, Wysokińska A. Effect of age on the dimensions and shape of spermatozoa of Large White Polish boars. *Arch Anim Breed*. 2011;54(5):504–14.
12. Gray RC, Day BN, Lasley JF, Tribble LF. Testosterone levels of boars at various ages. *J Anim Sci*. 1971;33(1):124–6.
13. Maistrelli C, Schmicke M, Hoedemaker M, Siebert U. An approach for investigating sexual maturity in wild boar males: testosterone and 17 β -estradiol analysis. *Animals (Basel)*. 2022;12(17):2295.
14. Zduńczyk S, Janowski T, Raś A, Barański W. Concentrations of oestrogens in blood plasma and seminal plasma of boars during the postpuberal period. *Pol J Vet Sci*. 2011;14(4):539–44.
15. Zhang B, Yan Z, Wang P, Yang Q, Huang X, Shi H, et al. Identification and characterization of lncRNA and mRNA in testes of Landrace and Hezuo boars. *Animals (Basel)*. 2021;11(8):2263.
16. Flowers WL. Management of boars for efficient semen production. *J Reprod Fertil Suppl*. 1997;52:67–78.
17. Colenbrander B, Kemp B. Factors influencing semen quality in pigs. *J Reprod Fertil Suppl*. 1990;40:105–15.
18. Audet I, Bérubé N, Bailey JL, Laforest JP, Quesnel H, Matte JJ. Effects of dietary vitamin supplementation and semen collection frequency on hormonal profile during ejaculation in the boar. *Theriogenology*. 2009;71(2):334–41.
19. Larsson K, Einarsson S, Lundström K, Hakkarainen J. Endocrine effects of heat stress in boars. *Acta Vet Scand*. 1983;24(3):305–14.
20. Popwell JM, Flowers WL. Variability in relationships between semen quality and estimates of in vivo and in vitro fertility in boars. *Anim Reprod Sci*. 2004;81(1):97–113.
21. Howroyd PC, Peter B, de Rijk E. Review of sexual maturity in the minipig. *Toxicol Pathol*. 2016;44(4):607–11.
22. Kasinathan P, Wei H, Xiang T, Molina JA, Metzger J, Broek D, et al. Acceleration of genetic gain in cattle by reduction of generation interval. *Sci Rep*. 2015;5:8674.
23. Ashokan M, Rana E, Sneha K, Namith C, Naveen Kumar GS, Azharuddin N, et al. Metabolomics—a powerful tool in livestock research. *Anim Biotechnol*. 2023;34(7):3237–49.
24. Goldansaz SA, Guo AC, Sajed T, Steele MA, Plastow G, Wishart DS. Livestock metabolomics and the livestock metabolome: a systematic review. *PLoS ONE*. 2017;12(5):e0177675.
25. Zhao J, Pan H, Zhao W, Li W, Li H, Tian Z, et al. Untargeted metabolomics revealed potential biomarkers of small yellow follicles of chickens during sexual maturation. *Metabolites*. 2023;13(2):176.
26. Menezes EB, Velho ALC, Santos F, Dinh T, Kaya A, Topper E, et al. Uncovering sperm metabolome to discover biomarkers for bull fertility. *BMC Genomics*. 2019;20:714.
27. Narud B, Klinkenberg G, Khezri A, Zeremichael TT, Stenseth E-B, Nordborg A, et al. Differences in sperm functionality and intracellular metabolites in Norwegian Red bulls of contrasting fertility. *Theriogenology*. 2020;157:24–32.
28. Li Z, Sun J, Li K, Qin J, Sun Y, Zeng J, et al. Metabolomic analysis reveals spermatozoa and seminal plasma differences between Duroc and Liang Guang Small-spotted pig. *Front Vet Sci*. 2023;9:1078929.
29. Rodriguez-Martinez H, Roca J, Alvarez-Rodriguez M, Martinez-Serrano CA. How does the boar epididymis regulate the emission of fertile spermatozoa? *Anim Reprod Sci*. 2022;246:106829.
30. Velho ALC, Menezes E, Dinh T, Kaya A, Topper E, Moura AA, et al. Metabolomic markers of fertility in bull seminal plasma. *PLoS ONE*. 2018;13(4):e0195279.
31. Guo Y, Li J, Hao F, Yang Y, Yang H, Chang Q, et al. A new perspective on semen quality of aged male: the characteristics of metabolomics and proteomics. *Front Endocrinol (Lausanne)*. 2023;13:1058250.
32. Shrestha A, Gaustad AH, Øiaas JB, Kommisrud E, van Son M, Nordborg A, et al. A metabolomic study uncovering key amino acids and amines in Duroc boar semen as biomarkers of sexual maturity. *Anim Reprod Sci*. 2025;279:107800.
33. Coman C, Solari FA, Hentschel A, Sickmann A, Zahedi RP, Ahrends R. Simultaneous metabolite, protein, lipid extraction (simplex): a combinatorial multimolecular omics approach for systems biology. *Mol Cell Proteomics*. 2016;15(4):1453–66.
34. Djoumbou Feunang Y, Eisner R, Knox C, Chepelev L, Hastings J, Owen G, et al. ClassyFire: automated chemical classification with a comprehensive, computable taxonomy. *J Cheminform*. 2016;8:61.
35. Pang Z, Lu Y, Zhou G, Hui F, Xu L, Viau C, et al. MetaboAnalyst 6.0: towards a unified platform for metabolomics data processing, analysis and interpretation. *Nucleic Acids Res*. 2024;52(W1):W398–406.
36. Pang Z, Xu L, Viau C, Lu Y, Salavati R, Basu N, et al. MetaboAnalyst 4.0: a unified LC-MS workflow for global metabolomics. *Nat Commun*. 2024;15:3675.
37. R Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2024.
38. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. 2015;43(7):e47.
39. Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. *J Stat Softw*. 2015;67(1):1–48.
40. Lenth R. Emmeans: estimated marginal means, aka least-squares means. R package version 1.11.1–00001; 2025. Available from: <https://rvinth.github.io/emmeans/>.
41. Hartig F. DHARMA: residual diagnostics for hierarchical (multi-level/mixed) regression models. R package version 0.4.7; 2024. Available from: <https://github.com/florianhartig/dharma>.
42. Brown-Woodman PD, White IG. Amino acid composition of semen and the secretions of the male reproductive tract. *Aust J Biol Sci*. 1974;27(4):415–22.
43. Argov-Argaman N, Mahgrefthe K, Zeron Y, Roth Z. Variation in lipid profiles within semen compartments—the bovine model of aging. *Theriogenology*. 2013;80(7):712–21.
44. Yien YY, Perfetto M. Regulation of heme synthesis by mitochondrial homeostasis proteins. *Front Cell Dev Biol*. 2022;10:895521.
45. Li Y, Peng Q, Shang J, Dong W, Wu S, Guo X, et al. The role of taurine in male reproduction: physiology, pathology and toxicology. *Front Endocrinol (Lausanne)*. 2023;14:1017886.
46. Asano A, Roman HB, Hirschberger LL, Ushiyama A, Nelson JL, Hinchman MM, et al. Cysteine dioxygenase is essential for mouse sperm osmoadaptation and male fertility. *FEBS J*. 2018;285(10):1827–39.
47. Alvarez JG, Storey BT. Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. *Biol Reprod*. 1983;29(3):548–55.
48. Partyka A, Rodak O, Bajzert J, Kochan J, Nizański W. The effect of L-carnitine, hypotaurine, and taurine supplementation on the quality of cryopreserved chicken semen. *Biomed Res Int*. 2017;2017:7279341.
49. Jang H, Kong H, Park C, Oh J, Lee S-G, Cheong H, et al. Effects of taurine on sperm characteristics during in vitro storage of boar semen. *Asian-Australas J Anim Sci*. 2006;19(11):1561–5.
50. Am-in N, Kirkwood RN, Techakumphu M, Tantasuparuk W. Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility. *Theriogenology*. 2011;75(5):897–903.
51. Sato H, Taketomi Y, Isogai Y, Miki Y, Yamamoto K, Masuda S, et al. Group III secreted phospholipase A2 regulates epididymal sperm maturation and fertility in mice. *J Clin Invest*. 2010;120(5):1400–14.
52. Chabory E, Damon C, Lenoir A, Henry-Berger J, Vernet P, Cadet R, et al. Mammalian glutathione peroxidases control acquisition and maintenance of spermatozoa integrity. *J Anim Sci*. 2010;88(4):1321–31.
53. Li T-K. The glutathione and thiol content of mammalian spermatozoa and seminal plasma. *Biol Reprod*. 1975;12(5):641–6.
54. Song C, Chang L, Wang B, Zhang Z, Wei Y, Dou Y, et al. Seminal plasma metabolomics analysis of differences in liquid preservation ability of boar sperm. *J Anim Sci*. 2023;101:skad392.
55. Wersinger SR, Rissman EF. Dopamine activates masculine sexual behavior independent of the estrogen receptor α . *J Neurosci*. 2000;20(11):4248–54.
56. Melis MR, Sanna F, Argiolas A. Dopamine, erectile function and male sexual behavior from the past to the present: a review. *Brain Sci*. 2022;12(7):826.
57. Tai A, Sawano T, Ito H. Antioxidative properties of vanillic acid esters in multiple antioxidant assays. *Biosci Biotechnol Biochem*. 2012;76(2):314–8.
58. Salau VF, Erukainure OL, Ibeji CU, Olasehinde TA, Koobanally NA, Islam MS. Vanillin and vanillic acid modulate antioxidant defense system via amelioration of metabolic complications linked to Fe²⁺-induced brain tissues damage. *Metab Brain Dis*. 2020;35(5):727–38.
59. Pastor-Soler N, Piétrement C, Breton S. Role of acid/base transporters in the male reproductive tract and potential consequences of their malfunction. *Physiology (Bethesda)*. 2005;20(6):417–28.

60. Dai P, Zou M, Cai Z, Zeng X, Zhang X, Liang M. pH homeodynamics and male fertility: a coordinated regulation of acid-based balance during sperm journey to fertilization. *Biomolecules*. 2024;14(6):685.
61. Vedelek V, Vedelek B, Lőrincz P, Juhász G, Sinka R. A comparative analysis of fruit fly and human glutamate dehydrogenases in *Drosophila melanogaster* sperm development. *Front Cell Dev Biol*. 2023;11:1281487.
62. Kayode OT, Rotimi DE, Kayode AAA, Olaolu TD, Adeyemi OS. Monosodium glutamate (MSG)-induced male reproductive dysfunction: a mini review. *Toxics*. 2020;8(1):7.
63. Endo D, Kon S, Sato T, Toyama F, Katsura Y, Nakauchi Y, et al. NMDA-type glutamate receptors mediate the acrosome reaction and motility initiation in newt sperm. *Mol Reprod Dev*. 2019;86(9):1106–15.
64. Momeni HR, Etemadi T, Alyasin A, Eskandari N. A novel role for involvement of N-methyl-D-aspartate (NMDA) glutamate receptors in sperm acrosome reaction. *Andrologia*. 2021;53(10):e14203.
65. Breitbart H, Grinshtein E. Mechanisms that protect mammalian sperm from the spontaneous acrosome reaction. *Int J Mol Sci*. 2023;24(23):17005.
66. Tang W, Wu J, Jin S, He L, Lin Q, Luo F, et al. Glutamate and aspartate alleviate testicular/epididymal oxidative stress by supporting antioxidant enzymes and immune defense systems in boars. *Sci China Life Sci*. 2020;63(1):116–24.
67. Ni H, Lu L, Deng J, Fan W, Li T, Yao J. Effects of glutamate and aspartate on serum antioxidant enzyme, sex hormones, and genital inflammation in boars challenged with hydrogen peroxide. *Mediators Inflamm*. 2016;2016:4394695.
68. PubChem. O-decanoyl-L-carnitine; CID 11953821. National Library of Medicine (US). Available from: <https://pubchem.ncbi.nlm.nih.gov/compound/11953821>. Accessed 18 June 2025.
69. Sukemore S, Watanabe T, Odo S. Relationship between serum L-carnitine levels and sperm parameters in boars. *Open J Anim Sci*. 2022;12(3):360–5.
70. Fang L, Jitian L, Jinzhong T. Study on the relationship between sperm motility and seminal plasma carnitine in Simmental bulls based on two LC-MS techniques. *Acta Vet Zootech Sin*. 2019;50(11):2357–64.
71. Jeulin C, Lewin L. Role of free L-carnitine and acetyl-L-carnitine in post-gonadal maturation of mammalian spermatozoa. *Hum Reprod Update*. 1996;2(2):87–102.
72. Mateus FG, Moreira S, Martins AD, Oliveira PF, Alves MG, Pereira ML. L-carnitine and male fertility: is supplementation beneficial? *J Clin Med*. 2023;12(18):5796.
73. Otuokere IE, Anyanwu JC, Igwe KK. Synthesis, characterization and antibacterial studies of 4-[(E)-Phenylmethylidene]amino-N-(1,3-thiazol-2-yl)benzenesulfonamide and its Mn(II) complex. *ChemSearch J*. 2020;11(1):44–51.
74. Shalai YR, Popovych MV, Mandzynets SM, Hreniukh VP, Finiuk N, Babysky AM. Prooxidant and antioxidant processes in the liver homogenate of healthy and tumor-bearing mice under the action of thiazole derivatives. *Ukr Biochem J*. 2021;93(3):61–7.
75. Horishny VY, Drapak IV, Chaban TI, Ostapiuk YV, Matyichuk VS. Synthesis and antitumor properties of some new N-(5-R-benzyl-1,3-thiazol-2-yl)-4,5-dihydro-1H-imidazole-2-carboxamides. *Indones J Pharm*. 2020;31(3):150–60.
76. Zhang Y, Liu Y, Liang H, Xu Q, Liu Z, Weng X. Metabolomic differences of seminal plasma between boars with high and low average conception rates after artificial insemination. *Reprod Domest Anim*. 2021;56(1):161–71.
77. Matsugo S, Nakamura Y. Pyrrole-2-carboxaldehydes: origins and physiological activities. *Molecules*. 2023;28(6):2599.
78. Jia Q, Farrow TM. 7-hydroxychromones as potent antioxidants. US patent US008110555B2. 2012.
79. Barranco I, Rubio CP, Tvarijonavičiute A, Rodríguez-Martínez H, Roca J. Measurement of oxidative stress index in seminal plasma can predict in vivo fertility of liquid-stored porcine artificial insemination semen doses. *Antioxidants (Basel)*. 2021;10(8):1203.
80. Oh YT, Lee JY, Lee J, Lee JH, Kim J-E, Ha J, et al. Oleamide suppresses lipopolysaccharide-induced expression of iNOS and COX-2 through inhibition of NF-κB activation in BV2 murine microglial cells. *Neurosci Lett*. 2010;474(3):148–53.
81. Divito EB, Cascio M. Metabolism, physiology, and analyses of primary fatty acid amides. *Chem Rev*. 2013;113(10):7343–53.
82. Collodel G, Castellini C, Lee JC-Y, Signorini C. Relevance of fatty acids to sperm maturation and quality. *Oxid Med Cell Longev*. 2020;2020:7038124.
83. Ghaemi M, Seighali N, Shafiee A, Beiky M, Kohandel Gargari O, Azarboo A, et al. The effect of myo-inositol on improving sperm quality and IVF outcomes: a systematic review and meta-analysis. *Food Sci Nutr*. 2024;12(11):8515–24.
84. Osman R, Lee S, Almubarak A, Han JI, Yu JJ, Jeon Y. Antioxidant effects of myo-inositol improve the function and fertility of cryopreserved boar semen. *Antioxidants (Basel)*. 2023;12(9):1673.
85. Condorelli R, La Vignera S, Di Bari F, Unfer V, Calogero A. Effects of myoinositol on sperm mitochondrial function in-vitro. *Eur Rev Med Pharmacol Sci*. 2011;15(2):129–34.
86. De Luca MN, Colone M, Gambioli R, Stringaro A, Unfer V. Oxidative stress and male fertility: role of antioxidants and inositols. *Antioxidants (Basel)*. 2021;10(8):1283.
87. Parrilla I, Martínez EA, Gil MA, Cuello C, Roca J, Rodríguez-Martínez H, et al. Boar seminal plasma: current insights on its potential role for assisted reproductive technologies in swine. *Anim Reprod*. 2020;17(3):e20200022.
88. Shan S, Xu F, Hirschfeld M, Brenig B. Sperm lipid markers of male fertility in mammals. *Int J Mol Sci*. 2021;22(16):8767.
89. Wang J, Marks J, Fortenberry R, Kaiser R. Interstellar formation of glyceric acid [HOCH₂CH(OH)COOH]—the simplest sugar acid. *Sci Adv*. 2024;10(11):ead3236.
90. Hirvonen OP, Kyröläinen H, Lehti M, Kainulainen H. Randomized trial: D-glyceric acid activates mitochondrial metabolism in 50–60-year-old healthy humans. *Front Aging*. 2021;2:752636.
91. Tilly JL, Kolesnick RN. Sphingolipid signaling in gonadal development and function. *Chem Phys Lipids*. 1999;102(1–2):149–55.
92. Cross NL. Sphingomyelin modulates capacitation of human sperm in vitro. *Biol Reprod*. 2000;63(4):1129–34.
93. Wang D, Tang Y, Wang Z. Role of sphingolipid metabolites in the homeostasis of steroid hormones and the maintenance of testicular functions. *Front Endocrinol (Lausanne)*. 2023;14:1170023.

3

Influence of sexual maturity on sperm quality in Duroc boars

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ABSTRACT

The objective of this study was to evaluate sperm quality of Duroc boars during sexual maturation and examine whether these parameters were related to metabolites in semen. Ejaculates were collected longitudinally from 28 Duroc boars at approximately 7 months of age (Age 1), 6 weeks later (Age 2) and 12 weeks later (Age 3). Motility characteristics, acrosome integrity, and viability were assessed on collection day and after five days storage at 18°C using computer-assisted sperm analysis and flow cytometry, while DNA fragmentation was measured on day five. Amino acid and amine concentrations in semen were measured by liquid chromatography-mass spectrometry and correlated with sperm quality parameters. Motility, rapid progressive motility, and acrosome intact live sperm increased significantly (adj $P < 0.05$) with boar maturation. Mature boars (Age 2 and Age 3) maintained superior sperm quality compared to younger boars after storage. The DNA fragmentation index was low in all samples but declined significantly from 1% at Age 1 to 0.64% at Age 2 (adj $P < 0.05$). Correlation analysis showed significant relationships between sperm quality and specific metabolites. Cystine, glutamate, aspartate, choline and taurine were inversely correlated with progressive and rapid progressive motility, while showing positive correlation with rapid non-progressive motility. These findings demonstrate that sperm quality continues improving beyond initial reproductive ability, especially between Age 1 and Age 3, with improvements in motility, viability, chromatin stability, and storage resilience. The observed relationships between metabolites and sperm quality parameters provide insights into biochemical mechanisms underlying sperm functionality during sexual maturation.

Keywords: Spermatozoa, Puberty, DNA-damage, Motility, Metabolites

1. Introduction

Boar sperm quality is an important determinant of reproductive success in swine, especially given the widespread use of artificial insemination (AI) in modern pig production (Kamanova et al., 2021). Efficient management of artificial insemination centres in porcine production is highly dependent on the semen collection from selected sires and the assessment of sperm quality. With the advent of genomic selection that facilitates the early identification of elite sires, breeding companies aim to incorporate these boars into semen production at the earliest opportunity to shorten the generation interval and accelerate genetic gain (Samorè and Fontanesi, 2016). This interest makes it essential to understand how sperm functional traits

develop from the first ejaculation to sexual maturity and how early young boars can meet commercial AI standards without compromising fertility.

Most studies have reported that ejaculate volume, sperm concentration, total sperm number and motility increase after 8-9 months of age, peaking during 18-25 months, thereafter, diminishing in commercial boars such as Duroc, Landrace, Pietrain, Hampshire and Yorkshire (Knecht et al., 2017; Wang et al., 2017). Boars that enter production at 8-9 months yield the highest lifetime functional sperm numbers, outperforming both very young (5-7 months) and later starting (≥ 10 months) according to Wang et al. (2017). Breed-specific studies in Polish Large White, cross-bred and Duroc boars echo this trajectory: improvements in ejaculate volume, sperm motility, plasma membrane integrity, normal sperm count and acrosome normality are documented from around 8 months of age through adulthood (19-30 months) (Kawęcka et al., 2008; Banaszewska and Kondracki, 2012; Fraser et al., 2016). Nonetheless, some discrepancies are reported. For instance, Duroc boars below 18 months produced smaller volumes of ejaculates, but higher sperm concentrations compared to adults aged 18-36 months (Hensel et al., 2023). Likewise, Kawęcka et al. reported marked reductions in spermatozoa with defects between 7.6 and 8.9 months in Duroc sires (Kawęcka et al., 2008). Yet, the literature is not entirely consistent, largely because most datasets span wide age categories, are cross-sectional or pool multiple breeds together (Abah et al., 2023). In addition, some boars will disappear from the dataset over time when culled due to reduced sperm quality, fertility or breeding value.

Reproductive success is also affected by sperm chromatin quality, with younger boars (7-10 months) exhibiting higher sperm-chromatin instability and substantially lower farrowing rates than mature adults (18-33 months) according to (Tsakmakidis et al., 2012). This suggests that reproductive performance may continue to improve well beyond the onset of puberty. The DNA fragmentation index (DFI) threshold level for boar fertility and litter size is reported to be between 2 and 6% (Evenson, 2016).

Seasonality represents another important factor influencing boar semen quality. Functional sperm numbers per ejaculate tend to be higher from October to April and lower from May to September (Wang et al., 2017). Studies have documented that ejaculate volume, total sperm count and total number of motile spermatozoa peak in the cooler autumn-winter with corresponding declines during spring-summer (April-September) (Fraser et al., 2016; Knecht et al., 2017; Hensel et al., 2023). In Duroc×Pietrain boars, total sperm count is ~12% higher in winter than summer. Young purebred Duroc boars of around 1 year old appear relatively heat-

77 tolerant and provide an equal amount of semen volume as in cool season, however, the capacity
78 to tolerate heat gradually diminished with increasing age (Huang et al., 2010). Testosterone and
79 total sperm count in Duroc boars are reported to peak in fall (Borg et al., 1993). Seasonal
80 patterns also affect sperm morphology, number of morphologically abnormal spermatozoa,
81 with increased occurrence of proximal cytoplasmic droplets apex in September-October while
82 head defects are most common between June and July (Kamanova et al., 2021).

83 In addition to sperm quality parameters, there is growing interest in identifying biochemical
84 markers in boar semen that could shed some light on sperm functionality (Memili et al., 2020;
85 Mateo-Otero et al., 2021b). Differences in amino acid levels in semen have been associated
86 with variation in sperm motility, viability, acrosome integrity, fertility and even tolerance to
87 semen preservation in boars (Mateo-Otero et al., 2021a; Mateo-Otero et al., 2021b; Mateo-
88 Otero et al., 2023). Earlier reports from our lab have shown that several amino acids in semen
89 changed with sexual maturation. Moreover, pathway analysis revealed that metabolic networks
90 involving alanine, aspartate, glutamate and histidine, were affected by age (Shrestha et al.,
91 2025). These findings highlight how semen composition of metabolites changes during the
92 transition from puberty to sexual maturity and could also be associated with sperm
93 functionality.

94 Despite these advances, several knowledge gaps remain regarding how sperm quality
95 changes as pubertal boars develop into the sexually mature phase. Most previous studies
96 examined broad age contrasts (young vs. adult vs. old) or were cross-sectional, yielding varied
97 results, making it difficult to characterize the trajectory of semen parameters from puberty to
98 sexual maturity in the same individuals. Moreover, to our knowledge, no study has earlier
99 correlated comprehensive sperm quality analysis with metabolomic profiling (amino acids and
100 amines) in a longitudinal design focused on the pubertal to sexual maturity transition in boars.

101 We hypothesized that sperm motility, viability, and chromatin stability would improve
102 between 7 months and 10 months of age, reflecting sexual maturation and that the influence of
103 age on semen traits would be observable across seasons. Moreover, we hypothesized that there
104 is a correlation between amino acid and amine concentrations and semen quality parameters.
105 The aim of the present study was therefore to examine the effect of sexual maturation on sperm
106 quality parameters in Duroc boars. Secondly, to investigate whether sperm quality was related
107 to the metabolomic state of the semen sample.

2. Materials and methods

2.1. Ethical approval

This study did not necessitate ethical approval since the boars were part of standard semen production procedures carried out in accordance with Norwegian legal frameworks. These encompassed the Animal Protection Act (December 20, 1974), the Animal Welfare Act (June 19, 2009), the Regulations for Pig Management in Norway (February 18, 2003), and the EU legislation governing pig semen production (Council Directive 90/429/EEC).

2.2. Experimental design

Sexual maturity-related changes in boar sperm functional parameters were investigated through two complementary experiments. Semen samples were collected from 28 Duroc boars across three maturation time-points to capture the transition during sexual maturation.

Experiment 1 examined temporal changes in sperm quality parameters. Semen samples from all 28 boars were assessed for motility, acrosome integrity and viability on the day of collection (Day 0) and following five days of storage at 18°C (Day 5). An aliquot of 500 µl from each semen sample was frozen at Day 5 and stored for later analysis of DNA damage.

Experiment 2 explored the relationship between sperm quality parameters and metabolite concentrations. Spermatozoa and seminal plasma were separated from 15 Duroc boars, and amino acids and amines were measured in sperm and seminal plasma separately using liquid chromatography-mass spectrometry, as detailed by Shrestha et al. (2025).

2.3. Animal material

Semen from 15 Duroc boars was collected from March to June 2023 (hereafter referred to as spring season), while semen samples from 13 boars were collected from September to December 2023 (hereafter referred to as autumn) randomly from the commercial breeding line of Norsvin SA. The average ages of 28 boars were 6.9 months (Age 1, 210 days), 8.2 months (Age 2, 251 days) and 9.7 months (Age 3, 295 days). Six-week intervals were maintained between each collection. These intervals were specifically chosen to capture the transitions during sexual maturation.

Initially, boars were housed in 4 m² pens in an isolation unit at Age 1 where they underwent quarantine and semen collection training. Semen collection was initiated once mounting behaviour was observed and collection procedures became feasible for individual boars. These animals were then relocated to AI centre before sampling started at Age 2, where

they were kept in 6 m² pens under standard housing conditions. Norsvin SA begins commercial distribution of semen from boars at Age 2, following Council Directive 90/429/EEC requirements.

Uniform management practices were implemented across both facilities (isolation unit and AI centre) encompassing standardized nutritional regimens, ventilation, temperature, and collection procedures. The gloved-hand technique with the same trained personnel throughout the study period was used to collect semen samples. This longitudinal sampling approach enabled the tracking of true changes with sexual maturity while minimizing confounding effects associated with inter-individual variation and facility management differences.

2.4. Sample processing and transport

Sperm concentrations (Cons) were determined using the Nucleocounter® SP-100 system (Chemometec, Allerød, Denmark), with results expressed in million cells per ml. Semen samples were subsequently diluted to a standardized concentration of 25 million spermatozoa per ml using Androstar® Plus extender (Minitube, 84184, Tiefenbach, Germany). Semen samples were transported from collection facilities to the University of Inland Norway laboratory maintaining stable temperatures through insulated Styrofoam packaging in 30 minutes.

2.5. Plasma membrane and acrosome integrity assessment

The sperm samples were stained with SYTO™ 60 Red Fluorescent Nucleic Acid Stain (S60, S11342, Invitrogen) for identification of sperm, propidium iodide (PI, P1304MP, Invitrogen, Paisley, UK) for dead sperm, and lectin peanut agglutinin (PNA) from *Arachis hypogaea*, linked with Alexa Fluor™ 488 (L32460, Invitrogen) for acrosome reacted sperm. Sperm samples were diluted to a concentration of 1 million sperm per sample in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄·2H₂O, 1.76 mM KH₂PO₄, pH 7.4) with fluorescent probes giving a final concentration of 0.1 µM S60, 1.2 µM PI and 0.05 µg/ml PNA, and incubated for 10 minutes. The samples were analysed by using the CytoFLEX Flow Cytometer (Beckman Coulter, Fullerton, CA, USA) and CytExpert software (version 2.5). For each sample, 10000 events were collected at slow rate. The CytoFLEX daily QC fluorophores (Beckman Coulter, Fullerton, CA, USA) were run daily for quality control. Unstained and single stained sperm samples were used for compensation control. The blue and red lasers (480 nm and 633 nm) were utilized, and the emission was collected by a 660/20 filter for S60, 525/40 filter for PNA-488, and a 610/20 filter for PI. Each sample was analysed in duplicate. Spermatozoa were separated from debris using a cytogram of SSC and S60 and a PI/S60

cytogram for further debris exclusion. The following subpopulations were subsequently defined by gating on sperm and a PI/PNA cytogram; acrosome intact live (AIL), acrosome intact dead (AID), and acrosome reacted dead (ARD).

2.6. Motility analysis by CASA

Sperm motility assessment was performed using computer-assisted sperm analysis (CASA) with Sperm Class Analyzer® (Microptic SL, Spain) equipped with a phase contrast Eclipse Ci-S/Ci-L microscope (Nikon, Japan), a BASLER Ace acA780-75 gc digital camera (Basler Vision Technologies, Germany) and the Sperm Class Analyzer software (version 6.1.0.0). Sperm samples were incubated at 38°C for 20 min, tilted gently five times before loading an aliquot of 3 µl into a pre-warmed Leja slide (Leja BV, Nieuw Venneep, The Netherlands). The analyses were performed in parallel for each sample. A minimum of eight microscope fields and at least 800 cells were examined for each sample. Camera settings were adjusted to capture 30 images at a rate of 45 frames per second. Spermatozoa were identified based on head area 20-80 µm². Every motility file was subjected to a quality control procedure by visually examining that spermatozoa were correctly identified. The kinematic parameters recorded for each sperm were velocity curved line (VCL, µm/s), velocity average path (VAP, µm/s), velocity straight line (VSL, µm/s), the straightness (STR) of the average path defined as the ratio VSL/VAP (%), linearity (LIN) of the curvilinear path (VSL/VCL (%)), Wobble (WOB), ratio VAP/VCL (%), amplitude of the lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz). The defined threshold for motility was VAP > 10 µm/s. Spermatozoa exhibiting STR > 45 % were considered progressive motile (PM), VAP 10-20 µm/s were considered slow (Slow), VAP 20-50 µm/s were considered medium motile (Medium), VAP > 50 µm/s and STR > 45 % as rapid progressive (RapidP), and VAP > 50 µm/s and STR < 45 % as rapid non-progressive (RapidNP). The mean values of the different kinematic parameters of the different sub populations were calculated from single cell data from 11368 spermatozoa.

2.7. DNA damage by SCSA

Analysis of DNA was performed by the Sperm Chromatin Structure Assay® (SCSA) procedure as described by Evenson and Jost (Evenson and Jost, 2001) with subsequent modifications implemented by Boe-Hansen et al. (Boe-Hansen et al., 2005). Analysis was performed using Cell Lab Quanta™ SC MPL flow cytometer with a 488 nm argon laser at 22 Mw power (Beckman Coulter, Software Version 1.0 A, USA). Sperm samples were thawed at 37°C for 1 minute and diluted to 2 million sperm/mL in 200 µL TNE buffer (10 mM Tris-HCL,

0.15 M NaCl, 1 mM EDTA, pH 7.4). Thereafter, 400 μ L acid detergent solution (0.38 M NaCl, 80 mM HCl, 0.1% Triton X-100, pH 1.2) was added which was incubated for 30 seconds. Acridine orange (AO) staining solution consisting of 6 μ g/mL AO (A3568, Life Technologies, USA) in a 37 mM citric acid-phosphate buffer (0.126 M Na₂HPO₄, 1.1 μ M EDTA, 0.15 M NaCl, pH 6) was used, from which 1.2 ml was added the sample. Samples were run in setup mode for 3 minutes before recording 5000 events/sample. Signals were separated with a 550 nm dichroic long pass mirror and fluorescence was detected using a 525 nm band pass filter and a 670 nm long pass filter. Prior to sample analysis, the instrument was saturated with AO by running an equilibration solution (1.2 mL AO staining solution + 400 μ L acid detergent solution) through the system for 5 minutes. To maintain laser stability, reference boar semen with a known DFI was used to adjust the mean green and red fluorescence signals to 425 ± 5 and 125 ± 5 , respectively, at the start and after every fifth sample. Further analysis was conducted using FCS Express 6 software (Denovo Software, Los Angeles, CA, USA) to determine the proportions of red (ssDNA) and green (dsDNA) fluorescence. The percentage of DNA fragmentation index (%DFI), the mean level of DNA fragmentation (mean DFI), and the variability in DNA fragmentation (SD DFI) was calculated from a histogram representing the fluorescence ratio, red / (red + green). To assess the percentage of spermatozoa with high DNA stainability (%HDS), a bivariate cytogram was utilized.

2.8. Statistical analysis

Statistical analyses were conducted in R 4.4.2 (R Core Team, Vienna, Austria) using the R package glmmTMB 1.1-11 (McGillucuddy et al., 2025). Data distributions were checked with the Shapiro–Wilk test, sample skewness and Q-Q plots. Variables measured as percentages (STR, LIN, WOB, AIL, motility, PM, Medium, Slow, RapidP and RapidNP) were displayed in the figures as percentages but converted to proportions ($0 < y < 1$) for analysis and modelled with beta-error generalized linear mixed models (GLMMs; logit link), whereas strictly positive continuous variables were fitted with Gaussian (identity) or Gamma (log) errors selected according to their observed distribution.

For parameters measured on both Day 0 and Day 5, two mixed-effects models were fitted to each outcome: (i) $Age \times Day + (1 | Boar)$ and (ii) $Season \times Age \times Day + (1 | Season/Boar)$, allowing age-related patterns to be assessed within and across seasons. For parameters measured only once (concentration on Day 0; %DFI, SD DFI, mean DFI and %HDS on Day 5), separate analyses were conducted. Parameters %DFI and %HDS were converted to proportions and modelled using beta regression with logit link: $Proportion \sim Age \times Season +$

(1 | BoarID). Concentration, SD DFI, and mean DFI were modelled using Gamma regression with log link: $Parameter \sim Age \times Season + (1 | Season/BoarID)$. Additional season-specific analyses were conducted using $Parameter \sim Age + (1 | BoarID)$ to examine age effects within each season independently. Model assumptions were verified with DHARMA 0.4.7 simulated residuals (Hartig, 2024). Post-hoc pairwise comparisons were performed using estimated marginal means with emmeans 1.11.0 (Lenth et al., 2025). *P*-values were adjusted using the Benjamini-Hochberg method, with adjusted $P < 0.05$ considered statistically significant.

Relationships between metabolites in spermatozoa ($n = 26$) and seminal plasma ($n = 27$) with sperm quality parameters ($n = 23$, encompassing motility, acrosome integrity and viability, and DNA fragmentation index described above) were assessed using repeated measures correlation analysis for 15 boars from spring collection. *P*-values were adjusted using the Benjamini-Hochberg correction method and correlations with adjusted *P*-values < 0.05 were considered statistically significant.

3. Results

The percentage of acrosome intact live (AIL) sperm was not significantly different due to age at Day 0. However, after storage for 5 days, younger boars (Age 1) had significantly lower AIL compared to more mature boars (Age 2, Age 3) ($P < 0.05$) (Figure 1, Table 1).

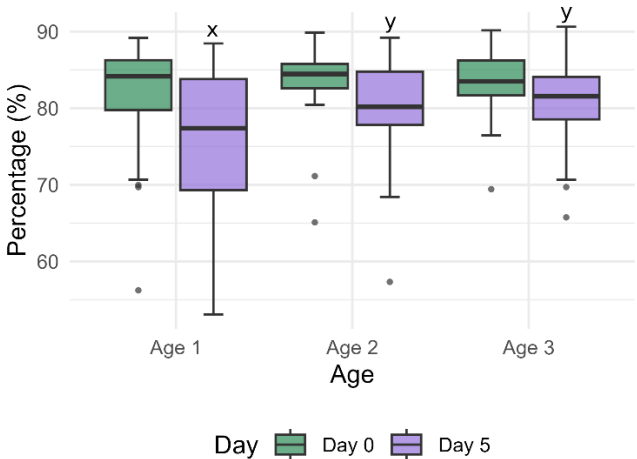


Figure 1: Acrosome intact live sperm percentage across boar sexual maturation and semen storage duration. Box plots display the percentage of acrosome intact live (AIL) spermatozoa in Duroc boars ($n = 28$) evaluated longitudinally at approximately 6.9 (Age 1), 8.2 months (Age 2) and 9.7 months (Age 3) of age. Semen samples were analyzed by flow cytometry on the day of collection (Day 0, green) and after five days of storage at 18°C (Day 5, purple). Different letters (x, y) indicate statistically significant differences between age groups within each storage time point (adj $P < 0.05$).

The influence of sexual maturity was evident in several sperm motility parameters. While total motility showed no age-related differences on Day 0, storage revealed that by Day 5, samples from Age 2 and Age 3 showed significantly higher motility than those from Age 1 (Figure 2, Table 1, Supplementary Table 1). Both PM and RapidP were significantly higher in samples from mature boars (Age 2 and Age 3) compared to Age 1 on both Day 0 and Day 5 ($P < 0.05$). Medium sperm decreased significantly in Age 2 and Age 3 samples on both days compared to Age 1 ($P < 0.05$), while RapidNP sperm also showed lower percentages in mature boars than in younger boars at day 0. Slow motile sperm decreased in Age 3 compared to Age 1 and Age 2 at Day 5 ($P < 0.05$).

Likewise, differences were observed for kinematic parameters (Figure 3, Table 1, Supplementary Table 2). VAP significantly increased with age at day 5, while VCL and ALH showed a significant reduction on day 0 in Age 2 and Age 3 compared to Age 1 ($P < 0.05$). VSL, LIN, STR, and WOB increased significantly with age on both measurement days ($P < 0.05$ for Age 1 vs Age 2 and Age 1 vs Age 3).

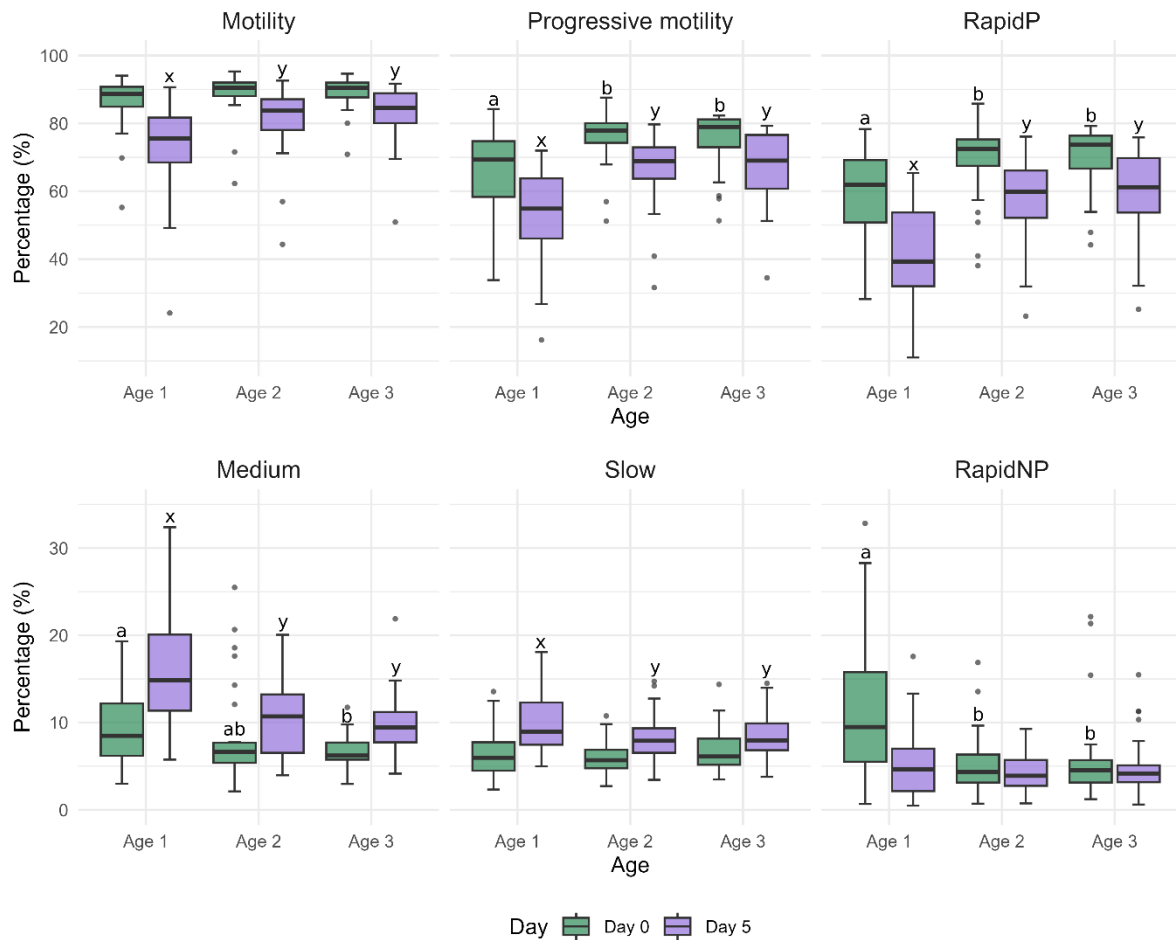
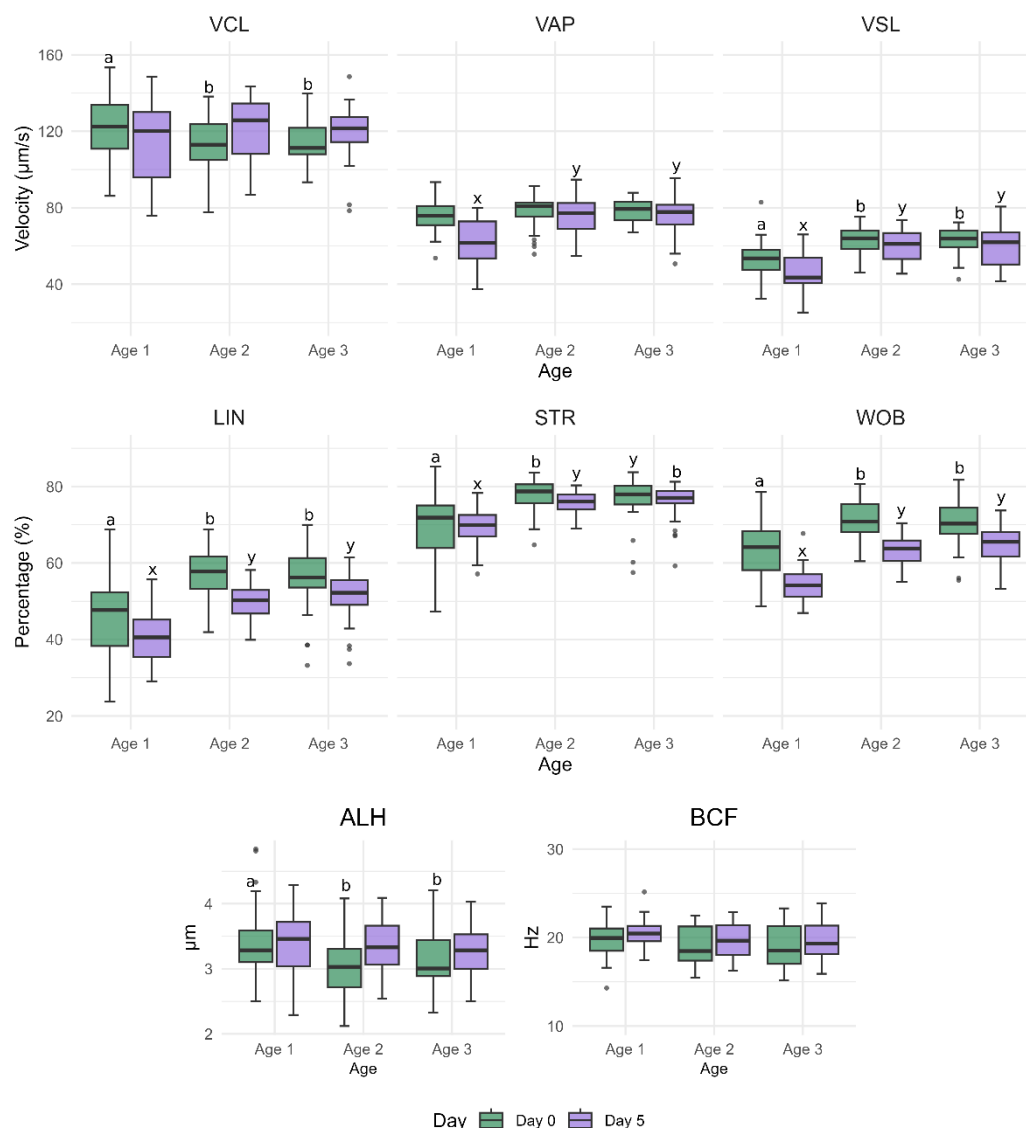


Figure 2: Computer-assisted sperm analysis (CASA) of motility subpopulations during boar sexual maturation and semen storage duration. Box plots display six motility parameters in Duroc boars ($n = 28$) evaluated longitudinally at approximately 6.9 (Age 1), 8.2 months (Age 2) and 9.7 months (Age 3) of age. Semen samples were analyzed by CASA on the day of collection (Day 0, green) and after five days of storage at 18°C (Day 5, purple). Motility subpopulations include motility, progressive motility, rapid progressive (RapidP), medium, slow, and rapid non-progressive (RapidNP) motility expressed as percentages within the total sperm population. Different letters indicate statistically significant differences between age groups within each storage time point: a, b for Day 0 comparisons and x, y for Day 5 comparisons (adj $P < 0.05$).



286
 287 Figure 3: Computer-assisted sperm analysis (CASA) of kinematic parameters during boar sexual maturation and
 288 semen storage duration. Box plots display sperm movement characteristics in Duroc boars ($n = 28$) evaluated
 289 longitudinally at approximately 6.9 (Age 1), 8.2 months (Age 2) and 9.7 months (Age 3) of age. Semen samples
 290 were analyzed by CASA on the day of collection (Day 0, green) and after five days of storage at 18°C (Day 5,
 291 purple). Kinematic parameters include velocity curved line (VCL, µm/s), velocity average path (VAP, µm/s),
 292 velocity straight line (VSL, µm/s), linearity (LIN) defined as the VSL/VCL (%) ratio, straightness (STR) defined
 293 as the VSL/VAP (%) ratio, Wobble (WOB) defined as the VAP/VCL (%) ratio, amplitude of lateral head
 294 displacement (ALH, µm) and beat cross-frequency (BCF, Hz). Different letters indicate statistically significant
 295 differences between age groups within each storage time point: a, b for Day 0 comparisons and x, y for Day 5
 296 comparisons (adj $P < 0.05$).

Table 1: Statistical comparisons of sperm quality parameters) between age groups during sexual maturation in Duroc boars. Pairwise contrasts showing estimated differences between Age 1 (6.9 months), Age 2 (8.2 months), and Age 3 (9.7 months) for sperm functional parameters evaluated on collection days (Day 0) and after five days of storage at 18°C (Day 5). Only comparisons that differ significantly are presented (adj $P < 0.05$).

Parameters	Contrast	estimate	SE	Adj. P .val
AIL (Day 5)	Age 1 - Age 2	-0.24	0.07	2.07×10^{-3}
	Age 1 - Age 3	-0.30	0.07	1.15×10^{-4}
Motility (Day 5)	Age 1 - Age 2	-0.52	0.09	7.43×10^{-8}
	Age 1 - Age 3	-0.63	0.10	2.07×10^{-10}
PM (Day 0)	Age 1 - Age 2	-0.48	0.10	1.00×10^{-6}
	Age 1 - Age 3	-0.43	0.09	7.27×10^{-6}
PM (Day 5)	Age 1 - Age 2	-0.57	0.09	6.39×10^{-10}
	Age 1 - Age 3	-0.60	0.09	9.06×10^{-11}
RapidP (Day 0)	Age 1 - Age 2	-0.46	0.10	6.84×10^{-6}
	Age 1 - Age 3	-0.50	0.10	1.73×10^{-6}
RapidP (Day 5)	Age 1 - Age 2	-0.72	0.10	6.44×10^{-13}
	Age 1 - Age 3	-0.78	0.10	2.03×10^{-14}
RapidNP (Day 0)	Age 1 - Age 2	0.82	0.10	2.73×10^{-16}
	Age 1 - Age 3	0.71	0.09	1.90×10^{-13}
Medium (Day 0)	Age 1 - Age 3	0.37	0.11	9.71×10^{-4}
Medium (Day 5)	Age 1 - Age 2	0.47	0.09	1.20×10^{-7}
	Age 1 - Age 3	0.57	0.09	6.25×10^{-10}
Slow (Day 5)	Age 1 - Age 2	0.18	0.07	1.36×10^{-2}
	Age 1 - Age 3	0.15	0.07	3.33×10^{-2}
VAP (Day 5)	Age 1 - Age 2	-14.16	1.76	7.52×10^{-13}
	Age 1 - Age 3	-14.40	1.76	6.80×10^{-13}
VCL (Day 0)	Age 1 - Age 2	11.75	2.62	1.22×10^{-4}
	Age 1 - Age 3	8.83	2.62	2.79×10^{-3}
VSL (Day 0)	Age 1 - Age 2	-9.41	1.81	1.86×10^{-6}
	Age 1 - Age 3	-9.22	1.81	2.27×10^{-6}
VSL (Day 5)	Age 1 - Age 2	-14.58	1.81	8.16×10^{-13}
	Age 1 - Age 3	-14.88	1.81	6.29×10^{-13}
STR (Day 0)	Age 1 - Age 2	-0.42	0.05	0.00
	Age 1 - Age 3	-0.36	0.05	0.00
STR (Day 5)	Age 1 - Age 2	-0.31	0.05	0.00
	Age 1 - Age 3	-0.31	0.05	0.00
LIN (Day 0)	Age 1 - Age 2	-0.46	0.05	2.02×10^{-17}
	Age 1 - Age 3	-0.39	0.05	2.03×10^{-13}

LIN (Day 5)	Age 1- Age 2	-0.40	0.05	1.24×10^{-13}
	Age 1- Age 3	-0.44	0.05	3.62×10^{-16}
WOB (Day 5)	Age 1- Age 2	-0.39	0.05	6.36×10^{-16}
	Age 1- Age 3	-0.32	0.05	1.56×10^{-11}
WOB (Day 5)	Age 1- Age 2	-0.37	0.05	4.41×10^{-16}
	Age 1- Age 3	-0.42	0.05	1.42×10^{-19}
ALH (Day 0)	Age 1- Age 3	0.40	0.07	2.01×10^{-6}
	Age 1- Age 3	0.31	0.07	1.54×10^{-4}

Note: AIL, acrosome intact live spermatozoa; PM, progressive motile spermatozoa (straight-line velocity [STR] > 45%); Rapid, rapid motile spermatozoa (velocity average path [VAP] > 50 $\mu\text{m/s}$); RapidP, rapid progressive spermatozoa (VAP > 50 $\mu\text{m/s}$ and STR > 45%); RapidNP, rapid non-progressive spermatozoa (VAP > 50 $\mu\text{m/s}$ and STR < 45%); Medium, medium motile spermatozoa (VAP 20-50 $\mu\text{m/s}$); Slow, slow spermatozoa (VAP 10-20 $\mu\text{m/s}$); VAP, velocity average path; VCL, velocity curved line; VSL, velocity straight line; STR, straightness (VSL/VAP $\times$ 100); LIN, linearity (VSL/VCL $\times$ 100); WOB, wobble (VAP/VCL $\times$ 100); ALH, amplitude of lateral head displacement. Statistical analysis performed using generalized linear mixed models with boar identity as random effect. Post-doc comparisons conducted using estimated marginal means with Benjamini-Hochberg correction for multiple testing. Estimates represent differences between age groups with corresponding standard errors (SE).

Significant age-related improvements were observed in sperm DNA fragmentation parameters as well. %DFI decreased from Age 1 to Age 3 ($P < 0.05$, Table 2). SD DFI followed a similar pattern, indicating not just lower fragmentation but also higher homogeneity in older boar sperm. %HDS decreased significantly from Age 1 to Age 3 ($P < 0.05$), while mean DFI showed a significant reduction in Age 3 compared to Age 2 ($P < 0.05$).

Table 2: Sperm quality parameters measured on day 0 only by age group. Values represent mean $\pm$ standard deviation, with median and range provided for each parameter. DFI, MeanDFI, SD DFI and %HDS were measured on frozen samples after five days storage at 18°C. Sperm concentration was measured on the day of collection. Values with different letters differ significantly (adj $P < 0.05$).

Parameter	Age	Mean $\pm$ SD	Median	Min	Max
%DFI	Age 1	0.96 $\pm$ 0.76 ^a	0.80	0.38	4.66
	Age 2	0.75 $\pm$ 0.20 ^{ab}	0.76	0.37	1.20
	Age 3	0.64 $\pm$ 0.21 ^b	0.60	0.37	1.32
MeanDFI	Age 1	228.39 $\pm$ 5.41 ^{ab}	226.47	220.10	237.80
	Age 2	229.81 $\pm$ 5.64 ^a	229.32	222.24	244.84
	Age 3	226.77 $\pm$ 6.48 ^b	227.54	207.50	237.50
SD DFI	Age 1	20.77 $\pm$ 3.32 ^a	20.89	15.72	32.05
	Age 2	19.77 $\pm$ 2.96 ^{ab}	19.10	15.14	25.58
	Age 3	18.66 $\pm$ 2.93 ^a	18.22	15.55	30.56
%HDS	Age 1	2.36 $\pm$ 0.81 ^a	2.26	1.09	4.23
	Age 2	2.11 $\pm$ 0.66 ^{ab}	2.01	1.05	3.74
	Age 3	1.97 $\pm$ 0.60 ^a	2.10	0.84	3.00
Concentration	Age 1	379.12 $\pm$ 206.05	379.90	67.70	757.10
	Age 2	325.45 $\pm$ 136.64	312.95	45.80	709.70
	Age 3	313.46 $\pm$ 142.25	311.15	81.00	651.40

Note: %DFI, percentage DNA fragmentation index; MeanDFI, mean DNA fragmentation index; SD DFI, standard deviation of DNA fragmentation index; %HDS, percentage high DNA stainability.

Table 3: Statistical comparisons of chromatin integrity parameters between age groups during sexual maturation in Duroc boars. Pairwise contrasts showing estimated differences between Age 1 (6.9 months), Age 2 (8.2 months), and Age 3 (9.7 months) for chromatin integrity parameters measured after five days storage at 18°C. Only comparisons that differ significantly are presented (adj $P < 0.05$).

Parameters	Contrast	estimate	SE	Adj P .val
%DFI	Age 1 vs. Age 3	0.29	0.10	0.0119
%HDS	Age 1 vs. Age 3	0.19	0.05	0.0007
SD DFI	Age 1 vs. Age 3	0.10	0.04	0.0131
MeanDFI	Age 2 vs. Age 3	0.01	0.00	0.0095

Note: %DFI, percentage DNA fragmentation index; MeanDFI, mean DNA fragmentation index; SD DFI, standard deviation of DNA fragmentation index; %HDS, percentage high DNA stainability. Statistical analysis performed using generalized linear mixed models with boar identity as random effect. Post-doc comparisons conducted using estimated marginal means with Benjamini-Hochberg correction for multiple testing. Estimates represent differences between age groups with corresponding standard errors (SE).

Across all age groups, five days storage at 18°C induced a significant reduction in motility, progressive motility, RapidP, RapidNP, VAP, WOB and STR, accompanied by increased proportions of Slow and Medium sperm ($P < 0.05$; Supplementary Table 3).

In general, the improvements with increasing age in motility, progressive motility, RapidP, VAP, VSL, STR, LIN, WOB, and AIL remained consistent across seasons (adj $P <$

336 0.05) (Table 4, Table 5). Similarly, age-related decreases in RapidNP, Medium, and VCL were
337 significant in both seasonal cohorts (adj $P < 0.05$) (Table 4, Table 5).

Table 4: Sperm quality parameters during sexual maturation by season, day and age. Semen samples were collected from 28 Duroc boars (N = 15 in spring, N = 13 in autumn) at approximately 7 months (Age 1), 6 weeks later (Age 2) and 12 weeks later (Age 3) of first collection. Samples were analysed immediately after collection on day 0 and after 5 days of storage at 18°C (Day 5) in spring and autumn. Motility analysis was conducted using Computer Assisted Sperm Analysis (CASA). Data are presented as mean $\pm$ standard deviation. Within day and between age, values sharing a letter are not significantly different (GLMM post hoc, $P \geq 0.05$).

Season	Day	Age	AIL (%)	Motility (%)	PM (%)	RapidP	RapidNP	Medium	Slow
Spring	Day 0	Age 1	79.42 $\pm$ 7.81	85.87 $\pm$ 9.38 ^a	61.34 $\pm$ 12.71 ^a	55.09 $\pm$ 13.93 ^a	16.43 $\pm$ 8.82 ^a	8.28 $\pm$ 4.27 ^a	6.08 $\pm$ 2.72 ^{ab}
		Age 2	80.50 $\pm$ 5.79	91.97 $\pm$ 1.59 ^b	78.73 $\pm$ 5.08 ^b	74.56 $\pm$ 5.47 ^b	6.78 $\pm$ 3.88 ^b	5.42 $\pm$ 1.64 ^b	5.22 $\pm$ 1.30 ^a
		Age 3	79.67 $\pm$ 4.26	90.73 $\pm$ 3.22 ^b	74.49 $\pm$ 9.57 ^b	69.52 $\pm$ 10.39 ^b	7.96 $\pm$ 6.35 ^b	6.43 $\pm$ 2.03 ^{ab}	6.83 $\pm$ 2.57 ^b
Autumn	Day 5	Age 1	70.33 $\pm$ 7.12 ^x	75.64 $\pm$ 12.92 ^x	56.42 $\pm$ 13.66 ^x	46.04 $\pm$ 14.41 ^x	7.60 $\pm$ 3.56 ^x	13.95 $\pm$ 4.70 ^x	8.06 $\pm$ 1.73
		Age 2	76.15 $\pm$ 7.29 ^y	85.20 $\pm$ 4.26 ^y	70.99 $\pm$ 5.15 ^y	63.24 $\pm$ 7.93 ^y	5.35 $\pm$ 2.46 ^y	9.69 $\pm$ 4.19 ^y	6.94 $\pm$ 1.71
		Age 3	76.59 $\pm$ 5.45 ^y	84.90 $\pm$ 3.79 ^y	68.95 $\pm$ 7.80 ^y	61.84 $\pm$ 8.88 ^y	6.14 $\pm$ 3.95 ^y	9.05 $\pm$ 2.48 ^y	7.88 $\pm$ 1.36
	Day 0	Age 1	82.90 $\pm$ 7.71	86.46 $\pm$ 6.68	71.31 $\pm$ 8.62	62.42 $\pm$ 10.77 ^a	5.62 $\pm$ 3.10 ^a	10.88 $\pm$ 3.89 ^a	7.54 $\pm$ 2.98
		Age 2	86.03 $\pm$ 1.82	85.03 $\pm$ 8.44	72.99 $\pm$ 8.97	62.60 $\pm$ 12.91 ^a	3.37 $\pm$ 2.06 ^b	11.89 $\pm$ 6.76 ^a	7.17 $\pm$ 2.34
		Age 3	86.08 $\pm$ 2.75	87.36 $\pm$ 5.80	75.94 $\pm$ 7.41	70.28 $\pm$ 9.14 ^b	3.29 $\pm$ 1.49 ^{ab}	6.90 $\pm$ 2.24 ^b	6.89 $\pm$ 2.56
	Day 5	Age 1	80.53 $\pm$ 8.65 ^x	67.36 $\pm$ 16.29 ^x	49.52 $\pm$ 15.45 ^x	34.21 $\pm$ 14.50 ^x	2.16 $\pm$ 1.13 ^x	19.15 $\pm$ 7.56 ^x	11.84 $\pm$ 3.66 ^x
		Age 2	83.34 $\pm$ 3.71 ^y	76.17 $\pm$ 12.87 ^y	61.11 $\pm$ 13.35 ^y	51.36 $\pm$ 15.16 ^y	3.42 $\pm$ 2.35 ^y	11.69 $\pm$ 4.38 ^y	9.71 $\pm$ 3.09 ^y
		Age 3	84.86 $\pm$ 4.09 ^y	79.46 $\pm$ 12.04 ^y	65.01 $\pm$ 14.04 ^y	56.14 $\pm$ 16.61 ^y	3.60 $\pm$ 1.99 ^y	10.57 $\pm$ 4.41 ^y	9.14 $\pm$ 3.38 ^y

Note. AIL, acrosome intact live spermatozoa; PM, progressive motile spermatozoa (straight-line velocity [STR] $> 45\%$); Rapid, rapid motile spermatozoa (velocity average path [VAP] $> 50 \mu\text{m/s}$); RapidP, rapid progressive spermatozoa (VAP $> 50 \mu\text{m/s}$ and STR $> 45\%$); RapidNP, rapid non-progressive spermatozoa (VAP $> 50 \mu\text{m/s}$ and STR $< 45\%$); Medium, medium motile spermatozoa (VAP 20-50 $\mu\text{m/s}$); Slow, slow spermatozoa (VAP 10-20 $\mu\text{m/s}$).

Table 5: Kinematic parameters of sperm motility during sexual maturation of Duroc boars by season, day and age. Semen samples were collected from 28 Duroc boars (N = 15 in spring, N = 13 in autumn) at approximately 7 months (Age 1), 6 weeks later (Age 2) and 12 weeks later (Age 3) of first collection. Samples were analysed immediately after collection on day 0 and after 5 days of storage at 18°C (Day 5) in spring and autumn. Sperm kinematic parameters assessed by Computer Assisted Sperm Analysis (CASA). Data are presented as mean $\pm$ standard deviation. Within day and between age, values sharing a letter are not significantly different (GLMM post hoc, $P \geq 0.05$).

Season	Day	Age	VAP ($\mu\text{m/s}$)	VCL ($\mu\text{m/s}$)	VSL ($\mu\text{m/s}$)	STR (%)	LIN (%)	WOB (%)	ALH (μm)	BCF (Hz)
Spring	Day 0	Age 1	74.70 $\pm$ 7.62 ^a	132.24 $\pm$ 18.51 ^a	48.68 $\pm$ 8.56 ^a	64.09 $\pm$ 8.32 ^a	39.29 $\pm$ 8.84 ^a	58.55 $\pm$ 6.57 ^a	3.64 $\pm$ 0.60 ^a	20.84 $\pm$ 1.50 ^a
		Age 2	82.33 $\pm$ 4.94 ^b	118.75 $\pm$ 10.39 ^b	64.91 $\pm$ 6.51 ^b	76.28 $\pm$ 4.55 ^b	55.35 $\pm$ 6.68 ^b	70.41 $\pm$ 5.27 ^b	3.19 $\pm$ 0.42 ^b	19.41 $\pm$ 2.14 ^b
		Age 3	79.78 $\pm$ 5.45 ^b	120.84 $\pm$ 10.56 ^b	61.37 $\pm$ 9.17 ^b	73.96 $\pm$ 7.10 ^b	51.43 $\pm$ 8.75 ^c	67.08 $\pm$ 6.32 ^c	3.32 $\pm$ 0.45 ^b	19.50 $\pm$ 2.21 ^b
Autumn	Day 5	Age 1	66.01 $\pm$ 8.16 ^x	126.12 $\pm$ 11.22	46.44 $\pm$ 7.64 ^x	66.80 $\pm$ 5.05 ^x	37.03 $\pm$ 4.81 ^x	52.81 $\pm$ 3.35 ^x	3.65 $\pm$ 0.31 ^x	21.17 $\pm$ 1.73 ^x
		Age 2	80.00 $\pm$ 8.76 ^y	129.02 $\pm$ 10.12	62.95 $\pm$ 7.23 ^y	75.02 $\pm$ 3.16 ^y	48.46 $\pm$ 4.75 ^y	62.31 $\pm$ 4.81 ^y	3.55 $\pm$ 0.33 ^{xy}	20.18 $\pm$ 1.53 ^y
		Age 3	78.46 $\pm$ 7.59 ^y	124.81 $\pm$ 8.10	61.66 $\pm$ 10.37 ^y	74.61 $\pm$ 6.23 ^y	49.43 $\pm$ 7.90 ^y	63.42 $\pm$ 6.00 ^y	3.41 $\pm$ 0.36 ^x	20.06 $\pm$ 2.14 ^y
	Day 0	Age 1	74.61 $\pm$ 10.23	111.81 $\pm$ 16.77 ^a	59.16 $\pm$ 9.60	75.66 $\pm$ 4.19 ^a	53.49 $\pm$ 6.81 ^a	67.91 $\pm$ 5.26 ^a	3.18 $\pm$ 0.59 ^a	18.37 $\pm$ 2.05
		Age 2	73.34 $\pm$ 10.88	102.07 $\pm$ 16.76 ^b	60.69 $\pm$ 9.57	79.23 $\pm$ 3.28 ^b	59.28 $\pm$ 5.38 ^b	72.62 $\pm$ 4.04 ^b	2.84 $\pm$ 0.47 ^b	18.73 $\pm$ 2.27
		Age 3	77.33 $\pm$ 5.76	105.95 $\pm$ 9.76 ^{ab}	64.36 $\pm$ 5.02	79.85 $\pm$ 2.60 ^b	60.15 $\pm$ 4.82 ^b	73.28 $\pm$ 4.26 ^b	2.88 $\pm$ 0.33 ^b	18.46 $\pm$ 2.23
Day 5	Age 1	Age 1	56.84 $\pm$ 12.19 ^x	102.31 $\pm$ 20.80 ^x	45.42 $\pm$ 11.37 ^x	72.72 $\pm$ 4.39 ^x	43.80 $\pm$ 5.60 ^x	56.46 $\pm$ 5.20 ^x	3.07 $\pm$ 0.49	19.48 $\pm$ 1.35
		Age 2	71.20 $\pm$ 11.75 ^y	110.77 $\pm$ 19.21 ^y	57.77 $\pm$ 8.98 ^y	76.28 $\pm$ 2.63 ^y	51.76 $\pm$ 4.05 ^y	64.90 $\pm$ 3.22 ^y	3.09 $\pm$ 0.39	19.21 $\pm$ 2.08
		Age 3	73.49 $\pm$ 12.64 ^y	113.05 $\pm$ 19.21 ^y	59.90 $\pm$ 10.14 ^y	77.11 $\pm$ 2.71 ^y	52.78 $\pm$ 4.50 ^y	65.80 $\pm$ 4.09 ^y	3.14 $\pm$ 0.39	19.07 $\pm$ 2.16

Note. VAP, velocity average path; VCL, velocity curved line; VSL, velocity straight line; STR, straightness (VSL/VAP $\times$ 100); LIN, linearity (VSL/VCL $\times$ 100); WOB, wobble (VAP/VCL $\times$ 100); ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Relationship between semen metabolites and sperm quality parameters

Repeated measure correlation analysis revealed a significant relationship between sperm and seminal plasma metabolites and several sperm quality parameters (Figure 4a and 4b, Supplementary Table 5, Supplementary Table 6).

For spermatozoa, cystine showed significant negative correlations (adjusted $P < 0.05$) with PM, RapidP, motility, and AIL, while positively correlating with RapidNP and %DFI. In addition, several kinematic parameters were correlated with cystine concentrations (Supplementary Table 5). Moreover, taurine, aspartate, glutamate and choline concentrations were all negatively correlated with PM and RapidP and positively associated with RapidNP (adjusted $P < 0.05$, Figure 4a). Hydroxyproline, however, was only inversely related to PM and RapidP. Regarding metabolites in seminal plasma, aspartate, glutamate, choline and L-carnitine were all negatively correlated with PM and RapidP while positively associated with RapidNP (adjusted $P < 0.05$). Taurine was positively correlated with RapidNP, while phenylalanine had a positive correlation with PM and RapidP. Cysteine and glutamine, on the other hand, showed a negative correlation with sperm quality parameters. Cystine showed an inverse relationship with PM and RapidP, and glutamine specifically correlated negatively with AIL (adjusted $P < 0.05$, Figure 4b).

The complete set of significant correlation coefficients for all 26 amino acids and 23 sperm parameters is provided in Supplementary Table 5 and Supplementary Table 6.

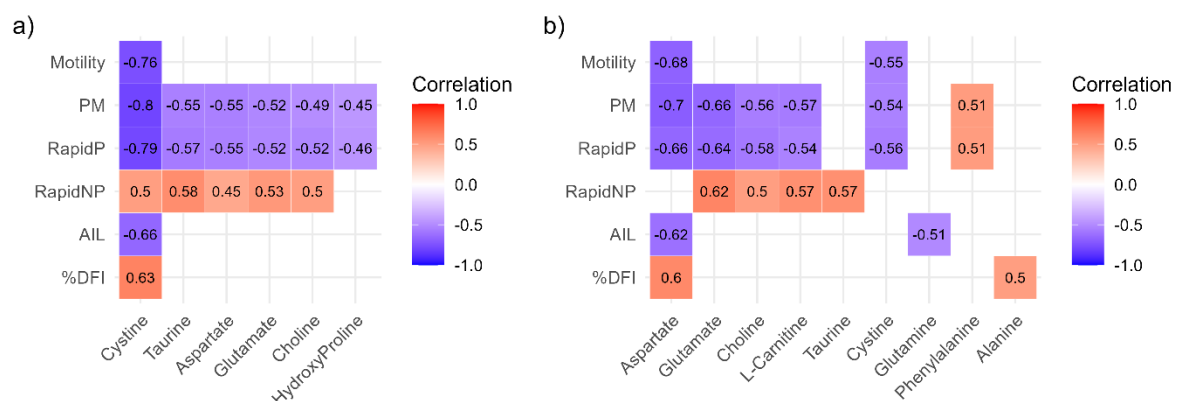


Figure 4: Heatmap displaying significant repeated measure correlation coefficients (r_{rm}) between key sperm quality parameters and a) sperm metabolites and b) seminal plasma metabolites in Duroc boars. Semen samples were collected from 15 Duroc boars at approximately 7 months (Age 1), and 6 (Age 2), and 12 weeks later (Age 3) of first collection. Samples were analysed immediately after collection. Blue indicates negative correlations, while red denotes positive correlations. Only statistically significant and interesting correlations (adj $P < 0.05$) are

displayed. Abbreviations: PM, progressive motility; RapidP, rapid progressive; RapidNP, rapid non-progressive; AIL, acrosome intact live; %DFI, percentage DNA fragmentation index.

4. Discussion

Our longitudinal investigation reveals that Duroc boars' sperm functional parameters undergo substantial improvements during sexual maturation. Several sperm quality parameters including acrosome intact live and rapid progressive motility increased significantly between the youngest boars (Age 1) and more mature boars (Age 2 and Age 3), with none of them showing significant difference between Age 2 and Age 3, indicating that these parameters stabilize at Age 2. After storage of 5 days at 18°C, this trend persisted and became even more pronounced, suggesting that including an analysis of motility after storage might increase differences between samples and provide more information for the selection of boars for semen production.

Kinematic analysis in our study revealed age-related swimming patterns. Young boars at Age 1 produced sperm with higher amplitude of lateral head displacement and more erratic swimming patterns (higher VCL), consistent with higher RapidNP, while mature boars (Age 3) achieved more linear trajectories (higher VSL, LIN, STR) and higher proportion of RapidP. This shift suggests that the reproductive system undergoes fine-tuning during sexual maturation with swimming efficiency rather than simply producing more motile cells. Earlier studies have shown relationships between rapid motility and linear swimming pattern with fertility in boars (Fernández-López et al., 2022; Bae et al., 2024). Including the measurements of percentage RapidP at AI stations could be valuable information in the selection of semen doses.

While several studies have investigated the effect of age on boar sperm quality (Knecht et al., 2017; Wang et al., 2017; Hensel et al., 2023), our study is the first longitudinal examination of this critical early maturation phase. Our results align with the previous observation by Kawęcka et al. (2008) and Knecht et al. (2017) which reported an increase in total motile spermatozoa and spermatozoa with normal acrosome after 8-9 months in several domestic breeds of pigs, including Duroc. Similarly, a study by Schulze et al. (2014) reported that ejaculates meeting commercial-grade standards are not produced reliably until at least 8 months of age with samples collected earlier, typically showed reduced sperm concentration, ejaculate volume, and motility, along with increased morphological defects, which cause them to fall below quality-control criteria for commercial application.

Another parameter important for fertility is chromatin integrity. In this study, Duroc boars at Age 3 showed significant improvement in sperm chromatin integrity with lower %DFI and %HDS compared to Age 1. This decline in %DFI and %HDS with advancing age reflects gradual chromatin packaging maturation. As boars mature, spermatogenesis produces sperm with more tightly packed DNA and fewer chromatin defects, reducing both DNA breaks and the proportion of immature sperm (Tsakmakidis et al., 2012). A previous study by Boe-Hansen et al. (2008) reported that boars with DFI above ~2% experienced slightly reduced litter sizes, while values exceeding 6% significantly compromise farrowing rates (Evenson, 2016). It is worth noting that average %DFI were very low in our study, around 1% in the youngest boars (Age 1) compared to Age 3 (< 0.7%), suggesting minimal DNA damage with most likely negligible biological impact on reproductive outcomes. This is supported by Godinho et al. (2025), who reported no effects on litter size and farrowing rate in boars with a DNA fragmentation index of 1.33%. These superior chromatin quality metrics in our boars could be attributed to their genetic background. It is important to consider that other breeds or non-selected lines may exhibit higher baseline DFI values (Boe-Hansen et al., 2008; Didion et al., 2009). These results show that the boars in this study enter puberty with high chromatin quality and gain only slight improvements as they mature.

Another interesting finding from our study is the differential response to storage stress across age groups. While all samples from all three ages showed some decline in their quality parameters after five days of storage at 18°C, which is in line with previous study by Paulenz et al. (2000), the magnitude of quality decline was most pronounced in samples from the youngest boars. Samples from Age 2 and Age 3 showed better storage tolerance in maintaining higher motility, acrosome integrity and viability than Age 1. This shows that with sexual maturity, storage tolerance capacity increases. From a practical standpoint, these findings suggest that semen from boars at Age 2 (mean 8.2 months) might offer superior performance in commercial AI programs, particularly when longer storage intervals are necessary.

The correlation analysis between amino acid concentrations and sperm quality parameters in our study reveals an interesting biochemical narrative of sexual maturation. The significant negative correlation between spermatozoal cystine, glutamate, aspartate, choline and taurine, with PM and RapidP suggest that metabolic efficiency improves with age. Rather than requiring higher concentration of these metabolites, sperm from mature boars appear to achieve superior function with more refined metabolic profiles. This pattern aligns well with the reported roles of these metabolites in sperm function. Glutamate influences sperm motility by modulating Ca^{2+} signals through N-methyl-D-aspartate receptors (Endo et al., 2019),

influences sperm chemotaxis (Frolikova et al., 2020), acrosome reaction (Momeni et al., 2021), enhances antioxidant capacity and stimulates spermatogonial cell proliferation during development (Santillo et al., 2019; Tang et al., 2020). However, excessive levels can compromise sperm quality and mitochondrial integrity as reported in mice and *Drosophila* spermatids respectively (Kayode et al., 2020; Vedelek et al., 2023). This could be the case in our study where the youngest boars had higher levels of glutamate, which could be too high, and the concentration need to stabilise before motility would be optimal, as we see at Age 2 and Age 3. Similarly, taurine maintains sperm membrane integrity and acts as an antioxidant protecting against oxidative stress (Li et al., 2023), while D-aspartate stimulates testosterone synthesis and improve sperm concentration and motility in human, however, higher aspartate concentrations is reported to suppress germ cell differentiation in mice testis cultures (D’Aniello et al., 2012; Tomita et al., 2016). Choline, essential for membrane structure and fluidity, promotes motility via cholinergic signaling through acetylcholine conversion (Bray et al., 2006; Johnson et al., 2012), yet excess choline can transiently impair spermatogenesis as reported in mice (Vachhrajani et al., 1993). The positive correlations between these metabolites and RapidNP suggest that too high metabolite concentrations could give more sperm with hyperactivated swimming patterns in younger animals compared to mature boars. The ability to be hyperactive *in vivo* in the oviduct is likely necessary for successful fertilization (Suarez and Ho, 2003), but having a high proportion of rapid non-progressive sperm in an insemination dose is presumably not beneficial. Seminal plasma glutamate, aspartate, choline, and taurine showed similar correlation results with sperm parameters. These metabolomic patterns extend our previous findings of declining concentration of sperm glutamate, alanine, aspartate, choline, taurine between 7 and 10 months (Shrestha et al., 2025), indicate direct relationships between metabolite concentrations and sperm functional properties.

While our primary focus was on age effects, we also observed noteworthy seasonal variations, however, and importantly, the sexual-maturity related improvements we documented remain consistent across both seasons, indicating that sexual maturation effects are robust and not merely caused by seasonal variation.

Longitudinal design represents a strength allowing us to track true developmental improvements within individuals rather than inferring patterns from cross-sectional comparisons. Comprehensive assessment combining traditional semen quality metrics and metabolites concentrations provides a wider picture of sexual maturation. These findings carry practical implications for breeding companies like Norsvin, which currently initiate semen collection after 8 months of age. Our data suggests that while 7-months old boars can produce

viable semen, the significant improvements documented between Age 1 and Age 2 indicate that earlier collection might compromise both semen quality and storage performance. However, knowing that boars at 7 months of age have lower sperm quality, but still quite high, it could be possible for the AI companies to increase the number of spermatozoa per AI dose used for insemination to get the same amounts of functional sperm during insemination, and most likely obtain the same level of fertility. By this strategy, it could be possible to use valuable boars earlier in the breeding program. The enhanced storage resilience of semen in mature boars could also be particularly valuable for commercial operations requiring extended storage periods or shipment to distant locations.

Our focus on a single breed (Duroc) with a moderate sample size of 28, collected during specific seasonal periods, may limit the generalizability of our findings to other swine breeds and environmental conditions. Additionally, in this study, there was an absence of fertility data from Age 1 and limited data from Age 2 and Age 3, as several of the boars were culled with age. This limits our ability to assess reproductive outcomes directly. While the sperm quality improvements we documented align with parameters known to influence fertility (Broekhuijse et al., 2012), we cannot definitively establish that these changes translate to improved conception rates or litter sizes in commercial settings. The casual relationships through field fertility data and underlying enzymatic pathways remain an important future objective.

Future research directions should also include validation across multiple breeds and correlation of these sperm quality parameters and metabolites with field fertility. Moreover, the metabolites studied herein require further experimentation to determine their potential as biomarkers of sexual maturity. Developing rapid, cost-effective assays for key metabolites, to be used together with motility assessment by CASA, could enable breeding companies to assess individual young boar readiness for semen production and their integration in breeding programs. Experiments with feed intervention could also be performed to analyze the effects of specific metabolites on sperm quality in younger boars. Our storage findings suggest opportunities for optimizing semen preservation protocols based on boar age. Investigating whether age-specific extender formulation or storage conditions could further enhance the preservation of semen from younger animals might help bridge the gap between early genetic selection goals and optimal reproductive performance.

5. Conclusion

Our study demonstrates that sexual maturation in Duroc boars is associated with improvements in sperm quality, including motility, viability, acrosome integrity, and storage resilience. Key sperm quality parameters, such as progressive motility and kinematic traits, stabilize around 8.2 months of age, supporting current industry practices regarding optimal timing for semen collection. Furthermore, the observed correlation between glutamate, aspartate, choline, and taurine concentrations with sperm quality parameters suggests that sexual maturation involves biochemical optimization. These findings provide opportunities for developing precise assessment tools to evaluate sperm quality better and optimize the use of younger boars in breeding programs.

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CRedit authorship contribution statement

Soudabeh Rozebehani: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. Asmita Shrestha: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. Nazia Haque: Methodology, Investigation. Syeda Afsana Nushrat: Methodology, Investigation. Tewoldebrhan Zeremichael: Methodology, Investigation. Anna Nordborg: Methodology, Investigation, Writing – review & editing. Ann Helen Gaustad: Conceptualization, Resources, Writing – review & editing. Anne Hege Alm-Kristiansen: Conceptualization, Resources, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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References

- Abah, K.O., Fontbonne, A., Partyka, A., Nizanski, W., 2023. Effect of male age on semen quality in domestic animals: potential for advanced functional and translational research? *Veterinary Research Communications* 47, 1125-1137.
- Bae, J.W., Hwang, J.M., Lee, W.J., Kim, D.H., Yi, J.K., Ha, J.J., Oh, D.Y., Kwon, W.S., 2024. Application of sperm motion kinematics and motility-related proteins for prediction of male fertility. *Theriogenology* 218, 223-230.
- Banaszewska, D., Kondracki, S., 2012. An Assessment of the Breeding Maturity of Insemination Boars Based on Ejaculate Quality Changes. *Folia Biologica* 60, 151-162.
- Boe-Hansen, G.B., Christensen, P., Vibjerg, D., Nielsen, M.B., Hedeboe, A.M., 2008. Sperm chromatin structure integrity in liquid stored boar semen and its relationships with field fertility. *Theriogenology* 69, 728-736.
- Boe-Hansen, G.B., Morris, I.D., Ersbøll, A.K., Greve, T., Christensen, P., 2005. DNA integrity in sexed bull sperm assessed by neutral Comet assay and sperm chromatin structure assay. *Theriogenology* 63, 1789-1802.
- Borg, K.E., Lunstra, D.D., Christenson, R.K., 1993. Semen characteristics, testicular size, and reproductive hormone concentrations in mature duroc, meishan, fengjing, and minzhu boars. *Biol Reprod* 49, 515-521.
- Bray, C., Son, J.-H., Meizel, S., 2006. Acetylcholine causes an increase of intracellular calcium in human sperm. *Molecular Human Reproduction* 11, 881-889.
- Broekhuijse, M.L.W.J., Šoštarić, E., Feitsma, H., Gadella, B.M., 2012. Application of computer-assisted semen analysis to explain variations in pig fertility¹. *Journal of Animal Science* 90, 779-789.
- D’Aniello, G., Ronsini, S., Notari, T., Grieco, N., Infante, V., D’Angel, N., Mascia, F., Fiore, M.M.D., Fisher, G., D’Aniello, A., 2012. D-Aspartate, a Key Element for the Improvement of Sperm Quality. *Advances in Sexual Medicine* 02, 45-53.
- Didion, B.A., Kasperson, K.M., Wixon, R.L., Evenson, D.P., 2009. Boar Fertility and Sperm Chromatin Structure Status: A Retrospective Report. *Journal of Andrology* 30, 655-660.
- Endo, D., Kon, S., Sato, T., Toyama, F., Katsura, Y., Nakauchi, Y., Takayama-Watanabe, E., Watanabe, A., 2019. NMDA-type glutamate receptors mediate the acrosome reaction and motility initiation in newt sperm. *Molecular Reproduction and Development* 86, 1106-1115.
- Evenson, D., Jost, L., 2001. Sperm chromatin structure assay for fertility assessment. *Curr Protoc Cytom Chapter 7, Unit 7.13*.

569 Evenson, D.P., 2016. The Sperm Chromatin Structure Assay (SCSA®) and other sperm DNA
570 fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. *Anim*
571 *Reprod Sci* 169, 56-75.

572 Fernández-López, P., Garriga, J., Casas, I., Yeste, M., Bartumeus, F., 2022. Predicting fertility
573 from sperm motility landscapes. *Communications Biology* 5, 1027.

574 Fraser, L., Strzeżek, J., Filipowicz, K., Mogielnicka-Brzozowska, M., Zasiadczyk, L., 2016.
575 Age and seasonal-dependent variations in the biochemical composition of boar semen.
576 *Theriogenology* 86, 806-816.

577 Frolikova, M., Otcenaskova, T., Valasková, E., Postlerova, P., Stopkova, R., Stopka, P.,
578 Komrskova, K., 2020. The Role of Taste Receptor mTAS1R3 in Chemical Communication of
579 Gametes. *International Journal of Molecular Sciences* 21, 2651.

580 Godinho, R.M., Andersen-Ranberg, I., Gaustad, A.H., da Silva, C.L.A., Grindflek, E., Zak, L.J.,
581 2025. Fine-tuning the screening of young boars for production centers of artificial insemination
582 semen doses. *Anim Reprod Sci* 272, 107727.

583 Hartig, F., 2024. DHARMA: Residual Diagnostics for Hierarchical (Multi-Level / Mixed)
584 Regression Models, R package version 0.4.7.

585 Hensel, B., Pieper, L., Jung, M., Schulze, M., 2023. Influence of age, breed, and season on the
586 quality of boar semen stored at low-temperature. *Theriogenology* 208, 102-108.

587 Huang, Y.H., Lo, L.L., Liu, S.H., Yang, T.S., 2010. Age-related changes in semen quality
588 characteristics and expectations of reproductive longevity in Duroc boars. *Anim Sci J* 81, 432-
589 437.

590 Johnson, A.R., Lao, S., Wang, T., Galanko, J.A., Zeisel, S.H., 2012. Choline dehydrogenase
591 polymorphism rs12676 is a functional variation and is associated with changes in human sperm
592 cell function. *PLoS One* 7, e36047.

593 Kamanova, V., Nevrkla, P., Hadas, Z., Lujka, J., Filipcik, R., 2021. Changes of sperm
594 morphology in Duroc, Landrace and Large White boars depending on the ambient temperature
595 during the year. *Vet Med (Praha)* 66, 189-196.

596 Kawęcka, M., Pietruszka, A., Jacyno, E., Czarnecki, R., Kamyczek, M., 2008. Quality of semen
597 of young boars of the breeds Pietrain and Duroc and their reciprocal crosses. *Archives Animal*
598 *Breeding* 51, 42-54.

599 Kayode, O.T., Rotimi, D.E., Kayode, A.A.A., Olaolu, T.D., Adeyemi, O.S., 2020. Monosodium
600 Glutamate (MSG)-Induced Male Reproductive Dysfunction: A Mini Review. *Toxics* 8.

Knecht, D., Jankowska-Mąkosza, A., Duziński, K., 2017. The effect of age, interval collection and season on selected semen parameters and prediction of AI boars productivity. *Livestock Science* 201, 13-21.

Lenth, R.V., Banfai, B., Bolker, B., Buerkner, P., GinÃ©-VÃ¡zquez, I., Herve, M., Jung, M., Love, J., Miguez, F., Piaskowski, J., Riebl, H., Singmann, H., 2025. emmeans: Estimated Marginal Means, aka Least-Squares Means.

Li, Y., Peng, Q., Shang, J., Dong, W., Wu, S., Guo, X., Xie, Z., Chen, C., 2023. The role of taurine in male reproduction: Physiology, pathology and toxicology. *Front Endocrinol (Lausanne)* 14, 1017886.

Mateo-Otero, Y., Fernandez-Lopez, P., Delgado-Bermudez, A., Nolis, P., Roca, J., Miro, J., Barranco, I., Yeste, M., 2021a. Metabolomic fingerprinting of pig seminal plasma identifies in vivo fertility biomarkers. *Journal of Animal Science and Biotechnology* 12, 113.

Mateo-Otero, Y., Fernandez-Lopez, P., Ribas-Maynou, J., Roca, J., Miro, J., Yeste, M., Barranco, I., 2021b. Metabolite Profiling of Pig Seminal Plasma Identifies Potential Biomarkers for Sperm Resilience to Liquid Preservation. *Front Cell Dev Biol* 9, 669974.

Mateo-Otero, Y., Madrid-Gambin, F., Lllavanera, M., Gomez-Gomez, A., Haro, N., Pozo, O.J., Yeste, M., 2023. Sperm physiology and in vitro fertilising ability rely on basal metabolic activity: insights from the pig model. *Communications Biology* 6, 344.

McGillycuddy, M., Popovic, G., Bolker, B.M., Warton, D.I., 2025. Parsimoniously Fitting Large Multivariate Random Effects in glmmTMB. *Journal of Statistical Software* 112, 1 - 19.

Memili, E., Moura, A.A., Kaya, A., 2020. Metabolomes of sperm and seminal plasma associated with bull fertility. *Anim Reprod Sci* 220, 106355.

Momeni, H.R., Etemadi, T., Alyasin, A., Eskandari, N., 2021. A novel role for involvement of N-methyl-D-aspartate (NMDA) glutamate receptors in sperm acrosome reaction. *Andrologia* 53, e14203.

Paulenz, H., Kommisrud, E., Hofmo, P., 2000. Effect of Long-Term Storage at Different Temperatures on the Quality of Liquid Boar Semen. *Reproduction in Domestic Animals* 35, 83-87.

Samore, A.B., and Fontanesi, L., 2016. Genomic selection in pigs: state of the art and perspectives. *Italian Journal of Animal Science* 15, 211-232.

Santillo, A., Falvo, S., Fiore, M.M.D., Russo, F.D.G., Chieffi, P., Usiello, A., Pinelli, C., Baccari, G.C., 2019. *Journal of Cellular Biochemistry | Wiley Online Library*.

Schulze, M., Buder, S., Rudiger, K., Beyerbach, M., Waberski, D., 2014. Influences on semen traits used for selection of young AI boars. *Anim Reprod Sci* 148, 164-170.

Shrestha, A., Gaustad, A.H., Øiaas, J.B., Kommisrud, E., van Son, M., Nordborg, A., Alm-Kristiansen, A.H., 2025. A metabolomic study uncovering key amino acids and amines in Duroc boar semen as biomarkers of sexual maturity. *Anim Reprod Sci*, 107800.

Suarez, S.S., Ho, H.C., 2003. Hyperactivated Motility in Sperm. *Reproduction in Domestic Animals* 38, 119-124.

Tang, W., Wu, J., Jin, S., He, L., Lin, Q., Luo, F., He, X., Feng, Y., He, B., Bing, P., Li, T., Yin, Y., 2020. Glutamate and aspartate alleviate testicular/epididymal oxidative stress by supporting antioxidant enzymes and immune defense systems in boars. *Science China Life Sciences* 63, 116-124.

Tomita, K., Tanaka, H., Kageyama, S., Nagasawa, M., Wada, A., Murai, R., Kobayashi, K., Hanada, E., Agata, Y., Kawauchi, A., 2016. The Effect of d-Aspartate on Spermatogenesis in Mouse Testis1. *Biology of Reproduction* 94.

Tsakmakidis, I.A., Khalifa, T.A., Boscov, C.M., 2012. Age-related changes in quality and fertility of porcine semen. *Biol Res* 45, 381-386.

Vachhrajani, K.D., Sahu, A.P., Dutta, K.K., 1993. Excess choline availability: A transient effect on spermatogenesis in the rat. *Reproductive Toxicology* 7, 477-481.

Vedelek, V., Vedelek, B., Lőrincz, P., Juhász, G., Sinka, R., 2023. A comparative analysis of fruit fly and human glutamate dehydrogenases in *Drosophila melanogaster* sperm development. *Frontiers in Cell and Developmental Biology* 11.

Wang, C., Li, J.L., Wei, H.K., Zhou, Y.F., Tan, J.J., Sun, H.Q., Jiang, S.W., Peng, J., 2017. Linear growth model analysis of factors affecting boar semen characteristics in Southern China. *J Anim Sci* 95, 5339-5346.

Supplementary Table 1: Age-related changes in sperm quality parameters during sexual maturation by day and age. Semen samples were collected from 28 Duroc boars at approximately 7 months (Age 1), 8.5 months (Age 2) and 10 months (Age 3) of age. Samples were analyzed immediately after collection on day 0 and after 5 days of storage at 18°C (Day 5) in spring and autumn. Motility analysis was conducted using Computer Assisted Sperm Analysis (CASA). Data are presented as mean $\pm$ standard deviation. Within day and between age, values sharing a letter are not significantly different (GLMM post hoc, $P \geq 0.05$).

Day	Age	AIL (%)	Motility (%)	PM (%)	RapidP	RapidNP	Medium	Slow
Day 0	Age 1	86.15 $\pm$ 8.10	65.97 $\pm$ 11.94	81.29 $\pm$ 7.82 ^a	58.49 $\pm$ 12.89 ^a	11.41 $\pm$ 8.65 ^a	9.49 $\pm$ 4.24 ^a	6.76 $\pm$ 2.89
	Age 2	88.75 $\pm$ 6.74	76.06 $\pm$ 7.59	83.46 $\pm$ 4.95 ^b	69.01 $\pm$ 11.25 ^b	5.20 $\pm$ 3.57 ^b	8.42 $\pm$ 5.70 ^{ab}	6.12 $\pm$ 2.07
	Age 3	89.16 $\pm$ 4.82	75.16 $\pm$ 8.51	83.10 $\pm$ 4.76 ^b	69.87 $\pm$ 9.66 ^b	5.79 $\pm$ 5.24 ^b	6.65 $\pm$ 2.10 ^b	6.86 $\pm$ 2.52
Day 5	Age 1	71.80 $\pm$ 14.90 ^a	53.22 $\pm$ 14.67 ^a	75.79 $\pm$ 9.39 ^a	40.55 $\pm$ 15.40 ^a	5.08 $\pm$ 3.85	16.36 $\pm$ 6.62 ^a	9.82 $\pm$ 3.35 ^a
	Age 2	81.01 $\pm$ 10.20 ^b	66.40 $\pm$ 10.87 ^b	80.00 $\pm$ 6.64 ^b	57.72 $\pm$ 13.08 ^b	4.45 $\pm$ 2.56	10.62 $\pm$ 4.32 ^b	8.22 $\pm$ 2.78 ^b
	Age 3	82.37 $\pm$ 8.92 ^b	67.12 $\pm$ 11.10 ^b	81.02 $\pm$ 6.29 ^b	59.20 $\pm$ 13.11 ^b	4.96 $\pm$ 3.39	9.75 $\pm$ 3.53 ^b	8.47 $\pm$ 2.54 ^b

Note. AIL, acrosome intact live spermatozoa; PM, progressive motile spermatozoa (straight-line velocity [STR] > 45%); Rapid, rapid motile spermatozoa (velocity average path [VAP] > 50 μ m/s); RapidP, rapid progressive spermatozoa (VAP > 50 μ m/s and STR > 45%); RapidNP, rapid non-progressive spermatozoa (VAP > 50 μ m/s and STR < 45%); Medium, medium motile spermatozoa (VAP 20-50 μ m/s); Slow, slow spermatozoa (VAP 10-20 μ m/s).

Supplementary Table 2: Kinematic parameters of sperm motility during sexual maturation of Duroc boars by day and age. Semen samples were collected from 28 Duroc boars at approximately 7 months (Age 1), 8.5 months (Age 2) and 10 months (Age 3) of age. Samples were analysed immediately after collection on Day 0 and after 5 days of storage at 18°C (Day 5) in spring and autumn. Sperm kinematic parameters assessed by Computer Assisted Sperm Analysis (CASA). Data are presented as mean $\pm$ standard deviation. Within day and between age, values sharing a letter are not significantly different (GLMM post hoc, $P \geq 0.05$).

Day	Age	VAP ($\mu\text{m/s}$)	VCL ($\mu\text{m/s}$)	VSL ($\mu\text{m/s}$)	STR (%)	LIN (%)	WOB (%)	ALH (μm)	BCF (Hz)
Day 0	Age 1	74.66 $\pm$ 8.75	122.75 $\pm$ 20.26 ^a	53.54 $\pm$ 10.36 ^a	69.46 $\pm$ 8.84 ^a	45.88 $\pm$ 10.63 ^a	62.90 $\pm$ 7.57 ^a	3.43 $\pm$ 0.63 ^a	19.69 $\pm$ 2.15
	Age 2	78.16 $\pm$ 9.28	111.01 $\pm$ 15.89 ^b	62.95 $\pm$ 8.20 ^b	77.65 $\pm$ 4.22 ^b	57.17 $\pm$ 6.32 ^b	71.43 $\pm$ 4.79 ^b	3.03 $\pm$ 0.47 ^b	19.10 $\pm$ 2.19
	Age 3	78.64 $\pm$ 5.63	113.93 $\pm$ 12.54 ^b	62.76 $\pm$ 7.56 ^b	76.69 $\pm$ 6.17 ^b	55.48 $\pm$ 8.34 ^b	69.96 $\pm$ 6.22 ^b	3.12 $\pm$ 0.45 ^b	19.02 $\pm$ 2.24
Day 5	Age 1	61.75 $\pm$ 11.05 ^a	115.06 $\pm$ 20.10	45.96 $\pm$ 9.38 ^a	69.55 $\pm$ 5.55 ^a	40.17 $\pm$ 6.15 ^a	54.51 $\pm$ 4.61 ^a	3.38 $\pm$ 0.49	20.38 $\pm$ 1.76
	Age 2	75.92 $\pm$ 11.00 ^b	120.55 $\pm$ 17.41	60.54 $\pm$ 8.36 ^b	75.61 $\pm$ 2.95 ^b	49.99 $\pm$ 4.67 ^b	63.52 $\pm$ 4.28 ^b	3.33 $\pm$ 0.42	19.73 $\pm$ 1.84
	Age 3	76.15 $\pm$ 10.36 ^b	119.35 $\pm$ 15.29	60.84 $\pm$ 10.11 ^b	75.77 $\pm$ 5.00 ^b	50.98 $\pm$ 6.65 ^b	64.53 $\pm$ 5.25 ^b	3.28 $\pm$ 0.39	19.60 $\pm$ 2.17

Note. VAP, velocity average path; VCL, velocity curved line; VSL, velocity straight line; STR, straightness ($\text{VSL/VAP} \times 100$); LIN, linearity ($\text{VSL/VCL} \times 100$); WOB, wobble ($\text{VAP/VCL} \times 100$); ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Supplementary Table 3: Descriptive statistics for sperm quality parameters measured on Day 0 (D0) and Day 5 (D5) of storage at -18°C. Values are presented as mean ± SD and median.

Parameters	Mean ± SD (D0)	Mean ± SD (D5)	Median (D0)	Median (D5)	<i>P</i> -value (D5-D0)
Motility	88.02 ± 6.74	78.39 ± 12.42	89.89	81.47	3.90 × 10 ⁻⁷ *
PM	72.40 ± 10.4	62.25 ± 13.78	76.10	65.89	0.04568*
VAP	77.15 ± 8.15	71.27 ± 12.65	79.25	72.88	0.00015*
VCL	115.90 ± 17.09	118.32 ± 17.65	115.86	121.54	0.07970
VSL	59.75 ± 9.74	55.78 ± 11.55	61.11	58.25	0.31714
BCF	19.27 ± 2.19	19.90 ± 1.9	19.30	19.88	0.38453
ALH	3.19 ± 0.54	3.33 ± 0.44	3.12	3.36	0.91995
STR	74.60 ± 7.5	73.64 ± 5.43	76.66	75.60	0.0314*
LIN	52.84 ± 9.87	47.05 ± 7.61	54.28	47.77	0.1575
WOB	68.10 ± 7.26	60.85 ± 6.51	69.25	61.21	3.14 × 10 ⁻⁵ *
RapidP	65.79 ± 12.35	52.49 ± 16.16	69.84	56.11	0.00128*
RapidNP	7.47 ± 6.7	4.83 ± 3.28	5.06	4.16	1.12 × 10 ⁻¹⁶ *
Medium	8.19 ± 4.3	12.24 ± 5.75	6.76	11.13	1.07 × 10 ⁻⁶ *
Slow	6.58 ± 2.50	8.84 ± 2.96	5.92	8.19	0.00068*
AIL	82.62 ± 6.01	78.94 ± 7.81	83.94	80.31	0.13458

*denotes statistical significance. AIL, acrosome intact live spermatozoa; PM, progressive motile spermatozoa (straight-line velocity [STR] > 45%); Rapid, rapid motile spermatozoa (velocity average path [VAP] > 50 µm/s); RapidP, rapid progressive spermatozoa (VAP > 50 µm/s and STR > 45%); RapidNP, rapid non-progressive spermatozoa (VAP > 50 µm/s and STR < 45%); Medium, medium motile spermatozoa (VAP 20-50 µm/s); Slow, slow spermatozoa (VAP 10-20 µm/s); VAP, velocity average path; VCL, velocity curved line; VSL, velocity straight line; STR, straightness (VSL/VAP × 100); LIN, linearity (VSL/VCL × 100); WOB, wobble (VAP/VCL × 100); ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Supplementary Table 4: Descriptive statistics for sperm quality parameters measured in Spring and Autumn. Values are presented as mean $\pm$ SD and median. Within seasons, values sharing a letter are not significantly different (GLMM post hoc, $P \geq 0.05$).

Parameters	Mean $\pm$ SD (Spring)	Mean $\pm$ SD (Autumn)	Median (Spring)	Median (Autumn)
Motility	85.72 $\pm$ 8.68	80.31 $\pm$ 12.76	87.94	85.57
PM	68.49 $\pm$ 12.04 ^a	65.98 $\pm$ 14.43 ^b	70.93	69.75
VAP	76.88 $\pm$ 8.86	71.14 $\pm$ 12.42	78.07	73.09
VCL	125.30 $\pm$ 12.48 ^a	107.66 $\pm$ 17.46 ^b	124.34	106.98
VSL	57.67 $\pm$ 10.92 ^a	57.88 $\pm$ 10.81 ^b	59.01	59.77
BCF	20.19 $\pm$ 1.95 ^a	18.89 $\pm$ 2.02 ^b	20.48	18.66
ALH	3.46 $\pm$ 4.45 ^a	3.03 $\pm$ 0.45 ^b	3.47	3.01
STR	71.79 $\pm$ 7.43 ^a	76.81 $\pm$ 4.04 ^b	74.61	77.20
LIN	46.83 $\pm$ 9.57 ^a	53.54 $\pm$ 7.45 ^b	48.60	53.69
WOB	62.43 $\pm$ 7.82 ^a	66.83 $\pm$ 7.07 ^b	62.84	67.94
RapidP	61.71 $\pm$ 13.94	56.17 $\pm$ 17.35	65.03	59.08
RapidNP	8.38 $\pm$ 6.34 ^a	3.58 $\pm$ 2.29 ^b	6.19	3.09
Medium	8.80 $\pm$ 4.32	11.85 $\pm$ 6.23	7.58	10.37
Slow	6.84 $\pm$ 2.16	8.72 $\pm$ 3.42	6.62	8.15
AIL	83.96 $\pm$ 5.61	77.11 $\pm$ 7.09	84.71	79.00
%DFI	0.85 $\pm$ 0.628	0.712 $\pm$ 0.20	0.67	0.66
SD DFI	20.7 $\pm$ 3.62 ^a	18.62 $\pm$ 2.06 ^b	20.05	18.39
MeanDFI	225.55 $\pm$ 5.37 ^a	231.52 $\pm$ 4.86 ^b	225.3	231.29
%HDS	1.95 $\pm$ 0.64 ^a	2.37 $\pm$ 0.73 ^b	1.89	2.29
Concentration	378.7 $\pm$ 163.32	293.92 $\pm$ 157.40	355.7	301.2

Note. AIL, acrosome intact live spermatozoa; PM, progressive motile spermatozoa (straight-line velocity [STR] > 45%); Rapid, rapid motile spermatozoa (velocity average path [VAP] > 50 μ m/s); RapidP, rapid progressive spermatozoa (VAP > 50 μ m/s and STR > 45%); RapidNP, rapid non-progressive spermatozoa (VAP > 50 μ m/s and STR < 45%); Medium, medium motile spermatozoa (VAP 20-50 μ m/s); Slow, slow spermatozoa (VAP 10-20 μ m/s); VAP, velocity average path; VCL, velocity curved line; VSL, velocity straight line; STR, straightness (VSL/VAP $\times$ 100); LIN, linearity (VSL/VCL $\times$ 100); WOB, wobble (VAP/VCL $\times$ 100); ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Supplementary Table 5: Repeated measure correlation analysis between spermatozoa metabolites and sperm quality parameters in Duroc boars ($n = 15$) from spring collection. Only statistically significant correlations (Adj $P < 0.05$) are presented with correlation coefficient (r_value).

Metabolite	Sperm parameter	r_value	P -adjusted
Cystine	PM	-0.80	3.00×10^{-5}
Cystine	RapidP	-0.79	3.00×10^{-5}
Cystine	Motility	-0.76	1.28×10^{-4}
Cystine	WOB	-0.76	1.31×10^{-4}
Cystine	LIN	-0.75	1.42×10^{-4}
Glutamate	WOB	-0.73	3.36×10^{-4}
Taurine	WOB	-0.73	3.39×10^{-4}
Cystine	VSL	-0.71	5.03×10^{-4}
Taurine	LIN	-0.71	5.03×10^{-4}
Cystine	STR	-0.70	6.08×10^{-4}
Glutamate	LIN	-0.70	6.08×10^{-4}
Aspartate	WOB	-0.70	6.08×10^{-4}
Glutamate	ALH	0.70	6.08×10^{-4}
Glutamate	Concentration	0.70	6.08×10^{-4}
Choline	WOB	-0.69	7.06×10^{-4}
Aspartate	LIN	-0.67	1.49×10^{-3}
Cystine	AIL	-0.66	1.67×10^{-3}
Choline	LIN	-0.66	2.14×10^{-3}
Taurine	ALH	0.65	2.14×10^{-3}
Taurine	VSL	-0.65	2.45×10^{-3}
Threonine	WOB	-0.65	2.45×10^{-3}
Aspartate	Concentration	0.65	2.45×10^{-3}
Taurine	STR	-0.64	2.48×10^{-3}
Glutamate	VCL	0.64	2.64×10^{-3}
Glutamate	AID	0.64	2.96×10^{-3}
Cystine	%DFI	0.63	3.22×10^{-3}
Choline	ALH	0.63	3.55×10^{-3}
Glycine	BCF	0.63	3.64×10^{-3}
Aspartate	VSL	-0.62	4.44×10^{-3}
Asparagine	WOB	-0.62	4.44×10^{-3}
Glutamate	STR	-0.62	4.46×10^{-3}
Cystine	ARD	0.61	4.58×10^{-3}
Choline	AID	0.61	5.51×10^{-3}
Glutamate	VSL	-0.60	6.08×10^{-3}

Cystine	VAP	-0.60	6.21×10^{-3}
Choline	VSL	-0.60	6.21×10^{-3}
L-Carnitine	ALH	0.60	6.21×10^{-3}
Taurine	VCL	0.60	6.08×10^{-3}
Aspartate	STR	-0.59	7.12×10^{-3}
Alanine	WOB	-0.59	7.12×10^{-3}
Taurine	BCF	0.59	7.12×10^{-3}
Taurine	AID	0.59	7.12×10^{-3}
Cystine	ALH	0.59	7.12×10^{-3}
Threonine	LIN	-0.59	7.12×10^{-3}
Asparagine	ALH	0.59	7.12×10^{-3}
Aspartate	ALH	0.59	7.13×10^{-3}
Cystine	Concentration	0.58	7.50×10^{-3}
Threonine	ALH	0.58	7.84×10^{-3}
L-Carnitine	WOB	-0.58	7.84×10^{-3}
Taurine	Concentration	0.58	7.84×10^{-3}
Taurine	RapidNP	0.58	8.26×10^{-3}
Cystine	SDDFI	0.58	8.26×10^{-3}
Choline	STR	-0.57	9.58×10^{-3}
Aspartate	AID	0.57	9.62×10^{-3}
Taurine	RapidP	-0.57	9.83×10^{-3}
Choline	Concentration	0.57	9.83×10^{-3}
Glutamate	BCF	0.56	1.16×10^{-2}
Choline	VCL	0.56	1.24×10^{-2}
Taurine	PM	-0.55	1.24×10^{-2}
Aspartate	RapidP	-0.55	1.24×10^{-2}
Threonine	BCF	0.55	1.24×10^{-2}
Aspartate	PM	-0.55	1.25×10^{-2}
Asparagine	LIN	-0.55	1.26×10^{-2}
Alanine	LIN	-0.55	1.32×10^{-2}
L-Carnitine	VCL	0.54	1.54×10^{-2}
Threonine	Concentration	0.54	1.55×10^{-2}
Choline	BCF	0.54	1.67×10^{-2}
Glycine	VSL	-0.53	1.78×10^{-2}
Threonine	VSL	-0.53	1.78×10^{-2}
Glutamate	RapidNP	0.53	1.78×10^{-2}
L-Carnitine	LIN	-0.53	1.78×10^{-2}
Alanine	ALH	0.53	1.81×10^{-2}

HydroxyProline	LIN	-0.53	1.86×10^{-2}
Glutamate	RapidP	-0.52	2.25×10^{-2}
HydroxyProline	BCF	0.52	2.27×10^{-2}
Aspartate	VCL	0.52	2.29×10^{-2}
HydroxyProline	WOB	-0.52	2.32×10^{-2}
Glutamate	PM	-0.52	2.33×10^{-2}
Choline	RapidP	-0.52	2.34×10^{-2}
HydroxyProline	VSL	-0.51	2.34×10^{-2}
L-Carnitine	Concentration	0.51	2.34×10^{-2}
Aspartate	BCF	0.51	2.37×10^{-2}
Asparagine	BCF	0.51	2.53×10^{-2}
Asparagine	Concentration	0.51	2.53×10^{-2}
Glycine	LIN	-0.51	2.54×10^{-2}
Alanine	Concentration	0.51	2.66×10^{-2}
Aspartate	VAP	-0.50	2.69×10^{-2}
Alanine	BCF	0.50	2.69×10^{-2}
Cystine	RapidNP	0.50	2.76×10^{-2}
Asparagine	VSL	-0.50	2.85×10^{-2}
Cystine	Medium	0.50	2.94×10^{-2}
Isoleucine	ALH	0.50	2.96×10^{-2}
Isoleucine	WOB	-0.50	2.96×10^{-2}
Cystine	AID	0.50	2.96×10^{-2}
Proline	WOB	-0.50	2.96×10^{-2}
Choline	RapidNP	0.50	2.99×10^{-2}
Proline	ALH	0.49	2.99×10^{-2}
HydroxyProline	STR	-0.49	2.99×10^{-2}
L-Carnitine	AID	0.49	2.99×10^{-2}
Asparagine	VCL	0.49	2.99×10^{-2}
Lysine	Concentration	0.49	3.07×10^{-2}
Threonine	VCL	0.49	3.07×10^{-2}
Alanine	VSL	-0.49	3.07×10^{-2}
Glycine	STR	-0.49	3.08×10^{-2}
Arginine	ALH	0.49	3.14×10^{-2}
Choline	PM	-0.49	3.14×10^{-2}
Creatine	WOB	-0.49	3.16×10^{-2}
Proline	BCF	0.49	3.17×10^{-2}
Arginine	Concentration	0.49	3.23×10^{-2}
Creatine	LIN	-0.48	3.38×10^{-2}

Glycine	WOB	-0.48	3.38×10^{-2}
Leucine	BCF	0.48	3.41×10^{-2}
Taurine	VAP	-0.48	3.50×10^{-2}
Threonine	AID	0.48	3.59×10^{-2}
Alanine	VCL	0.48	3.59×10^{-2}
Asparagine	AID	0.48	3.59×10^{-2}
Choline	VAP	-0.48	3.66×10^{-2}
Lysine	ALH	0.47	3.66×10^{-2}
Threonine	VAP	-0.47	3.66×10^{-2}
Leucine	ALH	0.47	3.67×10^{-2}
Isoleucine	BCF	0.47	3.81×10^{-2}
Proline	Concentration	0.47	3.86×10^{-2}
Cystine	VCL	0.47	3.86×10^{-2}
L-Carnitine	BCF	0.47	3.86×10^{-2}
Leucine	WOB	-0.47	3.86×10^{-2}
Threonine	STR	-0.47	3.86×10^{-2}
Lysine	WOB	-0.47	3.91×10^{-2}
Lysine	BCF	0.47	3.91×10^{-2}
Choline	Medium	0.47	3.92×10^{-2}
Arginine	BCF	0.47	3.98×10^{-2}
Arginine	WOB	-0.46	4.05×10^{-2}
Alanine	STR	-0.46	4.53×10^{-2}
HydroxyProline	RapidP	-0.46	4.54×10^{-2}
Leucine	Concentration	0.46	4.57×10^{-2}
Serine	WOB	-0.46	4.61×10^{-2}
Ornithine	AID	0.45	4.62×10^{-2}
Isoleucine	VCL	0.45	4.62×10^{-2}
Methionine	ALH	0.45	4.62×10^{-2}
Serine	Concentration	0.45	4.70×10^{-2}
Aspartate	RapidNP	0.45	4.76×10^{-2}
Alanine	AID	0.45	4.80×10^{-2}
HydroxyProline	PM	-0.45	4.96×10^{-2}

Supplementary Table 6: Repeated measure correlation analysis between seminal plasma metabolites and sperm quality parameters in Duroc boars (n = 15) from spring collection. Only statistically significant correlations (Adj $P < 0.05$) are presented with correlation coefficient (r_value).

Metabolite	Sperm Parameter	r_value	P -adjusted
Glutamate	Concentration	0.87	9.96×10^{-8}
L-Carnitine	Concentration	0.85	3.10×10^{-7}
Glutamate	WOB	-0.81	7.08×10^{-6}
Glutamate	ALH	0.81	7.08×10^{-6}
Glutamate	LIN	-0.78	2.32×10^{-5}
L-Carnitine	ALH	0.75	1.52×10^{-4}
Glutamate	VCL	0.72	3.90×10^{-4}
Aspartate	Concentration	0.72	3.90×10^{-4}
Glutamate	STR	-0.71	4.90×10^{-4}
Choline	Concentration	0.70	6.79×10^{-4}
Aspartate	PM	-0.70	6.88×10^{-4}
Choline	WOB	-0.70	6.88×10^{-4}
L-Carnitine	WOB	-0.69	7.67×10^{-4}
Taurine	Concentration	0.68	1.02×10^{-3}
Choline	ALH	0.68	1.02×10^{-3}
L-Carnitine	LIN	-0.68	1.11×10^{-3}
Aspartate	Motility	-0.68	1.11×10^{-3}
L-Carnitine	VCL	0.67	1.12×10^{-3}
Aspartate	WOB	-0.66	1.55×10^{-3}
Aspartate	LIN	-0.66	1.58×10^{-3}
Aspartate	RapidP	-0.66	1.58×10^{-3}
Choline	LIN	-0.66	1.64×10^{-3}
Glutamate	PM	-0.66	1.78×10^{-3}
Glutamate	RapidP	-0.64	2.62×10^{-3}
Glutamate	VSL	-0.64	2.65×10^{-3}
L-Carnitine	STR	-0.63	3.30×10^{-3}
Taurine	LIN	-0.63	3.69×10^{-3}
Aspartate	STR	-0.63	3.72×10^{-3}
Taurine	WOB	-0.62	4.02×10^{-3}
Aspartate	AIL	-0.62	4.26×10^{-3}
Glutamate	RapidNP	0.62	4.42×10^{-3}
Aspartate	ARD	0.62	4.45×10^{-3}
Aspartate	DFIpercent	0.60	7.20×10^{-3}
Taurine	ALH	0.59	9.28×10^{-3}

Taurine	STR	-0.59	9.65×10^{-3}
Cystine	WOB	-0.58	1.06×10^{-2}
Choline	STR	-0.58	1.06×10^{-2}
Choline	RapidP	-0.58	1.09×10^{-2}
Glutamate	AID	0.58	1.12×10^{-2}
L-Carnitine	RapidNP	0.57	1.21×10^{-2}
L-Carnitine	PM	-0.57	1.22×10^{-2}
Choline	VSL	-0.57	1.26×10^{-2}
Taurine	RapidNP	0.57	1.33×10^{-2}
Aspartate	ALH	0.57	1.33×10^{-2}
Cystine	RapidP	-0.56	1.35×10^{-2}
Taurine	VCL	0.56	1.47×10^{-2}
Taurine	VSL	-0.56	1.47×10^{-2}
Choline	PM	-0.56	1.47×10^{-2}
Aspartate	VSL	-0.56	1.47×10^{-2}
Choline	VCL	0.55	1.55×10^{-2}
Cystine	Motility	-0.55	1.67×10^{-2}
Cystine	PM	-0.54	1.90×10^{-2}
Cystine	LIN	-0.54	1.93×10^{-2}
L-Carnitine	RapidP	-0.54	1.98×10^{-2}
Taurine	AID	0.53	2.39×10^{-2}
Phenylalanine	Concentration	-0.53	2.44×10^{-2}
Cystine	VSL	-0.53	2.68×10^{-2}
L-Carnitine	VSL	-0.52	2.90×10^{-2}
Valine	Concentration	-0.52	2.90×10^{-2}
Glutamine	AIL	-0.51	3.19×10^{-2}
Phenylalanine	PM	0.51	3.19×10^{-2}
Cystine	Concentration	0.51	3.19×10^{-2}
Cystine	VAP	-0.51	3.65×10^{-2}
Phenylalanine	RapidP	0.51	3.65×10^{-2}
Alanine	DFIpercent	0.50	3.73×10^{-2}
Glutamate	BCF	0.50	4.07×10^{-2}
Choline	RapidNP	0.50	4.32×10^{-2}
Phenylalanine	WOB	0.49	4.93×10^{-2}

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Sperm RNA landscape during sexual maturation in Duroc boars

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Abstract

Background: Sexual maturation in boars impacts reproductive efficiency in swine production, yet the molecular mechanisms underlying this developmental transition remain poorly understood. This study aimed to investigate the transcriptomic changes in sperm from Duroc boars during sexual maturation, conducting a longitudinal analysis. The total RNA and miRNA profiles from the same individuals at puberty (~7 months) and sexual maturity (~10 months) were compared, identifying molecular signatures associated with reproductive development. Total RNA sequencing and miRNA sequencing were performed, followed by differential expression analysis using a paired statistical model in DESeq2 to account for repeated measures.

Results: Differential expression analysis identified 60 differentially expressed genes using stringent criteria ($\text{adj } P < 0.05$, $|\log_2\text{FC}| \geq 0.5$), with 65% upregulated in sperm of boars 10-months versus 7-months of age. Key upregulated genes included *NCLN*, *RGS12*, *CIB2*, *FOXP4*, *PHC1*, *CDC25B* and *AKAP1*, while *HSP90AA1*, *EVI5*, *FSIP2*, *VDAC3*, and *ALMS1* were key downregulated genes. Furthermore, Gene ontology analysis revealed significant enrichment of 11 biological processes, mostly related to reproductive development, and four molecular functions (transferase activity, transferring phosphorus-containing groups, protein serine/threonine kinase activity, protein kinase activity and signal sequence binding). Additionally, our miRNA analysis identified six differentially expressed miRNAs ($\text{adj } P < 0.05$ & $|\log_2\text{FC}| \geq 0.5$); ssc-miR-193a-5p, ssc-miR-574-3p, ssc-miR-126-3p, ssc-miR-196a, ssc-miR-210 were upregulated, and ssc-miR-338 showed downregulation in 10-months age. Integrated analysis identified 17 miRNA-mRNA regulatory pairs, enriched in pathways related to cell cycle processes and chromatin binding, suggesting coordinated regulation across RNA biotypes in sperm during sexual maturation.

Conclusions: Our study reveals coordinated transcriptomic shifts in boar sperm during sexual maturation. Most genes show increased expression at 10 months of age, with enrichment of terms related to reproductive development. Several miRNAs appear to regulate these changes through targeted mRNA interactions. These findings expand our understanding of the biological processes underlying sexual maturation in pigs.

Keywords

Differential gene expression, miRNA, Porcine spermatozoa, Sexual maturation, Spermatozoal RNA

Introduction

Sexual reproduction in mammalian males depends on producing functionally competent spermatozoa through spermatogenesis and their subsequent maturation [1]. Spermatogenesis begins in the testes, where haploid sperm develops morphologically but remains functionally immature. These developing sperm only acquire progressive motility and fertilization capacity during their transit through the epididymis from the testis [1]. Within the epididymis, sperm undergo biochemical and physiological transformations, including membrane remodeling, protein modifications and the development of motility and fertilization potential [2]. This maturation process is essential for producing sperm capable of navigating within the female reproductive tract to reach, penetrate and fertilize an egg.

In boars, sperm production begins at puberty (around 5-6 months of age), while sexual maturity and optimal semen functionality develop gradually from 8-9 months and proceed until approximately 24 months, though individual and breed variations exist [3-5]. The progression toward sexual maturity is reflected in several measurable parameters. Duroc boars show an increase in ejaculate volume, sperm concentration, total sperm count, motility, normal morphology, and sperm count with normal acrosome as they mature from 230 days to 270 days, while the percentage of morphologically defective sperm decreases during this period [6]. Similar improvements continue in most pig breeds (Polish Large White, Polish Landrace, Pietrain, Hamshire and Duroc), with a progressive rise in ejaculate volume and sperm concentration from 9 months onward [5].

Histological examination provides further support of this developmental timeline. In Duroc testes, spermatozoa and seminiferous tubule lumina become detectable at 150 days, with a large number of spermatocytes visible at 270 days, while these structures are absent at earlier stages (75 and 130 days) [3, 4]. Seminiferous tubule diameter also increases between 75 and 270 days, reflecting continuous testicular maturation [3].

Alongside these age-related macroscopic improvements in sperm quality parameters, there is an increasing interest in the underlying molecular changes during the transition from puberty to sexual maturity. Transcriptomic analysis offers valuable insights into these molecular-level changes in sperm. While spermatozoa were traditionally considered merely carriers of paternal genetic material and believed to be transcriptionally silent due to minimal cytoplasm and absence of intact ribosomal RNAs (rRNAs), accumulating evidence has revealed a rich and functional RNA landscape within these cells that influences sperm functionality and reproductive outcomes [7, 8]. Boar sperm contain diverse RNA populations that likely contribute to multiple biological functions beyond being mere remnants of spermatogenesis, including sperm maturation, functionality and fertility outcomes [9]. These include messenger RNAs (mRNAs), micro RNAs (miRNAs), short noncoding RNAs (sncRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), Piwi interacting RNAs (piRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), mitochondrial ribosomal RNAs (MtrRNAs), and mitochondrial transfer RNAs (MttRNAs) [10-12].

Early characterization of porcine sperm RNA by Yang and colleagues in 2009 [11], using expressed sequence tags (ESTs), revealed a fragmented RNA profile containing transcripts encoding proteins involved in spermatogenesis such as protamine 1 (*PRM1*) and protamine 2 (*PRM2*), as well as early embryonic development, including sperm-specific antigen 2 (*SSFA2*) and sestrin 1 (*SESNI*). This pioneering work was expanded through RNA sequencing (RNA-Seq) approaches, which identified 4436 genes expressed in boar sperm that reflect seasonal changes, including highly abundant transcripts of mitochondrial origin and key spermatogenesis regulators like *PRM1* and ornithine decarboxylase antizyme 2 (*OAZ3*) [13, 14].

RNA-Seq analysis has further identified many genes functionally relevant to sperm motility, morphology, fertilization capacity, and freezability. For instance, comparison between fresh and frozen-thawed boar sperm revealed 567 mRNAs and 135 miRNAs to be differentially expressed [15]. Similarly, Alvarez-Rodriguez et al. [16] observed 347 up-regulated and 174 down-regulated RNA transcripts in high-fertility boars versus low-fertility boars, including transcripts encoding proteins involved in sperm motility, such as cation channel sperm associated auxiliary subunit gamma (*CATSPERG*).

Non-coding RNAs, especially miRNAs, have attracted considerable research interest due to their involvement in regulating boar sperm quality and function. Curry et al. [17] reported differential expression of miRNAs (including miR-22, let-7a, let-7d, and let-7e) in sperm with abnormal morphology or reduced motility. Chang et al. [18] identified 15 differentially expressed miRNAs between fresh ejaculated and cauda epididymal spermatozoa in Landrace boars, with eight being downregulated (including ssc-let-7 family members, ssc-miR-26a, ssc-miR-98) and seven upregulated (including ssc-miR-19b, ssc-miR-34c). These differentially expressed miRNAs target genes involved in key signaling pathways such as cell-cycle regulation, and apoptosis [18].

Martinez et al. [19] found four differentially expressed miRNAs (miR-1285, miR-92a, miR-34c, and miR-30) between ejaculate and cauda epididymal spermatozoa, while also observing varying abundance levels of miR-182, miR-1285, miR-191, and miR-96 between high and low fertility boars, suggesting these miRNAs are involved in fertility. In a comprehensive profiling study, Luo et al. [20] revealed distinct miRNA expression patterns across reproductive tissues, with approximately 15% of miRNAs co-expressed in Landrace testis, epididymis and sperm, mirroring the physiological relationship between these tissues. Particularly, miR-34 showed remarkably higher abundance in sperm, compared to the other reproductive tissues [20].

Gòdia et al. [10] have revealed correlations between RNA abundance and 25 sperm quality parameters in Pietrian boars, demonstrating the complex molecular basis of sperm function and fertility. This examination identified highly abundant mRNAs (*PRM1*, *OAZ3*, DnaJ heat shock protein family member B8 (*DNAJB8*), tripeptidyl peptidase (*TPP2*), transition protein 1 (*TNPI1*)) and miRNAs (ssc-miR-30d, ssc-miR-34c, ssc-miR-30c-5p, ssc-miR-191, members of the let-7 family, and ssc-miR-425-5p) in boar sperm associated with sperm biology [10].

Despite considerable progress in understanding sperm biology through transcriptomics, there remain significant gaps in our knowledge concerning the transcriptomic changes that characterize the transition from puberty to sexual maturity in boars. Previous studies have primarily examined RNA expression in boar sperm in relation to season, anatomy, sperm quality, fertility, and cryopreservation using a cross-sectional design comparing different individuals. Limited understanding exists about which genes become systematically enriched or depleted as boars mature sexually, and whether these molecular shifts actively contribute to enhanced sperm performance or are simply developmental byproducts. Understanding these developmental

changes at a molecular level has gained interest as breeding companies are increasingly motivated to introduce younger boars earlier in breeding programs based on genomic selection [21].

This study aimed to perform comparative transcriptomic profiling of sperm collected from the same Duroc boars at both puberty (~7 months) and sexual maturity (~10 months) using a longitudinal study design. Our primary objectives were to: (i) identify differentially expressed genes (DEGs) and miRNAs in sperm between these two developmental time points, and (ii) explore the biological pathways and molecular mechanisms associated with sexual maturation. We hypothesized that sperm from sexually mature boars would exhibit distinct transcriptomic signatures compared to those from the same boars at puberty, reflecting molecular adaptations that enhance reproductive function. These insights would provide a unique window into developmental changes in boar sperm at the molecular level, which might be utilized to optimize selection criteria in breeding programs.

Materials and methods

Experimental design

This study examined 6 *Sus scrofa* boars of the Duroc breed over a three-month time span from March to June 2023. The boars were sourced from a commercial breeding line used by Norsvin SA, the pig breeding company in Norway. To track transcriptomic transitions linked to sexual maturity, semen was collected from the same individuals at two time points, at approximately 7 months (pubertal) and 10 months (mature) of age.

At the start of the study, the pubertal boars were placed in individual 4 m² pens within an isolation unit, adhering to guidelines set by the Norwegian Food Safety Authority for animals of this size. This phase served as a quarantine period and an opportunity to train boars for semen collection procedures. Semen samples were collected once the animals showed interest in mounting and were physically capable of producing semen. The boars were transferred to an Artificial Insemination (AI) center at around 8 months old, where they were housed in larger 6 m² pens. Semen collection was performed using the gloved-hand technique, always carried out by the same trained personnel to ensure consistency. For collection, the boars were briefly moved from their home pens to a separate area. Environmental conditions, including ventilation, lighting, and temperature, were kept consistent across both the isolation unit and the AI center to avoid introducing factors that

might affect metabolic or physiological measurements. All boars were provided with the same balanced commercial diet throughout the study.

Sample pre-processing

A fresh semen sample containing 200 million sperm per ml was centrifuged at 600×g for 10 min at room temperature (RT) to separate sperm from the seminal plasma. After discarding the supernatant (seminal plasma), the sperm pellets were washed with 1 mL phosphate buffer solution (PBS) (centrifuged at 600×g for 10 min at RT). Next, the somatic cell lysis was performed in the sample as per Goodrich et al. [22] with slight modification. The samples were kept on ice for 30 min with 1 mL of somatic cell lysis buffer (0.1 % SDS, 0.5 % Triton X-100 in nuclease-free water) and thereafter washed by centrifuging at 200 × g for 15 min at 4°C. The samples were thereafter examined using phase-contrast microscopy to ensure the absence of somatic cells. Following this examination, the sperm were washed twice with 1 mL PBS, each time centrifuged at 600×g for 10 min at 4°C. Finally, the sperm pellets were snap-frozen and stored in liquid nitrogen until further analysis.

Total RNA isolation and quality assessment

Frozen spermatozoa were thawed in hands and immediately processed for lysis. To lyse the sperm cells, 1 ml of Qiazol (Qiagen, Germany) and 120 µL of tris(2-carboxyethyl)phosphine (TCEP) (Sigma-Aldrich, Saint-Louis, MO, USA) were added to the sperm pellet. The mixture was vortexed for 5 min, incubated at 56°C for 60 min and then incubated at RT for another 5 min. To confirm successful lysis of the spermatozoa, phase-contrast microscopy was used. For RNA isolation, the manufacturer's kit protocol (miRNeasy Mini Kit, Qiagen, Germany) was followed with slight modifications. To each sample lysate, 200 µL of chloroform was added before centrifugation at 12000×g at 4°C for 15 min. The upper aqueous phase was transferred to a new collection tube. 100% ethanol (1.5 times recovered aqueous phase) was added to the aqueous phase and mixed thoroughly. The sample was then loaded into the RNeasy Mini spin column and centrifuged at 8000×g for 20 seconds at RT. Washing was performed with Buffer RWT (prepared with isopropanol) centrifuged at 8000×g for 20 seconds at RT. On-column DNase digestion was performed by adding 10 µL DNase I stock solution to 70 µL Buffer RDD as instructed in the kit RNase-Free DNase Set (Qiagen, Germany). Washing of the column was performed subsequently by 350 µL Buffer RWT, and 500 µL Buffer RWT with centrifugation at 8000×g for 20 seconds at

RT. Additional washing of the spin column by 500 μ L Buffer RPE was done by centrifuging at 8000 $\times$ g for 2 min to dry the spin column membrane at RT. To eliminate any possible carryover of Buffer RPE or residual flow-through, the spin column was placed into a new 2 mL collection tube and centrifuged at full speed for 1 min. Finally, total RNA elution was done with 40 μ L of RNase-free water. The quantity of RNA was measured using QubitTM 4 fluorometer with QubitTM High Sensitivity (HS) kit (Thermo Fisher Scientific, USA). Assessment of RNA quality was performed using 4150 TapeStation system and TapeStation Analysis Software (4.1.1) with High Sensitivity RNA ScreenTape (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's protocol. Lack of somatic cell contamination was confirmed by the non-detection of 18S and 28S rRNA peaks in the TapeStation analysis [11, 23]. Similarly, absence of DNA contamination was confirmed using *PRMI* TaqManTM Gene Expression Assay (Applied Biosystems, CA, USA, Assay ID: Ss03383652_u1) by comparing RT-qPCR reactions with and without reverse transcriptase (-RT controls) following the protocol described in [24].

Total RNA Sequencing

Using 4 μ L RNA (8.8-61.2 ng), rRNA was blocked from cDNA extension using the QiaSeq FastSelect HMR reagents (Qiagen, Germany), following the “-rRNA and/or -Globin with the NEBNext Ultra II Directional Library Prep Kit” procedure in the manufacturer's handbook v12/21, including the “partially degraded RNA (RIN 2-6)” variations. Sequencing libraries were thereafter prepared with the Ultra II Directional Library Prep Kit (NEB) according to manufacturer's instructions version 5.0_12/22 from step 4.2 onwards, with dilution of the NEB adapter 1:25 and indexed adapters. Libraries were amplified for 16 PCR cycles. Sequencing was performed on a NovaSeq-6000 instrument (Illumina) on an SP flowcell running RTA v3.4.4 with 1% PhiX added, with 150 bp paired end sequencing. Demultiplexing into individual sample FASTQ datasets was performed with bcl2fastq v2.20.0.422.

miRNA Sequencing

miRNA libraries were prepared using the QiaSeq miRNA library kit (Qiagen, Germany). Inputs ranged from 5.5-38.3 ng RNA. Adapters were diluted accordingly, following the manufacturer's recommendations, and samples were amplified with 18 cycles of PCR. Samples were indexed with unique adapters to allow pooling on a single sequencing run. Sequencing was performed on a NextSeq-500 instrument (Illumina) running RTA v2.11.3 with 1% PhiX added, with 82 cycles of

sequencing. Demultiplexing into individual sample FASTQ datasets was performed with bcl2fastq v2.20.0.422.

Total RNA seq and miRNA seq analysis

Raw fastq files were subjected to quality control using FastQC (v0.12.1) [25]. Adapter sequences and low-quality bases were removed using Trim Galore (v0.6.10) [26] with the following parameters: quality cutoff of 20, stringency of 3, and a minimum read length of 35 bases. The trimming was executed in paired-end mode, retaining unpaired reads and trimming ambiguous “N” bases. Post-trimming quality was again confirmed by running FastQC on the trimmed sequences. The *Sus scrofa* (Sscrofa 11.1) reference genome and its corresponding GTF annotation were obtained from Ensembl release 112 [27]. The reference genome was indexed for alignment using HISAT2’s (v2.2.1) hisat2-build [28]. Trimmed paired-end reads were aligned to the *Sus scrofa* genome using HISAT2 (v2.2.1) [28]. Alignment output (SAM files) was converted to BAM files using SAMtools (v1.16.1), then sorted and indexed. To account for PCR duplicates, duplicate marking was performed using Picard (v3.0.0) [29]. The duplicate-marked BAM files were indexed, and post-marking alignment statistics were obtained. Gene-level counts matrix were generated using featureCounts (Subread v2.0.4) [30] with the *Sus scrofa* ensembl GTF annotation file [27]. Parameters for featureCounts included paired-end mode, counting reads mapping to exons (using -t exon and -g gene_id), and a strandedness option (-s 2).

The standalone version of sRNAbench from the sRNAtoolbox suite [31] was used to annotate *Sus scrofa* small non-coding RNAs (sncRNAs), including miRNAs (based on miRBase v21). All annotations were performed using default parameters.

Differential expression analysis of total RNA-seq and miRNA-seq data

The DESeq2 package (v 1.46.0) from Bioconductor [32] was used to perform differential expression analysis for both total RNA-seq and small RNA-seq in R [33]. For both datasets, a paired design (~ Individual + Age') was implemented to account for individual variation while focusing on changes between Pubertal and Mature boar groups. To ensure robust analysis, we filtered both datasets to include only genes with a minimum of 10 read counts in at least 6 samples following recommended practice [34]. Differentially expressed genes were identified using the following criteria: adjusted *P*-values < 0.05 (Benjamini-Hochberg correction) and absolute log₂ fold change ≥ 0.5. For total RNA-seq data, differentially expressed Ensembl gene IDs were

converted to gene names using biomaRt (v 2.62.0), by querying the Ensembl BioMart database ('sscrofa_gene_ensembl') to retrieve external gene names, Entrez IDs, gene descriptions, and biotypes [35].

For miRNA analysis, target gene prediction was performed using the multiMiR package (v 2.4.0) [36]. Due to limited validated miRNA-target annotation in pigs, differentially expressed porcine miRNAs meeting the criteria ($\text{adj } P < 0.05$ and $|\log_2\text{FC}| \geq 0.5$) were manually mapped to human orthologs based on sequence conservation. Target prediction was conducted using the get_multimir() function with the following parameters: org = "hsa", table = "validated" to retrieve experimentally validated miRNA target interactions. The resulting human gene targets were then mapped back to porcine orthologs using the gorth() function from gprofiler2 (v 0.2.3) [37] by querying the pig genome database ('sscrofa_gene_ensembl').

Gene ontology (GO) enrichment analysis for both datasets was performed using clusterProfiler (v 4.14.4) [38] in conjunction with the *Sus scrofa* OrgDb (org.Ss.eg.db) (v 3.20.0) [39, 40]. Differentially expressed genes from total RNA-seq and predicted target genes from miRNA-seq were tested for enrichment in biological processes, molecular functions, and cellular components. GO enrichment analysis was performed using Benjamini-Hochberg correction, with significantly enriched terms identified at $q\text{-value} < 0.05$.

For integrative analysis to identify miRNA-mRNA regulatory pairs, predicted miRNA targets were intersected with differentially expressed mRNAs from our dataset ($\text{adj } P < 0.05$ and $|\log_2\text{FC}| \geq 0.5$), showing inverse and positive expression patterns. Specifically, predicted mRNA targets of downregulated and upregulated miRNAs were matched with downregulated and upregulated miRNAs. The resulting miRNA-mRNA regulatory pairs were annotated with gene symbols using Ensembl BioMart [35].

Results

RNA landscape and differential expression analysis

Initial characterization of our total RNA-Seq data revealed a diverse transcriptional landscape in boar sperm. From the 15,637 genes of all biotypes detected after quality filtering, protein-coding genes constituted the majority (86.91%), followed by long non-coding RNAs (11.56%), with

smaller proportions of small nucleolar RNA (0.61%), pseudogenes (0.33%), and other RNA classes (Additional file: Table S1).

Differential expression analysis between sperm samples from boars at 10-months and 7-months of age initially identified 230 genes with significant P -values ($\text{adj } P < 0.05$) (Additional file: Table S2). Applying additional biological significance criteria ($|\log_2\text{FC}| \geq 0.5$) refined this to 60 differentially expressed genes (DEGs). These included 39 upregulated genes (65%) and 21 downregulated genes (35%) in mature boars (Figure 1), suggesting that sexual maturation in boar sperm involves primarily transcriptional activation rather than repression.

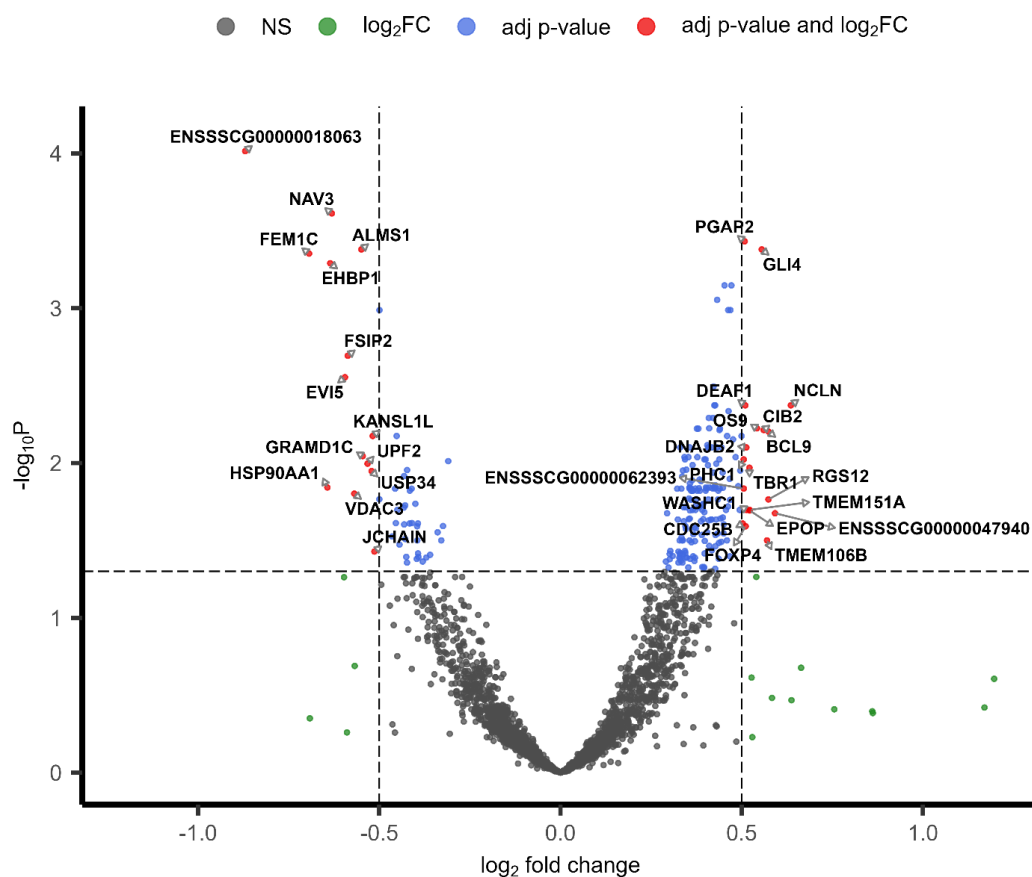


Figure 1: Volcano plot of differential gene expression between sperm collected from boars at 10 months and 7 months of age. $\log_2$ fold change (x-axis) is plotted against statistical significance ($-\log_{10}$ adj P -value, y-axis) for expressed genes. Red dots represent significantly differentially expressed genes meeting both significance ($\text{adj } P < 0.05$) and fold change ($|\log_2\text{FC}| > 0.5$) criteria. Blue dots indicate genes with significant P -values ($\text{adj } P < 0.05$) but below the fold change threshold ($|\log_2\text{FC}| < 0.5$). Green dots show genes with fold change above threshold ($|\log_2\text{FC}| > 0.5$) but lacking statistical significance ($\text{adj } P > 0.05$). Grey dots represent genes which are non-significant (NS).

The majority of DEGs were protein-coding genes (221, 96.08%) with smaller contributions from five lncRNAs (2.17%), three pseudogenes (1.30%), and one mitochondrial rRNA (0.43%). This distribution notably differs from the overall transcriptome composition, indicating selective enrichment of protein-coding genes in maturation-associated transcriptional changes.

Hierarchical clustering of all the DEGs showed an age-dependent expression profile, with samples clustering by age rather than by individual boar, with one exception (Figure 2).

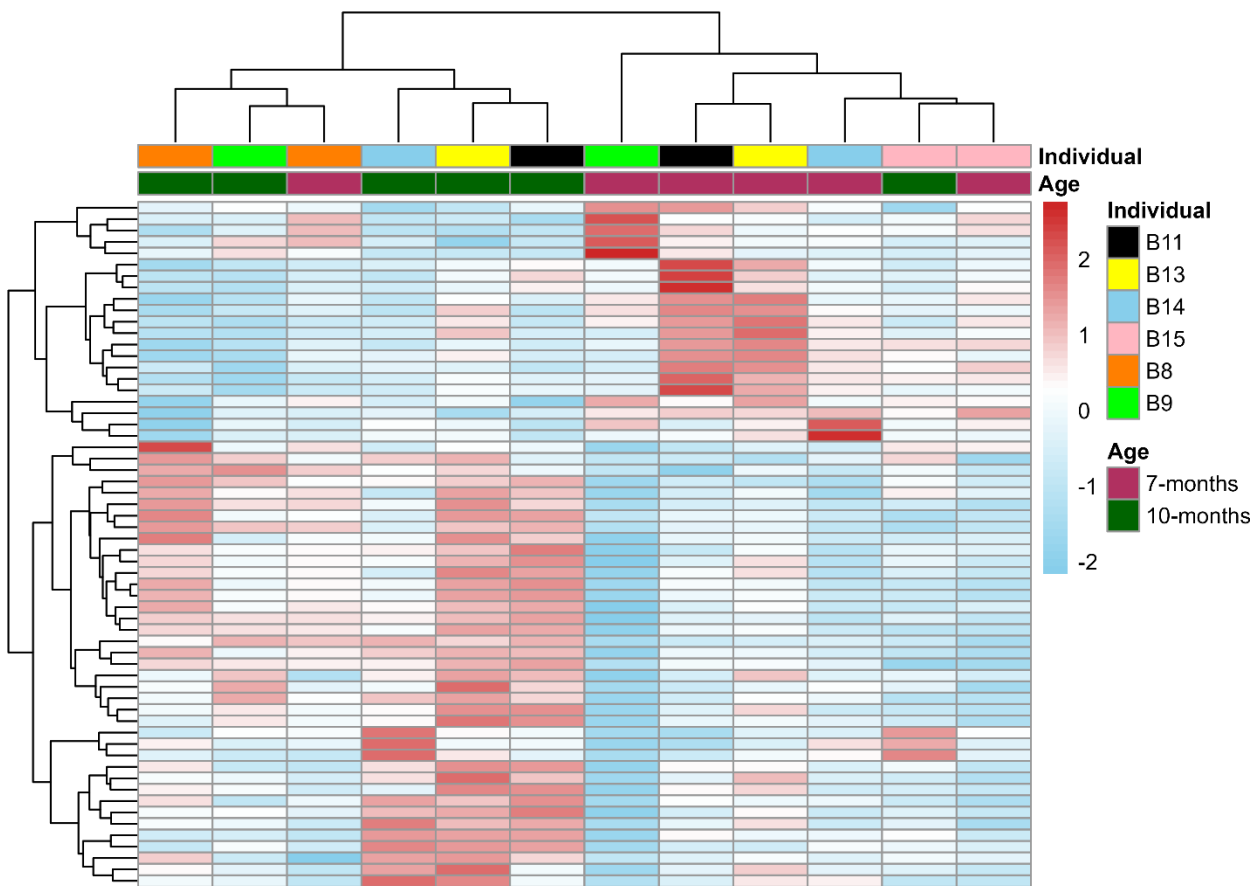


Figure 2: Hierarchical clustering of differentially expressed genes in sperm collected from boars at 7-months and 10-months of age. Heatmap shows expression patterns of all 60 differentially expressed genes ($\text{adj } P < 0.05$ & $|\log_2\text{FC}| > 0.5$) across sperm samples from six Duroc boars at two ages. Each column represents an individual sample with color bars at the top indicating the boar's identity and age (7-months in maroon, 10-months in dark green). Gene expression values were variance-stabilized and row-scaled, with red indicating higher expression and blue indicating lower expression relative to the mean across samples.

Key genes identified in sperm maturation

Analysis revealed a mitochondrial rRNA (ENSSSCG00000018063) as the most significantly altered differentially expressed gene (DEG) (Table 1) ($\log_2FC = -0.9$, adj $P = 9.67 \times 10^{-5}$). Other highly significant DEGs included neuron navigator 3 (*NAV3*) ($\log_2FC = -0.6$), post-GPI attachment to proteins 2 (*PGAP2*) ($\log_2FC = 0.5$), ALMS1 centrosome and basal body associated protein (*ALMS1*) ($\log_2FC = -0.5$), and GLI family zinc finger 4 (*GLI4*) ($\log_2FC = 0.6$), all with adj $P < 4.17 \times 10^{-4}$. Several of these top-ranked genes exhibited contrasting expression patterns, with both up- and down-regulated genes represented among the most significant changes (Table 1), highlighting the complex transcriptional remodeling that accompanies sperm maturation.

Table 1: Results from DESeq2 expression analysis for the top 10 most significantly differentially expressed genes in sperm samples from boars at 10-months, compared to 7-months of age. Genes are ranked by adjusted P -value, showing both up-regulated and down-regulated genes. All genes are protein coding genes except ENSSSCG00000018063 (Mt_rRNA). The table includes Ensembl gene ID, external gene name, base mean normalized values (baseMean), $\log_2$ fold change ($\log_2FC$), standard error of the fold change (lfcSE), Wald test statistics (stat) and adjusted P -value (adj P -value).

Ensgene ID	Gene Name	baseMean	$\log_2FC$	lfcSE	stat	adj P
ENSSSCG00000018063	<i>Novel gene</i>	3780.19	-0.9	0.16	-5.48	9.67×10^{-5}
ENSSSCG00000029249	<i>NAV3</i>	5125.87	-0.6	0.12	-5.19	2.44×10^{-4}
ENSSSCG00000035653	<i>PGAP2</i>	20008.42	0.5	0.10	5.03	3.70×10^{-4}
ENSSSCG00000037977	<i>ALMS1</i>	10215.12	-0.5	0.11	-4.91	4.17×10^{-4}
ENSSSCG00000039881	<i>GLI4</i>	4372.87	0.6	0.11	4.91	4.17×10^{-4}
ENSSSCG00000014217	<i>FEM1C</i> ¹	1686.26	-0.7	0.14	-4.86	4.43×10^{-4}
ENSSSCG00000008372	<i>EHBPI</i> ²	4150.54	-0.6	0.13	-4.80	5.12×10^{-4}
ENSSSCG00000034994	<i>Novel gene</i>	23962.23	0.5	0.10	4.69	7.12×10^{-4}
ENSSSCG00000037391	<i>AKAP1</i> ³	39884.72	0.5	0.10	4.69	7.12×10^{-4}
ENSSSCG00000001689	<i>ZNF644</i> ⁴	3683.40	0.5	0.10	-4.54	1.03×10^{-4}

¹Fem-1 Homolog C; ²EH Domain Binding Protein 1; ³A-Kinase Anchoring Protein 1; ⁴Zinc finger protein 644

We next examined genes showing the most pronounced upregulation in sperm collected from boars at 10 months compared to the same boars at 7 months of age (Table 2, adj $P < 0.05$ & $|\log_2FC| \geq$

0.5). *Nicalin* (*NCLN*) was the most upregulated gene, followed by regulator of G protein signaling 12 (*RGS12*), *BCL9* transcription coactivator (*BCL9*), transmembrane protein 106B (*TMEM106B*), calcium and integrin binding family member 2 (*CIB2*), *GLI4*, OS9 endoplasmic reticulum lectin (*OS9*), elongin BC and polycomb repressive complex 2 associated protein (*EPOP*), and transmembrane protein 151A (*TMEM151A*) with $\log_2FC$ values of 0.5 to 0.6. Other important upregulated genes in mature boars identified were polyhomeotic homolog 1 (*PHC1*), cell division cycle 25B (*CDC25B*), and A-kinase anchoring protein 1 (*AKAP1*) (Additional file: Table S3, adj $P < 0.05$, $\log_2FC = 0.5$).

The most downregulated genes (Table 3, adj $P < 0.05$ & $|\log_2FC| \geq 0.5$) revealed a different functional profile. An uncharacterized mitochondrial rRNA showed the highest downregulation followed by *FEMIC*, heat shock protein 90 alpha family class A member 1 (*HSP90AA1*), *EHBPI*, *NAV3*, ectropic viral integration site 5 (*EVI5*), fibrous sheath interacting protein 2 (*FSIP2*), voltage dependent anion channel 3 (*VDAC3*), *ALMS1*, GRAM domain containing 1C (*GRAMD1C*), and UPF2 regulator of nonsense mediated mRNA decay (*UPF2*).

Additional file: Table S3 provides comprehensive expression data for all differentially expressed genes.

Table 2: Results from DESeq2 expression analysis for the top 15 upregulated genes in sperm samples from boars at 10-months, compared to 7-months of age. Genes with $\log_2FC > 0.5$, ranked by magnitude of fold change. All genes are protein-coding. The table includes Ensembl gene ID, external gene name, base mean normalized values (baseMean), $\log_2$ fold change ($\log_2FC$), standard error of the fold change (lfcSE), Wald test statistics (stat) and adjusted P -value (adj P -value).

Ensgene ID	Gene Name	baseMean	$\log_2FC$	lfcSE	stat	adj P -value
ENSSSCG00000013475	<i>NCLN</i>	2068.17	0.6	0.15	4.13	4.23×10^{-3}
ENSSSCG000000047940		2466.01	0.6	0.18	3.23	2.11×10^{-2}
ENSSSCG000000008698	<i>RGS12</i>	1310.16	0.6	0.17	3.34	1.72×10^{-2}
ENSSSCG000000006698	<i>BCL9</i>	1518.39	0.6	0.15	3.93	6.26×10^{-3}
ENSSSCG000000032740	<i>TMEM106B</i>	1087.56	0.6	0.19	3.03	3.16×10^{-2}
ENSSSCG000000001762	<i>CIB2</i>	1555.84	0.6	0.14	3.94	6.12×10^{-3}
ENSSSCG000000039881	<i>GLI4</i>	4372.87	0.6	0.11	4.91	4.17×10^{-4}
ENSSSCG000000035875	<i>OS9</i>	1461.11	0.5	0.14	4.00	5.97×10^{-3}
ENSSSCG000000035677	<i>EPOP</i>	4434.57	0.5	0.16	3.24	2.02×10^{-2}
ENSSSCG000000012952	<i>TMEM151A</i>	1087.56	0.5	0.16	3.25	2.01×10^{-2}
ENSSSCG000000015891	<i>TBRI</i> ¹	5918.55	0.5	0.14	3.63	1.07×10^{-2}
ENSSSCG000000000746	<i>WASHC1</i> ²	1780.84	0.5	0.16	3.27	2.01×10^{-2}
ENSSSCG000000016217	<i>DNAJB2</i> ³	2408.58	0.5	0.14	3.80	7.93×10^{-3}
ENSSSCG000000001619	<i>FOXP4</i> ⁴	1453.92	0.5	0.16	3.12	2.56×10^{-2}
ENSSSCG000000012850	<i>DEAF1</i> ⁵	16686.87	0.5	0.12	4.13	4.24×10^{-3}

¹T-box brain transcription factor 1; ²WASH complex subunit 1; ³DnaJ heat shock protein family (Hsp40) member B2;

⁴Forkhead box P4; ⁵DEAF1 transcription factor

Table 3: Results from DESeq2 differential expression analysis for the top 15 downregulated genes in sperm samples from boars at 10-months, compared to 7-months of age. Genes with $\log_2FC > 0.5$, ranked by magnitude of fold change. All genes are protein-coding except ENSSSCG000000018063 (Mt_rRNA). The table includes Ensembl gene ID, external gene name, base mean normalized values (baseMean), $\log_2$ fold change ($\log_2FC$), standard error of the fold change (lfcSE), Wald test statistics (stat) and adjusted P -value (adj P -value).

Ensgene ID	Gene Name	baseMean	log ₂ FC	lfcSE	stat	adj <i>P</i> -value
ENSSSCG00000018063		3780.19	-0.9	0.16	-5.48	9.67×10^{-5}
ENSSSCG00000014217	<i>FEM1C</i>	1686.26	-0.7	0.14	-4.86	4.43×10^{-4}
ENSSSCG00000002535	<i>HSP90AA1</i>	1644.41	-0.6	0.19	-3.47	1.44×10^{-2}
ENSSSCG00000008372	<i>EHBP1</i>	4150.54	-0.6	0.13	-4.80	5.12×10^{-4}
ENSSSCG00000029249	<i>NAV3</i>	5125.87	-0.6	0.12	-5.19	2.44×10^{-4}
ENSSSCG00000006901	<i>EVI5</i>	5227.88	-0.6	0.14	-4.29	2.79×10^{-3}
ENSSSCG00000060494	<i>FSIP2</i>	2378.55	-0.6	0.13	-4.37	2.03×10^{-3}
ENSSSCG00000007026	<i>VDAC3</i>	1578.56	-0.6	0.17	-3.38	1.58×10^{-2}
ENSSSCG00000037977	<i>ALMS1</i>	10215.12	-0.5	0.11	-4.91	4.17×10^{-4}
ENSSSCG00000011915	<i>GRAMD1C</i>	1048.11	-0.5	0.15	-3.74	9.02×10^{-3}
ENSSSCG00000011117	<i>UPF2</i>	1407.68	-0.5	0.15	-3.66	1.01×10^{-2}
ENSSSCG00000008382	<i>USP34</i> ¹	4334.07	-0.5	0.14	-3.60	1.13×10^{-2}
ENSSSCG00000022830	<i>KANSL1L</i> ²	7569.74	-0.5	0.13	-3.90	6.69×10^{-3}
ENSSSCG00000035379	<i>JCHAIN</i> ³	1025.87	-0.5	0.17	-2.95	3.73×10^{-2}
ENSSSCG00000035136	<i>SDCCAG8</i> ⁴	1286.10	-0.5	0.15	-3.35	1.71×10^{-2}

¹Ubiquitin specific peptidase 34; ²KAT8 regulatory NSL complex subunit 1 like; ³Joining chain of multimeric IgA and IgM; ⁴SHH Signaling And Ciliogenesis Regulator SDCCAG8

Functional enrichment analysis

To gain insights into the biological significance of the observed transcriptional changes, we performed functional enrichment analyses using GO on differentially expressed genes. Our GO analysis revealed significant enrichment of 11 biological processes (adj *P* < 0.05), directly related to reproductive development and four molecular functions being transferase activity, transferring phosphorus-containing groups, protein serine/threonine kinase activity, protein kinase activity and signal sequence binding (Figure 3, Additional file: Table S4).

Within the biological processes (BP) category, we identified eight significant enrichments of terms directly related to male reproduction (adj *P* < 0.05). The most significantly enriched terms included germ cell development and cellular processes involved in reproduction in multicellular organisms, followed by gamete generation, developmental processes involved in reproduction,

363 spermatogenesis, spermatid development, male gamete generation and spermatid differentiation.
364 We also identified significant enrichment of protein targeting processes, including SRP-dependent
365 cotranslational protein targeting to membrane, cotranslational protein targeting to membrane, and
366 intracellular protein transmembrane transport (adj $P < 0.05$).

367 Molecular function (MF) analysis revealed significant enrichment of kinase-related activities (adj
368 $P < 0.05$), with protein serine/threonine kinase activity showing the highest significance. Other
369 significantly enriched terms (adj $P < 0.05$) included protein kinase activity, signal sequence
370 binding and transferase activity transferring phosphorus-containing groups.

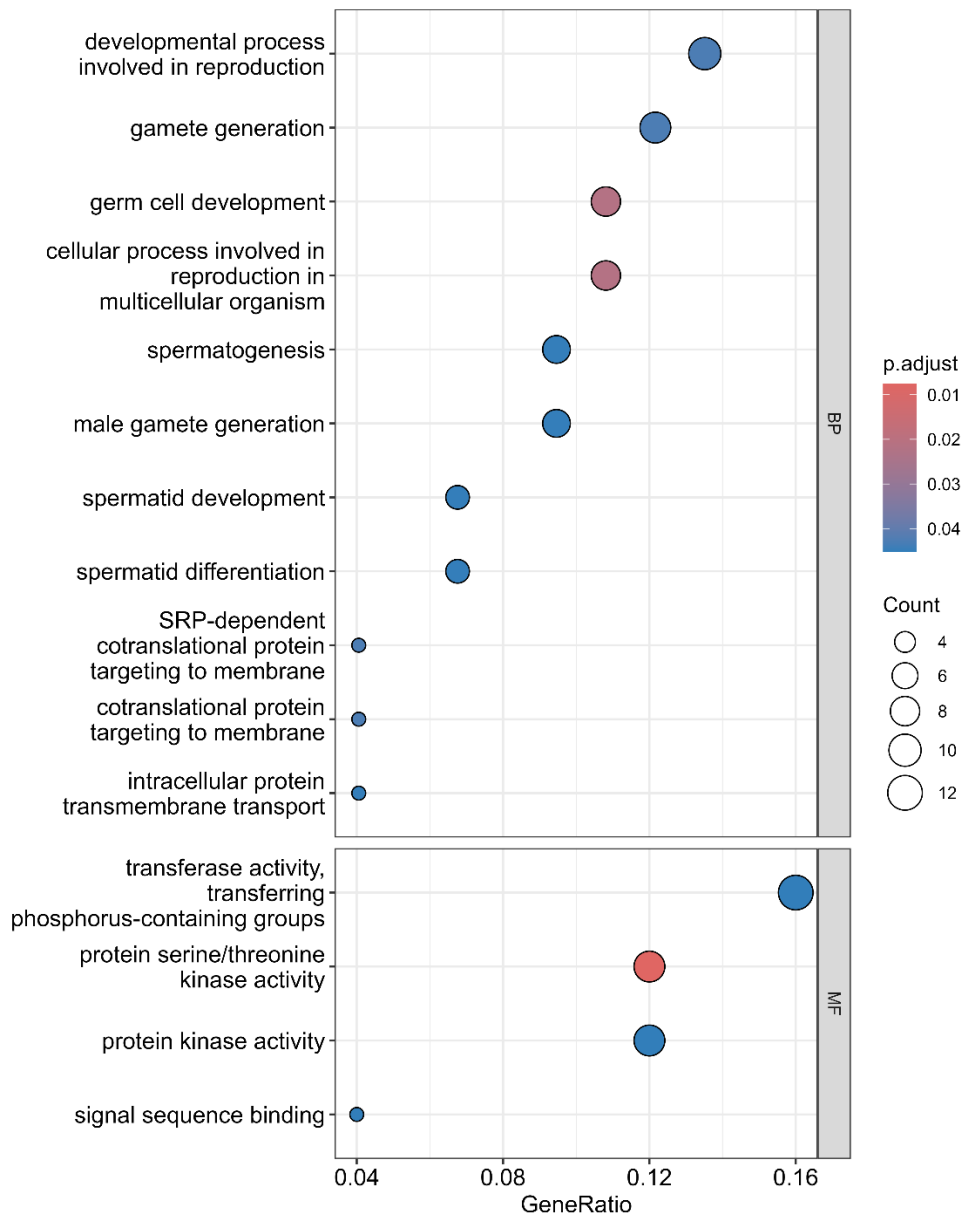


Figure 3: Enriched gene ontology terms among differentially expressed genes in sperm collected from 10-month versus 7-month boars. The dot plot displays significantly enriched biological process (BP) and molecular function (MF) terms ($\text{adj } P < 0.05$) with gene ratio (proportion of differentially expressed genes belonging to each term) on the x-axis. Dot size represents the number of genes per term, while color represents statistical significance ($\text{adj } P$), with red representing more significant values (lower $\text{adj } P$ -values) and blue representing less significant values (higher $\text{adj } P$ -values).

Differential miRNA expression during sexual maturation

Following total RNA profiling, we examined the miRNA landscape in boar sperm samples to understand the small RNA regulatory network during sexual maturation. Differential expression analysis identified six significantly altered miRNAs between pubertal and mature boars (adj $P < 0.05$, $|\log_2FC| \geq 0.5$), with five upregulated and one downregulated miRNA in mature boar samples, compared to pubertal boar samples (Figure 4, Table 4).

ssc-miR-193a-5p showed the highest statistical significance among upregulated miRNAs ($\log_2FC = 0.9$, adj $P = 1.09 \times 10^{-5}$), while ssc-miR-126-3p displayed the greatest fold change ($\log_2FC = 1.0$). The remaining upregulated miRNAs, ssc-miR-196a, ssc-miR-574-3p, and ssc-miR-210 showed moderate but significant increases, while ssc-miR-338 was downregulated in mature boars.

Table 4: Differentially expressed miRNAs in sperm collected from boars at 7-months and 10-months of age. Positive $\log_2FC$ values denote higher expression in sperm in mature boars (upregulated), and negative values denote lower expression in sperm in mature boars (downregulated). The table includes miRNA ID, base mean normalized values (baseMean), $\log_2$ fold change ($\log_2FC$), standard error of the fold change (lfcSE), Wald test statistics (stat), and adjusted P -value (adj P -value).

	miRNA	baseMean	$\log_2FC$	lfcSE	stat	adj P -value
Upregulated	ssc-miR-193a-5p	230.21	0.9	0.16	5.39	1.09×10^{-5}
	ssc-miR-196a	846.34	0.7	0.19	3.49	2.46×10^{-2}
	ssc-miR-126-3p	332.48	1	0.29	3.34	3.17×10^{-2}
	ssc-miR-574-3p	187.21	0.9	0.27	3.31	3.17×10^{-2}
	ssc-miR-210	674.89	0.7	0.21	3.16	3.87×10^{-2}
Downregulated	ssc-miR-338	177.17	-0.6	0.18	-3.62	2.46×10^{-2}

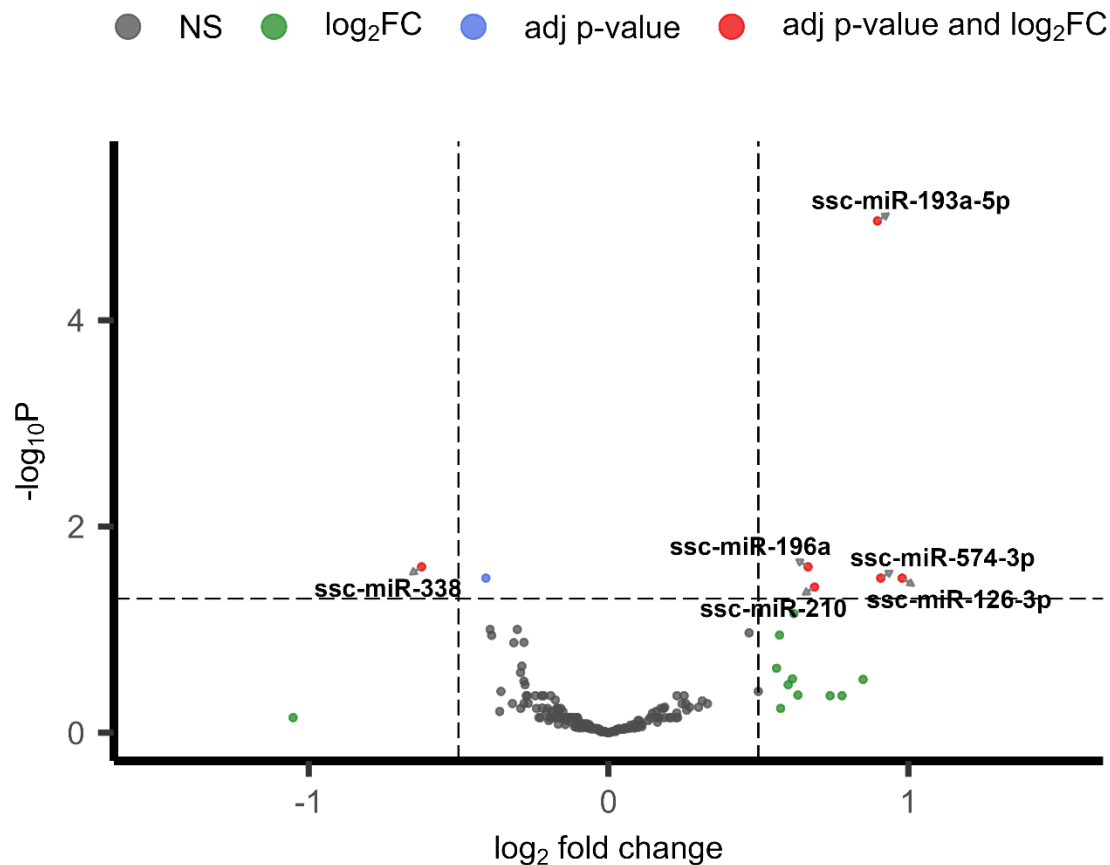


Figure 4: Differential expression of miRNAs in sperm collected from boars at 7-months and 10-months of age. Volcano plot displaying log₂ fold change (x-axis) versus statistical significance (-log₁₀ adjusted p-value, y-axis) for 179 miRNAs. Red dots represent miRNAs meeting both significance (adj $P < 0.05$) and fold change ($|\log_2FC| > 0.5$) thresholds and are labelled. Blue dots indicate miRNAs with significant P -values (adj $P < 0.05$) but insufficient fold change ($|\log_2FC| < 0.5$), and green dots show miRNAs with substantial fold change ($|\log_2FC| > 0.5$) but lacking statistical significance (adj $P > 0.05$). Grey dots show miRNAs which are non-significant (NS).

miRNA target prediction and functional enrichment analysis

Our target prediction analysis revealed extensive regulatory potential for the differentially expressed miRNAs in mature boar sperm. Among the upregulated miRNAs, ssc-miR-126-3p targeted 1187 genes, followed by ssc-miR-193a-5p (821 targets) and ssc-miR-574-3p (752 targets) (Additional file: Table S5). Collectively, these three miRNAs were predicted to regulate a network of 2459 unique genes, suggesting widespread post-transcriptional regulation during sexual

maturation. We observed a considerable overlap between the target genes of these three miRNAs. Specifically, ssc-miR-126-3p and ssc-miR-193a-5p shared 109 common targets, while ssc-miR-126-3p and ssc-miR-574-3p shared 117 targets. Additionally, ssc-miR-193a-5p and ssc-miR-574-3p converged on 100 common genes. Most notably, 25 genes were predicted to be regulated by all three upregulated miRNAs. This pattern of overlapping regulation suggests coordinated control of key developmental pathways during sperm maturation.

GO enrichment analysis of the miRNA targets identified 232 significantly enriched terms (Biological Processes = 167, Molecular Function = 36, Cellular Component = 29) (adj $P < 0.05$) (Figure 5, Additional file: Table S6). Among biological processes, cell cycle processes (adj $P = 3.69 \times 10^{-7}$), mitotic cell cycle processes (adj $P = 2.62 \times 10^{-5}$), and the mitotic cell cycle (adj $P = 2.62 \times 10^{-5}$) were significant. For molecular functions, mRNA binding (adj $P = 3.03 \times 10^{-6}$) chromatin binding (adj $P = 4.15 \times 10^{-4}$), and protein-macromolecule adaptor activity (adj $P = 4.15 \times 10^{-4}$) were significant, suggesting active roles of these miRNAs in mRNA and chromatin organization during maturation. Cellular components related to chromosome structure and function were also significant, including chromosome (adj $P = 1.14 \times 10^{-13}$), chromosomal region (adj $P = 3.77 \times 10^{-9}$), and chromosome, centromeric region (adj $P = 3.77 \times 10^{-9}$).

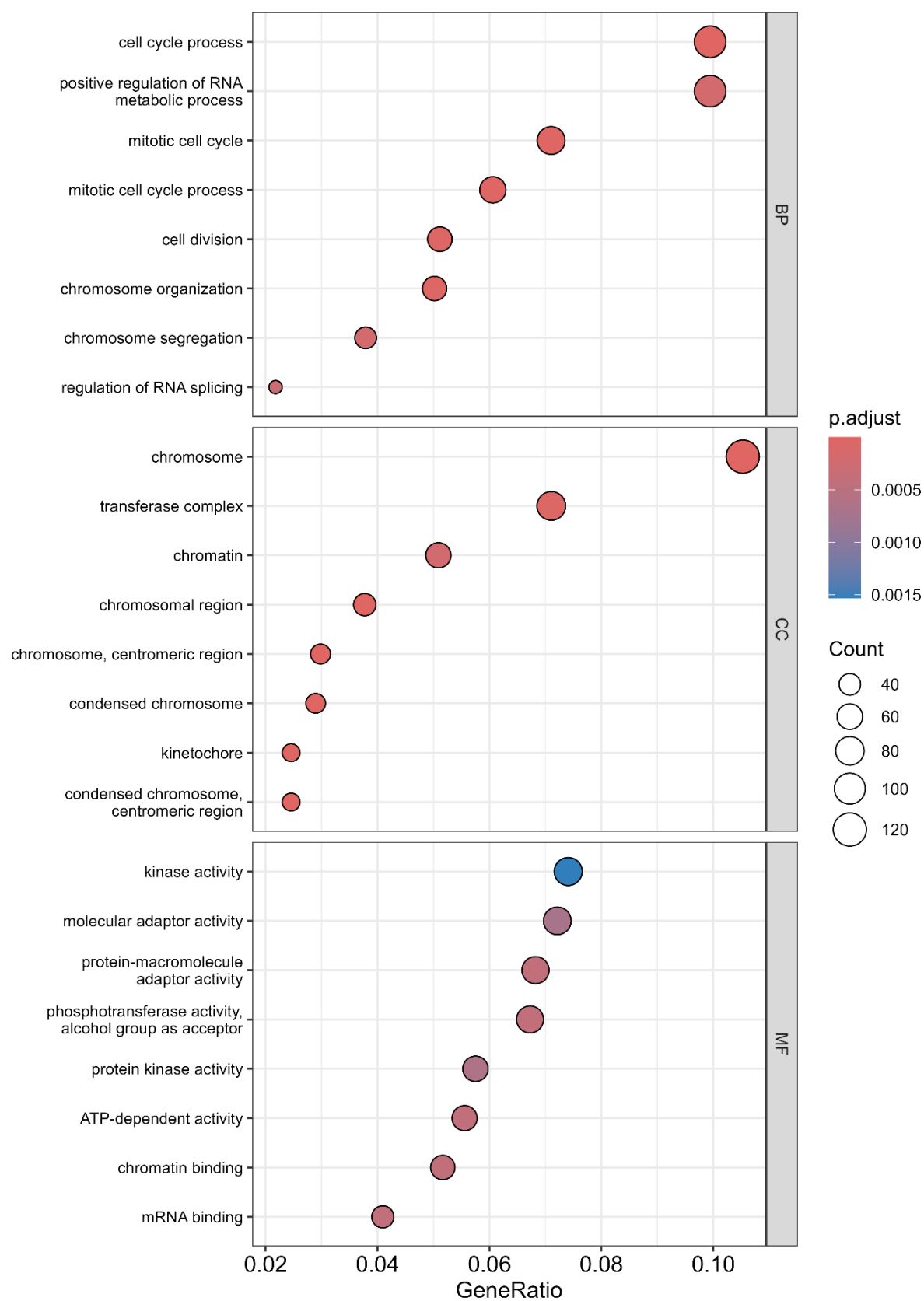


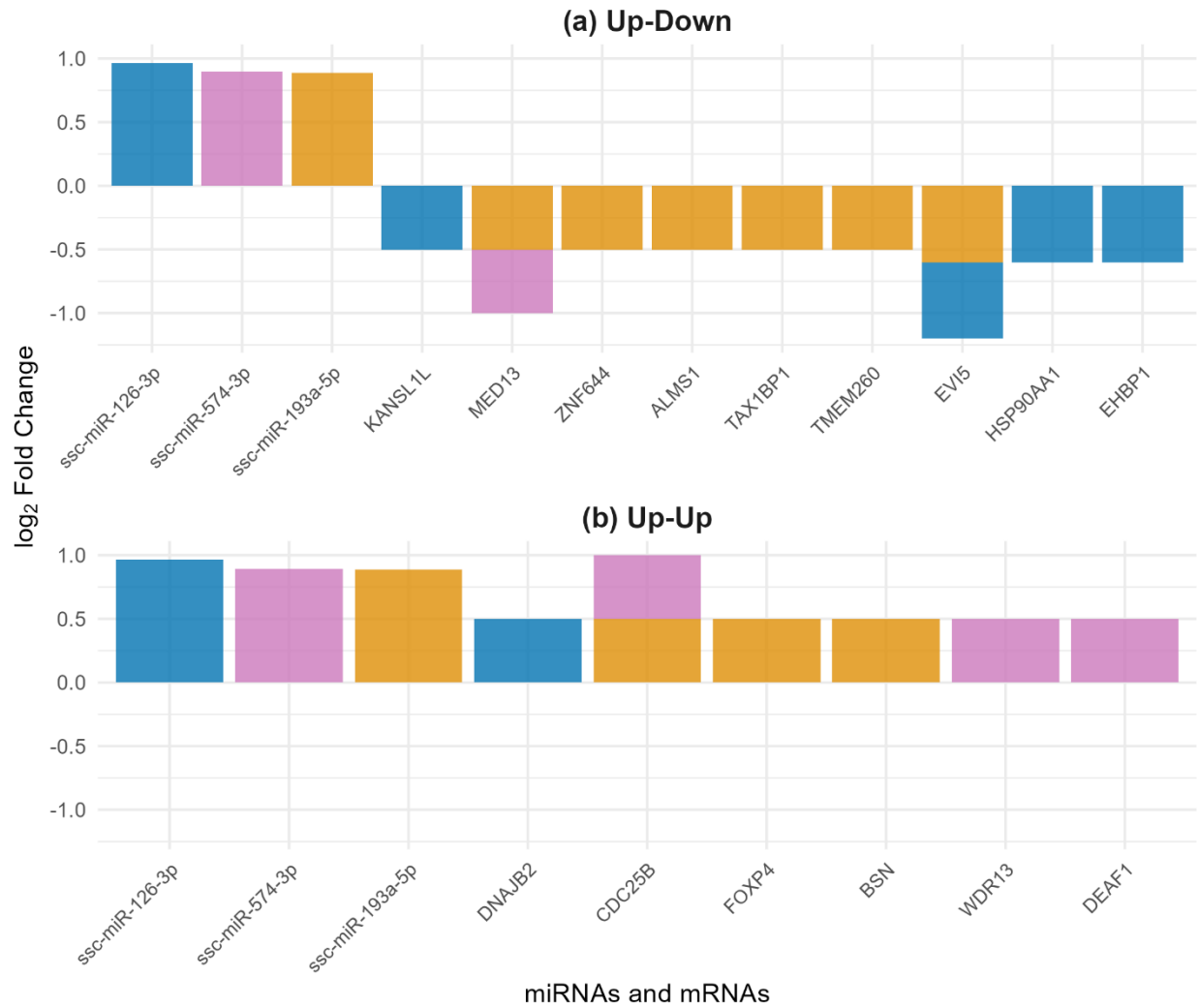
Figure 5: Gene ontology enrichment analysis of predicted target genes for differentially expressed miRNAs in sperm collected from boars at 7-months and 10-months of age. Top enriched biological process (BP), molecular function (MF), and cellular component terms (adj $P < 0.05$) are shown with gene ratio (proportion of target genes in each term) on the x-axis. Dot size represents the number of genes per term, while color represents statistical significance (adj P), with red representing more significant values (lower adj P -values) and blue representing less significant values (higher adj P -values).

Integrated miRNA-mRNA analysis

To identify functionally relevant miRNA-mRNA interactions during sexual maturation, we performed an integrated analysis of our miRNA and mRNA datasets. This analysis revealed both positive and negative expression relationships between miRNAs and their target mRNAs (Figure 6).

Three miRNAs dominated these regulatory interactions: ssc-miR-193a-5p, ssc-miR-126-3p, and ssc-miR-574-3p. The most significantly upregulated miRNA, ssc-miR-193a-5p (Table 4), showed targeting relationships with six downregulated mRNAs, including mediator complex subunit 13 (*MED13*), zinc finger protein 644 (*ZNF644*), *ALMS1*, tax1 binding protein 1 (*TAX1BP1*), and transmembrane protein 260 (*TMEM260*). Additionally, ssc-miR-193a-5p displayed positive expression with three upregulated targets: *CDC25B*, *FOXP4*, and bassoon presynaptic cytomatrix protein (*BSN*). Similarly, ssc-miR-126-3p showed targeting relationships with four downregulated mRNAs, including *EVI5*, *HSP90AA1*, *EHBP1*, and KAT8 regulatory NSL complex subunit 1 like (*KANSL1L*) while exhibiting a positive relationship with *DNAJB2*. The third upregulated miRNA, ssc-miR-574-3p, displayed a negative relationship with one downregulated mRNA (*MED13*) and positive relations with three upregulated targets (*CDC25B*, WD repeat domain 13 (*WDR13*), and *DEAF1*).

Notably, several genes were targeted by multiple upregulated miRNAs, suggesting coordinated regulation. For instance, *EVI5* was predicted as a target for both ssc-miR-126-3p and ssc-miR-193a-5p, while *MED13* was targeted by both ssc-miR-193a-5p and ssc-miR-574-3p. This convergence of multiple miRNAs on common targets highlights potential synergistic regulation of key genes during sexual maturation.



457

458 Figure 6: miRNA-mRNA regulatory relationships in sperm collected from boars at 7-months and 10-
 459 months of age. Bar plots displaying log₂ fold change expression patterns for miRNAs and their predicted
 460 target mRNAs. Panel (a) shows inverse regulatory relationships with upregulated miRNAs and their
 461 downregulated target mRNAs. Panel (b) displays positive regulatory relationships with upregulated
 462 miRNAs and their upregulated target mRNAs. Colors indicate different miRNA regulatory networks: blue
 463 bars represent ssc-miR-126-3p and its targets, orange bars represent ssc-miR-193a-5p and its targets, and
 464 purple bars represent ssc-miR-574-3p and its targets. miRNAs are positioned first (left side of each panel),
 465 followed by their target mRNAs. Only differentially expressed genes meeting both significance (adj $P <$
 466 0.05) and fold change ($|\log_2FC| \geq 0.5$) thresholds are shown.

Discussion

Our study presents the first comprehensive characterization of transcriptomic changes in boar sperm during the transition from puberty to sexual maturity. Using paired sperm samples collected from the same Duroc boars at approximately 7 and 10 months of age with combined total RNA-seq and miRNA-seq analysis, we identified distinctive transcriptional signatures that characterize this important reproductive transition. Our findings reveal a predominant pattern of upregulation in both protein-coding genes and miRNAs, along with coordinated mRNA-miRNA pairs that collectively regulate sperm maturation in Duroc boars.

Transcriptomic landscape transformation

The most striking feature of the transcriptomic landscape during sexual maturation is the predominant upregulation of gene expression, with 65% of DEGs showing increased expression in mature, compared to pubertal boars. This activation bias suggests that sexual maturation is characterized by systematic genetic programming rather than transcriptional silencing [7, 41]. Hierarchical clustering analysis revealed a clear separation of samples by age, establishing a reproducible molecular signature of maturity that transcends individual variation. Notably, two boars appeared as outliers in the clustering analysis; this observation may reflect individual biological variation. While previous studies have examined transcriptomic profiles of sperm of sexually mature animals across different species, such as boars [12], bulls, rams, and stallions, our work uniquely captures the dynamic changes occurring during the developmental window from puberty to sexual maturity in Duroc boars.

Enhanced sperm function through protein-coding gene modulation

Our analysis identified *NCLN* as the top differentially expressed upregulated gene in mature boars. *NCLN* encodes nicalin, a protein that functions as a component of the multi-pass translocon complex that mediates insertion of membrane proteins into lipid bilayers [42]. This significant upregulation suggests fundamental changes in membrane protein organization during sperm maturation. Several other prominently upregulated genes appear to support multiple aspects of sperm functionality. For instance, *RGS12*, coding for a regulator of G protein signaling, is involved in Ca^{2+} oscillation required for oocyte activation after fertilization in humans [43]. Similarly, *CIB2* produces a calcium and integrin binding family member that regulates intracellular calcium homeostasis [44] and has been implicated in mouse spermatogenesis [45]. In yak, *CIB2* transcripts

were hypermethylated at m⁶A sites in the adult testis compared to the juvenile testis, contributing to spermatogenesis [46], supporting our findings for sexual maturity.

Additionally, we observed the significant upregulation of genes related to chromatin remodeling and gametogenesis, including *FOXP4* and *PHCI* [47, 48]. Transcript levels of *FOXP4* are reported to increase during the development of spermatogonial stem cells, promoting their proliferation and supporting normal sperm production [47]. *PHCI* produces a protein that acts as a central regulator of differentially expressed genes in mouse spermatogonial stem cells, maintaining balance between self-renewal and differentiation during spermatogenesis [48]. Our analysis also revealed significant upregulation of other genes critical for sperm functionality during sexual maturation. *CDC25B* encodes the CDC25B protein, which is found to localize to the midpiece, principal piece and head cytosol of human spermatozoa, suggesting a role in non-cycle functions in terminally differentiated germ cells [49]. AKAP1 anchors protein kinase A to the outer mitochondrial membrane, facilitating localized phosphorylation events that support mitochondrial energy production and sperm motility in human spermatozoa [50]. Levels of *AKAP1* transcripts were significantly higher in normozoospermic men than in those with reproductive disorders [51] and moreover, *Akap1* has been linked to spermatogenesis in mice [52]. The coordinated upregulation of these genes during sexual maturation suggests enhanced regulation of energy metabolism, phosphorylation cascades, and chromatin organization as boars transition from puberty to sexual maturity.

Downregulation of developmental and metabolic genes during sexual maturation

We observed significant downregulation of *HSP90AA1* in our study, a gene that encodes a molecular chaperone involved in protein maturation and regulation [53]. Interestingly, previous studies have found HSP90AA1 protein to be overrepresented in low-fertility bull sperm, suggesting that its downregulation in mature boar sperm may be beneficial for optimal functionality. HSP90AA1 has also been implicated in regulating the acrosome reaction in yaks [54]. The downregulation of *HSP90AA1* in our study may therefore represent a finely tuned optimization of protein folding and stability required for mature boar sperm function.

Our analysis also revealed that mitochondrial rRNA was significantly downregulated in mature boars. It indicates the remodeling of energy production pathways during maturation [55]. FSIP2, a protein associated with the sperm fibrous sheath and specific to spermatogenic cells [56], showed

decreased expression in sperm from mature boars in our study. FSIP2 has been identified in the phosphoproteomics studies in yak as part of a protein network with AKAP3, AKAP4, and CABYR, suggesting roles in protein kinase A binding, cytoskeletal organization, and sperm motility [57]. Mouse models with *FSIP2* mutations demonstrate multiple morphological abnormalities of sperm flagella, while its overexpression produces elongated tail structures and altered acrosome [56]. This suggests that precise regulation of FSIP2 levels is critical for proper sperm development. The downregulation observed herein for mature boars could reflect a developmental fine-tuning, where lower levels are optimal for the maintenance of properly formed flagellar and acrosomal structures in fully matured sperm.

VDAC3 encodes the voltage-dependent anion channel 3 protein, which localizes to the acrosomal region and midpiece of spermatozoa. In mouse models, VDAC3 protein deficiency caused significant decrease in sperm motility, viability, acrosome reaction, capacitation, fertilization, and embryo development. Proper regulation of VDAC3 is essential for proper mitochondrial sheath formation during spermatogenesis, sperm function and male fertility [58, 59]. Similarly, upregulation of gene family member *VDAC1* was reported in Duroc boars with high DNA fragmentation index [60]. The downregulation of *VDAC3* in the sperm of 10-months old in our study may indicate fine-tuning of mitochondrial dynamics as sperm reach functional maturity. *ALMS1* was also significantly downregulated in the sperm of 10-months old individuals. Loss of ALMS1 protein function in mouse resulted in primary spermatogenic defects characterized by disrupted progression from round to elongating spermatids, abnormal sperm tail and mitochondrial structure, and testicular atrophy leading to male infertility [61]. Likewise, conditional knockout of *Upf2* in mouse germ cells causes severe spermatogenic defects and male infertility, highlighting *UPF2*'s important role in fertility [62].

Functional integration through biological pathways

The GO enrichment analysis provides a functional context for understanding the transcriptomic changes occurring during boar sexual maturation. The predominant enrichment of reproduction-related biological processes aligns with the developmental transition being studied and highlights the molecular underpinnings of sexual maturation.

The significant enrichment of the spermatid development and differentiation terms suggests that even in ejaculated sperm, the transcriptomic signature of developmental processes remains

detectable and undergoes dynamic regulation from puberty to sexual maturity. Several genes driving this enrichment were common across multiple reproduction-related GO terms, including testis specific serine kinase 2 (*TSSK2*), testis specific serine kinases 6 (*TSSK6*), *AKAP1*, spermatogenesis associated 20 (*SPATA20*), AT-rich interaction Domain 4A (*ARID4A*), cytochrome P450 19A3 (*CYP19A3*), *CDC25B*, proprotein convertase subtilisin/kexin type 4 (*PCSK4*), *VDAC3*, and neutralized E3 ubiquitin protein ligase 1 (*NEURL1*) (Figure 7).

Notably, many of these genes have established roles in sperm function that we have previously discussed in this paper. For instance, the upregulation of *AKAP1* and *CDC25B* in sperm of 10-months old boars likely supports mitochondrial function and non-cell cycle functions, respectively [49, 50] while the downregulation of *VDAC3* might represent a refinement in mitochondrial dynamics during spermatogenesis [59].

The identification of protein targeting to the membrane as an enriched process, represented by signal recognition particle 54 (*SRP54*), SEC61 translocon subunit alpha 2 (*SEC61A2*), and translocon associated membrane protein 1 (*TRAM1*) highlights the importance of proper protein localization in mature sperm. Membrane protein organization is crucial for acrosome reaction, and sperm-egg fusion, and our findings suggest that these processes undergo refinement during sexual maturation.

Our analysis also revealed significant enrichment of protein kinase activity terms, driven by a diverse set of genes including AKT serine/threonine kinase 2 (*AKT2*), protein kinase C delta (*PRKCD*), cyclin dependent kinase 16 (*CDK16*), dual specificity tyrosine phosphorylation regulated kinase 1B (*DYRK1B*), mitogen-activated protein kinase 6 (*MAPK6*), CaM kinase like vesicle associated (*CAMKV*), integrin linked kinase (*ILK*), choline phosphotransferase 1 (*CHPT1*), terminal nucleotidyltransferase 5C (*TENT5C*), *TSSK2*, *TSSK6*, and *AKAP1*. The enrichment of this functional category underscores the importance of phosphorylation-dependent signaling in regulating sperm maturation and function.

Our network analysis also indicates distinct yet interconnected functional clusters (Figure 7). Notably, the connection between kinase activity and structural processes, specifically spermatid development, is mediated by *TSSK2*, *TSSK6*, and *AKAP1*. This interconnection demonstrates how regulatory networks orchestrate the functional optimization of sperm as boars transition from puberty to sexual maturity.

Collectively, these findings provide a systems-level perspective on boar sperm maturation. The enrichment of both developmental and signaling pathways supports a model where the final stages of sexual maturation involve not just quantitative changes in gene expression but qualitative shifts in pathway activation and coordination.

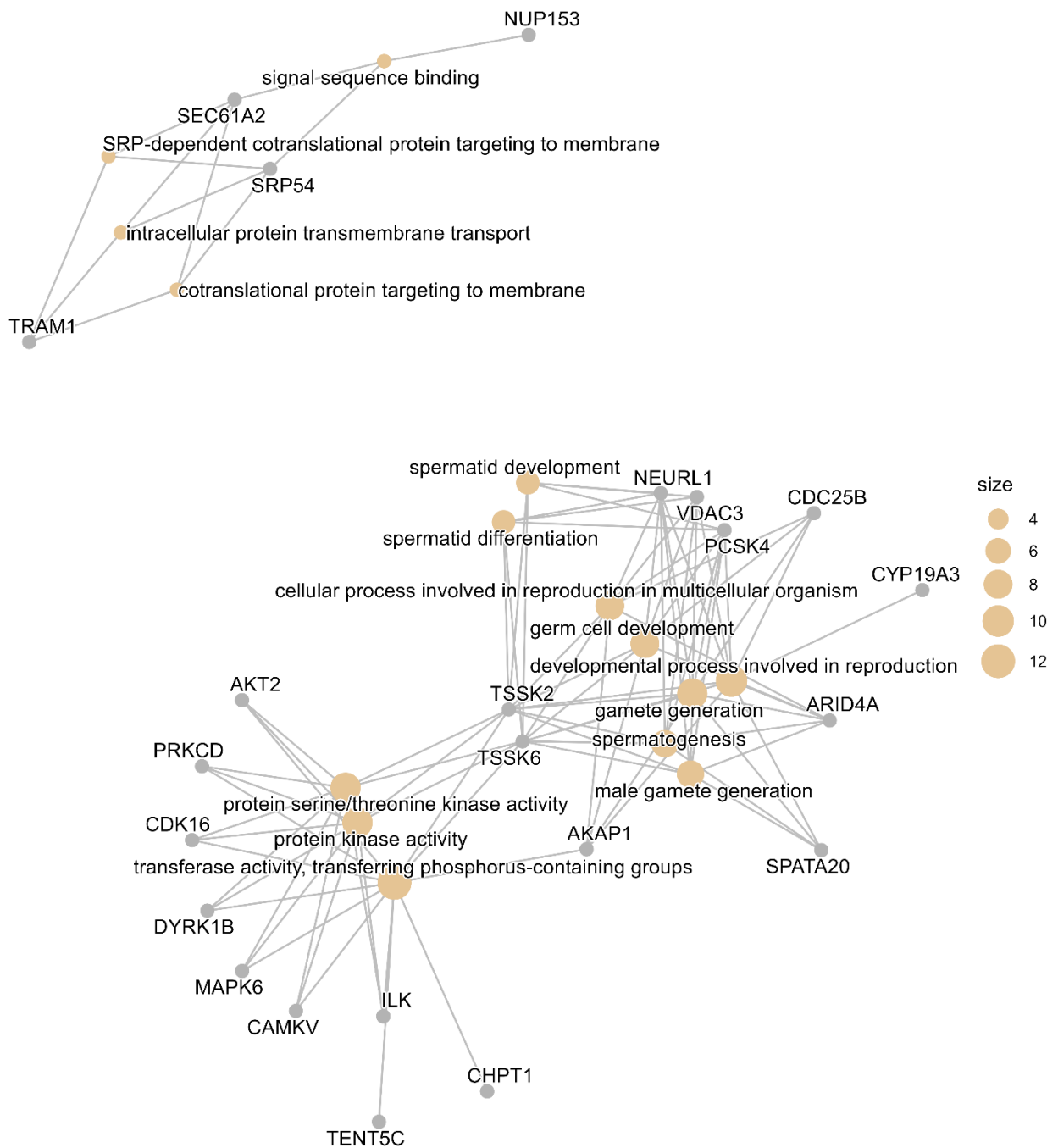


Figure 7: Gene-concept network analysis of differentially expressed genes in sperm of 10-month old versus 7-month old boars. This cnetplot depicts the relationships between differentially expressed genes (grey nodes) and enriched Gene Ontology (GO) terms (colored nodes). The size of GO term nodes indicates the number of associated genes. There are three distinct functional clusters: the upper cluster represents membrane protein transport pathways, the central cluster represents reproductive development processes, and the lower cluster represents kinase signaling networks. Several genes like TSSK2, TSSK6, and AKAP1 connect the two clusters. Line connections represent associations between GO terms and genes based on GO annotations.

miRNA regulation during sexual maturation

Beyond protein-coding genes, our study revealed significant changes in miRNA expression during sexual maturation. Five out of six differentially expressed miRNAs were upregulated in mature boars, mirroring the trend observed in protein-coding genes. Among the upregulated miRNAs, we found ssc-miR-193a-5p, which has been previously implicated in sperm function across multiple species. In bovine studies, miR-193a-3p was overexpressed in both sperm and seminal plasma of high-fertility bulls, directly connecting this miRNA to reproductive success [63]. Human literature further supports this connection, identifying miR-193a as a key regulator in asthenozoospermia influencing sperm motility [64].

ssc-miR-196a has been identified as evolutionarily conserved between amphibians and mammals, critical for spermatogenesis across all tetrapods, spanning approximately 350 million years, and misregulation of this miR-196 contributes to male sterility [65]. Intriguingly, Lian et al. [66] found miR-196 expression restricted to immature porcine testes (1 month), contrasting with our finding of increased expression in sperm of mature vs pubertal boars. This apparent discrepancy may reflect the different regulatory roles of miR-196 during testicular development versus sperm maturation. Zhang et al. [67] demonstrated that miR-196a enhances Sertoli cell proliferation and reduces apoptosis by targeting regulator of chromosome condensation 2 (*RCC2*) and ATP binding cassette subfamily B member 9 (*ABCB9*) genes in pigs, suggesting its importance in establishing optimal cellular environments for sperm production.

The ssc-miR-126-3p has been extensively characterized in reproductive contexts across species. In pigs, miR-126 has been reported to stimulate cell proliferation and suppress apoptosis in immature Sertoli cells targeting *PIK3R2* and activating the PI3k/AKT signaling pathways [68]. In

humans, reduced miR-126 expression is documented in patients with a complete absence of sperm due to impaired spermatogenesis [69].

The upregulation of ssc-miR-574-3p connects directly to energy metabolism regulation. Ma et al. [70] demonstrated that miR-574 directly targets mt-ND5, a component of the mitochondrial respiratory chain complex I, thereby regulating mitochondrial function and ATP generation in human sperm. Elevated levels of miR-210 have been observed in testicular tissues with maturation arrest/hypospermatogenesis and in the seminal plasma of varicocele patients [71, 72]. These findings underscore the context-dependent roles of miR-210, with its upregulation in sperm from mature boars potentially reflecting a specific regulatory adaptation during sexual maturity.

The downregulation of ssc-miR-338 represents an equally important aspect of the sexual maturation process. Madison-Villar and Michalak [65] identified miR-338 among their evolutionarily conserved set of miRNAs critical for spermatogenesis across tetrapods, with misexpression correlating with testicular dysfunction in sterile fish hybrids. Our analysis further revealed that three miRNAs: ssc-miR-193a-5p, ssc-miR-126-3p, and ssc-miR-574-3p, emerged as central regulators, collectively targeting 2,459 unique genes.

Integrative Analysis of miRNA-mRNA Interactions

Our integrated analysis identified 10 and seven miRNA-mRNA pairs with negatively and positively correlated expression, respectively. The convergence of multiple miRNAs on common targets suggests redundant control mechanisms ensuring proper sexual maturation. It might also indicate a fine-tuning of gene expression or cooperative effects of the miRNAs. For instance, *EVI5* was targeted by both ssc-miR-126-3p and ssc-miR-193a-5p; *MED13* and *CDC25B* were targeted by both ssc-miR-193a-5p and ssc-miR-574-3p.

Our analysis identified six target genes that were downregulated and three target genes upregulated by ssc-miR-193a-5p, including *EVI5*, *ALMS1*, and *CDC25B*. Proteins encoded by *EVI5* are involved in cell cycle progression [73], *ALSM1* protein has a role in spermatogenesis, sperm tail, and mitochondrial structure in mice [61], and *CDC25B* protein localizes to the human sperm midpiece and head [49]. This suggests that ssc-miR-193a-5p coordinates the fine-tuning of both structural and functional components of sperm.

Similarly, upregulated ssc-miR-126-3p potentially targeted one upregulated gene and four downregulated genes in our study, including *EVI5*, and *HSP90AA1*. Overexpression of *HSP90AA1* protein is linked to poor fertility in bulls [74] and it has also been implicated in regulating the acrosome reaction in yaks [54] and protein folding in human [53]. The downregulation of *HSP90AA1* in our sperm samples from mature boars may therefore represent a finely tuned optimization of protein folding and stability required for mature sperm function. Taken together, our miRNA findings complement and extend the mRNA results. Concurrently, they provide a deeper understanding of the molecular mechanisms driving the transition from puberty to sexual maturity in Duroc boars.

Limitations and future directions

Our sample size of six boars and focus on a single breed, though adequate for detecting major transcriptomic shifts, may have limited our ability to identify more subtle changes with lower effect sizes and applicability to other breeds. We applied a lower fold-change cutoff ($|\log_2FC| \geq 0.5$) than typically used in somatic tissue studies, since mature spermatozoa carry 600 times less RNAs than somatic cell [75] and even modest expression changes can be biologically meaningful during the maturation process.

Future studies can expand our findings in several directions. Confirmation of the top 15-20 differentially expressed genes with RT-qPCR on larger cohorts across multiple swine breeds to validate these transcriptomic signatures would enhance their applicability as biomarkers of sexual maturity. Moreover, integrating additional omics data, including metabolomics, proteomics, and epigenetics, would offer a more comprehensive understanding of the regulatory networks governing sexual maturation.

Conclusion

This study provides the first comprehensive transcriptomic profile of sperm between pubertal (~7 months) and mature (~10 months) boars. Our paired analysis revealed predominantly upregulated genes during maturation, including *NCLN*, *RGS2*, *CIB2*, *FOXP4*, *PHC1*, *CDC25B*, and *AKAP1* involved in membrane organization, calcium homeostasis, protein kinase signaling, and reproductive processes. Concurrently, genes associated with sperm development and metabolism, such as *HSP90AA1*, *EVI5*, *FSIP2*, *VDAC3*, and *ALMS1* were downregulated during maturation.

679 We identified six differentially expressed miRNAs and predicted 17 miRNA-mRNA regulatory
680 pairs. Functional enrichment analysis identified biological processes such as reproduction, cell
681 cycle regulation, and chromatin organization, indicating coordinated transcriptional regulation
682 during sexual maturation.

683 These findings advance our understanding of boar sexual maturation and provide a first step
684 towards potential biomarkers for assessing reproductive development, with practical implications
685 for swine breeding programs where genomic selection drives earlier introduction of young boars.

686 **List of abbreviations**

687 *ABCB9*: ATP binding cassette subfamily B member 9

688 AI: Artificial insemination

689 *AKAP1*: A-kinase anchoring protein 1

690 *AKT2*: AKT serine/threonine kinase 2

691 *ALMS1*: ALMS1 centrosome and basal body associated protein

692 *ARID4A*: AT-rich interaction Domain 4A

693 BAM: Binary alignment/map

694 *BCL9*: BCL9 transcription coactivator

695 BP: Biological processes

696 *BSN*: Bassoon presynaptic cytomatrix protein

697 *CAMKV*: CaM kinase like vesicle associated

698 *CATSPERG*: Cation channel sperm associated auxiliary subunit gamma

699 *CDC25B*: Cell division cycle 25B

700 *CDK16*: Cyclin dependent kinase 16

701 *CHPT1*: Choline phosphotransferase 1

702 *CIB2*: Calcium and integrin binding family member 2

703 *CYP19A3*: Cytochrome P450 19A3

704 *DEAF1*: DEAF1 transcription factor

705 DEGs: Differentially expressed genes

706 *DNAJB2*: DnaJ heat shock protein family (Hsp40) member B2

707 *DNAJB8*: DnaJ heat shock protein family member B8

708 *DYRK1B*: Dual specificity tyrosine phosphorylation regulated kinase 1B

709 *EHBP1*: EH domain binding protein 1

710 *EPOP*: Elongin BC and polycomb repressive complex 2 associated protein

711 ESTs: Expressed sequence tags

712 *EVI5*: Ectropic viral integration site 5

713 *FEM1C*: Fem-1 homolog C

714 *FOXP4*: Forkhead box P4

715 *FSIP2*: Fibrous sheath interacting protein 2

716 *GLI4*: GLI family zinc finger 4

717 GO: Gene ontology

718 *GRAMD1C*: GRAM domain containing 1C

719 GTF: Gene transfer format

720 HS: High sensitivity

721 *HSP90AA1*: Heat shock protein 90 alpha family class A member 1

722 *ILK*: Integrin linked kinase

723 *JCHAIN*: Joining chain of multimeric IgA and IgM

724 *KANSL1L*: KAT8 regulatory NSL complex subunit 1 like

725 *KANSL1L*: KAT8 regulatory NSL complex subunit 1 like

726 lncRNAs: Long non-coding RNAs

727 *MAPK6*: Mitogen-activated protein kinase 6

728 *MED13*: Mediator complex subunit 13

729 MF: Molecular function

730 miRNAs: micro RNAs

731 mRNAs: messenger RNAs

732 Mt_rRNA: mitochondrial rRNA

733 MtrRNAs: Mitochondrial ribosomal RNAs

734 MttRNAs: Mitochondrial transfer RNAs

735 *NAV3*: Neuron navigator 3

736 *NCLN*: Nicalin

737 *NEURL1*: Neutralized E3 ubiquitin protein ligase 1

738 NS: Non-significant

739 *OAZ3*: Ornithine decarboxylase antizyme 2

740 *OS9*: OS9 endoplasmic reticulum lectin

741 PBS: Phosphate buffer solution

742 PCR: Polymerase chain reaction

743 *PCSK4*: Proprotein convertase subtilisin/kexin type 4

744 *PGAP2*: Post-GPI attachment to proteins 2

745 *PHC1*: Polyhomeotic homolog 1

746 piRNAs: Piwi interacting RNAs

747 *PRKCD*: Protein kinase C delta

748 *PRM1*: Protamine 1

749 *PRM2*: Protamine 2

750 *RCC2*: Regulator of chromosome condensation 2

751 *RGS12*: Regulator of G protein signaling 12

752 RNA-Seq: RNA sequencing

753 rRNAs: ribosomal RNAs

754 RT: Room temperature

755 SAM: Sequence alignment/map

756 *SDCCAG*: SHH Signaling And Ciliogenesis Regulator SDCCAG8

757 *SEC61A2*: SEC61 translocon subunit alpha 2

758 *SESN1*: Sestrin 1

759 sncRNAs: short noncoding RNAs

760 snoRNAs: small nucleolar RNAs

761 snRNAs: small nuclear RNAs

762 *SPATA20*: Spermatogenesis associated 20

763 SRP: Signal recognition particle

764 *SRP54*: Signal recognition particle

765 *SSFA2*: Sperm-specific antigen 2

766 *TAX1BP1*: Tax1 binding protein 1

767 *TBRI*: T-box brain transcription factor 1

768 TCEP: Tris(2-carboxyethyl)phosphine

769 *TENT5C*: Terminal nucleotidyltransferase 5C

770 *TMEM106B*: Transmembrane protein 106B

771 *TMEM151A*: Transmembrane protein 151A

772 *TMEM260*: Transmembrane protein 260

773 *TNPI*: Transition protein 1

774 *TPP2*: Tripeptidyl peptidase

775 *TRAM1*: Translocon associated membrane protein 1

776 tRNAs: transfer RNAs

777 *TSSK2*: Testis specific serine kinase 2

778 *TSSK6*: Testis specific serine kinase 6

779 *UPF2*: UPF2 regulator of nonsense mediated mRNA decay

780 *USP34*: Ubiquitin specific peptidase 34

781 *VDAC3*: Voltage dependent anion channel 3

782 *WASHC1*: WASH complex subunit 1

783 *WDR13*: WD repeat domain 13

784 *ZNF644*: Zinc finger protein 644

785 **Declarations**

786 **Ethics approval and consent to participate**

787 Ethical approval was not required for this study, as the boars were involved in routine semen
 788 production practices consistent with Norwegian laws and regulations. These include the Animal
 789 Protection Act (December 20, 1974), the Animal Welfare Act (June 19, 2009), the Regulations for
 790 the Management of Pigs in Norway (February 18, 2003), and the European Union's legislation on
 791 pig semen production (Council Directive 90/429/EEC).

792 **Consent for publication**

793 Not applicable

Availability of data and material

The datasets used and/or analyzed during the current study have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series number GSE305494 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?&acc=GSE305494>).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AS conceptualized the study, designed the methodology, collected the samples, isolated and performed quality control of RNA for sequencing, performed bioinformatic and statistical analysis and the interpretation of data, created visualization, and was a major contributor in writing the original draft and reviewing and editing the manuscript. MS contributed to guiding bioinformatic and statistical analysis and reviewing and editing the manuscript. AH performed miRNA seq bioinformatic analysis, guided bioinformatic analysis, and reviewed and edited the manuscript. SR contributed to the sampling and initial optimization of the RNA isolation and quality assessment protocol. GDG and UB conducted total RNA sequencing and miRNA sequencing and reviewed and edited the manuscript. EK contributed to the conceptualization and acquisition of funding for the project, as well as reviewing and editing the manuscript. AHAK conceptualized the study, provided resources, supervised the project, and contributed to writing, reviewing, and editing the manuscript. All authors read and approved the final manuscript.

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References

1. Toshimori K. Testicular and epididymal maturation of mammalian spermatozoa. In: *Introduction to mammalian reproduction*. edn. Edited by Tulsiani DRP. Boston, MA: Springer US; 2003: 93-111.
2. Souza APBd, Schorr-Lenz ÂM, Lucca F, Bustamante-Filho IC. The epididymis and its role on sperm quality and male fertility. *Animal reproduction*. 2017;14:1234-44.
3. Ding H, Luo Y, Liu M, Huang J, Xu D. Histological and transcriptome analyses of testes from duroc and meishan boars. *Scientific Reports*. 2016;6(1):20758.
4. Zheng Y, Gao Q, Li T, Liu R, Cheng Z, Guo M, et al. Sertoli cell and spermatogonial development in pigs. *J Anim Sci Biotechnol*. 2022;13(1):45.
5. Banaszewska D, Kondracki S. An assessment of the breeding maturity of insemination boars based on ejaculate quality changes. *Folia Biologica*. 2012;60(3-4):151-62.
6. Kawęcka M, Pietruszka A, Jacyno E, Czarnecki R, Kamyczek M. Quality of semen of young boars of the breeds pietrain and duroc and their reciprocal crosses. *Archives Animal Breeding*. 2008;51:42-54.
7. Gòdia M, Swanson G, Krawetz SA. A history of why fathers' rna matters†. *Biology of Reproduction*. 2018;99(1):147-59.
8. Gur Y, Breitbart H. Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes. *Genes Dev*. 2006;20(4):411-6.
9. Sa P, Gòdia M, Lewis N, Lian Y, Clop A. Genomic, transcriptomic and epigenomic analysis towards the understanding of porcine semen quality traits. Past, current and future trends. *Animal Reproduction Science*. 2024;269:107543.
10. Gòdia M, Reverter A, González-Prendes R, Ramayo-Caldas Y, Castelló A, Rodríguez-Gil J-E, et al. A systems biology framework integrating gwas and rna-seq to shed light on the molecular basis of sperm quality in swine. *Genetics Selection Evolution*. 2020;52(1):72.
11. Yang CC, Lin YS, Hsu CC, Wu SC, Lin EC, Cheng WTK. Identification and sequencing of remnant messenger rnas found in domestic swine (*sus scrofa*) fresh ejaculated spermatozoa. *Animal Reproduction Science*. 2009;113(1):143-55.
12. Gòdia M, Castelló A, Rocco M, Cabrera B, Rodríguez-Gil JE, Balasch S, et al. Identification of circular rnas in porcine sperm and evaluation of their relation to sperm motility. *Scientific Reports*. 2020;10(1).

13. Gòdia M, Mayer FQ, Nafissi J, Castelló A, Rodríguez-Gil JE, Sánchez A, Clot A. A technical assessment of the porcine ejaculated spermatozoa for a sperm-specific rna-seq analysis. *Syst Biol Reprod Med*. 2018;64(4):291-303.
14. Gòdia M, Estill M, Castelló A, Balasch S, Rodríguez-Gil JE, Krawetz SA, et al. A rna-seq analysis to describe the boar sperm transcriptome and its seasonal changes. *Front Genet*. 2019;10:299.
15. Dai DH, Qazi IH, Ran MX, Liang K, Zhang Y, Zhang M, et al. Exploration of mirna and mrna profiles in fresh and frozen-thawed boar sperm by transcriptome and small rna sequencing. *Int J Mol Sci*. 2019;20(4).
16. Alvarez-Rodriguez M, Martinez C, Wright D, Barranco I, Roca J, Rodriguez-Martinez H. The transcriptome of pig spermatozoa, and its role in fertility. *Int J Mol Sci*. 2020;21(5).
17. Curry E, Safranski TJ, Pratt SL. Differential expression of porcine sperm micrnas and their association with sperm morphology and motility. *Theriogenology*. 2011;76(8):1532-39.
18. Chang Y, Dai D-h, Li Y, Zhang Y, Zhang M, Zhou G-b, Zeng C-j. Differences in the expression of micrnas and their predicted gene targets between cauda epididymal and ejaculated boar sperm. *Theriogenology*. 2016;86(9):2162-71.
19. Martinez CA, Roca J, Alvarez-Rodriguez M, Rodriguez-Martinez H. Mirna-profiling in ejaculated and epididymal pig spermatozoa and their relation to fertility after artificial insemination. *Biology (Basel)*. 2022;11(2).
20. Luo Z, Liu Y, Chen L, Ellis M, Li M, Wang J, et al. Microrna profiling in three main stages during porcinespermatogenesis. *Journal of Assisted Reproduction and Genetics*. 2015;32(3):451-60.
21. Meuwissen T, Hayes B, Goddard M. Genomic selection: A paradigm shift in animal breeding. *Animal Frontiers*. 2016;6(1):6-14.
22. Goodrich R, Johnson G, Krawetz SA. The preparation of human spermatozoal rna for clinical analysis. *Archives of Andrology*. 2007;53(3):161-67.
23. Card CJ, Anderson EJ, Zamberlan S, Krieger KE, Kaproth M, Sartini BL. Cryopreserved bovine spermatozoal transcript profile as revealed by high-throughput ribonucleic acid sequencing. *Biol Reprod*. 2013;88(2):49.

24. Haug LM, Wilson RC, Alm-Kristiansen AH. Epigenetic-related transcriptional reprogramming elucidated by identification and validation of a novel reference gene combination for rt-qpcr studies in porcine oocytes of contrasting quality. *Mol Biol Rep.* 2024;51(1):368.
25. Fastqc: A quality control tool for high throughput sequence data [<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>]
26. Krueger FJ, F.; Ewels, P.; Afyounian, E.; Weinstein, M.; Schuster-Boeckler, B.; Hulselmans, G.; Selamons. Felixkrueger/trimgalore: V0.6.10 - add default decompression path. In: Zenodo; 2023.
27. Harrison PW, Amode MR, Austine-Orimoloye O, Azov Andrey G, Barba M, Barnes I, et al. Ensembl 2024. *Nucleic Acids Research.* 2023;52(D1):D891-D99.
28. Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with hisat2 and hisat-genotype. *Nature Biotechnology.* 2019;37(8):907-15.
29. Picard tools [<https://broadinstitute.github.io/picard/>]
30. Liao Y, Smyth GK, Shi W. Featurecounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics.* 2013;30(7):923-30.
31. Aparicio-Puerta E, Lebrón R, Rueda A, Gómez-Martín C, Giannoukakos S, Jaspez D, et al. Srnabench and srnatoolbox 2019: Intuitive fast small rna profiling and differential expression. *Nucleic Acids Res.* 2019;47(W1):W530-w35.
32. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for rna-seq data with deseq2. *Genome Biology.* 2014;15(12):550.
33. R Core Team. R: A language and environment for statistical computing. In. Vienna, Austria: R Foundation for Statistical Computing; 2022.
34. Bourgon R, Gentleman R, Huber W. Independent filtering increases detection power for high-throughput experiments. *Proceedings of the National Academy of Sciences.* 2010;107(21):9546-51.
35. Durinck S, Spellman PT, Birney E, Huber W. Mapping identifiers for the integration of genomic datasets with the r/bioconductor package biomart. *Nature Protocols.* 2009;4(8):1184-91.

36. Ru Y, Kechris KJ, Tabakoff B, Hoffman P, Radcliffe RA, Bowler R, et al. The multimir r package and database: Integration of microrna–target interactions along with their disease and drug associations. *Nucleic Acids Research*. 2014;42(17):e133-e33.
37. Kolberg L, Raudvere U, Kuzmin I, Vilo J, Peterson H. Gprofiler2 -- an r package for gene list functional enrichment analysis and namespace conversion toolset g:Profiler. *F1000Res*. 2020;9.
38. Yu G, Wang L-G, Han Y, He Q-Y. Clusterprofiler: An r package for comparing biological themes among gene clusters. *OMICS: A Journal of Integrative Biology*. 2012;16(5):284-87.
39. Carlson M. Org.Ss.Eg.Db: Genome wide annotation for pig. In.; 2023.
40. Huber W, Carey VJ, Gentleman R, Anders S, Carlson M, Carvalho BS, et al. Orchestrating high-throughput genomic analysis with bioconductor. *Nature Methods*. 2015;12(2):115-21.
41. Gur Y, Breitbart H. Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes. *Genes & Development*. 2006;20(4):411-16.
42. McGilvray PT, Anghel SA, Sundaram A, Zhong F, Trnka MJ, Fuller JR, et al. An er translocon for multi-pass membrane protein biogenesis. *Elife*. 2020;9.
43. Ma T, Zhou S, Xie X, Chen J, Wang J, Zhang G. A case report of a family with developmental arrest of human prokaryotic stage zygote. *Front Cell Dev Biol*. 2024;12:1280797.
44. Patel K, Giese AP, Grossheim JM, Hegde RS, Delio M, Samanich J, et al. A novel c-terminal cib2 (calcium and integrin binding protein 2) mutation associated with non-syndromic hearing loss in a hispanic family. *PLoS One*. 2015;10(10):e0133082.
45. Xu Z, Miyata H, Kaneda Y, Castaneda JM, Lu Y, Morohoshi A, et al. Cib4 is essential for the haploid phase of spermatogenesis in mice†. *Biol Reprod*. 2020;103(2):235-43.
46. Guo S, Pei J, Wang X, Cao M, Xiong L, Kang Y, et al. Transcriptome studies reveal the n(6)-methyladenosine differences in testis of yaks at juvenile and sexual maturity stages. *Animals (Basel)*. 2023;13(18).
47. Luo SW, Tang L, Zhou D, Bo H, Fan LQ. Foxp4 promotes proliferation of human spermatogonial stem cells. *Asian J Androl*. 2023;25(3):322-30.
48. Sisakhtnezhad S, Heshmati P. Comparative analysis of single-cell rna sequencing data from mouse spermatogonial and mesenchymal stem cells to identify differentially expressed

genes and transcriptional regulators of germline cells. *J Cell Physiol.* 2018;233(7):5231-42.

49. Teng YN, Chung CL, Lin YM, Pan HA, Liao RW, Kuo PL. Expression of various *cdc25b* isoforms in human spermatozoa. *Fertil Steril.* 2007;88(2):379-82.

50. Reinton N, Collas P, Haugen TB, Skålhegg BS, Hansson V, Jahnsen T, Taskén K. Localization of a novel human α -kinase-anchoring protein, *hakap220*, during spermatogenesis. *Developmental Biology.* 2000;223(1):194-204.

51. Dai Y, Kong X, Yao C, Xiong C, Li Z, Li H. Multi-stage screening cell-free seminal mrnas to diagnose completion of meiosis and predict testicular sperm retrieval in men with non-obstructive azoospermia. *Andrology.* 2022;10(4):749-57.

52. Sang Y, Liu J, Li X, Zhou G, Zhang Y, Gao L, et al. The effect of sinps on DNA methylation of genome in mouse spermatocytes. *Environmental Science and Pollution Research.* 2021;28(32):43684-97.

53. Forsythe HL, Jarvis JL, Turner JW, Elmore LW, Holt SE. Stable association of *hsp90* and *p23*, but not *hsp70*, with active human telomerase. *J Biol Chem.* 2001;276(19):15571-4.

54. Zhang R, Guo X, Liang C, Pei J, Bao P, Yin M, et al. Identification and validation of yak (*bos grunniens*) frozen-thawed sperm proteins associated with capacitation and the acrosome reaction. *J Proteome Res.* 2022;21(11):2754-70.

55. Brand MD, Orr AL, Perevoshchikova IV, Quinlan CL. The role of mitochondrial function and cellular bioenergetics in ageing and disease. *Br J Dermatol.* 2013;169 Suppl 2(0 2):1-8.

56. Fang X, Gamallat Y, Chen Z, Mai H, Zhou P, Sun C, et al. Hypomorphic and hypermorphic mouse models of *fsip2* indicate its dosage-dependent roles in sperm tail and acrosome formation. *Development.* 2021;148(11).

57. Zhang R, Liang C, Guo X, Bao P, Pei J, Wu F, et al. Quantitative phosphoproteomics analyses reveal the regulatory mechanisms related to frozen-thawed sperm capacitation and acrosome reaction in yak (*bos grunniens*). *Front Physiol.* 2022;13:1013082.

58. Kwon WS, Park YJ, Mohamed el SA, Pang MG. Voltage-dependent anion channels are a key factor of male fertility. *Fertil Steril.* 2013;99(2):354-61.

971 59. Mise S, Matsumoto A, Shimada K, Hosaka T, Takahashi M, Ichihara K, et al. Kastor and
972 polluks polypeptides encoded by a single gene locus cooperatively regulate vdac and
973 spermatogenesis. *Nat Commun.* 2022;13(1):1071.

974 60. van Son M, Tremoen NH, Gaustad AH, Myromslien FD, Våge DI, Stenseth EB, et al. Rna
975 sequencing reveals candidate genes and polymorphisms related to sperm DNA integrity in
976 testis tissue from boars. *BMC Vet Res.* 2017;13(1):362.

977 61. Arsov T, Silva DG, O'Bryan MK, Sainsbury A, Lee NJ, Kennedy C, et al. Fat aussie--a new
978 alström syndrome mouse showing a critical role for *alms1* in obesity, diabetes, and
979 spermatogenesis. *Mol Endocrinol.* 2006;20(7):1610-22.

980 62. Bao J, Tang C, Yuan S, Porse BT, Yan W. Upf2, a nonsense-mediated mrna decay factor, is
981 required for prepubertal sertoli cell development and male fertility by ensuring fidelity of
982 the transcriptome. *Development.* 2015;142(2):352-62.

983 63. Kasimanickam V, Kumar N, Kasimanickam R. Investigation of sperm and seminal plasma
984 candidate micrnas of bulls with differing fertility and in silico prediction of mirna-mrna
985 interaction network of reproductive function. *Animals (Basel).* 2022;12(18).

986 64. Li L, Chen S. Screening, identification and interaction analysis of key micrnas and genes
987 in asthenozoospermia. *Int J Med Sci.* 2021;18(7):1670-79.

988 65. Madison-Villar MJ, Michalak P. Misexpression of testicular micrna in sterile xenopus
989 hybrids points to tetrapod-specific micrnas associated with male fertility. *J Mol Evol.*
990 2011;73(5-6):316-24.

991 66. Lian C, Sun B, Niu S, Yang R, Liu B, Lu C, et al. A comparative profile of the micrna
992 transcriptome in immature and mature porcine testes using solexa deep sequencing. *Febs*
993 *j.* 2012;279(6):964-75.

994 67. Zhang S, Guo J, Liang M, Qi J, Wang Z, Jian X, et al. Mir-196a promotes proliferation and
995 inhibits apoptosis of immature porcine sertoli cells. *DNA Cell Biol.* 2019;38(1):41-48.

996 68. Tang X, Chen Y, Luo H, Bian Q, Weng B, Yang A, et al. Mir-126 controls the apoptosis
997 and proliferation of immature porcine sertoli cells by targeting the *pik3r2* gene through the
998 *pi3k/akt* signaling pathway. *Animals (Basel).* 2021;11(8).

999 69. Dabaja AA, Mielnik A, Robinson BD, Wosnitzer MS, Schlegel PN, Paduch DA. Possible
1000 germ cell-sertoli cell interactions are critical for establishing appropriate expression levels

for the sertoli cell-specific microrna, mir-202-5p, in human testis. *Basic Clin Androl.* 2015;25:2.

70. Ma J, Chen Q, Wang S, Ma R, Jing J, Yang Y, et al. Mitochondria-related mir-574 reduces sperm atp by targeting nd5 in aging males. *Aging (Albany NY).* 2020;12(9):8321-38.

71. Tang D, Huang Y, Liu W, Zhang X. Up-regulation of microrna-210 is associated with spermatogenesis by targeting igf2 in male infertility. *Med Sci Monit.* 2016;22:2905-10.

72. Xu YW, Ou NJ, Song YX, Wang XH, Kang JQ, Yang YJ, et al. Seminal plasma mir-210-3p induces spermatogenic cell apoptosis by activating caspase-3 in patients with varicocele. *Asian J Androl.* 2020;22(5):513-18.

73. Lim YS, Tang BL. The evi5 family in cellular physiology and pathology. *FEBS Lett.* 2013;587(12):1703-10.

74. D'Amours O, Calvo É, Bourassa S, Vincent P, Blondin P, Sullivan R. Proteomic markers of low and high fertility bovine spermatozoa separated by percoll gradient. *Molecular Reproduction and Development.* 2019;86(8):999-1012.

75. Zhao Y, Li Q, Yao C, Wang Z, Zhou Y, Wang Y, et al. Characterization and quantification of mrna transcripts in ejaculated spermatozoa of fertile men by serial analysis of gene expression. *Human Reproduction.* 2006;21(6):1583-90.

1018 Additional Files

1019 Table S1: Gene biotype distribution in boar sperm transcriptome. The table shows the relative abundance of different
 1020 RNA biotypes among 15,637 genes detected by total RNA sequencing after quality filtering (≥ 10 reads in ≥ 6 samples).

Biotype	Count	Percentage
protein_coding	13580	86.91
lncRNA	1807	11.56
snoRNA	96	0.61
psuedogene	51	0.33
snRNA	36	0.23
scaRNA	16	0.1
miRNA	8	0.05
misc_RNA	6	0.04
processed_pseudogene	6	0.04
unitary_pseudogene	4	0.03
Mt_tRNA	3	0.02
Y_RNA	3	0.02
Mt_rRNA	2	0.01
ribozyme	2	0.01
rRNA	2	0.01
transcribed_unprocessed_pseudogene	1	0.01
translated_unprocessed_pseudogene	1	0.01
vault_RNA	1	0.01

1021 Table S2: Complete list of significantly expressed genes in boar sperm at 10 months versus 7 months age (adj $P <$
 1022 0.05). The table includes Ensembl gene ID (ensgene), Ensembl gene symbol (external_gene_name), gene biotype,

1023 base mean normalized values (baseMean), log₂ fold change (log₂FC), standard error of the fold change (lfcSE), Wald
1024 test statistics (stat), raw *P*-value, adjusted *P*-value (adj *P*-value), Entrez gene ID, chromosome name, strand, gene
1025 description.

1026 Excel file is provided.

1027 Table S3: Complete list of differentially expressed genes in boar sperm at 10 months versus 7 months age (adj *P* <
1028 0.05 & |log₂FC| ≥ 0.5). The table includes Ensembl gene ID, Ensembl gene symbol, gene biotype, base mean
1029 normalized values (baseMean), log₂ fold change (log₂FC), standard error of the fold change (lfcSE), Wald test statistics
1030 (stat), raw *P*-value and adjusted *P*-value (adj *P*-value).

1031 Excel file is provided.

1032 Table S4: Gene Ontology (GO) enrichment analysis of differentially expressed genes in boar sperm at 10 months
1033 versus 7 months age. The table lists the ontology category (biological process (BP), molecular function (MF)), GO
1034 ID, term description, the ratio of target genes versus total targets (GeneRatio), the background ratio (BgRatio), the
1035 enrichment factor (RichFactor), fold enrichment, z-score, raw p-value, Benjamini-Hochberg adjusted *P*-value (adj *P*),
1036 q-value, NCBI (Entrez) gene IDs, and the count of genes in the term.

1037 Excel file is provided.

1038 Table S5: Target genes for differentially expressed miRNAs in boar sperm at 10 months versus 7 months age. Tables
1039 include miRNAs, the number of target genes (Count) and the Ensembl gene IDs of those targets.

1040 Excel file is provided.

1041 Table S6: Gene ontology (GO) enrichment analysis of target genes for differentially expressed miRNAs in boar sperm
1042 at 10 months versus 7 months age. The table lists the ontology category , GO ID, term description, the ratio of target
1043 genes versus total targets (GeneRatio), the background ratio (BgRatio), the enrichment factor (RichFactor), fold
1044 enrichment, z-score, raw p-value, Benjamini-Hochberg adjusted *P*-value (adj *P*), q-value, matching ensembl gene
1045 symbols and the count of target genes in the term, and ontology category (biological process (BP), molecular function
1046 (MF), cellular component (CC)).

1047 Excel file is provided.



In modern pig breeding, artificial insemination is essential for improving genetics. Early semen collection from boars can lower costs and speed up genetic progress, but there's no precise way to determine sexual maturity in young boars. Standard tests like sperm count, concentration, motility, and morphology don't capture deeper biological changes. This research aimed to map the molecular and functional changes in boar semen during maturation.

We studied the same boars over several months, collecting semen at three time points: around 7 months of age, then 6 and 12 weeks after the first collection. This longitudinal approach captured genuine maturation changes while controlling for genetic differences. We used metabolomics to identify metabolite changes, transcriptomics to understand molecular mechanisms, and functional tests to assess sperm quality.

Our research identified key metabolites in sperm, such as glutamate, alanine, aspartate, choline and taurine that changed significantly with age and could serve as potential biomarkers. We found that as boars mature, there were notable improvements in sperm motility, viability, DNA integrity, and storage tolerance. These functional gains were directly linked to specific metabolites. Transcriptomic analysis revealed changes in several genes and miRNAs in expression related with sperm development.

This study is the first comprehensive longitudinal examination of sexual maturation in boars, laying a foundation for developing molecular biomarkers that could help determine optimal breeding readiness, which may reduce costs and accelerating genetic progress in pig breeding programs.