Asian Journal of Agriculture and Biology
https://doi.org/10.35495/ajab.2025.174

Original Article

Development and proteomic assessment of a freeze-dried albumin gradient technique for sperm sexing in Bali bull

Muhammad Yusuf[1*], Sigit Bintara[2], Widjiati Widjiati [3], Athhar Manabi Diansyah[1], Sahiruddin Sahiruddin[1], Masturi Masturi [1], Kurniawan Dwi Prihantoko[2], Aeni Nurlatifah[2], Suryo Kuncorojakti[3], Suhernik Susilowati[3], Tulus Maulana[4], Syahruddin Said[4]

[1]Faculty of Animal Science, Hasanuddin University, Jl. Perintis Kemerdekaan Km. 10 Tamalanrea Makassar, South Sulawesi, Indonesia

[2]Faculty of Animal Science, Gadjah Mada University, Depok, Sleman Regency, Special Region of Yogyakarta, Indonesia

[3]Faculty of Veterinary Medicine, Airlangga University, Campus C, Mulyorejo St., Surabaya, East Java, Indonesia

[4]Research Center for Applied Zoology, National Research and Innovation Agency, Jl. Raya Bogor Km. 46, Cibinong, West Java, Indonesia

*****Corresponding author's email:* myusuf@unhas.ac.id

Received: 06 August 2025 / Revised: 11 October 2025 / Accepted: 14 October 2025 / Published Online: 18 November 2025

Abstract

Reproductive efficiency in livestock can be enhanced through sperm sexing technologies; however, conventional methods such as flow cytometry are expensive, technically demanding, and impractical for field use. Simpler, affordable, and biologically validated alternatives are crucial, especially for indigenous breeds like Bali cattle. This study evaluated the effectiveness of a freeze-dried albumin (egg white) gradient (10%–30%) method for separating X- and Y-bearing spermatozoa in Bali bulls, alongside proteomic profiling to confirm the biological distinctiveness of each fraction. Semen from three mature Bali bulls (*Bos javanicus*) (n = 3; 5 ejaculates per bull; total 15 ejaculates) underwent separation via the freeze-dried albumin gradient. Sperm quality and kinematic parameters were analyzed using Computer-Assisted Sperm Analysis (CASA), while morphometric assessment estimated sexed sperm proportions. Proteomic analysis using liquid chromatography–tandem mass spectrometry (LC-MS/MS) was followed by gene ontology enrichment, hierarchical clustering, and Pearson correlation, with identified proteins validated against literature. The method enriched X-spermatozoa in the upper layer (69.67%) and Y-spermatozoa in the lower layer (73.50%), with significant differences $p < 0.05$ in motility, viability, and membrane integrity. Proteomics identified 418 proteins, including 45 unique to X-sperm and 159 unique to Y-sperm. GO enrichment linked X-sperm proteins to nuclear and structural roles, while Y-sperm proteins were associated with mitochondrial and motility processes. Clustering distinctly separated the two sperm types, and several proteins correlated strongly with functional traits. Literature validation confirmed sex-specific markers. This method represents a low-cost, biologically validated alternative for practical sperm sexing in Bali cattle, combining sperm quality assessment with proteomics to support sex-preferential breeding

Keywords: Bali bull, Bos javanicus, Sperm sexing, Albumin gradient, Proteomics, Sex-specific proteins

How to cite this article:

Yusuf M, Bintara S, Widjiati W, Diansyah AM, Sahiruddin S, Masturi M, Prihantoko KD, Nurlatifah A, Kuncorojakti S, Susilowati S, Maulana T and Said S. Development and proteomic assessment of a freeze-dried albumin gradient technique for sperm sexing in Bali bull. Asian J. Agric. Biol. 2026: e2025174. DOI: https://doi.org/10.35495/ajab.2025.174

Introduction

Reproductive efficiency is a key driver of livestock productivity and genetic progress, particularly in local breeds such as Bali cattle (Bos javanicus), which serve as an integral component of Indonesia's national breeding programs. However, reproductive outcomes in these indigenous breeds remain suboptimal, with conception rates a frequently below benchmark targets of 50–60% per artificial insemination (AI) (Said et al., 2024; Hasrin et al., 2022). One of the underlying challenges is the lack of effective sex-preselection methods, which limits the strategic optimization of herd structure and production goals.

Sperm sexing has emerged as a solution to this issue, allowing for the preselection of X- or Y-bearing spermatozoa to produce the offspring of the preferred sex. Flow cytometry, which sorts sperm based on DNA content differences between X and Y chromosomes, is the most widely used technique and has been shown to achieve high accuracy in various livestock species (Maulana et al., 2022). In reality, the technique has not become routine. The reasons are quite practical: it costs a lot to run, depends on advanced laboratory tools that are not always available, and can even harm the sperm when lasers or chemical stains are used (Diansyah et al., 2020). Because of these drawbacks, many in the field have started to look for alternatives that are simpler, less invasive, and, most importantly, affordable enough to be applied under farm conditions in developing countries.

Albumin-based gradient separation methods offer a potentially viable alternative. These techniques exploit subtle differences in sperm surface charge, mass, or sedimentation properties to achieve partial separation of X and Y spermatozoa without chemical staining. Previous studies have reported moderate success with fresh albumin gradients (Afriani et al., 2022; Sahiruddin et al., 2021); however, their consistency, scalability, and molecular validation remain areas of concern. Recently, freeze-dried albumin has been introduced as a more stable and storable alternative with improved separation efficiency (Rahmat et al., 2024), yet comprehensive studies validating its molecular outcomes—particularly regarding proteomic profiling of sexed spermatozoa, are still lacking.

Proteomic profiling has the potential to elucidate the functional and biological characteristics that differentiate X- and Y-bearing spermatozoa. Several studies using advanced proteomics approaches have revealed significant differences in protein composition between X and Y sperm fractions, which are believed to underlie differences in motility, fertilization potential, and oxidative stress resistance (Quelhas et al., 2021; Pozdyshev et al., 2023). Despite these advances, most proteomic studies to date have relied on flow cytometry–sorted spermatozoa, with limited exploration of alternative sexing methods such as albumin gradients. Moreover, the integration of proteomic findings with sperm quality and kinematic characteristics remains rare, particularly in native cattle breeds like Bali cattle.

Given these gaps, this study was designed to investigate the proteomic landscape of X- and Y-bearing spermatozoa separated using the freeze-dried albumin gradient method in Bali cattle. By combining sperm quality evaluation, kinematic analysis, high-resolution liquid chromatography–tandem mass spectrometry (LC-MS/MS) profiling, gene ontology enrichment, and literature validation, this research aims to characterize the biological differences between sexed sperm fractions and assess the effectiveness of a field-applicable sexing method. The results are expected to enhance the biological foundation for developing accurate, low-cost sexing technologies and inform future reproductive strategies in local cattle populations.

Material and Methods

Experimental design

This study used semen from three healthy, sexually mature (bulls' age range in years) Bali bulls (Bos javanicus) kept at the Faculty of Animal Science, Hasanuddin University. Five replicates of ejaculate were collected from each bull using an artificial vagina. Sperm sexing was performed using a freeze-dried albumin gradient to separate X- and Y-bearing spermatozoa. Sample collection and sexing were conducted at the Laboratory of Animal Reproduction, Faculty of Animal Science, Universitas Hasanuddin, while proteomic analysis of the sorted sperm was carried out at the Laboratory of Genomics, National Research and Innovation Agency (BRIN).

Spermatozoa sexing using freeze-dried albumin gradient

The separation of X- and Y-chromosome-bearing spermatozoa was performed following the protocol of

Diansyah et al. (2025a). Fresh semen samples collected from Bali bulls were first evaluated macroscopically Specify which parameters. Egg albumin was manually separated from the yolk under sterile conditions, frozen at −20°C, and subsequently lyophilized using a freeze dryer at alternating temperatures of 45°C and −10°C under 25 Pa using an alternating shelf-temperature schedule: 4 h at 45 °C (primary drying) followed by 2h at −10 °C (re-freeze stabilization), repeated for 8 cycles (total 48 h); temperature ramp rate 1 °C/min between phases, with 4 mm sample depth in sterile Petri dishes. The resulting freeze-dried albumin powder was stored in sterile containers mention temperature or duration of storage. To prepare the sexing medium, the lyophilized albumin was reconstituted in sterile distilled water and mixed with semen extender to obtain 10% and 30% (v/v) concentrations, representing the upper and lower layers of the gradient, respectively. Each layer was placed into sterile test tubes, and 1 mL of diluted semen was carefully layered on top. Tubes were incubated at room temperature (~25°C) for 30 minutes (Diansyah et al., 2020). to facilitate differential sedimentation. Afterwards, 2 mL of sperm suspension were aspirated from each gradient layer and centrifuged at at 700 × g (2,500 rpm; r= 10 cm) for 15 minutes. Pellets were resuspended in extender, and semen quality parameters specifying which semen quality parameters were assessed. Based on sedimentation profile, sperm fractions were classified as putative X- or Y-bearing.

Semen quality analysis

Motility and kinematics: Sperm motility and kinematics were evaluated using the SpermVision® computer-assisted semen analysis (CASA) system (Minitube, Germany) integrated with a phase-contrast microscope (Carl Zeiss) and a thermostatically controlled stage at 38°C. A 5–10 µL semen sample was placed on a pre-warmed slide with a cover slip. At least 200 sperm cells were analysed across five fields (Nirmala et al., 2025).

Sperm concentration: Concentration was determined using a photometer (SDM 6, Minitube, Germany) following Alfian et al. (2025) according to manufacturer's protocol. Semen was diluted in 0.9% NaCl solution and measured according to manufacturer instructions.

Viability and morphology: 10 µL of semen was mixed with 2% eosin-nigrosin??, smeared on a slide, and examined at 400× magnification under a Primo Star microscope (Zeiss). Red-stained sperm were considered non-viable. Morphological abnormalities were recorded from 200 sperm cells per sample.

Membrane and acrosome integrity: HOST was conducted using hypo-osmotic solution (osmolarity value) (0.179 g NaCl/100 mL water), incubated at 37°C for 30 min. Sperm with coiled tails indicated intact membranes. Acrosome integrity was assessed using formol-saline (1:4), with intact acrosomes displaying dark apical staining (Diansyah et al., 2021).

Determination of X and Y spermatozoa proportions

The proportion of X and Y spermatozoa was estimated based on sperm head morphometry. Smear preparations of fresh semen were analysed under a trinocular microscope (AxioCam Erc 5s, Zeiss, Germany). Head size measurements followed the method of Rahmat et al. (2024), with classification criteria as follows: X-spermatozoa = head size ≥ mean + SE; Y-spermatozoa = head size ≤ mean − SE; and uncategorised if within the SE range. We acknowledge that morphology-based inference can misclassify individual spermatozoa due to overlapping head-size distributions; therefore, confirmatory molecular assays such as fluorescence in situ hybridization (FISH) or qPCR targeting sex-chromosome markers are recommended where feasible, and enrichment estimates are interpreted accordingly.

Protein profiling and LC-MS/MS analysis

Proteomic analysis of sexed spermatozoa was carried out using one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS-PAGE). Total protein concentration was determined via the Bradford assay, using bovine serum albumin (BSA) as the reference standard. Protein separation was performed on polyacrylamide gels consisting of 12% resolving and 4% stacking components, run at 60 V and 20 mA for 3.5 hours. Separated proteins were visualised by staining with Coomassie Brilliant Blue. Band size estimations were based on comparison with a molecular weight marker (ExcelBand PM2700, SMOBIO) as described by Diansyah et al. (2022a). Target protein bands were excised from the gel and subjected to destaining using a solution of ammonium bicarbonate and acetonitrile (ACN). Proteins were then reduced with TCEP and alkylated using iodoacetamide (IAA) include concentrations and

incubation times). Dehydration was conducted using 100% ACN, followed by enzymatic digestion with trypsin (10 ng/μL) at 37°C for 4 hours. Peptide purification was achieved using Pierce C18 Spin Columns, with elution performed using 70% ACN. The eluates were dried and prepared for LC-MS/MS (Parrilla et al., 2024; Zoca et al., 2022). The dried peptides were reconstituted in 2% ACN containing 0.1% formic acid and analysed using a Nano LC Ultimate 3000 system coupled to a Q Exactive Plus Orbitrap mass spectrometer (Thermo Scientific). Chromatographic separation was carried out on a C18 trap and analytical column at a flow rate of 300 nL/min using a multi-step gradient of solvent B (0.1% formic acid in ACN). Mass spectral acquisition was conducted over a mass-to-charge (m/z) range of up to 2000, with trypsin used as the protease and standard variable modifications considered, including N-terminal acetylation, methionine oxidation, and cysteine carbamidomethylation (Diansyah et al., 2025b).

Statistical analysis and bioinformatics

Semen quality parameters and sperm kinematic data were statistically analysed using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). Data were tested for normality and homogeneity using the Shapiro–Wilk and Levene's tests, respectively. Differences between X- and Y-bearing spermatozoa were assessed using independent samples t-tests, and results were expressed as mean ± standard deviation (SD). A significance level of $p < 0.05$ was used throughout. For proteomic analysis, protein identification was performed using Proteome Discoverer 2.2 (Thermo Scientific) against a curated bovine database comprising the UniProt reference proteome for *Bos taurus* supplemented with UniProtKB TrEMBL entries annotated as *Bos indicus* × *Bos taurus* (release 2025_03). This choice maximizes sequence coverage and annotation quality while remaining highly orthologous to *Bos javanicus*. Peptide–spectrum matches and proteins were controlled at FDR < 1% (target–decoy), and proteins were retained only if supported by ≥ 2 unique peptides. Identified proteins were functionally annotated using Gene Ontology (GO) classifications, encompassing Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) categories. Functional enrichment and pathway analyses were performed using the PANTHER Classification System(http://www.pantherdb.org) and the STRING database (version 12.0; https://string-db.org), with a confidence interaction score threshold of 0.7 (high confidence). GO terms and enriched pathways with $p < 0.05$ were considered statistically significant. Heatmap clustering and Pearson correlation analyses between sperm quality and kinematic parameters, X/Y spermatozoa proportions, and protein abundance were performed using Chiplot (https://www.chiplot.online). Z-score normalization was applied to abundance values prior to visualization.

Results

Effectiveness of sperm separation using freeze-dried albumin gradient

Table 1 presents the comparison of semen quality parameters between X- and Y-bearing spermatozoa obtained using the freeze-dried albumin gradient method. Statistical analysis showed significant differences ($p < 0.05$) in motility, viability, membrane integrity, and acrosome integrity between the two groups. No significant difference was observed in abnormality percentage.

Table 2 summarizes the kinematic parameters of X- and Y-bearing spermatozoa following separation using the freeze-dried albumin gradient. Significant differences ($p < 0.05$) were observed in DCL, DSL, VSL, LIN, and STR values between the two sperm populations. No significant differences were found in DAP, VCL, VAP, or WOB. Superscript letters indicate statistically significant differences within rows.

The separation of spermatozoa using the freeze-dried albumin gradient resulted in distinct distributions across the gradient layers. The upper fraction was enriched with X-bearing spermatozoa, while the lower layer predominantly contained Y-bearing spermatozoa. In contrast, the composition of the unprocessed fresh semen showed a nearly balanced proportion between X and Y sperm. The percentage of unclassified cells remained minimal across all fractions, indicating efficient separation and classification following the gradient procedure.

Table-1. Semen Quality of Separated X and Y Spermatozoa Using Freeze-Dried Albumin Gradient.

Parameter	Sexed Sperm (Mean ± SD)	
	X-Sperm	Y-Sperm
Motility (%)	76.31±1.65[a]	81.65±1.79[b]
Viability (%)	93.23±1[a]	89.58±1.41[b]
Abnormality (%)	6.09±0.88	6.13±0.89
Membrane Integrity (%)	81.76±1.4[b]	85.43±1.63[a]
Acrosome Integrity (%)	88.7±1.41[a]	84.89±0.86[b]

Note: n = 15; test = independent-samples t-test; α = 0.05. Values with different superscript letters (a, b) within the same row indicate a statistically significant difference between X- and Y-bearing spermatozoa ($p < 0.05$).

Table-2. Semen kinematic of separated X and Y spermatozoa using freeze-dried albumin gradient.

Parameter	Sexed Sperm (Mean ± SD)	
	X-Sperm	Y-Sperm
DCL (μm)	48.83±1.89[b]	57.96±1.91[a]
DAP (μm)	24.47±4.78	27.64±3.14
DSL (μm)	15.51±1.31[b]	18.39±1.43[a]
VCL (μm/s)	107.33±28.24	132.93±15.09
VAP (μm/s)	59.82±11.4	67.56±5.07
VSL (μm/s)	39.88±1.65[b]	43.22±1.78[a]
LIN (%)	57.45±9.12[a]	35.36±3.24[b]
STR (%)	80.18±0.08[a]	66.22±6.93[b]
WOB (%)	61.28±12.15	52.35±6.19

Note: n = 15; test = independent-samples t-test; α = 0.05. Values with different superscript letters (a, b) within the same row indicate a statistically significant difference between X- and Y-bearing spermatozoa ($p < 0.05$).

Table-3. Proportion of X- and Y-Bearing Spermatozoa Across Albumin Gradient Fractions.

Layer	X-Sperm (%)	Y-Sperm (%)	Unclassified (%)
Fresh Semen	47.67±1.89	49.83±0.58	2.50±1.32
Upper	69.67±3.82	28.50±3.12	1.83±0.76
Lower	24.17±4.34	73.50±4.29	2.33±1,25

Proteomic profiling of sexed spermatozoa

Figure 1 illustrates the distribution of identified proteins between X- and Y-bearing spermatozoa following separation using the freeze-dried albumin gradient method. A total of 418 proteins were identified, of which 214 proteins (51.2%) were shared by both X- and Y-sperm. Additionally, 45 proteins (10.8%) were uniquely found in X-sperm, while 159 proteins (38.0%) were unique to Y-sperm.

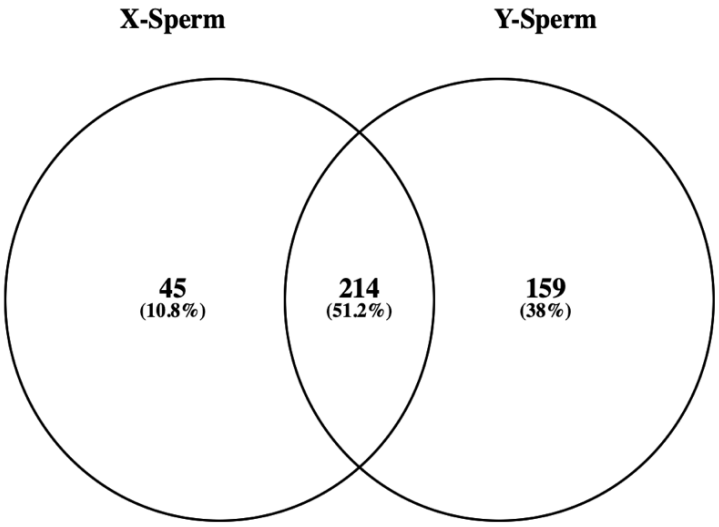

Figure-1. Venn diagram of unique and shared proteins in sexed spermatozoa separated by a freeze-dried albumin gradient. UniProt Bos taurus + TrEMBL Bos indicus × Bos taurus (rel. 2025_03); criteria: protein FDR < 1%, ≥ 2 unique peptides; Numbers denote protein counts (X-unique = 45, Y-unique = 159, shared = 214).

Gene ontology (GO) functional enrichment

Gene Ontology enrichment analysis was performed separately for the unique proteins identified in X- and Y-bearing spermatozoa to determine their associated biological processes, molecular functions, and cellular components. The results are summarized in Figure 2. In X-bearing spermatozoa, unique proteins were enriched in biological processes such as *metabolic-based process*, *mitochondrial cristae organization*, *cell cycle*, and *cytoskeleton organization*. Molecular function analysis indicated significant enrichment in *structural constituent of cytoskeleton*, *ATP binding*, *purine ribonucleotide binding*, and *RNA binding*. Cellular component analysis revealed dominant localization in *mitochondria*, *supramolecular complex*, *nucleoplasm*, and *sperm fibrous sheath*, suggesting a role in structural stability and intracellular regulation. In contrast, Y-bearing spermatozoa showed enrichment in biological processes including *microtubule-based processes*, *motile cilium organization*, *pyruvate metabolic process*, *oxidative phosphorylation*, and *organelle organization*. Molecular functions of Y-sperm proteins were associated with *oxidoreductase activity*, *GTP binding*, *structural molecule activity*, and *cytoskeletal protein binding*. Cellular components were predominantly linked to *mitochondria*, *axoneme*, *sperm tail*, *sperm principal piece*, and *inner mitochondrial membrane*, reflecting the energy-demanding functions related to motility. These results suggest that X-sperm protein functions are more closely related to nuclear, metabolic, and structural roles, while Y-sperm proteins are more involved in energy metabolism and motility-related functions, consistent with the physiological differences observed between the two sperm types.

https://doi.org/10.35495/ajab.2025.174

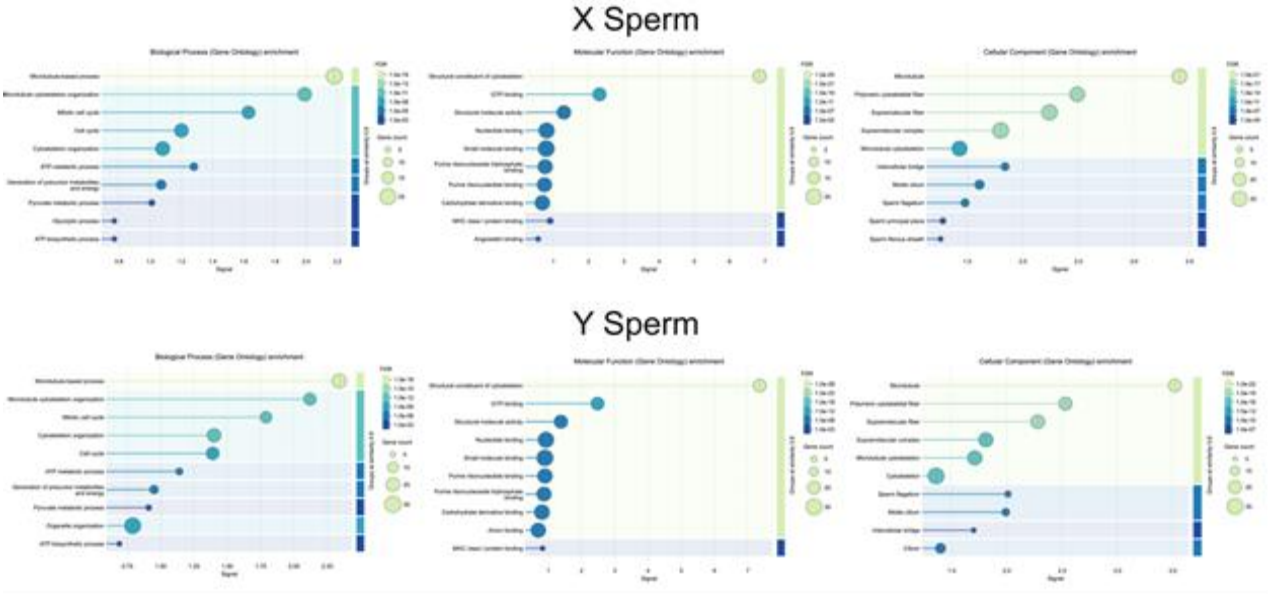

Figure-2. Gene Ontology (GO) Functional Enrichment of Sexed Spermatozoa.

Unique proteins identified in sexed spermatozoa

Table 4 lists the proteins uniquely identified in X-bearing spermatozoa following separation using the freeze-dried albumin gradient method. Five proteins were found to be exclusive or dominantly expressed in X-sperm, including SPA17, FXR1, ZPBP, PARP6, and PKM. These proteins are associated with biological functions such as zona pellucida binding, ATP metabolism, and spermatid development.

Table 5 presents the list of proteins uniquely identified in Y-bearing spermatozoa following separation using the freeze-dried albumin gradient method. A total of fifteen proteins were found to be exclusive or dominantly expressed in the Y-sperm fraction. These include Tektin-5 (TEKT5), IZUMO family member 4 (IZUMO4), Interaction protein for cytohesin exchange factors 1 (IPCEF1), E3 ubiquitin-protein ligase (UBR1), Glutamine--fructose-6-phosphate transaminase (GFPT2), cAMP-dependent protein kinase type II-alpha regulatory subunit (PRKAR2A), Actin related protein T2 (ACTRT2), Acrosomal vesicle protein 1 (ACRV1), Peroxiredoxin-5 (PRDX5), Asparaginase-like protein 1 (ASRGL1), Malate dehydrogenase (MDH2), Voltage-dependent anion-selective channel protein 2 (VDAC2), Aconitate hydratase (ACO2), Triosephosphate isomerase (TPI1). And cytochrome c oxidase subunit 5a (COX5A).

Table-4. Unique Proteins Identified in X-Bearing Spermatozoa.

Accession	Protein	ID Gene	MW (kDa)	Function
A0A4W2D7R3	Pyruvate kinase	PARP6	64.2	Pyruvate kinase activity
Q3SYS3	Sperm surface protein Sp17	SPA17	16.9	Binding of sperm to zona pellucida
Q2TBT7	RNA-binding protein FXR1	FXR1	69.7	Spermatid development
A0AAA9RZ12	Zona pellucida binding protein	ZPBP	36.7	Binding of sperm to zona pellucida
A0A4W2ILV6	Pyruvate kinase	PKM	71.8	ATP Binding

Note: UniProt Bos taurus + TrEMBL Bos indicus × Bos taurus (rel. 2025_03); criteria: protein FDR < 1%, ≥ 2 unique peptides.

Table-5. Unique Proteins Identified in Y-Bearing Spermatozoa.

Accession	Protein	ID Gene	MW (kDa)	Function
A0A3Q1MR16	Tektin-5	TEKT5	46.7	Flagellated sperm motility
A0A8B9WCP3	IZUMO family member 4	IZUMO4	27.4	Sperm-egg penetration
A0A3Q1MN04	Interaction protein for cytohesin exchange factors 1	IPCEF1	52.9	Cytohesin Activity
A0AAA9SPQ7	E3 ubiquitin-protein ligase	UBR1	187.4	Zinc ion binding
A0A3Q1MD36	glutamine--fructose-6-phosphate transaminase	GFPT2	77.7	Carbohydrate derivative binding
A0AAF6YZS2	cAMP-dependent protein kinase type II-alpha regulatory subunit	PRKAR2A	45.7	Protein domain specific binding
A0A4W2HT18	Actin related protein T2	ACTRT2	41.9	Filament actin activity
Q32KR2	Acrosomal vesicle protein 1	ACRV1	28.9	Acetylcholine receptor binding
A0A6P5B0L1	Peroxiredoxin-5	PRDX5	26.1	Hydrogen peroxide-mediated signaling
A0AAF6YQE1	Asparaginase-like protein 1	ASRGL1	40.3	L-asparaginase activity
A0AAF6YTP4	Malate dehydrogenase	MDH2	33	ATP binding
A0A4W2FV49	Non-selective voltage-gated ion channel VDAC2	VDAC2	31.6	Porin activity
A0A3Q1M6K6	Aconitate hydratase, mitochondrial	ACO2	84.7	ATP binding
Q5E956	Triosephosphate isomerase	TPI1	26.7	Gluconeogenesis
Q6QRN2	Cytochrome c oxidase subunit 5A, mitochondrial	COX5A	16.7	Flagellated sperm motility

Note: UniProt Bos taurus + TrEMBL Bos indicus × Bos taurus (rel. 2025_03); criteria: protein FDR < 1%, ≥ 2 unique peptides.

Clustering of protein expression patterns

Figure 3 shows a heatmap of hierarchical clustering based on the Z-score normalized abundance of 20 uniquely expressed proteins across X- and Y-bearing spermatozoa samples. The heatmap reveals a clear separation between X-sperm (left cluster, purple) and Y-sperm (right cluster, green), indicating distinct expression patterns of the selected proteins. Proteins highly abundant in Y-sperm samples are shown in red shades, while those more abundant in X-sperm are represented in blue-green shades. These distinct clustering patterns support the consistency and specificity of protein expression profiles associated with sperm sex.

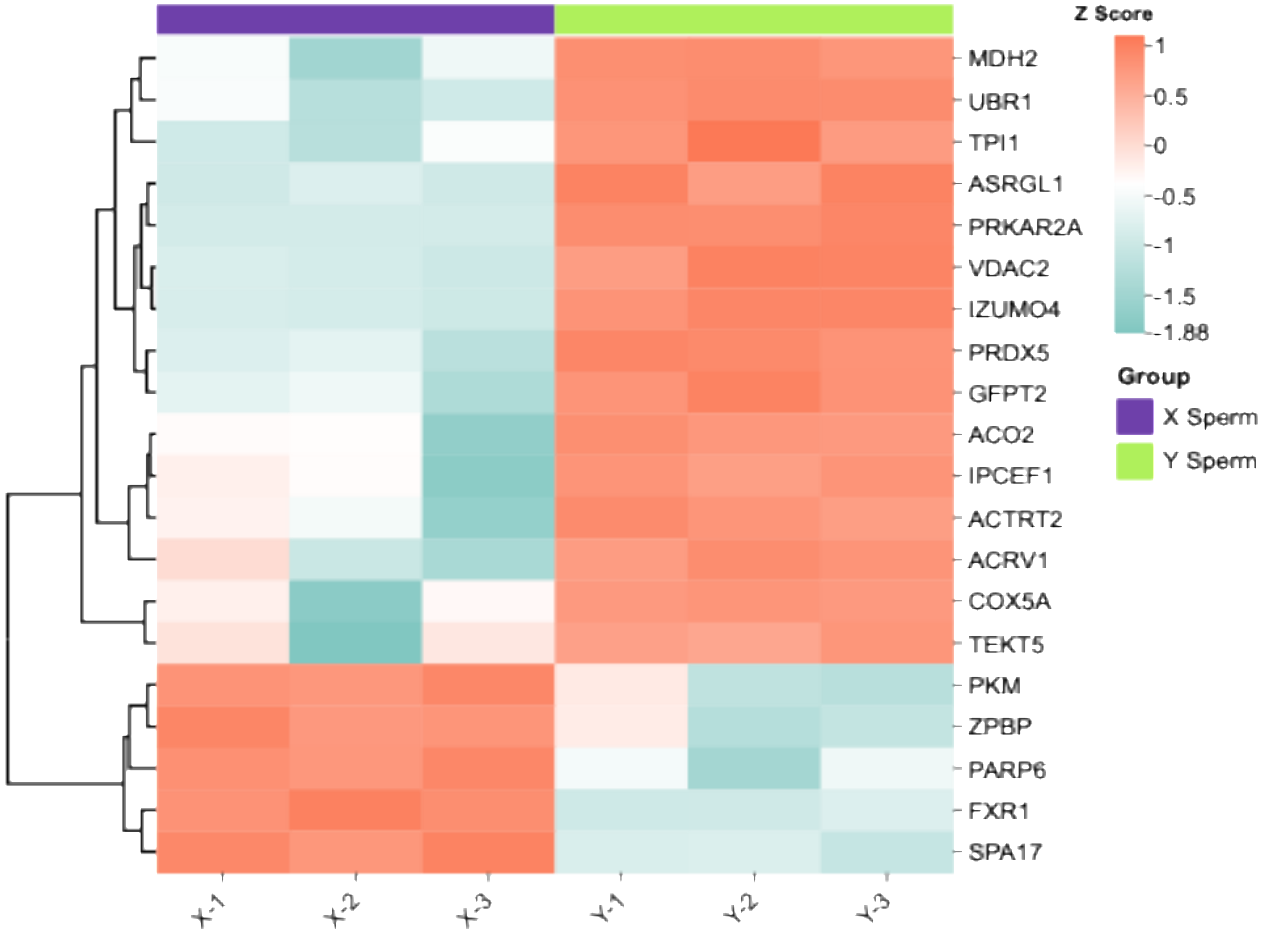

Figure-3. Heatmap Clustering of Unique Protein Expression in X- and Y-Bearing Spermatozoa.

Correlation between sperm quality, proportions and protein abundance

Figure 4 illustrates a heatmap of Pearson correlation coefficients between the expression levels of selected proteins (PARP6, SPA17, FXR1, ZPBP, PKM) and various sperm quality and kinematic parameters in X-Sperm. Notably, PARP6 displayed a strong positive correlation with VSL (r = 0.834), DCL (r = 0.678), and Membrane Integrity (r = 0.750), indicating its potential association with linear sperm velocity and membrane function. SPA17 exhibited very strong correlations with VSL (r = 0.640). FXR1 was positively correlated with STR (r = 0.991) and DSL (r = 0.990). Similarly, PKM demonstrated strong positive correlations with DCL (r = 0.797), Membrane Integrity (r = 0.855), and VSL (r = 0.918).

Figure 5 presents a heatmap of Pearson correlation coefficients between uniquely expressed proteins, sperm quality parameters, and the proportion of sexed spermatozoa in Y-Sperm. COX5A exhibited perfect correlation with STR (r = 1.000), and very high correlations with Proportion (r = 0.989), TPI1 (r = 0.989), Motility (r = 0.980), VAP (r = 0.996), and VSL (r = 0.929). TPI1 showed high correlations with COX5A (r = 0.989), Proportion (r = 0.987), and Motility (r = 0.939).

PRKAR2A was positively correlated with DAP (r = 0.929), DSL (r = 0.915), VAP (r = 0.903), Motility (r = 0.832), and Proportion (r = 0.863). TEKT5 showed positive associations with Membrane Integrity (r = 0.955), DAP (r = 0.785), and DSL (r = 0.762). IZUMO4 exhibited very high correlations with UBR1 (r = 0.998), VDAC2 (r = 0.999), VSL (r = 0.972), VCL (r = 0.918), and Proportion (r = 0.816). GFPT2 demonstrated significant correlations with Motility (r = 0.993), COX5A (r = 0.997), TPI1 (r = 0.974), and STR (r = 0.998).

https://doi.org/10.35495/ajab.2025.174

Additionally, ASRGL1 was positively correlated with COX5A (r = 1.000), STR (r = 1.000), and VSL (r = 0.998). UBR1correlated positively with VCL (r = 0.888), Proportion (r = 0.774), and DSL (r = 0.698). VDAC2 showed strong positive associations with Proportion (r = 0.787), VSL (r = 0.959), and UBR1 (r = 1.000). ACO2 was positively correlated with LIN (r = 0.929) and COX5A (r = 0.999). ACTRT2 exhibited strong correlations with LIN (r = 0.999) and WOB (r = 0.960). ACRV1 showed high correlations with UBR1 (r = 0.911), VSL (r = 0.744), and VCL (r = 0.619). MDH2 was associated positively with Motility (r = 0.964) and Membrane Integrity (r = 0.964).

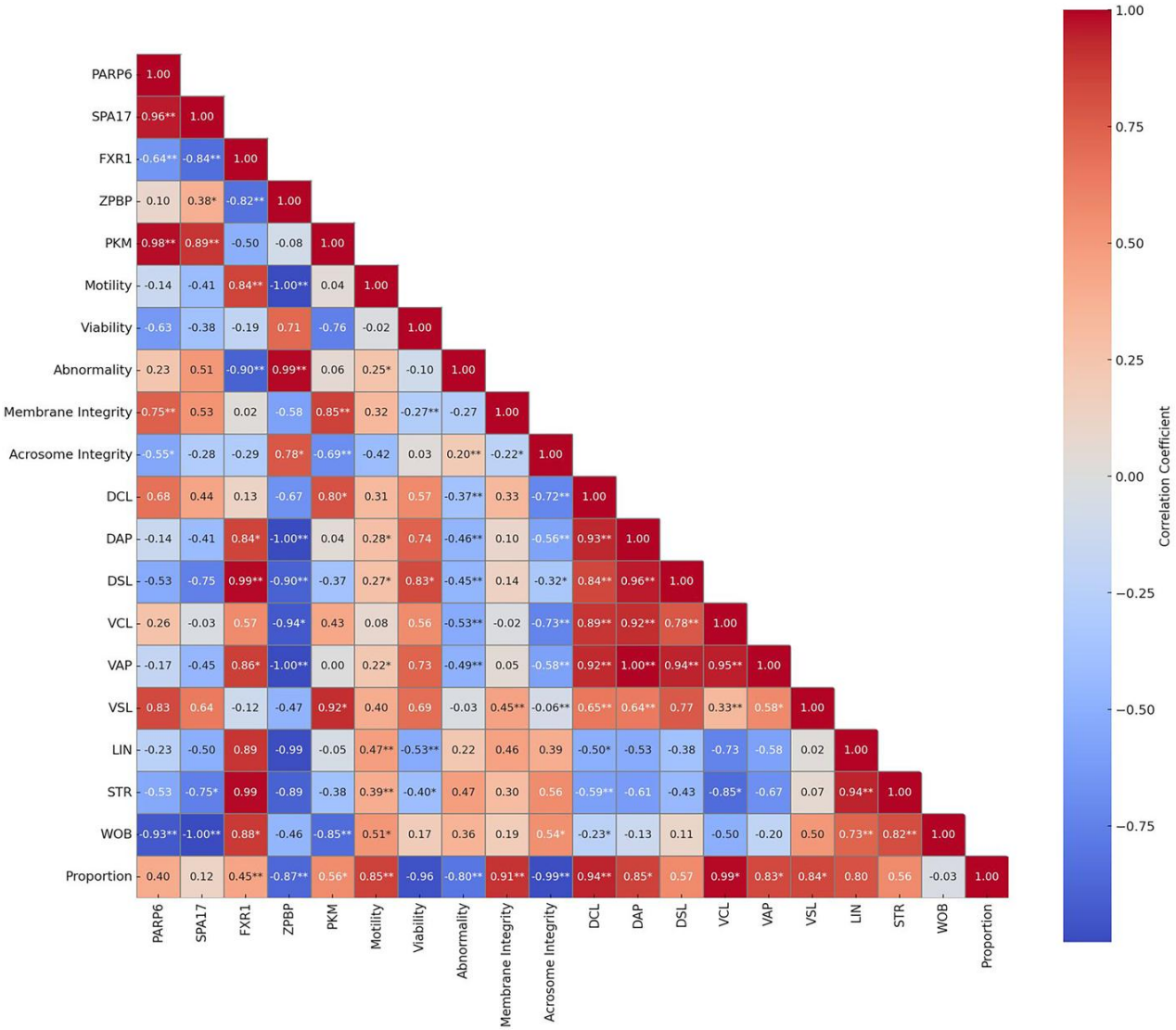

Figure-4. Heatmap of Pearson Correlation between Sperm Quality, Proportions, and Protein Abundance in X-Sperm.

https://doi.org/10.35495/ajab.2025.174

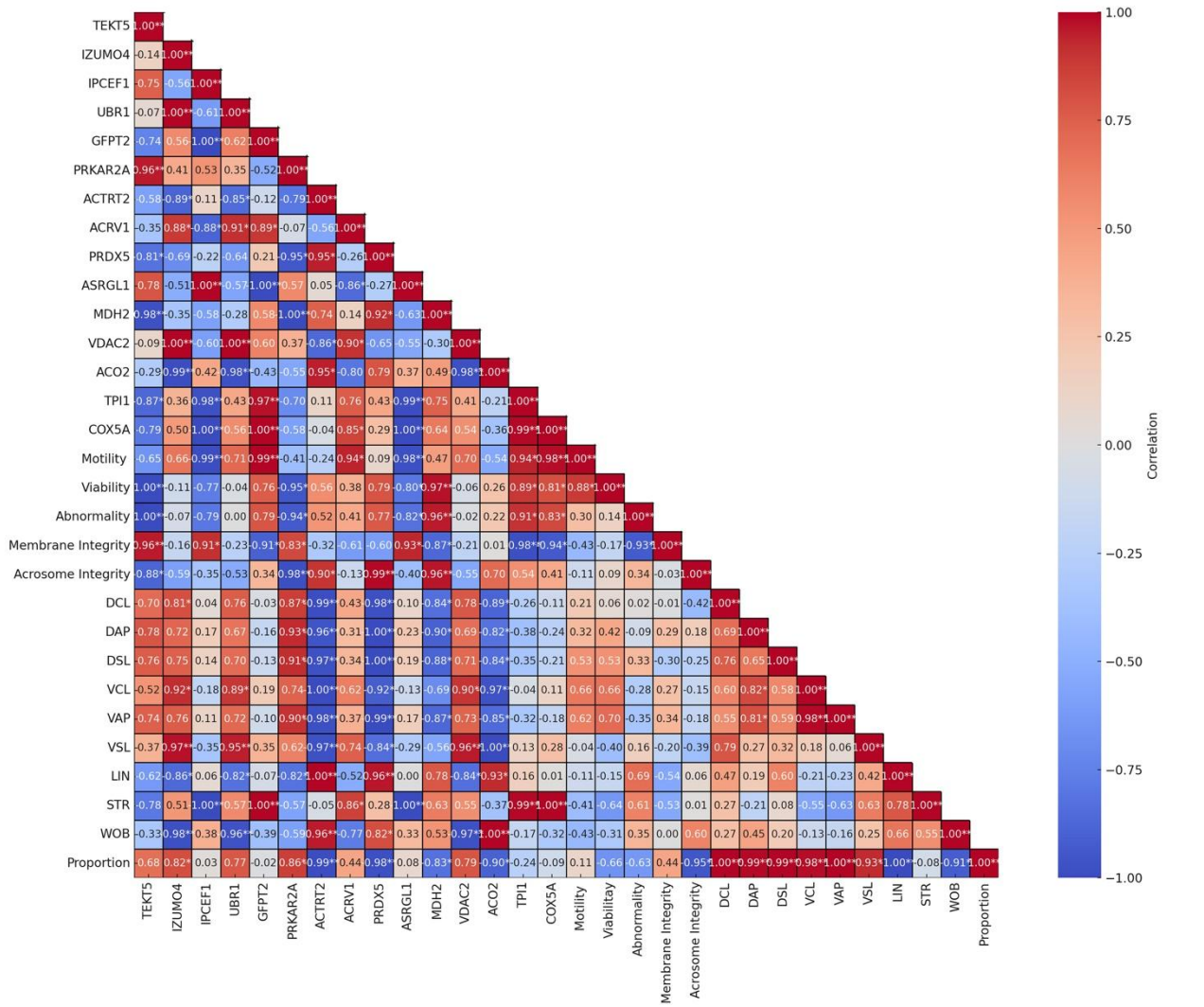

Figure-5. Heatmap of Pearson Correlation between Sperm Quality, Proportions, and Protein Abundance in Y-Sperm.

Validation of identified proteins from existing literature

Table 6 presents literature-based evidence supporting the uniqueness and functional relevance of the identified proteins in X- and Y-bearing spermatozoa. Several proteins detected in the X-sperm fraction—such as PARP6, SPA17, FXR1, ZPBP, and PKM—have been previously reported as either exclusive to X-sperm or more abundantly expressed in X-bearing spermatozoa, particularly in cattle and pigs. These proteins are associated with functions including zona pellucida binding, spermatid differentiation, and energy supply. Likewise, all proteins identified in the Y-sperm fraction—including TEKT5, IZUMO4, IPCEF1, UBR1, GFPT2, PRKAR2A, ACTRT2, ACRV1, PRDX5, ASRGL1, MDH2, VDAC2, ACO2, TPI1, and COX5A have been validated in previous proteomic studies as Y-sperm enriched or specific. These proteins play roles in sperm motility, fertilization, mitochondrial function, and oxidative stress resistance. The consistency between current findings and prior literature enhances the biological validity of the observed protein expression patterns and reinforces their potential roles as molecular markers for sperm sex differentiation.

Table-6. Literature Validation of Identified Unique Proteins in Sexed Spermatozoa.

Protein	Evidence from Literature	Reference
PARP6	Reported as a higher-abundance/exclusive protein in X-sperm in bovine proteomic studies	Said and Khosravi, 2012 (PMID: 34009990)
SPA17	Exclusive to X-sperm in cattle and pigs; plays a role in sperm–oocyte interaction	Shangguan et al., 2025 (PMID: 34009990)
FXR1	Identified in X-sperm fraction, associated with spermatid differentiation	Kang et al., 2022 (PMID: 31170143)
ZPBP	Exclusive to X-sperm in cattle in iTRAQ-based analysis	Mostek-Majewska et al., 2023 (PMID: 34009990)
PKM	More abundant in X-sperm than Y-sperm; supports energy supply	Xue et al., 2025 (PMID: 34009990)
TEKT5	Identified as Y-sperm enriched protein in bovine proteomics	Geng et al., 2024 (PMID: 34009990)
IZUMO4	Reported as Y-sperm specific in cattle; role in sperm–oocyte interaction	Lai et al., 2022 (PMID: 34009990)
IPCEF1	Identified in Y-sperm proteome; possible role in membrane trafficking	Marks et al., 2021 (PMID: 31170143)
UBR1	Found in Y-sperm; may regulate protein turnover during spermatogenesis	Xie et al., 2023 (PMID: 34009990)
GFPT2	Y-sperm enriched; influence glycoprotein modification in sperm membrane	Cavarocchi et al., 2025 (PMID: 34009990)
PRKAR2A	Enriched in Y-sperm; linked to cAMP signaling in sperm	Zhang et al., 2022 (PMID: 34009990)
ACTRT2	Reported in Y-sperm fraction; structural role in sperm head formation	Jin et al., 2024 (PMID: 34009990)
ACRV1	Y-sperm enriched; involved in zona pellucida penetration	Dai et al., 2025 (PMID: 34009990)
PRDX5	Found in Y-sperm; may contribute to oxidative stress resistance	Agnieszka et al., 2025 (PMID: 31170143)
ASRGL1	Y-sperm enriched; possible role in maintaining sperm protein integrity	Bisconti et al., 2021 (PMID: 31170143)
MDH2	Y-sperm enriched; supports energy supply for motility	Ali et al., 2023 (PMID: 34009990)
VDAC2	Found in Y-sperm; role in mitochondrial function	Ali et al., 2023 (PMID: 34009990)
ACO2	Y-sperm enriched; supports mitochondrial ATP production	Irigoyen et al., 2024 (PMID: 34009990)
TPI1	Found in Y-sperm; supports glycolytic ATP production	An et al., 2022 (PMID: 34009990)
COX5A	Y-sperm enriched; critical for mitochondrial ATP production	Guan et al., 2023 (PMID: 34009990)

Note: UniProt Bos taurus + TrEMBL Bos indicus × Bos taurus (rel. 2025_03); criteria: protein FDR < 1%, ≥ 2 unique peptides.

Discussion

This study demonstrated that spermatozoa separated using a freeze-dried albumin gradient exhibited significant differences ($P < 0.05$) in semen quality attributes, notably in motility, viability, membrane integrity, and acrosome integrity. X-bearing spermatozoa presented higher viability and acrosome integrity, while Y-bearing sperm showed enhanced motility and membrane integrity. These findings are in agreement

with previous studies that X- and Y-chromosome-bearing spermatozoa differ in structural and physiological aspects due to variations in DNA content and associated metabolic demands (Maulana et al., 2022; Paitoon et al., 2024; Rahman and Pang, 2020). The superior membrane and acrosome integrity in X-sperm may be linked to greater cytoplasmic volume and antioxidative capacity, offering structural resilience during transit (Wang et al., 2025). Conversely, the increased motility of Y-spermatozoa aligns with their lower DNA content and smaller head size, allowing more efficient propulsion (Kumar et al., 2024; El-Naby et al., 2021). These physiological characteristics are not only relevant for understanding natural fertilization dynamics but also hold implications for optimizing assisted reproductive technologies, as sperm subpopulation traits directly affect fertilization rates and embryo development (Guilherme et al., 2020; Tanga et al., 2021).

These structural and metabolic differences are further reflected in the sperm kinematic profiles, as assessed by CASA, which revealed significant variation in motion characteristics including curvilinear distance (DCL), straight-line distance (DSL), straight-line velocity (VSL), linearity (LIN), and straightness (STR). Y-bearing sperm displayed greater curvilinear and straight-line velocities, indicative of high-energy movement, while X-bearing spermatozoa exhibited superior trajectory linearity, suggesting more energy-efficient locomotion. These kinematic variations mirror intrinsic metabolic and structural specializations previously documented in bovine and porcine models (Raafi et al., 2021; Rahmat et al., 2024; Diansyah et al., 2025a). The high velocity observed in Y-sperm is often attributed to elevated mitochondrial density and enhanced oxidative phosphorylation, facilitating greater ATP availability to support flagellar activity (Guo et al., 2017) although this remains debated across species. In contrast, the linear, conservative movement of X-sperm may support prolonged survivaland functionality within the female reproductive tract (Saint-Dizier et al., 2020). These findings further support the utility of sperm kinematics as reliable biomarkers of fertilization competence in bovines (Raafi et al., 2021; Diansyah et al., 2022b).

Importantly, the freeze-dried albumin gradient technique was effective in separating sperm populations based on sex, as demonstrated by morphometric analysis. X-spermatozoa were enriched in the upper albumin layer (69.67%), while Y-spermatozoa predominated in the lower layer (73.50%). These results confirm the physical separation mechanism of the technique, which leverages differences in sedimentation velocity and membrane properties (Diansyah et al., 2025b; Rahmat et al., 2024). The use of freeze-dried albumin provides advantages over fresh gradients, including improved storage stability, better reproducibility, and greater practicality under field conditions (Hutapea et al., 2023; Silambarasan and Rajalakshmi, 2022). While flow cytometry remains the gold standard for sperm sexing in terms of accuracy, its limitations include high cost, requirement for advanced infrastructure, and potential reduction in sperm viability due to laser exposure (Afriani et al., 2022; Maulana et al., 2022). Previous implementations of albumin-based methods in goats and pigs also support the robustness and species-translatability of this approach (Pongprayoon et al., 2025), underscoring its potential role in cost-effective and scalable reproductive management strategies for cattle production systems in developing regions.

Proteomic analysis using LC-MS/MS identified a total of 418 proteins across the sexed spermatozoa, with 45 proteins uniquely expressed in X-bearing sperm and 159 proteins exclusively detected in Y-bearing spermatozoa. This proteomic divergence underscores the molecular distinction between the two sperm types, consistent with previous findings in bovine and porcine models (Gacem et al., 2023; Ashwitha et al., 2023; Fan et al., 2025). Gene Ontology (GO) enrichment analysis further revealed divergent biological specializations (Figure 2). Proteins identified in X-sperm were significantly associated with cytoskeletal organization, nucleic acid binding, and mitochondrial cristae structure, predominantly localized in the nucleoplasm, fibrous sheath, and supramolecular complexes (Wang et al., 2021; Ecovoiu et al., 2022; Hejazian et al., 2023). These enrichments suggest structural and regulatory functions that may enhance chromosomal stability and fertilization capacity. In contrast, proteins unique to Y-sperm were enriched in microtubule-based processes, oxidative stress responses, and energy metabolism, with major localization in the mitochondrial membrane, axoneme, and sperm tail. This architecture supports the high motility and metabolic demands of Y-bearing spermatozoa (Leung et al., 2022; Ureña et al., 2022; Cavarocchi et al., 2025), with specific involvement of TPI1, ACO2, and MDH2 in glycolytic and oxidative pathways (Gacem et al., 2023; Irigoyen et al., 2024).

Specific proteins in X-spermatozoa such as SPA17, FXR1, ZPBP, PARP6, and PKM (spelling out acronyms) have been implicated in zona binding, transcriptional regulation, and energy production (Rosyada et al., 2023; Sanderson and Cohen, 2020; Wang et al., 2024). While SPA17 and ZPBP are functionally important in zona

pellucida recognition, the correlation analysis highlights FXR1 as particularly significant due to its strong associations with trajectory parameters, suggesting a potential role in directing sperm motility. These proteins may collectively facilitate oocyte interaction and contribute to the structural and nuclear stability of X-spermatozoa. Conversely, Y-spermatozoa exhibited elevated expression of proteins such as TEKT5, COX5A, IZUMO4, PRKAR2A, and VDAC2, which are associated with flagellar function, oxidative phosphorylation, and sperm-oocyte fusion—reflecting a phenotype optimized for rapid and energy-intensive fertilization strategies (Lin et al., 2024; Parkes and Garcia, 2024; Mostek-Majewska et al., 2023). Hierarchical clustering using Z-score normalized abundance (Figure 3) clearly segregated X- and Y-bearing sperm populations. X-specific proteins like SPA17, ZPBP, and FXR1 grouped distinctly from Y-specific proteins including COX5A, TEKT5, and VDAC2. This supports a consistent and sex-specific proteomic signature, highlighting the effectiveness of the freeze-dried albumin gradient method in separating molecularly distinct sperm subpopulations (Amaliah et al., 2023).

Correlation analysis (Figures 4 and 5) revealed that proteins in X-sperm such as PARP6, PKM, SPA17, and FXR1 were positively associated with key motility and membrane integrity parameters. These relationships underscore the role of these proteins in maintaining functional competence, particularly for progressive motility and structural stability. Notably, FXR1 showed the strongest associations with directional parameters, suggesting its importance in orienting sperm movement consistent with previous findings that linked FXR1 to cytoskeletal regulation and spermatid development (Kang et al., 2022; Huang et al., 2023). Likewise, Y-sperm proteins including COX5A, GFPT2, PRKAR2A, and TPI1 showed strong associations with sperm velocity, trajectory, and proportion of sexed spermatozoa, reflecting their mitochondrial and glycolytic function in supporting high-energy demand. COX5A and TPI1 have previously been implicated in mitochondrial oxidative phosphorylation and glycolysis, which are essential for ATP supply during high-speed flagellar motion (Jin et al., 2022; Cai et al., 2023).

Table 6 presents a cross-validation of these uniquely identified proteins with previous high-throughput proteomic studies in livestock. All proteins—whether in X- or Y-sperm fractions—have been independently validated in bovine or porcine sperm, reinforcing the biological reliability and potential applicability of the findings (Shen et al., 2021; Wang et al., 2024).

The outcomes of this study hold significant implications for reproductive technology and livestock management. The freeze-dried albumin gradient offers a cost-effective, instrument-light alternative to flow cytometry, allowing for practical implementation in resource-limited settings such as smallholder cattle systems. This aligns with the growing interest in non-cytometric sexing methods that are accessible, scalable, and maintain sperm viability (Dhangada et al., 2024; Amaliah et al., 2023). This is particularly valuable for preferential sexed breeding programs targeting improved herd structure and productivity.

In addition to practical relevance, the study provides biological insight into sperm sex differentiation. Several proteins identified here—such as SPA17, FXR1, COX5A, and PRKAR2A represent promising biomarkers for molecular diagnostics, with potential use in assay development or fertility screening. These markers are consistent with prior research showing differential abundance in sexed sperm and their association with fertilization success and embryonic development potential (Ribeiro et al., 2025; Quelhas et al., 2021; Vallet-Buisan et al., 2023).

The integration of proteomic data with sperm quality parameters and kinematic traits presents a multi-dimensional approach to functional sperm evaluation. Such integrative frameworks have been proposed as future standards in precision livestock breeding (Guo et al., 2023) This comprehensive profiling could enhance semen selection criteria in artificial insemination programs and reduce economic inefficiencies from failed fertilizations.

Nevertheless, some limitations must be acknowledged. The lack of chromosomal validation (e.g., via FISH or PCR-based sexing) represents a critical gap in confirming sperm identity. Furthermore, functional roles of the identified proteins remain hypothetical due to the absence of experimental assays such as gene knockdown or overexpression. Expanding future research to include molecular confirmation and functional validation will clarify causal mechanisms. The small sample size (n = 3 bulls) may limit the generalizability of findings. Broader validation across diverse breeds and environments is essential to assess robustness and reproducibility. Finally, as a proteomics-only approach, the study does not capture the complexity of post-translational modifications or regulatory networks. Integrating other omics layers—such as phosphoproteomics, transcriptomics, or metabolomics could yield a more holistic understanding of sperm sex-specific physiology and function.

Conclusion

This study demonstrated that the freeze-dried albumin gradient method is an effective, low-cost, and biologically valid technique for separating X- and Y-bearing spermatozoa in Bali cattle. Significant differences in semen quality, sperm kinematics, and morphometric proportions confirmed the physical separation of sexed sperm populations, which was further supported by distinct proteomic profiles. The identification of sex-specific proteins validated through clustering analysis, gene ontology enrichment, trait correlation, and literature references highlight their potential as biomarkers for sperm sexing and functional evaluation. These findings provide a practical, field-adaptable workflow for sex-preferential breeding in Bali cattle that could be piloted in other livestock species with local validation.

Acknowledgments

The authors gratefully acknowledge the support from Indonesia Collaborative Research with Contract Number: 01319/UN4.22/PT.01.03/2025 for funding and facilitating this research.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: This research was supported by Indonesia Collaborative Research with Contract Number: 01319/UN4.22/PT.01.03/2025.

Ethical Approval Statement

Ethical approval was granted by the Ethics Committee of the Faculty of Animal Science, Universitas Hasanuddin (Approval No. 015/UN4.12/EC/VI/2025).

Contribution of Authors

Yusuf M, Bintara S, Widjiastuti W, Maulana T, Sahiruddin S, Said S & Diansyah AM: Conceived and designed the experiment and manuscript editing.

Yusuf M, Bintara S, Widjiastuti W, Diansyah AM, Sahiruddin S & Masturi M: Performed the experimental procedures and collected data.

Prihantoko KD, Nurlatifah A & Susilowati S: Supervised and coordinated the research and collected and analysed clinical data.

Diansyah AM, Maulana T & Nurlatifah A: Conducted statistical analysis.

Yusuf M, Bintara S, Diansyah AM, Said S, Maulana T, Kuncorojakti S & Nurlatifah A: Prepared the initial draft of the manuscript.

All authors read and approved final draft of the manuscript.

References

Afriani T, Udin Z, Hellyward J, Purwati E, Rastosari A and Wahyudi D, 2022. Separation of bull spermatozoa bearing x- and y-chromosome by using albumin gradient and swim-up technique in pesisir cattle. J. Anim. Health Prod. 10(3): 337-343.

Agnieszka MM, Magdalena BN, Mariola S and Andrzej C, 2025. PRDX5 and PRDX6 translocation and oligomerization in bull sperm: A response to cryopreservation-induced oxidative stress. Cell Commun. Signal. 23(1): 15.

Alfian A, Yusuf M, Ako A, Toleng A, Diansyah A, Amrullah M, Rahmat R and Hasrin H, 2025. The effects of mineral mix supplementation on reproductive performance of Bali bulls. Egypt. J. Vet. Sci.: 1-8.

Ali MA, Qin Z, Dou S, Huang A, Wang Y, Yuan X and Zeng C, 2023. Cryopreservation induces acetylation of metabolism-related proteins in boar sperm. Int. J. Mol. Sci. 24(13): 10983.

Amaliah R, Yusuf M and Toleng AL, 2023. The quality of Bali bull sexed semen using freeze-dried albumin. Proc. 4th Int. Conf. Anim. Sci. Technol. (ICAST 2021). AIP Publ. 2628(1): 070013.

An X, Li T, Chen N, Wang H, Su M, Shi H and Ma Y, 2022. miR-1285-3p targets TPI1 to regulate the glycolysis metabolism signaling pathway of Tibetan sheep Sertoli cells. PLoS One 17(9): e0270364.

Ashwitha A, Ramesha KP, Ramesh P, Kootimole CN, Devadasan MJ, Ammankallu S and Prasad TSK, 2023. Quantitative proteomics profiling of spermatozoa and seminal plasma reveals proteins associated with semen quality in Bos indicus bulls. J. Proteomics 273: 104794.

Bisconti M, Simon JF, Grassi S, Leroy B, Martinet B, Arcolia V and Hennebert E, 2021. Influence of risk factors for male infertility on sperm protein composition. Int. J. Mol. Sci. 22(23): 13164.

Cai M, Qin Y, Wan A, Jin H, Tang J and Chen Z, 2023. COX5A as a potential biomarker for disease activity and organ damage in lupus. Clin. Exp. Med. 23(8): 4745-4756.

Cavarocchi E, Drouault M, Ribeiro JC, Simon V, Whitfield M and Touré A, 2025. Human asthenozoospermia: Update on genetic causes, patient management, and clinical strategies. Andrology.

Dai C, Wu H and Lin G, 2025. P-082 The functional role of Acrv1 in sperm-egg binding. Hum. Reprod. 40(Suppl. 1): deaf097-391.

Dhangada VR, Gaikwad US, Dhage SA, Deokar DK, Lokhande AT and Kamble DK, 2024. Innovative reproductive technology in animal breeding: A review. J. Adv. Biol. Biotechnol. 27(6): 544-532.

Diansyah AM, Santoso S, Herdis H, Yusuf M, Priyatno TP, Maulana T, Toleng AL, Dagong MIA, Said S, Iskandar H, Nurlatifah A, Lestari P, Affandy L and Baharun A, 2025b. Identification of reproductive performance in Bali-polled bulls using computer-assisted semen analysis and plasma seminal proteomics. Vet. World 18(1): 102-109.

Diansyah AM, Yusuf M and Kaiin EM, 2020. The quality of sperm post-immobilization at some parts of FH sperm using laser diodes. IOP Conf. Ser. Earth Environ. Sci. 492(1): 012074.

Diansyah AM, Yusuf M, Rahmat R and Alfian AM, 2025a. Enhanced sperm sexing efficiency and quality preservation in Bali bulls using freeze-dried albumin separation media. Open Vet. J. 15(6): 2416-2426.

Diansyah AM, Yusuf M, Toleng AL and Dagong MIA, 2022b. Characteristic and kinematics of Bali-polled bull sperms. Adv. Anim. Vet. Sci. 10(8): 1787-1796.

Diansyah AM, Yusuf M, Toleng AL, Dagong MIA and Maulana T, 2022a. The expression of plasma protein in Bali-polled bulls using 1D-SDS-PAGE. World Vet. J. 12(3): 316-322.

Diansyah AM, Yusuf M, Tolleng AL, Surahman S, Raafi M and Sahiruddin, 2021. The quality of intact plasma membrane of bull frozen sperm in different breeds. IOP Conf. Ser. Earth Environ. Sci. 788(1): 012134.

Ecovoiu AA, Ratiu AC, Micheu MM and Chifiriuc MC, 2022. Inter-species rescue of mutant phenotype—The standard for genetic analysis of human genetic disorders in Drosophila melanogaster model. Int. J. Mol. Sci. 23(5): 2613.

El-Naby SHH, Kandiel MMM, Mahmoud KGM, El-Sisy GA, Soliman WTM and Ahmed YF, 2021. Morphological comparison in spermatozoa from Italian and Egyptian buffalo bulls, and their IVF outcome. Adv. Anim. Vet. Sci. 9(9): 1449-1455.

Fan S, Xie F, Wang Q, Ye H, Tian Y, Liu Y and Zhang X, 2025. Proteomic analysis reveals the difference between the spermatozoa of young and old Sus scrofa. Sci. Rep. 15(1): 1433.

Gacem S, Castello-Ruiz M, Hidalgo CO, Tamargo C, Santolaria P, Soler C and Silvestre MA, 2023. Bull sperm SWATH-MS-based proteomics reveals link between high fertility and energy production, motility structures, and sperm–oocyte interaction. J. Proteome Res. 22(11): 3607-3624.

Geng XY, Jin HJ, Xia L, Wang BB and Chen SR, 2024. Tektin bundle interacting protein, TEKTIP1, functions to stabilize the tektin bundle and axoneme in mouse sperm flagella. Cell. Mol. Life Sci. 81(1): 118.

Guan H, Sun J, Liang X and Yao W, 2023. Protective role of cytochrome C oxidase 5A (COX5A) against mitochondrial disorder and oxidative stress in VSMC phenotypic modulation and neointima formation. Curr. Vasc. Pharmacol. 21(2): 128-142.

Guilherme C, dos Santos Filho A, Vieira J, Fantin B, Nascimento P, Guerra M, Batista A and Wischral A, 2020. Correlation between sperm kinetics and in vitro fertilisation potential of Girolando bulls. Rev. Agrar. Acad. 3: 44-53.

Guo H, Gong Y, He B and Zhao R, 2017. Relationships between mitochondrial DNA content, mitochondrial activity, and boar sperm motility. Theriogenology 87: 276-283.

Guo Y, Li J, Hao F, Yang Y, Yang H, Chang Q and Teng X, 2023. A new perspective on semen quality of aged male: The characteristics of metabolomics and proteomics. Front. Endocrinol. 13: 1058250.

Hasrin H, Toleng ALT and Hasbi H, 2022. Kebuntingan sapi Bali yang diinseminasi buatan setelah menunjukkan birahi alami. AVES J. Ilmu Peternak. 16(1): 11-16.

Hejazian SM, Ardalan M, Hosseiniyan Khatibi SM, Rahbar Saadat Y, Barzegari A, Gueguen V and Pavon-Djavid G, 2023. Biofactors

regulating mitochondrial function and dynamics in podocytes and podocytopathies. J. Cell. Physiol. 238(10): 2206-2227.

Huang Q, Chen X, Yu H, Ji L, Shi Y, Cheng X and Yu J, 2023. Structure and molecular basis of spermatid elongation in the Drosophila testis. Open Biol. 13(11): 230136.

Hutapea TPH, Madurani KA, Syahputra MY, Hudha MN, Asriana AN, Suprapto and Kurniawan F, 2023. Albumin: Source, preparation, determination, applications, and prospects. J. Sci. Adv. Mater. Devices 8(2): 100549.

Irigoyen P, Mansilla S, Castro L, Cassina A and Sapiro R, 2024. Mitochondrial function and reactive oxygen species production during human sperm capacitation: Unraveling key players.

Jin HJ, Fan Y, Yang X, Dong Y, Zhang XZ, Geng XY and Chen SR, 2024. Disruption in CYLC1 leads to acrosome detachment, sperm head deformity, and male in/subfertility in humans and mice. Elife 13: RP95054.

Jin X, Wang D, Lei M, Guo Y, Cui Y, Chen F and Chen X, 2022. TPI1 activates the PI3K/AKT/mTOR signaling pathway to induce breast cancer progression by stabilizing CDCA5. J. Transl. Med. 20(1): 191.

Kang JY, Wen Z, Pan D, Zhang Y, Li Q, Zhong A and Liu MF, 2022. LLPS of FXR1 drives spermiogenesis by activating translation of stored mRNAs. Science 377(6607): eabj6647.

Kumar S, Magotra A, Kumar M, Dalal DS and Kumari S, 2024. Semen sexing and its impact on fertility and genetic gain in cattle. Zygote 32(2): 109-118.

Lai BS, Ruan GR, Liu M, Liu YH, Huang XH and Lin QM, 2022. Effect of NAGase on sperm motility related to Izumo4 protein in vitro. Indian J. Anim. Health 61(2): 250-263.

Leung MR, Roelofs MC, Chiozzi RZ, Hevler JF, Heck AJ and Zeev-Ben-Mordehai T, 2022. Unraveling the intricate microtubule inner protein networks that reinforce mammalian sperm flagella. bioRxiv: 2022-09.

Lin YH, Su CH, Chen HM, Wu MS, Pan HA, Chang CN and Teng YN, 2024. MicroRNA-320a enhances LRWD1 expression through the AGO2/FXR1-dependent pathway to affect cell behaviors and the oxidative stress response in human testicular embryonic carcinoma cells. Aging (Albany NY) 16(4): 3973.

Marks KE, Flaherty S, Patterson KM, Stratton M, Martinez GJ and Reynolds JM, 2021. Toll-like receptor 2 induces pathogenicity in Th17 cells and reveals a role for IPCEF in regulating Th17 cell migration. Cell Rep. 35(13): 109303.

Maulana T, Ridwan R, Gunawan M, Agung PP, Afiati F, Kaiin EM and Said S, 2022. Successful separation of X- and Y-spermatozoa Ongole crossbreed using a nano-albumenalbumin gradient column. Trop. Anim. Sci. J. 45(4): 397-404.

Mostek-Majewska A, Majewska A, Janta A and Ciereszko A, 2023. New insights into posttranslational modifications of proteins during bull sperm capacitation. Cell Commun. Signal. 21(1): 72.

Nirmala A, Toleng AL, Yusuf M, Herdis H, Diansyah AM, Amrullah MF, Rahmat R, Rajamuddin R, Alfian AM and Hasrin H, 2025. Effect of adding kasumba turate (Carthamus tinctorius L.) extract in tris-egg yolk diluent on sperm quality in Bali bulls. J. Anim. Health Prod. 13(1): 154-159.

Paitoon P, Sartsook A, Thongkham M, Sathanawongs A, Lumsangkul C, Pattanawong W, Hongsibsong S and Sringarm K, 2024. Sperm quality variables of sex-sorted bull semen produced by magnetic-activated cell sorting coupled with recombinant antibodies targeting Y-chromosome-bearing sperm. Theriogenology 219: 11-21.

Parkes R and Garcia TX, 2024. Bringing proteomics to bear on male fertility: Key lessons. Expert Rev. Proteomics 21(4): 181-203.

Parrilla I, Cambra JM, Cuello C, Rodriguez-Martinez H, Gil MA and Martinez EA, 2024. Cryopreservation of highly extended pig spermatozoa remodels its proteome and counteracts polyspermic fertilization in vitro. Andrology 12(6): 1356-1372.

Pongprayoon P, Kuntip N, Suwanasopee T, Jattawa D, Niramitranon J, Japrung D and Koonawootrittriron S, 2025. Comparative studies of structure and dynamics of caprine, leporine, ovine, and equine serum albumins. J. Biomol. Struct. Dyn. 43(6): 2772-2780.

Pozdyshev DV, Kombarova NA and Muronetz VI, 2023. Biochemical features of X- or Y-

chromosome-bearing spermatozoa for sperm sexing. Biochemistry (Moscow) 88(5): 655-666.

Quelhas J, Santiago J, Matos B, Rocha A, Lopes G and Fardilha M, 2021. Bovine semen sexing: Sperm membrane proteomics as candidates for immunological selection of X- and Y-chromosome-bearing sperm. Vet. Med. Sci. 7(5): 1633-1641.

Raafi M, Yusuf M, Toleng AL, Diansyah AM, Surahman S and Sahiruddin S, 2021. Movement patterns of sperms at different bull breeds using computer-assisted sperm analysis (CASA). IOP Conf. Ser. Earth Environ. Sci. 788: 012137.

Rahman MS and Pang MG, 2020. New biological insights on X- and Y-chromosome-bearing spermatozoa. Front. Cell Dev. Biol. 7: 388.

Rahmat R, Yusuf M, Toleng AL, Herdis H, Diansyah AM and Hasrin H, 2024. The quality of Bali bull sexed sperms using freeze-dry albumin at different concentrations of sexing medium. Adv. Anim. Vet. Sci. 12(2): 249-258.

Ribeiro DG, de Oliveira Carvalho J, Sartori R, Monteiro PLJ, Fontes W, de Souza Castro M and Mehta A, 2025. The presence of sexed sperm in bovine oviduct epithelial cells alters the protein profile related to stress and immune response. Res. Vet. Sci. 184: 105522.

Rosyada ZNA, Pardede BP, Kaiin EM, Gunawan M, Maulana T, Said S and Purwantara B, 2023. A proteomic approach to identifying spermatozoa proteins in Indonesian native Madura bulls. Front. Vet. Sci. 10: 1287676.

Sahiruddin, Widjiati, Madyawati SP, Toleng AL, Yusuf M, Masturi, Ako A and Amrullah MF, 2021. The quality of Bali bull sexed sperms at different incubation time using egg white sedimentation method. IOP Conf. Ser. Earth Environ. Sci. 788: 012142.

Said S, Maulana T, Iskandar H, Kaiin EM, Khaerunnisa I, Putra WPB, Hasan F and Arifiantini RI, 2024. Sperm protein profile and their correlation with frozen semen quality of indigenous Indonesian buffalo bulls. J. Adv. Vet. Anim. Res. 11(4): 846-855.

Said TM and Khosravi F, 2012. Role of caspase, PARP, and oxidative stress in male infertility. In: Studies on men's health and fertility. Humana Press, Totowa, NJ, pp. 237-254.

Saint-Dizier M, Mahé C, Reynaud K, Tsikis G, Mermillod P and Druart X, 2020. Sperm interactions with the female reproductive tract: A key for successful fertilization in mammals. Mol. Cell. Endocrinol. 516: 110956.

Sanderson DJ and Cohen MS, 2020. Mechanisms governing PARP expression, localization, and activity in cells. Crit. Rev. Biochem. Mol. Biol. 55(6): 541-554.

Shangguan A, Ding F, Ding R, Sun W, Li X, Bao X and Zhang S, 2025. Whole-genome bisulfite sequencing of X and Y sperm in Holstein bulls reveals differences in autosomal methylation status. BMC Genomics 26(1): 282.

Shen D, Zhou C, Cao M, Cai W, Yin H, Jiang L and Zhang S, 2021. Differential membrane protein profile in bovine X- and Y-sperm. J. Proteome Res. 20(6): 3031-3042.

Silambarasan I and Rajalakshmi AN, 2022. A review on freeze-drying: A stability enhancement technique. Res. J. Pharm. Technol. 15(10): 4841-4846.

Tanga BM, Qamar AY, Raza S, Bang S, Fang X, Yoon K and Cho J, 2021. Semen evaluation: Methodological advancements in sperm quality-specific fertility assessment – A review. Anim. Biosci. 34(8): 1253-1270.

Ureña I, González C, Ramón M, Gòdia M, Clop A, Calvo JH and Serrano M, 2022. Exploring the ovine sperm transcriptome by RNAseq techniques: Effect of seasonal conditions on transcripts abundance. PLoS One 17(3): e0264978.

Vallet-Buisan M, Mecca R, Jones C, Coward K and Yeste M, 2023. Contribution of semen to early embryo development: Fertilization and beyond. Hum. Reprod. Update 29(4): 395-433.

Wang J, Li R, Huang F, Niu P, Zheng Y, Yuan D, Du Y, Li C, Yang M, Suo J and Gao Q, 2025. Comparative analysis of AQP7 expression and cryotolerance in X- and Y-chromosome bearing bovine sperm. Front. Cell Dev. Biol. 13: 1582961.

Wang J, Wen S, Chen M, Xie J, Lou X, Zhao H and Shi G, 2024. Regulation of endocrine cell alternative splicing revealed by single-cell RNA sequencing in type 2 diabetes pathogenesis. Commun. Biol. 7(1): 778.

Wang K, Jiao H, Cheng X, Zhang L, Zhang S, Liu G and Yang F, 2024. Proteomic analysis of differences in the freezability of porcine sperm identifies α-amylase as a key protein. J. Proteome Res. 23(7): 2641-2650.

Wang W, Tian S, Nie H, Tu C, Liu C, Li Y and Tan YQ, 2021. CFAP65 is required in the acrosome biogenesis and mitochondrial sheath assembly during spermiogenesis. Hum. Mol. Genet. 30(23): 2240-2255.

Xie SM, Lai JX, Liu CQ, Zhang XX, Lin YM, Lan QW and Mao YL, 2023. UBR4 deficiency causes male sterility and testis abnormal in Drosophila. Front. Endocrinol. 14: 1165825.

Xue Q, Ren X, Xu T, Yang T, Sun L, Luo X and Li X, 2025. Comparative proteomics and phosphoproteomics analysis reveals differential sperm motility in Mediterranean buffalo semen. J. Proteomics 315: 105401.

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

Zoca SM, Northrop-Albrecht EJ, Walker JA, Cushman RA and Perry GA, 2022. Proteomics dataset of epididymal fluid, seminal plasma, and proteins loosely attached to epididymal and ejaculated sperm from Angus bulls. Data Brief 42: 108150.