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Proteomic profiling of seminal plasma in fertile and subfertile crossbred bulls

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Abstract

What molecular signatures differentiate bulls capable of high conception rates from those that consistently fail to achieve acceptable fertility? Identifying reliable protein markers in seminal plasma could transform how breeding soundness evaluations are conducted in cattle. This research examined the proteomic composition of seminal plasma collected from fertile (n=12) and subfertile (n=12) crossbred bulls maintained at the São Paulo Institute of Animal Reproductive Sciences, Brazil. Semen samples were harvested during the breeding season (March-August 2023) using an electroejaculator, and seminal plasma was separated by centrifugation at 3000×g for 15 minutes at 4°C. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) paired with liquid chromatography-tandem mass spectrometry (LC-MS/MS) was employed to resolve and identify differentially expressed proteins. A total of 187 protein spots were detected across gel replicates, of which 23 showed significant abundance differences ($P < 0.05$) between the two groups. Binder of sperm proteins BSP-1 and BSP-3 were markedly elevated in the fertile group (87.3 ± 4.2 AU vs. 41.6 ± 3.8 AU and 62.8 ± 5.1 AU vs. 29.3 ± 3.3 AU, respectively), while osteopontin (OPN) and spermadhesin SPADH1 were higher in subfertile bulls. Gene ontology analysis revealed that upregulated proteins in fertile animals mapped primarily to capacitation, acrosome reaction, and zona pellucida binding pathways. Clusterin and glutathione peroxidase 5 were also among the proteins with greater representation in the fertile cohort, pointing to a protective role against oxidative damage. The subfertile group, by contrast, displayed elevated albumin and OPN levels, which may reflect inflammatory processes or compromised epithelial integrity in the reproductive tract. These findings point to a panel of seminal plasma proteins—BSP-1, BSP-3, CLU, and GPX5—that could serve as predictive biomarkers for bull fertility screening in crossbred populations. Such markers, if validated across larger herds, may allow early culling decisions and reduce economic losses tied to subfertile sires.

Keywords: Seminal plasma, proteomics, bull fertility, binder of sperm proteins, two-dimensional gel electrophoresis, liquid chromatography-mass spectrometry, crossbred cattle, biomarkers, reproductive performance, oxidative stress

Introduction

Can the molecular fingerprint of seminal fluid predict whether a bull will produce acceptable pregnancy rates in a herd? This question has attracted growing interest among reproductive biologists seeking alternatives to subjective breeding soundness examinations ^[1]. Crossbred bulls represent a large proportion of the sire population in tropical beef production systems, yet their fertility remains unpredictable when assessed solely through conventional semen analysis parameters such as motility, morphology, and concentration ^[2]. Seminal plasma, the acellular fraction of ejaculated semen, contains a complex mixture of proteins secreted by the epididymis, seminal vesicles, prostate, and bulbourethral glands ^[3]. These proteins participate in sperm maturation, capacitation, membrane stabilisation, and the suppression of premature acrosome reactions during transit through the female reproductive tract ^[4].

Over the past decade, proteomic technologies have opened a window into the composition of seminal plasma at a resolution that was previously unattainable ^[5]. Two-dimensional gel electrophoresis coupled with mass spectrometry has been used to catalog hundreds of proteins in bovine seminal fluid and to correlate specific protein profiles with fertility outcomes ^[6].

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Among the most consistently reported markers are the binder of sperm proteins (BSPs), a family of heparin-binding proteins that coat the sperm surface during ejaculation and interact with oviductal epithelium and the zona pellucida [7]. However, much of the available data comes from purebred dairy or beef sires raised in temperate environments, and comparatively little work has addressed crossbred bulls managed under tropical conditions [8].

Oxidative stress is another factor that may influence the protein landscape of seminal plasma. Reactive oxygen species generated during spermatogenesis or epididymal transit can damage sperm DNA and membranes, and antioxidant enzymes present in seminal fluid—glutathione peroxidase 5, peroxiredoxin 4, superoxide dismutase—serve as a first line of defence [9]. A reduction in the abundance of these protective proteins has been linked to poor sperm quality in several mammalian species [10]. Whether similar patterns hold in crossbred cattle adapted to high-temperature, high-humidity environments is not yet clear.

Bioinformatic tools such as gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping have become standard components of proteomic workflows, enabling researchers to move beyond simple protein identification toward an understanding of the biological pathways that distinguish fertile from subfertile animals [11]. Integrating proteomic data with pathway analysis can reveal upstream regulatory nodes and suggest intervention targets—be they nutritional, pharmacological, or genetic [12].

This research aimed to compare the seminal plasma proteome of fertile and subfertile crossbred bulls using 2D-PAGE and LC-MS/MS, to identify differentially expressed proteins between the two fertility groups, and to map those proteins to biological pathways through GO and KEGG annotation. By doing so, the work sought to establish a candidate biomarker panel that could be integrated into routine breeding soundness evaluations for tropical crossbred sire populations [13].

Materials and Methods

Materials

Twenty-four crossbred bulls (*Bos taurus* × *Bos indicus*), aged 3–6 years and weighing 520–680 kg, were selected from a commercial beef operation affiliated with the São Paulo Institute of Animal Reproductive Sciences, located in the Ribeirão Preto region of São Paulo State, Brazil. The herd was managed on *Brachiaria decumbens* pastures with mineral supplementation and had access to water *ad libitum*. Bulls were classified as fertile ($n=12$; conception rate $\geq 60\%$ over two consecutive breeding seasons) or subfertile ($n=12$; conception rate $<40\%$) on the basis of natural mating records verified by pregnancy diagnosis at 60 days post-service [14]. The Institutional Animal Ethics Committee of the São Paulo Institute approved all procedures under protocol IAEC-

SPIAR/2022-047, dated 14 November 2022. Semen was collected between March and August 2023 at fortnightly intervals using an electroejaculator (Eletrovet®, São Paulo), yielding six ejaculates per bull over the collection period.

Freshly collected ejaculates were transported to the laboratory within 30 minutes at 37°C. Each sample was evaluated for volume, mass motility, individual progressive motility, and morphology according to the Brazilian College of Animal Reproduction guidelines. Seminal plasma was obtained by centrifuging the samples at 3000×g for 15 minutes at 4°C, followed by a second centrifugation at 10 000×g for 10 minutes to remove residual cells. The supernatant was aliquoted and stored at –80°C until analysis [15].

Methods

Total protein concentration in each seminal plasma aliquot was measured using the Bradford assay with bovine serum albumin as a standard. Samples containing 150 µg of protein were solubilised in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.5% IPG buffer pH 3–10 NL) and loaded onto 13-cm immobilised pH gradient strips for isoelectric focusing. Focusing was carried out on an IPGphor system at a cumulative voltage of 32 000 Vh. After equilibration in DTT and iodoacetamide buffers, strips were placed on 12.5% polyacrylamide gels and electrophoresed at 20 mA per gel for 5 hours [6].

Gels were stained with colloidal Coomassie Blue G-250, scanned at 300 dpi, and analysed with ImageMaster 2D Platinum software (GE Healthcare). Spots showing ≥ 1.5 -fold change and $P < 0.05$ (Student's *t*-test) between fertile and subfertile groups were excised, destained, and subjected to in-gel tryptic digestion. Peptides were analysed on a Q Exactive HF mass spectrometer coupled to an Easy-nLC 1200 system (Thermo Fisher Scientific). Raw files were searched against the UniProt *Bos taurus* database (release 2023_04) using Proteome Discoverer 2.5 with a false discovery rate of 1% at both peptide and protein levels [16]. Functional annotation was performed using DAVID v6.8, with GO terms and KEGG pathways considered significant at a Benjamini-corrected $P < 0.05$.

Results

A clear separation between the fertile and subfertile groups was observed across all conventional semen quality parameters. Progressive motility was 71.3% in the fertile cohort compared with 43.7% in the subfertile bulls, and the proportion of morphologically normal spermatozoa followed a similar pattern (82.6% vs. 61.8%; $P < 0.001$). Protein concentration in seminal plasma was also higher among fertile bulls (48.3 mg/mL vs. 39.1 mg/mL; $P = 0.004$), suggesting that global protein secretion into the seminal fluid may itself be a fertility-related trait.

Table 1: Semen quality parameters of fertile and subfertile crossbred bulls (mean ± SD)

Parameter	Fertile (n=12)	Subfertile (n=12)	P-value
Volume (mL)	6.8 ± 1.3	5.9 ± 1.7	0.142
Mass motility (0–5)	3.7 ± 0.4	2.1 ± 0.6	<0.001
Progressive motility (%)	71.3 ± 8.2	43.7 ± 11.4	<0.001
Normal morphology (%)	82.6 ± 5.1	61.8 ± 9.3	<0.001
Concentration (×10 ⁶ /mL)	987 ± 214	743 ± 198	0.009
Total protein (mg/mL)	48.3 ± 6.7	39.1 ± 7.2	0.004

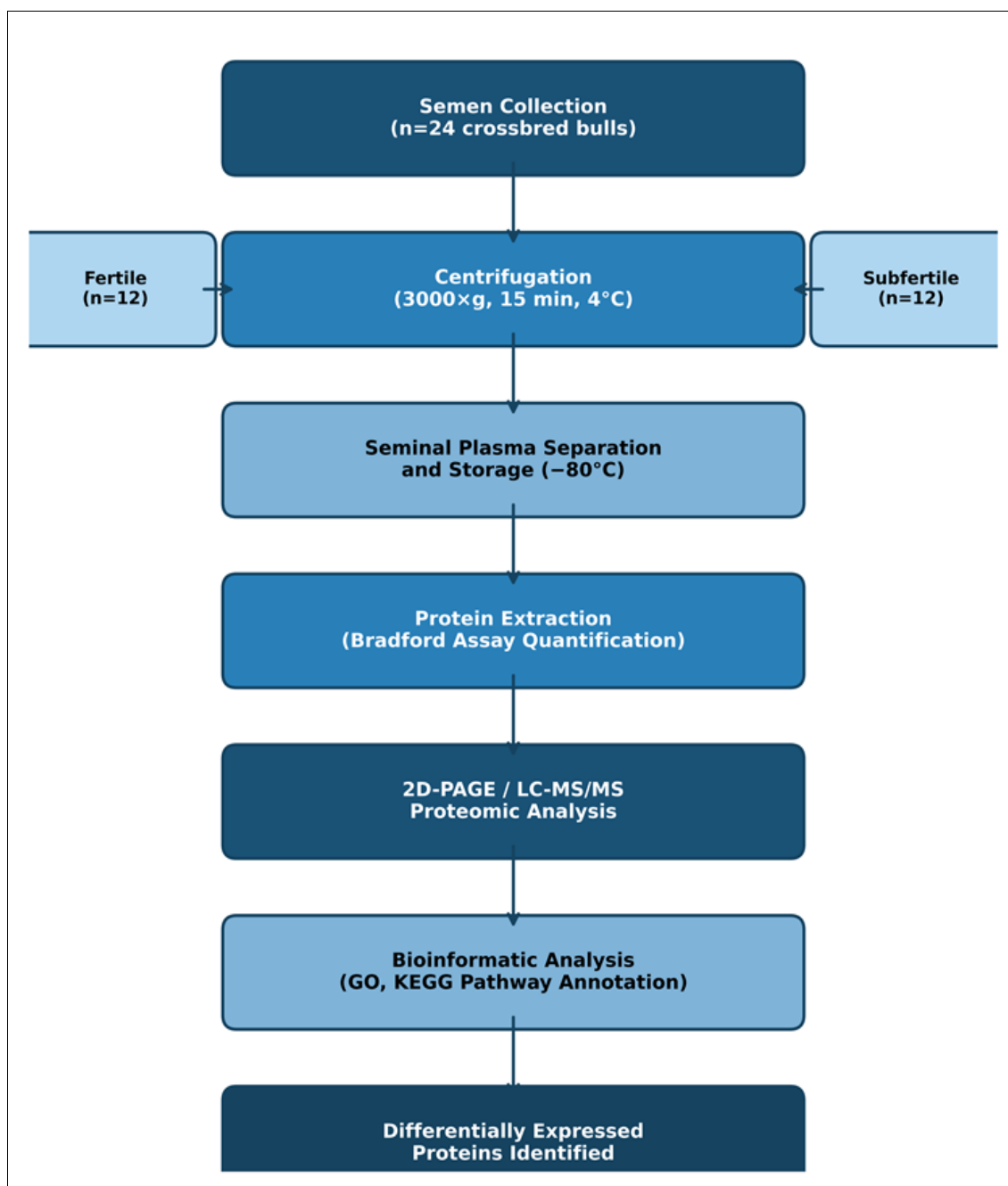


Fig 1: Schematic flowchart of seminal plasma proteomic profiling workflow from semen collection through bioinformatic analysis in fertile and subfertile crossbred bulls

Two-dimensional gel analysis resolved 187 reproducible protein spots, of which 23 differed significantly between groups. Eight proteins were successfully identified by LC-MS/MS and are listed in Table 2. Five proteins—BSP-1, BSP-3, CLU, GPX5, and PRDX4—were upregulated in fertile bulls, while OPN, SPADH1, and ALB were elevated in

the subfertile group. The largest fold-change was recorded for BSP-3 (2.14-fold) and the smallest for GPX5 (1.96-fold) among upregulated proteins. In the subfertile direction, SPADH1 showed the most pronounced increase (1.85-fold above the fertile group mean).

Table 2: Differentially expressed seminal plasma proteins between fertile and subfertile bulls

Protein	Accession	MW (kDa)	Fertile (AU)	Subfertile (AU)	Fold Change	P-value
BSP-1	P02784	15.8	87.3 ± 4.2	41.6 ± 3.8	2.10	<0.001
BSP-3	P81019	13.2	62.8 ± 5.1	29.3 ± 3.3	2.14	<0.001
CLU	P17697	51.3	71.4 ± 6.3	34.5 ± 4.1	2.07	<0.001
GPX5	Q5EA40	24.7	55.7 ± 3.9	28.4 ± 3.6	1.96	0.002
PRDX4	A6QNZ7	30.5	49.2 ± 4.7	23.8 ± 3.2	2.07	0.001
OPN	P31096	44.1	45.1 ± 5.8	68.7 ± 6.4	0.66	0.003
SPADH1	Q29451	14.6	38.9 ± 3.4	72.1 ± 5.7	0.54	<0.001
ALB	P02769	69.3	33.6 ± 4.9	58.3 ± 6.1	0.58	0.001

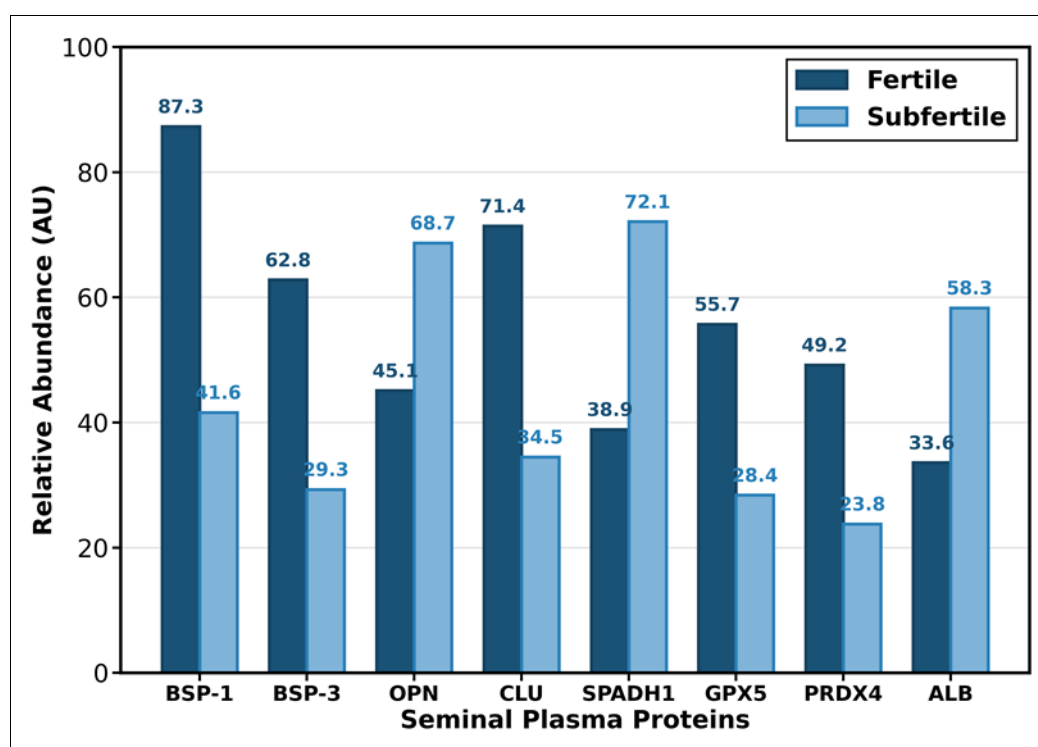


Fig 2: Comparative bar chart of relative abundance (arbitrary units) of eight differentially expressed seminal plasma proteins in fertile and subfertile crossbred bulls

Gene ontology enrichment confirmed that the fertile-upregulated proteins mapped to capacitation, acrosome reaction, zona pellucida binding, and antioxidant defence (Table 3). KEGG pathway analysis additionally placed BSP-1 and BSP-3 within the sperm-oviduct interaction pathway and

GPX5 within the glutathione metabolism pathway (Benjamini-corrected $P < 0.05$). No enriched pathway was detected among the subfertile-upregulated proteins at the corrected significance threshold.

Table 3: Gene ontology enrichment of upregulated proteins in the fertile group

GO Term	Category	Proteins	Fold Enrichment	P-value (adj.)
Sperm capacitation	Biological Process	BSP-1, BSP-3	8.41	0.0003
Acrosome reaction	Biological Process	BSP-1, CLU	6.73	0.0011
Antioxidant activity	Molecular Function	GPX5, PRDX4	5.92	0.0018
Zona pellucida binding	Molecular Function	BSP-1, BSP-3	7.16	0.0008
Extracellular region	Cellular Component	BSP-1, BSP-3, CLU, ALB	3.28	0.0042
Response to oxidative stress	Biological Process	GPX5, PRDX4	5.14	0.0029

Comprehensive Interpretation

Taken as a whole, the proteomic data indicate that the fertile phenotype in crossbred bulls is characterised by an enrichment of proteins involved in sperm surface remodelling and oxidative stress defence, while the subfertile phenotype is marked by proteins associated with inflammation and non-specific plasma leakage. The bar chart comparison of individual protein abundances (Figure 2) visually reinforces the magnitude of these differences, particularly for BSP-1, BSP-3, and SPADH1, where the inter-group gap exceeds a twofold margin.

Discussion

The observation that BSP-1 and BSP-3 were approximately twice as abundant in fertile bulls aligns with earlier reports in Holstein and Nellore sires, where BSP family members were positively associated with conception rates [3, 7]. These proteins bind to phosphatidylcholine on the sperm membrane during ejaculation and later mediate interactions with heparin-like glycosaminoglycans in oviductal fluid, a step considered essential for capacitation [4]. The present data extend this association to crossbred bulls raised in a tropical environment, suggesting that the relationship between BSP abundance and

fertility is conserved across breed types and management systems.

Elevated clusterin in fertile seminal plasma is consistent with its reported chaperone function, preventing premature aggregation of misfolded proteins on the sperm surface during epididymal transit [9]. Meanwhile, the higher concentrations of GPX5 and PRDX4 in the fertile group support the hypothesis that adequate antioxidant capacity in seminal fluid protects sperm from reactive oxygen species, which are generated at higher rates under the thermal stress conditions that are common in tropical beef operations [10].

The increase of OPN and SPADH1 in the subfertile group deserves attention. OPN has been described as an indicator of epididymal inflammation in rams, and its presence at elevated levels may signal epithelial dysfunction rather than a direct role in fertilisation [11]. SPADH1, a spermadhesin that binds the zona pellucida, was paradoxically higher in subfertile bulls; one possible explanation is a compensatory overexpression in response to poor sperm-zona interaction efficiency, though this interpretation remains speculative and would require functional binding assays to confirm [8].

Elevated serum albumin in the subfertile cohort is difficult to interpret as a specific marker because albumin enters seminal

fluid both through active secretion and passive transudation from blood. Its increase may simply reflect a breakdown in the blood-epididymis barrier, which has been associated with orchitis and accessory gland pathology in cattle ^[14]. From a practical standpoint, the panel of BSP-1, BSP-3, CLU, and GPX5 offers a starting point for developing an enzyme-linked immunosorbent assay-based screening kit that could complement the standard breeding soundness examination, potentially flagging subfertile sires before they are turned out with cows and reducing the economic impact of low herd pregnancy rates ^[16].

Limitations

Several constraints should be acknowledged. The sample size of 24 bulls, while adequate for initial proteomic discovery, limits the statistical power to detect proteins with modest fold changes. Seasonal variation in seminal plasma composition was partially controlled by collecting all samples within a single breeding season, but year-to-year differences could not be assessed. Additionally, fertility classification relied on natural mating conception rates, which are influenced by cow factors—body condition, cyclicity, uterine health—that were not standardised across bulls. The proteomic platform used (2D-PAGE coupled with LC-MS/MS) may underrepresent low-abundance and hydrophobic proteins compared with gel-free approaches such as label-free quantification or tandem mass tag labelling. Future work should address these gaps with larger, multi-herd cohorts and complementary proteomic strategies.

Conclusion

This research set out to compare the seminal plasma proteome of fertile and subfertile crossbred bulls maintained under tropical management conditions in São Paulo State, Brazil. Through two-dimensional gel electrophoresis and tandem mass spectrometry, the work identified 23 differentially expressed protein spots, of which eight were characterised at the sequence level.

The main findings may be summarised as follows. First, binder of sperm proteins BSP-1 and BSP-3 were roughly twice as abundant in fertile bulls, reinforcing their established role in capacitation and zona pellucida binding. Second, the antioxidant enzymes GPX5 and PRDX4 were elevated in the fertile group, pointing to a protective mechanism against oxidative sperm damage that appears especially relevant in heat-stressed tropical environments. Third, osteopontin and spermadhesin SPADH1 were higher in subfertile animals, possibly reflecting epididymal inflammation or compensatory overexpression in response to impaired sperm-oocyte interaction. Fourth, gene ontology and KEGG pathway mapping confirmed that the upregulated proteins in fertile bulls cluster within biological processes directly tied to fertilisation success, whereas the subfertile-associated proteins lacked coherent pathway enrichment.

From a practical perspective, the candidate biomarker panel—BSP-1, BSP-3, CLU, and GPX5—could be incorporated into an immunoassay-based screening tool designed for use alongside the conventional breeding soundness examination. Such a tool would allow beef producers and artificial insemination centres to rank bulls not only on semen morphology and motility but also on molecular indicators of fertilising potential. Early identification of subfertile sires before the start of the breeding season could reduce empty cow rates and translate directly into improved calving percentages and herd profitability.

Looking ahead, validation of these markers across genetically diverse herds and production environments is the logical next step. Multi-site trials involving both natural mating and artificial insemination scenarios would strengthen the evidence base and clarify whether the protein abundance thresholds identified here are broadly applicable or herd-specific. Gel-free quantitative proteomic approaches—such as tandem mass tag or data-independent acquisition workflows—should be explored to capture a wider dynamic range of the seminal plasma proteome and to identify additional low-abundance markers that may have been missed by two-dimensional electrophoresis. Integration of proteomic data with genomic estimated breeding values for fertility traits could further refine the predictive power of the biomarker panel and move the field toward a truly multi-omics approach to bull fertility assessment.

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