EKAETTE CHRIS UDOEKONG

PROTEOME OF BULL FERTILITY: A COMPARATIVE STUDY BETWEEN RESTING AND IN-SERVICE NELLORE BULLS

Dissertation submitted to Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Simone Eliza Facioni Guimarães

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"Esforce-se não para ser um sucesso, mas sim para ser de valor."

(Albert Einstein)

ABSTRACT

UDOEKONG, Ekaette Chris, M.Sc., Universidade Federal de Viçosa, June 2023. **Proteome of bull fertility: A comparative study between resting and in-service Nellore bulls.** Adviser: Simone Eliza Facioni Guimarães.

This study aimed to analyses sperm proteome of Nellore bulls by comparing their sperm protein profiles and identifying differentially abundant proteins during their in-service and resting periods. A total of twenty bulls aged between 19.8 and 22.7 months were investigated at their resting period and at the breeding season (in-service). Semen samples were collected from bulls by electroejaculatation and preserved in liquid nitrogen. A total of 155 sperm proteins were identified from the protein analysis using SDA-PAGE. 84 of these proteins were specific to both resting bulls and in-service bulls while 61 were found only in resting bulls and five found only in the samples from in-service bulls. The differentially expressed sperm proteins observed in sodium dodecyl sulfate polyacrylamide gel electrophoresis were identified using LC-MS/MS. In resting bulls, albumin was differentially expressed and significantly ($p<0.05$) lower, down regulated in in-service bulls. SRN, bovin proacrosine, GAPDHS, ASRGL1, BSP1, CES5A, SPACA3, SPAM1, ADAM32 and ACE were significantly ($p<0.05$) lower and down regulated in in-service bulls. SPADH1 and LOC101907989 were differentially expressed in in-service bulls, significantly ($p<0.05$) lower and down regulated in resting bulls; SPADH1 (spermadhesin-1) associates with seminal plasma proteins BSP-30k Da, seminal plasma PDC-109, seminal plasma A3, ANG1, RNASE4, SRN and carbohydrate-binding proteins (uncharacterized protein). These findings suggest that these sperm proteins play significant role and are associated with increased fertility in bull during in-service periods rather than when they are at rest. This study suggests prior clean-up of breeding bulls before breeding season to allow for biological processes and favorable correlations of other sperm protein like SPADH1 in the regulation of sperm maturation, functions, fertility and oocyte-sperm interaction.

Keywords: Fertility. Seminal plasma proteins. Spermadhesin-1.

RESUMO

UDOEKONG, Ekaette Chris, M.Sc., Universidade Federal de Viçosa, junho de 2023. **Proteoma da fertilidade de touros: um estudo comparativo entre touros Nelore em repouso e em serviço**. Orientadora: Simone Eliza Facioni Guimarães.

Este estudo teve como objetivo analisar o proteoma espermático de touro Nelore, comparando seus perfis proteicos espermáticos e identificando proteínas diferencialmente abundantes durante seus períodos de serviço e descanso. Um total de vinte touros com idades entre 19,8 e 22,7 meses foram investigados no período de descanso e na estação de monta (em serviço). Amostras de sêmen foram coletadas de touros por eletroejaculação e preservadas em nitrogênio líquido. Um total de 155 proteínas espermáticas foram identificadas a partir da análise de proteínas usando SDA-PAGE. 84 dessas proteínas eram comuns a touros em repouso e em serviço, enquanto 61 foram encontradas apenas em touros em repouso e cinco encontradas apenas em amostras de touros em serviço. As proteínas espermáticas diferencialmente expressas observadas na eletroforese em gel de dodecil sulfato de sódio e poliacrilamida foram identificadas usando LC-MS/MS. Em RB, a albumina foi expressa diferencialmente e significativamente ($p<0,05$) menor, regulada negativamente em touros em serviço. SRN, proacrosina bovina, GAPDHS, ASRGL1, BSP1, CES5A, SPACA3, SPAM1, ADAM32 e ACE foram significativamente ($p<0,05$) mais baixos e regulados negativamente em ISB. SPADH1 e LOC101907989 foram expressos diferencialmente em touros em serviço, significativamente ($p<0,05$) mais baixos e menos regulados em touros em repouso; SPADH1 (espermadesina-1) associa-se às proteínas do plasma seminal BSP-30k Da, plasma seminal PDC-109, plasma seminal A3, ANG1, RNASE4, SRN e proteínas de ligação a carboidratos (proteína não caracterizada). Esses achados sugerem que essas proteínas espermáticas desempenham um papel significativo e estão associadas ao aumento da fertilidade em touros durante os períodos de serviço, e não quando estão em repouso. Este estudo sugere uma limpeza prévia dos touros reprodutores antes da estação reprodutiva para permitir processos biológicos e correlações favoráveis de outras proteínas espermáticas com SPADH1 na regulação da maturação, funções, fertilidade e interação oócito-espermatozoide.

Palavras-chave: Fertilidade. Proteínas do plasma seminal. Espermadesina-1.

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LIST OF ACRONYMS AND ABBREVIATIONS

2DGE	Two-dimensional gel electrophoresis.
ACN	Acetonitrile.
BSA	Bovine serum albumin.
BLAST	Basic Local Alignment Search Tool.
CHAPS	3-3-cholamidopropyl dimethylammonium-1-propanesulfonate.
DAVID	Database for Annotation, Visualization, and Integrated Discovery.
DEP	Differentially Expressed Proteins.
DTT	Dithiothreitol**.**
emPAI	Exponentially Modified Protein Abundance Index.
FDR	False Discovery Rate.
RB	Resting Bulls.
ISB	In-Service Bulls.
KEGG	Kyoto Encyclopedia of Genes and Genomes.
LC-MS	Liquid Chromatography with tandem mass spectrometry**.**
MGF	Mascot generic format.
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.
TFA	Trifluoroacetic acid.
RPSBLAST	Reversed Position Specific Basic Local Alignment Search Tool.

LIST OF SYMBOLS

mL	Milliliter
g	Grams
%	Percentage
^{o}C	Degree Celsius
µL	Microliter
w/v	Weight per volume
mM	Millimetre
M	Metre
V	Volt
H	Hour
v/v	Volume per volume
L	Liter
ng/L	Nanogram per liter
min	Minute
µm	Micrometer
kV	Kilovolt
<	Less than

SUMMARY

1. INTRODUCTION

Bull fertility is a complex mechanism and is dependent on a number of chemical interactions. It begins with spermatogenesis right through sperm-oocyte penetration (Sutovsky, 2018) to embryonic development. A defect in this mechanism will result in infertility or subfertility (Jodar, et al. 2017; Özbek, et al. 2021). Fertility issues are common in breeding bulls especially among those kept commercially for sperm production and on field natural insemination (Khatun et al. 2013). The fertility of the bull is important as bulls may be disposed due to inability to achieve cumulative cow herd conception rates of 75% at the onset of the breeding season. According to Heverton et al. (2018), fertility level in each breeding animal is crucial for effective calf production as sub-fertile bulls may lead to longer calving intervals, fewer calves, higher feeding costs for non-pregnant cows and most common, the disposal of breeding bulls. These outcomes result in significant financial losses and failures in breeding program for any cattle industry (Abdollahi-Arpanahi, et al. 2017).

Reports are scare on the disposal of breeding bulls with inability to attain an optimum conception rate in cows at the start of the breeding season. Some male animals with seasonal breeding cycles produce poor quality sperm during the non-breeding season or reproductive rest periods (Martinez-Pastor, et al. 2005). In some mammals, the males exhibit testicular inactivity, poor viable sperm, variations in behaviors and other physiological conditions; and at the onset of breeding or in-service or reproductive active periods, they demonstrate testicular recrudescence and sexual activity (Monfort, et al. 1993). According to Mukhopadhyay et al. (2010), the poor reproductive potentials of such bulls can be attributed to factors related to genetics, environment and management of the animals. These variations however, are less notable in males of domestic animals, although changes in behavior and sperm qualities have been reported (Blottner, et al. 2001; Pérez, et al. 2002).

The spermatozoa are equipped with proteins connected to fertilizing ability and energy production for sperm metabolism during acrosomal process; for protection against apoptosis and oxidative stress, and other signaling pathways (Ciereszko, et al. 2000; Visconti, 2012; Sengupta, et al. 2020; Asadi, et al. 2021). Understanding the functioning of proteins responsible for spermatozoa production and differentiation, libido, sperm integrity such as poor sperm motility and viability

(Dogan et al. 2012; Aitken et al. 2012; Ferramosca, et al. 2013; O'Flaherty, 2014; Asadi, et al. 2021) sperm DNA damage (Zevnep, et al. 2022), as well as the presence of foreign cells in the seminal plasma due to inflammation in the reproductive system (Mukhopadhyay et. al, 2013; Khatun et. al, 2013) is imperative.

Proteomic approach has allowed for an extensive and comprehensive identification of proteins and their comparative analysis (Gaviraghi, et al. 2010; Kovac, et al. 2013; Westfalewicz, et al. 2021). Sperm proteome is a complex network of proteins that regulates key fertility processes, including sperm motility, capacitation, fertilization, and early embryonic development. (Luongo, et al. 2020). In recent time, functional data analysis from proteomic studies have helped researchers to generate knowledge to better understand the relationship and mechanism underlying male fertility and sperm proteome (Rahman, et al, 2013; Ashok, et. al, 2016; Mohammad, et. al, 2018). These studies have also enabled researchers to identify proteins in spermatozoa responsible for positive/negative regulations and modifications of sperm functions. Other works have focused on fertility-related proteins in semen (Gaviraghi, et al. 2010; Rego, et al. 2014; Da Ros, et al. 2015; Kasimanickam, et al. 2019; Pardede et al. 2020) as well as the connection between proteome and cryopreservation (Westfalewicz, et. al 2015). Other proteomic investigations, such as those conducted in humans, have revealed that sperm proteins may alter the transcription and post-transcriptional levels of gene expression during the early stages of embryogenesis. (Castilio, et al. 2018). Therefore, one may presume that the presence or absence of certain sperm proteins have impact on spermatozoa's ability to fertilize and enact embryonic development.

When these proteins are present, they are found in the sperm membrane, flagellum, cytoplasm, acrosome, lysosome and the nucleus, where they play key roles in sperm physiology and cellular functions (Dejarnette, 2005; Ashok, et al. 2016). Also, different male glands such as bulbourethral glands, the testis, the prostate gland, seminal vesicles, and epididymis secrete composite fluids which combines to form the seminal plasma (Camargo, et al. 2018; Viana, et.al, 2022). This seminal plasma contains complex molecules that are capable of regulating sperm functions pre- and post-fertilization including modulations in gene expression (Castillo, et al. 2018; Rodriguez-Martinez, et al. 2022). Understanding these integrated events with sperm production, maturation, fertilization and even apoptosis is key to understanding the functional integrity of the spermatozoa (Ashok, et al. 2016).

The abundance or absence of these sperm proteins in bulls may also have an effect on fertility by decreasing or increasing the developmental competence of embryogenesis at the molecular level (DeJarnette, 2005; Klein, et al. 2022), and influence later developmental phases of the embryo. A comprehensive analysis of proteins present in bull sperm can provide essential insight into their functional importance in the regulations of various processes of fertilization and reproduction (Peddinti et al. 2008; Rabaglino et al. 2022; Klien, et al. 2022). Therefore, helping cattle breeders make informed herd improvement decisions for reproductive efficiency and reduce premature disposal of breeding bulls.

There is a dearth of knowledge currently on the sperm proteome of both resting and in-service bulls and their comparative fertility potentials. As a result, breeders are unable to fully comprehend how rest and in-service periods impact bull fertility, leading to financial losses from premature bull disposal on account of reproductive incompetence. This study profiled sperm proteins of rest and in-service Nellore bulls. It identified their interactions, cellular components, biological processes and molecular functions as it impacts their fertility potential as well as compared results obtained for both rest and in-service periods.

1.1. Objectives

The broad aim of this study is to identify seminal plasma proteins associated with fertility/infertility in resting and in-service Nellore bulls.

The specific objectives are:

1. To identify protein components in the seminal plasma of resting and in-service Nellore bulls.
2. To characterize the identified protein into functional annotation and categories in both resting and in-service bulls.
3. To comparatively differentiate the proteins expressed in spermatozoa responsible for fertility/infertility in both resting and in-service bulls.

2. MATERIALS AND METHODS

2.1. Animal selection and semen collection

Twenty Nellore bulls between the ages of 19.8 and 22.7 were used in the study. A Breeding Soundness Evaluation was conducted on the bulls in accordance with the guidelines of the Colégio Brasileiro De Reprodução Animal Manual. Electroejaculation method (Siqueira, et al. 2012) was used to collect ejaculates from all twenty bulls. Samples of ejaculates were collected during their reproductive inactive period for once per week for a month prior to the start of the breeding season, once weekly during the initial month of the in-service duration. Samples collected from bulls at their rest period were identified as Resting Bulls (RB) while samples collected at the breeding season was labelled In-Service Bulls (ISB).

The semen physical characteristics were rated by estimating sperm mass motility on a scale of 0 to 5. An aliquot of semen was examined using conventional optical microscopy to determine the progressive rectilinear sperm motility (in percentage) and sperm vigor on a scale of 0 to 5. (CX31, Olympus, Tokyo, Japan). For the morphological analysis, we added semen aliquot taken from the sampled bulls into 1 ml of buffered saline formol. Pathologies were evaluated in wet preparation, between slide and coverslip at 1000x magnification under immersion, and assessed by phase contrast microscopy (BX41, Olympus, Tokyo, Japan). For each preparation, 200 cells were counted, and the percentage of defects sperm in acrosome, head, mid-piece, and tail was determined. Subsequently, three categories of pathologies were defined: major, minor and total defects (Hancoch, 1957).

2.2. Seminal plasma preparation for proteome analysis

To separate sperm from seminal plasma, the ejaculates of all the bulls were centrifuged using the Heraeus Multifuges X1R (from Thermo Scientific in Waltham, USA), at 10,000g for 10 minutes. We collected the seminal plasma fractions and conserved in 0.5 mL straws in liquid nitrogen at a temperature of -196 °C until they were processed. Subsequently, the seminal plasma from each bull was thawed and filtered through a 0.22 µm mesh membrane to yield the supernatant, which was used in further analysis (Blom, 1973).

2.3. Extraction and quantification of soluble proteins

We extracted the soluble proteins from 100 µL of filtered seminal plasma, precipitated by 600 µL of ice-cold solution acetone:ether (1:1) added with trichloroacetic acid (5%, w/v), and 1 mM DTT, and solubilized in 250 µL of 7 M urea, 2 M thiourea, 4% w/v CHAPS and 40 mM DTT. Then, seminal plasma proteins were quantified using Bradford method (Magalhães, et al. 2016) after calibrating a curve from known concentrations of BSA.

2.4. SDS-PAGE

The SDS-PAGE was conducted in a 2-staged process; separation at 14% T, 3.0% C and stacking: 5.1% T, 2.6% C using mini-Protean Tetra Cell, Bio-Rad (Hercules, USA), at 80 V for 15 minutes and 60 V for 4 h. Each sample used in the SDS-PAGE analysis contained 25 µg of soluble proteins and was compared to a molecular weight marker. (Broad Range, Bio-Rad, Hercules, USA). According to the standards of Laemmli, (1970), the gels were tainted with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, USA) for 2 h, and subsequently untainted for 10 h in 7.5% and 25% (v/v) of methanol solution and acetic acid respectively.

2.5. Sample pooling

Following SDS-PAGE protein separation, five biological replicates were pooled for each group of samples. For samples of RB, we had A1, A2, A3, A4 and A5; and for ISB samples, we had B1, B2, B3, B4 and B5. Each replicate contained 50 µg of proteins were subjected to electrophoresis for approximately one centimeter of resolution in the SDS-PAGE 12.5% gel. Then, gels were cut into two parts and placed in fixative solution (40% (v/v) methanol and 5% (v/v) acetic acid) for 2 h (Resjö, et. al, 2017). For 2 hours, a section of the gel was subjected to protein revelation using Coomassie Brilliant Blue R-250 technique (Bio-Rad, Hercules, USA). The remaining gel was kept in a 5% (v/v) acetic acid solution for additional enzymatic digestion.

2.6. Gel protein digestion

All parts of the gels were individually excised from the replicates (quintuplicate technique), pooled, cut into smaller fragments, and transferred to clean microtubes. We washed out the dye in the gel in three washes using 200 L of 50% v/v ACN and 25 mM ammonium bicarbonate (pH 8.0). Then we dehydrated the gel fragments, followed by vacuum drying using Concentrator Plus AG - 22331, Eppendorf, Hamburg, Germany with 200 L of 100% ACN for 5 minutes. Additionally, we conducted a protein reduction process on the gel fragments with the assistance of 100 uL of 65 mM DTT dissolved in 100 mM ammonium bicarbonate, exposing them to an incubation temperature of 56 °C for a duration lasting approximately 30 minutes. This was followed by gel portions been rinsed, hydrated, and dehydrated twice with ammonium bicarbonate and ACN, respectively. Finally, they were dried in a vacuum concentration system. Dried gel fragments were later rehydrated with Trypsin solution at a concentration of 25 ng/L (Sigma-Aldrich, Burlington, USA). The tubes containing the gel pieces also received 100 L of the activation solution (which was made up of 40 mM ammonium bicarbonate and 10% v/v CAN) following 45 minutes in an ice bath. The tubes were then placed in 37 °C water bath for 22 h. After the enzymatic digestion, the gels were sonicated for 10 min, vortexed for 20 sec, and the solutions were removed to clean microtubes. Subsequently, 200 µL of the recovery solution (5% (v/v) formic acid in 50% (v/v) ACN) was added to the remaining gel fragments.

Each microtube containing the gel fragments and recovery solution was vortexed for another 20 sec, and kept at room temperature for 15 min, and then sonicated for 2 min. Solution was removed and added to the previously reserved solution in the clean tube. This procedure was repeated twice, and the solution was transferred to clean microtubes. Solutions containing the tryptic peptides were concentrated in a vacuum centrifuge system (Shevchenko et al. 2006). After vacuum drying, 10 µL of 0.1% (v/v) trifluoroacetic acid (TFA) solution in ultrapure water was added to each microtube. Then, the tryptic peptides were desalted using C18 reversed-phase micro columns (Millipore, Burlington, USA) and eluted in a 50% ACN solution, acidified with 0.1% (v/v) TFA.

2.7. LC-MS/MS

The tryptic peptides from each biological replicate were solubilized in 20 µL of 0.1% (v/v) aqueous formic acid solution (LC-MS purity grade) and placed in appropriate tubes for application to the nano LC-MS/MS system. After sample preparation, 1µL of the solution was analyzed by nano LC-MS, using the UPLC nanoAcquity system (Waters, Milford, USA), containing a nanoAcquity UPLC® 2G-V/MTrap 5 µm Symmetry® C18 180 µm x 20 mm trap column, at a flow rate of 7 µL/min, for 3 min. Peptides were separated using a 1.7 µm BEH130 100 µm x 100 mm nanoAcquity UPLC® column, operating at a flow rate of 0.3 µL/min. The mobile phase of the chromatographic process had water acidified with 0.1% formic acid (solvent A); and ACN acidified with 0.1% formic acid (solvent B) as solvents. Chromatographic separation took place according to the following schedule: 2% B for 1 min; gradient from 2 to 30% B for 299 min; gradient from 30 to 85% B for 5 min; maintenance at 85% B for 5 min; gradient from 85 to 2% B for 5 min; and maintenance at 2% B for 5 min, totaling 320 min of chromatographic analysis.

The peptides were fed into a MAXIS 3G mass spectrometer (Bruker Daltonics, Billerica, USA) and configured to an online mode using a CaptiveSpray ionization source. An adequate technique (IE GCF 01-02-2017) was used to carry out the analysis of peptides, using a drying gas flow of 3 L/min, an ionization source temperature of 150 °C, and a transmission voltage of 2 kV. The raw data were converted into a list of masses in MGF and compared to a reference database.

2.8. Protein identification and functional classification of proteins

Mascot 2.4.0 (Matrix Science, London, UK) was used to compare the MGF files to the reference database. This reference database was a set of canonical protein sequences from the Bovidae family (Taxonomy ID 9895) available at the UniProt Knowledgebase database (UniProtKb; https://www.uniprot.org/ accessed in 28-03-2018). Peptide were then identified using parameters such as: methionine oxidation as a variable modification, trypsin enzymatic digestion (with one missed cleavage), and cysteine carbamidomethylation as a fixed modification. The error tolerance allowed for the acquired data was 30 ppm for the parental ion and 0.6 Da for the fragments, with the ion charge varying between +2 and +4.

Scaffold Q+ version 4.0 (Proteome Software Inc., Portland, USA) was used to confirm the Mascot results. Identified peptides were validated using the Peptide Prophet algorithm as reported by Keller, Nesvizhskii and Kolker, (2002), and proteins were verified using the Protein Prophet algorithm (Nesvizhskii, et al. 2003) and using the probability threshold for identification: 0.95, a false discovery rate of less than 1%, and a minimum number of peptides for protein identification. For this study we only considered proteins identified in at least two replicates.

For functional analysis, Gene Ontology (GO) was used. This term attributed to the identified proteins that were available at UniProtKB database. Functional enrichment analysis, GO terms was done by the Database for Annotation, Visualization, and Integrated Discovery platform (DAVID; https://david.ncifcrf.gov/home.jsp) (Sun, et al. 2003). Identified proteins were classified by the RPSBLAST tool of BLAST version 2.13.0 (Altschul et al. 1990) in categories of the EuKaryotic Orthologous Groups (KOG) database (Tatusov, et al. 2003), using an E-value threshold of $1e^{-10}$ for selecting the significant alignments.

2.9. Quantitative analysis of identified proteins

The Label-free quantitative analysis considered only proteins identified in at least three technical replicates of each treatment. The abundance of selected proteins was estimated by Exponentially Modified Protein Abundance Index (emPAI) using the Scaffold Q+ software version 4.0 (Pini, et al. 2018). Proteins with missing data were filtered out and not considered for differential expression analysis. Differentially expressed proteins were identified by msmsTests package (Gregori, et al. 2013) using R version 4.2.2 (https://cran.r-project.org) The calculated p-values were corrected using Benjamini-Hochberg procedure, and DEP proteins were those with FDR<0.05.

2.10. Venn diagram

A Venn diagram showing the relationship between identified sperm proteins from each RB and ISB was generated with library “venn” in R 4.2.2 (https://cran.r-project.org/)

2.11. STRING analysis: protein-protein interaction

A list of identified proteins corresponding to sperm interacting in both RB and ISB were uploaded to STRING and mapped to the bovine species database. The sperm proteins were mapped based on the existence of some fertility-related sperm proteins found in both RB and ISB and those upregulated in RB and ISB. The interaction was clustered using MCL algorithm (Enright, et al. 2002) with the inflation parament set to 3.

3. RESULTS

3.1. Protein identification

The LC-MS data analysis identified 155 proteins (Figure 1) in the samples from the bulls at their resting and in-service periods. 89 proteins were specific to bulls at both periods, 5 proteins were found only in bulls in-service, ISB; while 61 were found in the bulls at their rest period. Tables1a and 1b show proteins identified in both Groups A and B, and their corresponding gene names.

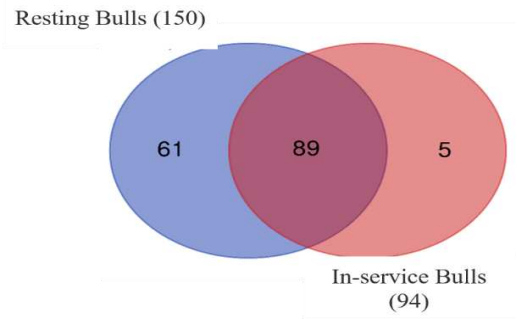


Figure 1 – Total number of proteins identified in RB and ISB

Table 1a – Protein specific to RB (Resting Bulls)

Entry	Protein names	Gene Names
E1B7S8	Acrosin-binding protein	ACRBP
Q2NKZ3	ADAM metallopeptidase domain 32	ADAM32
P00570	Adenylate kinase isoenzyme 1	AK1
P02769	Albumin	ALB

Q7SIH1	Alpha-2-macroglobulin	A2M
Q9XSJ4	Alpha-enolase	ENO1
F1MBJ3	Alpha-mannosidase	LOC107132586
Q58D55	Beta-galactosidase	GLB1
A3KMY8	Beta-glucuronidase	GUSB
P25326	Cathepsin S	CTSS
Q148D9	Cellular repressor of E1A-stimulated genes 1	CREG1
A7E3B2	Deoxyribonuclease-1	DNASE1L3
A6QNX2	DPP7 protein	DPP7
Q0VCX2	Endoplasmic reticulum chaperone BiP	HSPA5
E1B9P4	Epididymal sperm binding protein 1	ELSPBP1
Q3SZZ9	FGG protein	FGG
P02672	Fibrinogen alpha chain	FGA
P02676	Fibrinogen beta chain	FGB
F1MGU7	Fibrinogen gamma-B chain	FGG
Q3SZB7	Fructose-1,6-bisphosphatase 1	FBP1
B2KJ42	fructose-bisphosphatase	CDH2
D4QBB4	Globin A1	HBB
Q3ZBD7	Glucose-6-phosphate isomerase	GPI
Q28120	Glutaminyl-peptide cyclotransferase	QPCT
Q76LV2	Heat shock protein HSP 90-alpha	HSP90AA1
D4QBB3	Hemoglobin beta	HBB
P02070	Hemoglobin subunit beta	HBB
A0A3Q1MA45	Hensin	SRCR domain containing protein
P62803	Histone H4	
G3MXB5	IgA constant region	
A5D7Q2	Igh protein	
Q3SYR8	Immunoglobulin J chain	JCHAN
F1N191	Interleukin 4 induced 1	IL4I1
Q32LE5	Isoaspartyl peptidase/L-asparaginase	ASRGL1
F1MYA6	IZUMO family member 4	IZUMO4
A0A3Q1M1V3	Kazal-like domain-containing protein	LOC100296105
Q95114	Lactadherin	MFGE8
E1B6Z6	Lipocalin 2	LCN2
E1BDF3	Matrilin 4	MANTA
E1BG25	Melanotransferrin	MELTF
Q3T0K7	MFGE8 protein	MFGE8
A6QPE2	MGC148336 protein	MGC148336
E1BI74	NAD(P)(+)--arginine ADP-ribosyltransferase	LOC507756
P13696	Phosphatidylethanolamine-binding protein 1	PEBP1
Q32KN6	Phosphoglycerate kinase	PGK2
F1N2F2	Phosphoglycerate mutase	PGAM2
Q32KV0	Phosphoglycerate mutase 2	PGAM2
F1MB08	Phosphopyruvate hydratase	ENO1
A5D7U1	Placenta-expressed transcript 1 protein	PLET1
P0CG53	Polyubiquitin-B	UBB

P0CH28	Polyubiquitin-C	UBC
O02853	Prostaglandin-H2 D-isomerase	PTGDS
A6QQ77	Sperm acrosome membrane-associated protein 3	SPACA3
Q2PMM0	Sperm inner acrosomal membrane protein IAM38	
Q2KIM0	Tissue alpha-L-fucosidase	FUCA1
P62992	Ubiquitin-40S ribosomal protein S27a	RPS27A
P63048	Ubiquitin-60S ribosomal protein L40	UBA52
A0A3Q1M4K3	Ubiquitin-like domain-containing protein	LOC101902760
F1MNH9	WAP four-disulfide core domain protein 13	LOC112449384
Q32KZ7	Zona pellucida binding protein	ZPBP
E1BNL3	Zonadhesin	ZAN

Table 1b – Protein specific to ISB

Entry	Protein names	Gene Names
Q3T101	IGL@ protein	IGL@
A0A3Q1LWV8	Ig-like domain-containing protein	
P31096	Osteopontin	SPP1
P04557	Seminal plasma protein A3	
H7BWW2	Beta-hexosaminidase	HEXB

3.2. Quantitative analysis of identified proteins

Quantitative analysis using all identified sperm proteins (Figure 2). is shown in a correlation matrix revealing a positive correlation but 28 sperm proteins showed high correlation between 0.8 and 1 and where differentially abundant ($p<0.05$) in both RB and ISB. Beta-hexoaminidase (HEXB) and beta-hexoaminidase (E1BNL3) were significantly expressed ($p<0.05$) and only specific to samples from ISB. ADAM metallopeptidase domain 32 (ADAM32), glucose-6-phosphate isomerase (GPI), zonadhesin (ZAN), glutaminyl-peptide cyclotransferase (QPCT), fructose-bisphosphatase, cellular repressor of E1A-stimulated genes 1 (CREG1), adenylate kinase isoenzyme 1 (AK1), beta-glucuronidase (GUSB), albumin (ALB), zona pellucida binding protein (ZPBP), sperm acrosome membrane-associated protein 3 (SPACA3), isoaspartyl peptidase/L-asparaginase (ASRG1), and interleukin 4 induced 1 (IL4I1) were significantly abundant ($p<0.05$) in RB while the reminding eleven sperm proteins differentially expressed were specific to both Groups A and B.

The 28 differentially abundant protein were further analyzed using a heat map (Figure 3) to determine their relative reliability. Spermadhesin-1 (SPADH1) and uncharacterized protein (LOC101907989) were upregulated in ISB, albumin (ALB) was upregulated in A1 replicate sample of RB, the reminder 25 abundantly expressed ($p<0.05$) proteins were upregulated in RB and downregulated in ISB, this includes ACE, CPQ, IL4I1, AK1, CES5A, ASRGL1, GAPDHS, LOC112441537, ZPBP, bovin proacrosine, SPACA3, ZAN, HEXB, GPI and QPCT. Other protein was revealed zero-reliability for RB such as SRN and seminal plasma protein PDC-109 but were downregulated in ISB.

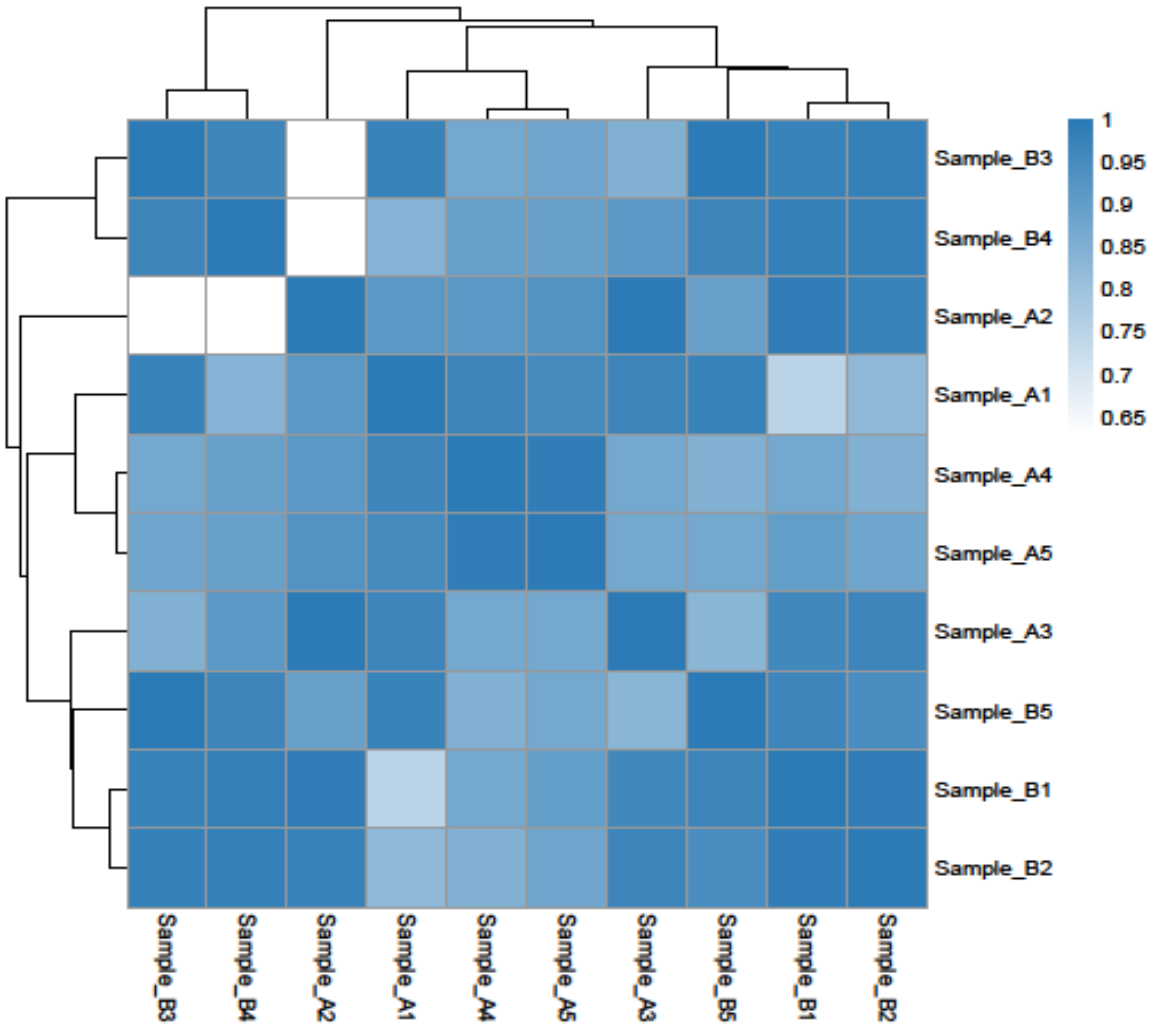


Figure 2 – Correlation matrix showing sperm protein samples from RB and ISB.

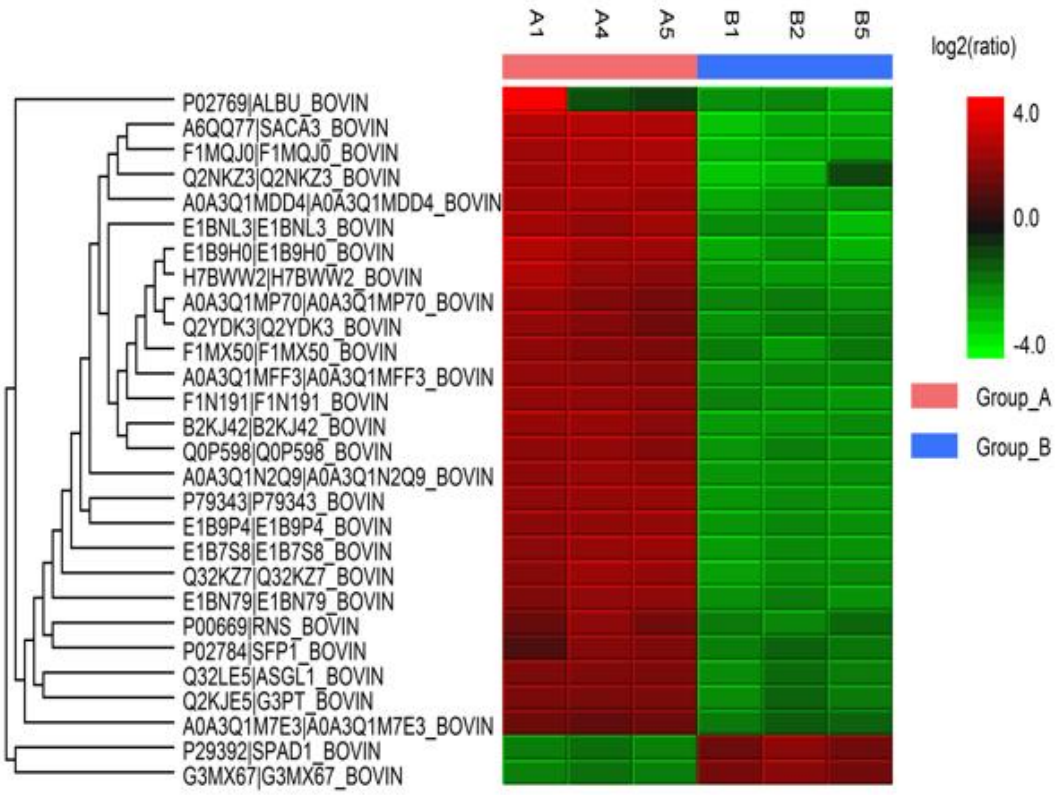
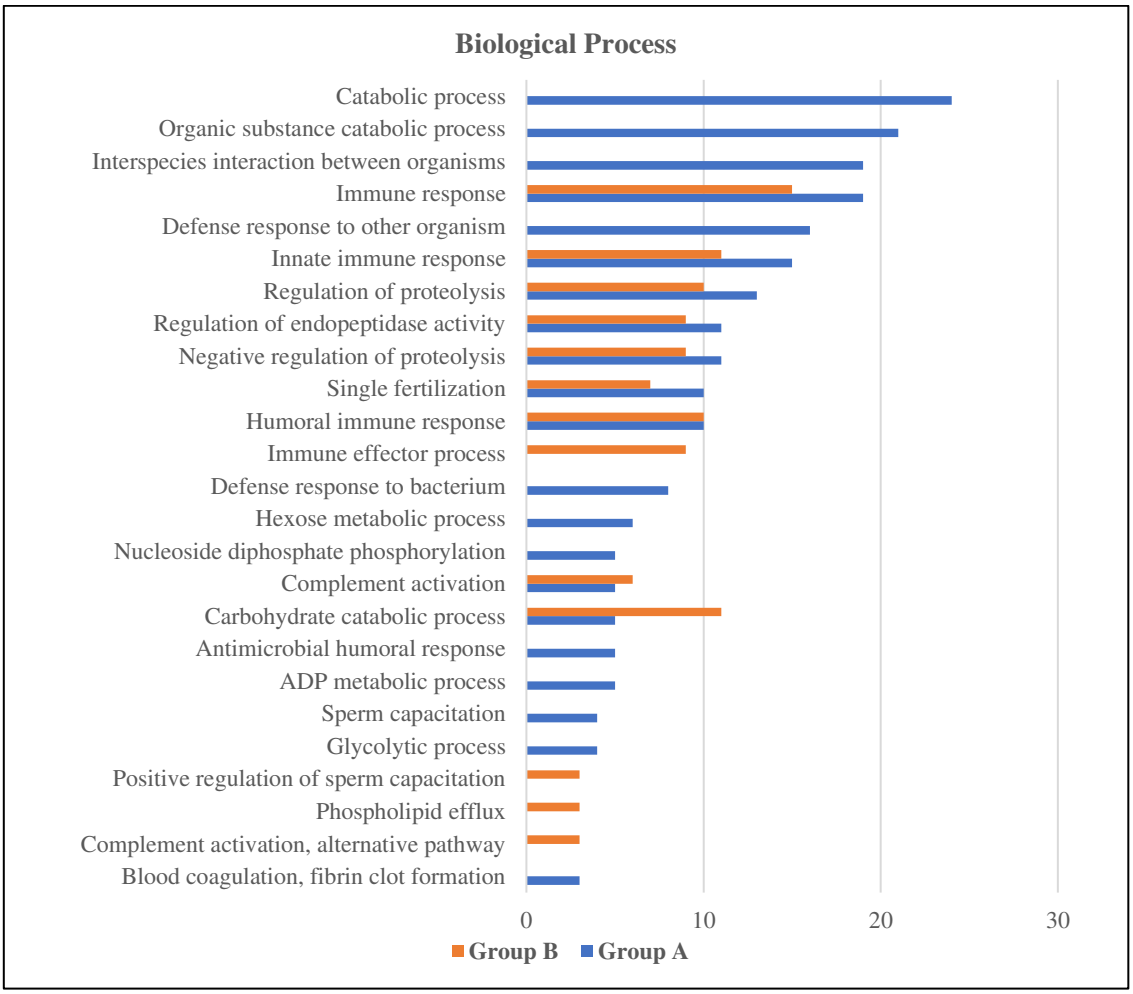


Figure 3 – Heat map showing 28 abundantly expressed sperm protein upregulated in red highlight, downregulated in green and zero reliability black in relationship with RB and ISB.

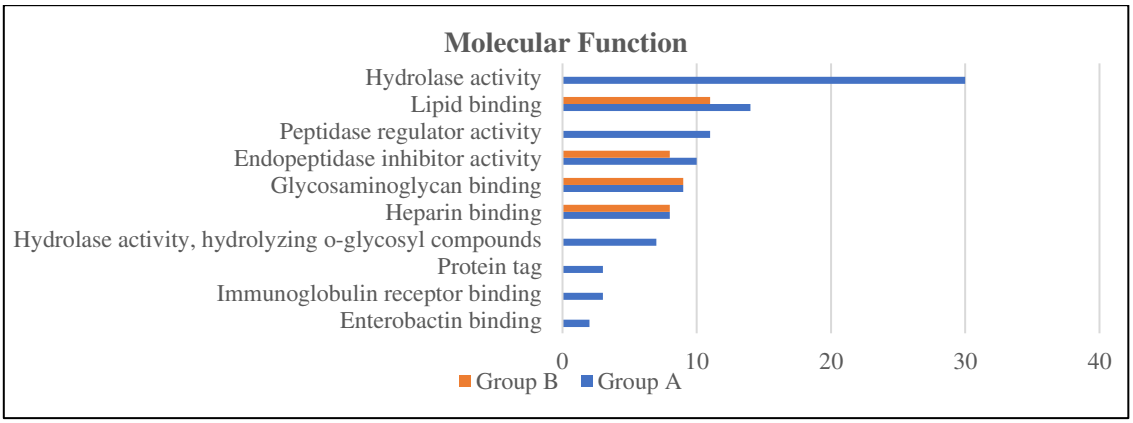
3.3. Functional classification of identified proteins

Based on the gene ontology, biological processes associate with only ISB were complement activation, alternative pathway, phospholipid pathway and positive regulation of sperm capacitation. Biological processes associated with RB were fibrinolysis, glycolytic processes, ADP metabolic processes, defense response to bacteria, nucleoside diphosphate phosphorylation and antimicrobial humoral responses among others (Figure 3A). The molecular function of these proteins mainly related to hydrolase activity, lipid binding, heparin binding, peptidase regulation activity, glycosaminoglycan binding among others (Figure 3B). The cellular component of these proteins was found to be fibrinogen complex, extracellular space and region, vacuole, intermediate filament and lysosome (Figure 3C).

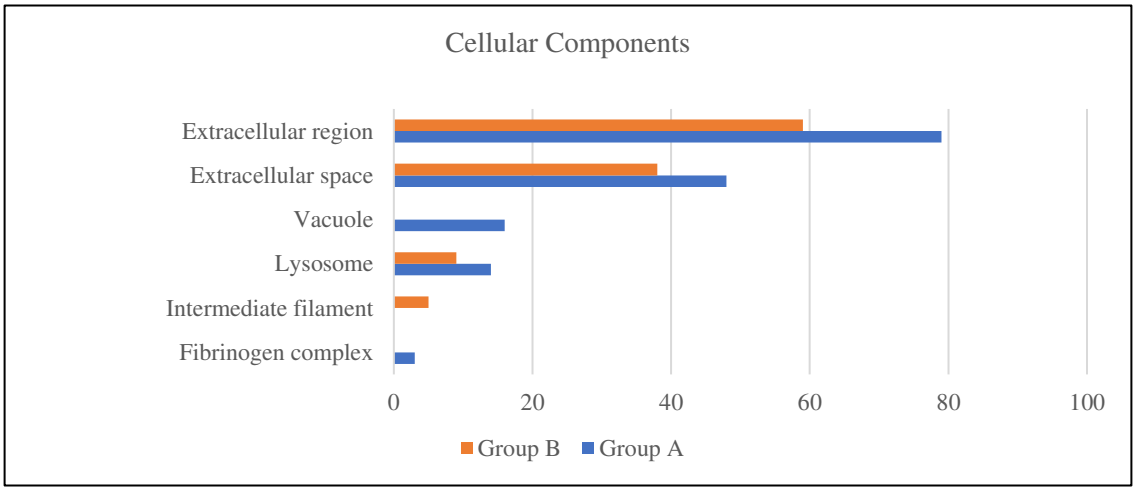
A


Biological Process
Catabolic process
Organic substance catabolic process
Interspecies interaction between organisms
Immune response
Defense response to other organism
Innate immune response
Regulation of proteolysis
Regulation of endopeptidase activity
Negative regulation of proteolysis
Single fertilization
Humoral immune response
Immune effector process
Defense response to bacterium
Hexose metabolic process
Nucleoside diphosphate phosphorylation
Complement activation
Carbohydrate catabolic process
Antimicrobial humoral response
ADP metabolic process
Sperm capacitation
Glycolytic process
Positive regulation of sperm capacitation
Phospholipid efflux
Complement activation, alternative pathway
Blood coagulation, fibrin clot formation
0
10
20
30
Group B
Group A

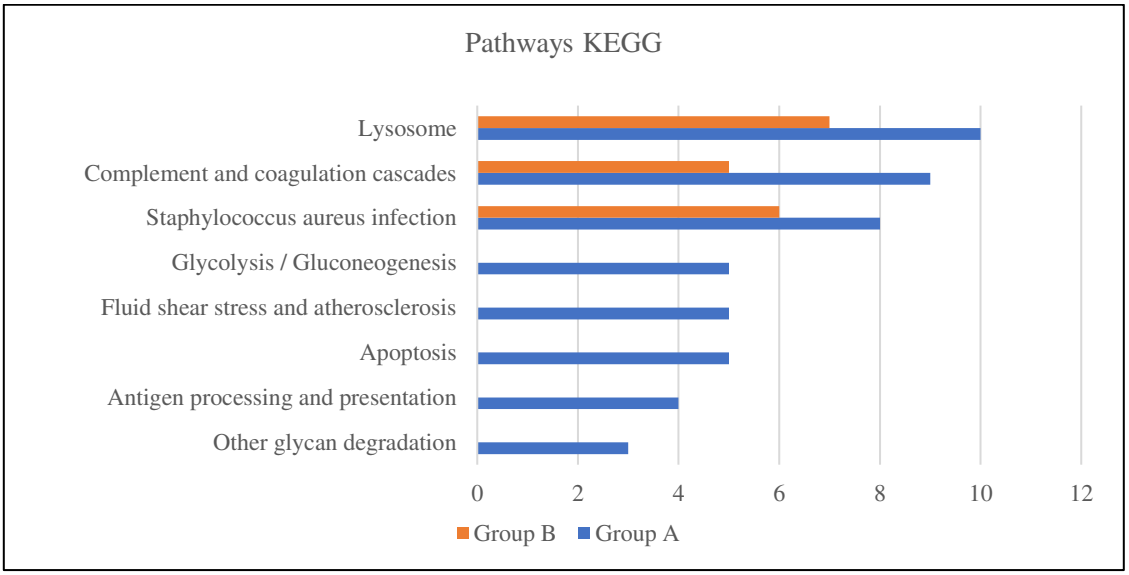
B


Molecular Function
Hydrolase activity
Lipid binding
Peptidase regulator activity
Endopeptidase inhibitor activity
Glycosaminoglycan binding
Heparin binding
Hydrolase activity, hydrolyzing o-glycosyl compounds
Protein tag
Immunoglobulin receptor binding
Enterobactin binding
0
10
20
30
40
Group B
Group A

C



D



Figures 4A, B, C, D – Graph representation of number of identified proteins. All proteins identified were classified by GO terms based on biological processes, molecular functions, cellular component and KEGG pathways.

KEGG pathway analysis performed (Figure 3D) demonstrated that differentially expressed ($p<0.05$) proteins of RB were primarily associated with glycan degradation, fluid shear stress and atherosclerosis; cellular apoptosis, gluconeogenesis, antigen processing and presentation. While proteins from both Groups A and B showed common connotation for lysosome, S*taphylococcus aureus* infection and complement and coagulation cascades with higher protein count from RB.

3.4. STRING analysis

The STRING analysis results (Fig. 5) showed interaction between fertility-related proteins (GAPDHS, TIMP2, SRN, RNASE4, BSP1, SPACA3, BSP5) identified in both RB and ISB and other proteins upregulated (Table:2) in both groups. The analysis for RB revealed spermadhesin-1 (SPADH1) network linages with fertility-related proteins such as SPADH1, NUCB1, RNASE4, BSP1, BSP3, BSP5, SRN and NPPC; while Osteopontin (SPP1) clustered in a network with GAPDHS, LALBA, MMP2, CD44, GAPDH, TIMPS, MMP14 and CLU which are proteins responsible for metabolic processes, glycolysis, spermatogenesis, sperm-oocyte adhesion and fusion as well as energy production. Other clusters include GAPDH, GAPDHS, PGAM1, ALDOA, GPI, ALDOC, PGK1, PGAM2, TPI1, FBP1 and ALDOB; F2, F2R, SERPINA5, FGA, SERPIND1 AND SERPINC1. These are responsible for metabolic processes, phospholipid efflux, energy production, glycolysis, spermatogenesis, sperm-oocyte adhesion and signaling processes, homeostasis, complement and coagulation cascades.

Interaction of proteins from RB showed a network of proteins that are responsible for metabolic processes, energy production, glycolysis, spermatogenesis, sperm-oocyte adhesion and signaling processes such as GAPDHS, PGK1, GPI, ALDOA, ALDOC, HK1, PFKL, PFKM, PFKP, PGK1 and AK1 (Fig.5) with cluster of bovine seminal plasma protein (BSP1, BSP5), TIMP2, and RNASE4. Other proteins that were upregulated in RB include albumin (ALB) which clustered with ACE and Matrix metalloproteinase-2 (MMP2) and in turn, clustered with SRN and SPACA3. The clusters of RB were similar to that of ISB by their functional annotation except for proteins such as ADAM32, CES5A, CPQ, CREG1, ASRGL2 and IL4I1 which share no link with other upregulated proteins in RB.

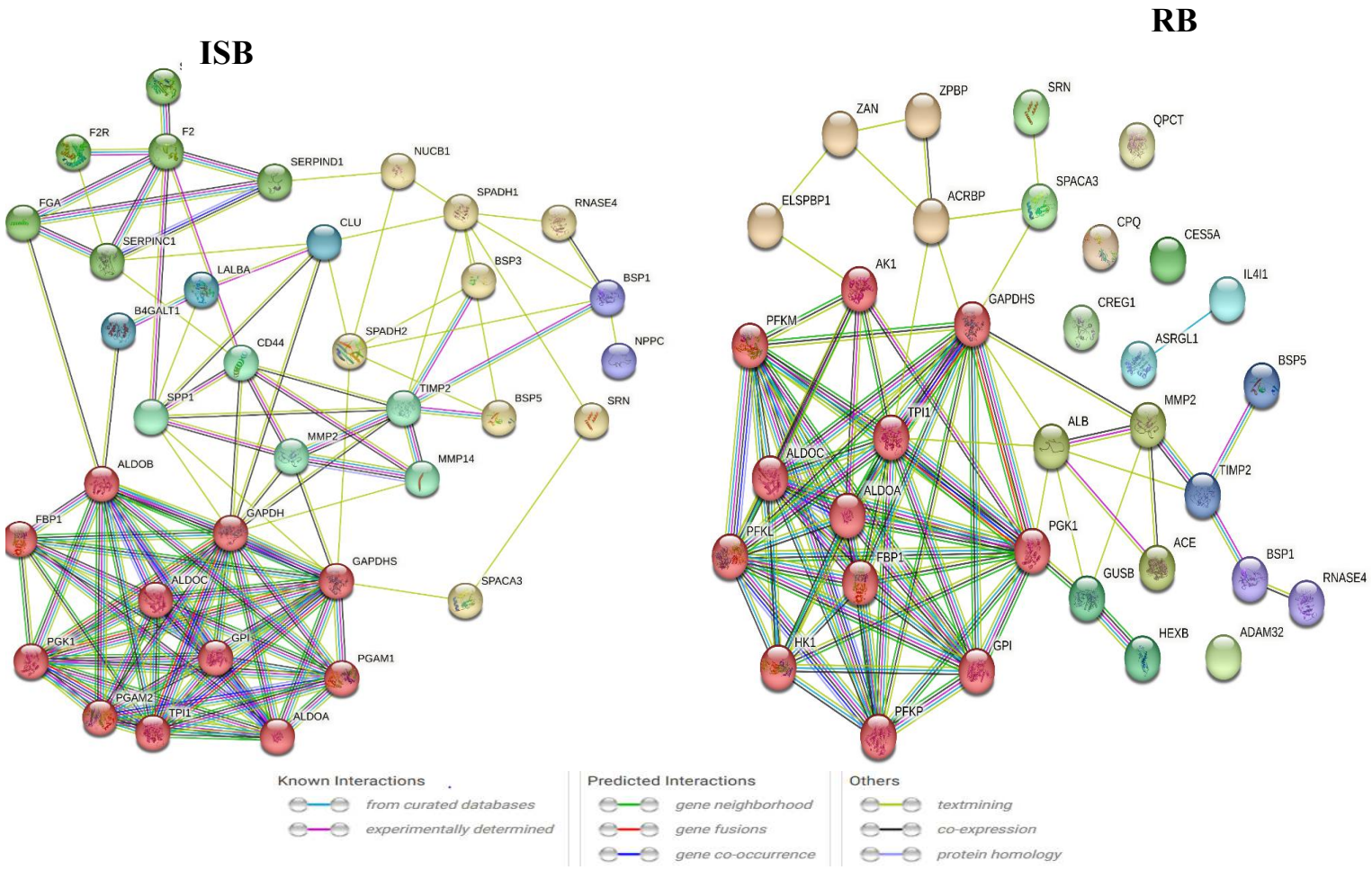


Figure 5 – STRING analysis genes with SPADH1 (upregulated in ISB) and sperm proteins unregulated in RB and other fertility-related sperm proteins. These proteins are linked with confidence network edges and the thickness of the line determines the degree of confidence in the linkages; The colored nodes represent query proteins and first shell of interactions. The filled nodes represent the known or predicted 3D protein structure. Protein names can be found in UniProtKB database (www.uniprot.org)

Table 2 - Differentially (p<0.05) expressed proteins between of RB and ISB, reliability and functional attributes

Accession entry	Protein description	Gene ID	Group reliability	Functional attribute (from UniProtKB database)
G3MX67	Uncharacterized protein	LOC101907989	ISB upregulated	Uncharacterized protein
P29392	Spermadhesin-1	SPADH1	ISB upregulated	This protein appears to be a potent growth factor with effects on ovarian granulosa cells; heparin binding; positive of sperm capacitation; participates in sperm-egg binding
Q28120	Glutaminyl-peptide cyclotransferase	QPCT	RB upregulated	Responsible for the biosynthesis of pyroglutamyl peptides. catalyzes N-terminal pyroglutamate formation
B2KJ42	fructose-bisphosphatase		ISB upregulated	Negative regulator of glycolysis; generating NADPH.
F1MX50	Cellular repressor of E1A-stimulated genes 1	CREG1	RB upregulated	Regulation of transcription, DNA-dependent
Q2YDK3	Hyaluronidase	SPAM1	RB upregulated	Involves in single fertilization, sperm-oocyte fusion
A0A3Q1MP70	Glucose-6-phosphate isomerase	GPI	RB upregulated	Positive regulation of immunoglobulin production, position regulation of endothelial cell migration, in utero embryonic development, part of pyruvate kinase activity
H7BWW2	Beta-hexosaminidase	HEXB	RB upregulated	Ganglioside catabolic process, beta-N-acetylhexosaminidase activity, Metabolic pathways,
E1B9H0	Beta-hexosaminidase	LOC786974	RB upregulated	Carbohydrate metabolic process
E1BNL3	Zonadhesin	ZAN	RB upregulated	Signaling and cellular processes

A0A3Q1MDD4	Beta-glucuronidase	GUSB	RB upregulated	Inhibited by L-aspartic acid; carbohydrate metabolic process, keratan sulfates degradation
Q2NKZ3	ADAM metallopeptidase domain 32	ADAM32	RB upregulated	Metalloendoprotease activity, peptidolysis,
F1MQJ0	Angiotensin-converting enzyme	ACE	RB upregulated	Testis development, regulates of angiotensin metabolism, post-transcriptional gene expression,
P02769	Albumin	ALB	RB upregulated	Preserve sperm against lipid peroxidation
A6QQ77	Sperm acrosome membrane-associated protein 3	SPACA3	RB upregulated	Sperm-oocyte adhesion and fusion (fertilization)
P79343	Acrosin	bovin proacrosine	RB upregulated	Spermatozoa protease
Q32KZ7	Zona pellucida binding protein	ZPBP	RB upregulated	Sperm-oocyte adhesion at oocyte zona pellucida glycoprotein
E1B9P4	Epididymal sperm binding protein 1	ELSPBP1	RB upregulated	Relates to sperm capacitation
E1B7S8	Acrosin-binding protein	ACRBP	RB upregulated	Acrosome formation and fertilization
A0A3Q1M7E3	Glutathione peroxidase	LOC112441537	RB upregulated	Responds to oxidative stress, peroxidase reaction
Q2KJE5	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	GAPDHS	RB upregulated	Causes alterations between different pathways for energy production during spermatogenesis and in the spermatozoon. Needed for sperm motility and male fertility. metabolic pathways, binds to sperm fibrous sheath
Q32LE5	Isoaspartyl peptidase/L-asparaginase	ASRGL1	RB upregulated	Metabolic pathways, alanine, aspartate and glutamate metabolism

P00669	Seminal ribonuclease	SRN	RB upregulated	Sperm capacitation, anti-oxidative function, immunosuppression, catalytic activity
P02784	Seminal plasma protein PDC-109		RB upregulated	Interacts with heparin-like glycosaminoglycan of the female genital tract, prevents premature capacitation; enhances spermatozoa fertilizing capacity.
E1BN79	Carboxylic ester hydrolase	CES5A	RB upregulated	Hydrolase activity, acting on glycosyl bonds
A0A3Q1N2Q9	Adenylate kinase isoenzyme 1	AK1	RB upregulated	Energy metabolism
F1N191	Interleukin 4 induced 1	IL4I1	RB upregulated	Regulation of T lymphocyte proliferation, catalytic processes.
A0A3Q1MFF3	Carboxypeptidase Q	CPQ	RB upregulated	Metalloexodipeptidase activity,

4. DISCUSSION

In this study, young Nellore bulls were used as model to compare fertility potential for RB and ISB. Previous investigations on bovine sperm proteomics revealed the presence of various proteins in spermatozoa which include GAPDH, ACE, and various seminal plasma proteins including C-type natriuretic peptide (NPPC), sulfhydryl oxidase (QSOX1), Osteopontin (SPP1), seminal plasma protein PDC-109, seminal plasma protein A3 (BSP3), seminal plasma protein-30kDa (BSP5), seminal ribonuclease (SRN) and metalloproteinase inhibitor 2 (TIMP2) to be proteins associated with fertility (Gwathmey, et al. 2006; Druart & De Graaf, 2018; Sutovsky, 2018; Özbek, et al. 2021; Ashwitha, et al. 2023), these results were similar to the proteins identified in our study.

RB expressed proteins which participate in inhibitory pathways, heparin-binding and metabolic processes such as glycolysis/gluconeogenesis, hexose metabolic processes and protein tag, proteolysis, fluid shear stress, antigen processing, oxidative phosphorylation, mast cell mediation, apoptosis or acrosome reaction and proteolysis, (Figure 3A, 3D). These proteins included AK1, ADAM32, PLA2G7, PTGDS, E1B9P4, FUCA1, SPACA3, PGAM2, A2M, HSPA5 (Table 1) among others (Valencia et al. 2017). The function of these protein however, are biological processes common to mammalian sperm physiology (Gomes, et. al. 2020) but in our study these proteins were only present in samples of RB.

RB samples also contained proteins responsible for ubiquitination which is critical in protein degradation (Grice and Nathan, 2016; Guo, et. al. 2023); a pathway targeting cell signaling, apoptosis, protein processing, stress and extracellular modulations, immune response and inflammations, and DNA repair. These interconnected ubiquitin proteins were identified in sample of RB in our study and were ubiquitin-60S ribosomal protein L40 (UBAS2), polyubiquitin-B (UBB), polyubiquitin-C (UBC) and ubiquitin-40S ribosomal protein S27a (RPS27A). According to Gomes, et al. (2020), sperm ubiquitinated during transit can be degraded and defective even after ejaculation in the female tract. This may suggest an impact of rest periods on fertility potential of bulls.

Samples from ISB were abundant in protein enriched with, sperm binding proteins, proteins unique in adaptive immune system, phospholipid efflux, energy metabolism, positive regulation of sperm capacitation and proteins involved in pathways that regulates cellular functions

such as transcription, translation, proliferation, cell signaling pathways, cell growth and survival (Figure 4A, 4D). These proteins include SPP1, seminal plasma protein PDC-109, seminal plasma protein A3, seminal plasma protein -30kDa, SRN, SPADH1, NPPC, CLU, SPADH2 and TIMP2.

The STRING analysis allowed us to further investigate key proteins that were upregulated for ISB and their interconnected proteins (Figure 5) allowing further details into specific functions of these protein in relation to fertility. SPADH1 appears to have broader connectivity with proteins that play major roles in phospholipid efflux, energy pathway, reproductive processes, sperm motility and capacitation, DNA synthesis, cell growth and survival and carbohydrate-binding protein with BSP1, BSP3, BSP5, ANG, RNASE4, SRN, CLU, TIMP2 and NUCB1; which in turn are linked to SPACA3, SPADH2, SPP1, SPAM1 and SERPINA5. The results from this analysis suggest that the proteins from ISB seems to be positively linked to fertility-related processes and fertilization.

Similarly, the STRING analysis for RB showed same result in comparism to those related to fertility in ISB. However, some proteins that were upregulated in RB such as ZAN, ADAM32, CES5A, CPQ, CREG1, ASRGL2 did not show network connection to fertility-related proteins in RB. Earlier investigations revealed that seminal plasma proteins such as PGAM2, GAPDHS, GPI, HSP90B1, HSPA5, ENO1, GLB1, QPCT and MANBA (Ozbek et. al, 2021) were associated with pathways and processes that causes asthenospermia (reduced motility in sperm) and were found highly differential in asthenozoospermic human male patients (Ozbek et. al, 2021).

Our study revealed that although samples of RB contained proteins that were linked to fertility-related processes, they also contained proteins that were critical to protein ubiquitination and other pathways which may impair spermatogenesis and high count of defective sperm during resting periods. In ISB, the sperm proteins spermadhesin-1 (SPADH1) was upregulated, well-known factor that stimulates cell division and the secretion of progesterone by bovine granulosa cells in vitro. This protein appears to also be a potent growth factor with effects on ovarian granulosa cells. It actively contributes to the binding and fusion of sperm and egg during fertilization and does this with other functional partners such as BSP1, BSP3, BSP5, ANG, RNASE4, NUCB1 and SRN. Perhaps, the presence or absence or abundance of these sperm proteins in the seminal plasma, their cellular interaction, molecular functions and biological processes influence the fertility potential of bull during in-service periods.

CONCLUSION

This study has revealed that specific pathways may affect fertility of resting bulls. The in-service bulls demonstrated alterations in various protein clusters from the STRING analysis which are relevant to sperm motility, capacitation-related events and sperm-egg fusion. The comparative analysis of the sperm proteome in in-service and resting bull cattle presents an opportunity to understand molecular mechanisms underlying male fertility and reproductive rest periods especially in male animals with a seasonal breeding cycle. This study suggests prior clean-up of breeding bulls to guarantee that the reproductive tract is cleansed of defective sperm cells in preparation for another breeding season; this will ensure favorable biological processes and positive correlations of other sperm proteins in the regulation of sperm maturation, functions, fertility and oocyte-sperm interaction.

The knowledge from this study would be valuable in developing strategies to improve reproductive efficiency in bull cattle and reduce premature disposal of breeding bulls due to subfertility; such as identifying potential targets for genetic interventions or nutritional enhancements that can boost sperm production and quality during reproductive rest periods. However, additional studies are needed in order to determine the specific roles played by sperm-specific proteins at resting and in-service periods that affect fertilization process.

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