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Proteomic analyses identify differences between bovine epididymal and ejaculated spermatozoa that contribute to longevity

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ABSTRACT

Sperm are stored for extended periods of time in the epididymis, but upon ejaculation motility is increased and lifespan is decreased. The objective of this study was to identify differences in proteins between epididymis and ejaculated samples that are associated with longevity. Ejaculated semen was collected from mature Angus bulls (n = 9); bulls were slaughtered and epididymal semen was collected. Epididymal and ejaculated semen were centrifuged to separate sperm and fluid. Fluids were removed and sperm pellets were resuspended in a high ionic solution and vortexed to remove loosely attached proteins. Sperm samples were centrifuged, and the supernatant was removed; both fluid and sperm samples were snap frozen in liquid nitrogen and stored at −80 °C. Protein analysis was performed by LCMS/MS. A different group of yearling Angus cross bulls (n = 40) were used for sperm cultures. Ejaculated (n = 20) and epididymal (n = 20) semen were diluted and cultured in a commercial media at pH 5.8, 6.8 and 7.3, at 4 °C. Sperm were evaluated for motility and viability every 24 h until motility was lower than 20%. There was an effect of pH, time and pH by time interaction for motility and viability for both ejaculated and epididymal sperm ($P \leq 0.05$). At 216 h of incubation epididymal sperm at pH 7.3 and ejaculated sperm at pH 6.8 reached motility below 20%. A total of 458 unique proteins were identified; 178, 298, 311, and 344 proteins were identified in ejaculated fluid, ejaculated sperm, epididymal fluid and epididymal sperm, respectively. There were 8, 24, 10, and 18 significant KEGG pathways (FDR <0.05) for ejaculated fluid, epididymal fluid, ejaculated sperm, and epididymal sperm, respectively. The metabolic pathway was identified as the most important KEGG pathway; glycolysis/gluconeogenesis, pentose phosphate, and glutathione metabolism pathways were significant among proteins only present in epididymal samples within the metabolic pathway. Other proteins identified that may be related to epididymal sperm's increased longevity were peroxidases and glutathione peroxidases for their antioxidant properties. In summary, energy metabolism in the epididymis appears to be more glycolytic compared to ejaculated and epididymis sperm have a larger number of antioxidants available which may be helping to maintain sperm in a quiescent state. Epididymal sperm remained viable (membrane integrity) longer than ejaculated sperm when cultured at the same pH.

1. Introduction

During spermatogenesis final maturation, spermatozoa lose their ability to biosynthesize, repair, grow, and divide, and become very simple in their metabolic function [1]. This results in spermatozoa becoming completely dependent on their external environment to survive and function. While in the epididymis, spermatozoa are stored for a long period of time in a relatively quiescent state. It is hypothesized that this is due to both quiescence and prevention of premature activation of the spermatozoa prior to ejaculation [2]. Upon ejaculation or dilution of the fluid of the caudal epididymis, motility is increased [3,4]. A consequence of this increased motility is a reduction in viability to only several hours in most species [5].

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Several studies have reported that the plasma membrane of spermatozoa is coated with glycoproteins [6–8], and several proteins that have been identified in the epididymal fluid are enzymes that are able to modify proteins or lipids at the spermatozoa surface. A subset of these proteins are implicated in spermatozoa protection (e.g. members of the Glutathione S-transferase family or peroxiredoxin isoforms) [9], and some of the proteins that are transferred to the spermatozoa are also proposed to modulate motility [10–12]. Macrophage migration inhibitory factor (MIF) protein, present in the epididymis, associates with the spermatozoa flagella, and may influence thiol; therefore impacting acquisition of spermatozoa motility [13], as a negative correlation had been reported between concentration of MIF and spermatozoa motility [2].

During ejaculation, epididymal sperm is mixed with seminal plasma from the accessory sex glands while epididymal fluid that kept sperm in a quiescent mode is diluted, consequently, sperm motility increased to a predominantly progressive motility pattern [3,4,14,15]. It has been reported that besides dilution of epididymal fluid, seminal plasma proteins, originated from the accessory sex glands, are attached to the sperm during ejaculation [16,17]. Thus, the objective of this experiment was to identify differences in proteins that are both in the environment (fluid) and loosely attached to spermatozoa in both the epididymis and following ejaculation measured by liquid chromatography with tandem mass spectrometry analysis (LCMS-MS). A secondary objective was to evaluate the effect of pH on sperm longevity. The hypotheses were that proteins differentially expressed between ejaculated and epididymis samples would be correlated to sperm longevity and sperm incubated in uterine pH at estrus (pH = 6.8) would have greater motility and longevity.

2. Materials and methods

All procedures were approved by the South Dakota State University Institutional Animal Care and Use Committee.

2.1. Study I

2.1.1. Experimental design

Semen from nine sexually mature (4-y old) Angus bulls with a history of successful breeding were collected by electro-ejaculation. Weekly ejaculates were collected for 2 wk and discarded (2 ejaculates); bulls were rested for one week before a third weekly ejaculate was collected. After the third semen collection bulls were rested for 6 wk to renormalize epididymal reserves and then slaughtered. Testes and epididymides were collected and transported back to the laboratory. Epididymides were dissected and epididymal semen was collected from the caudal section of the epididymis. Ejaculated and epididymal sperm were evaluated for motility, viability and mitochondrial membrane potential at the time of semen collection. Ejaculated sperm were evaluated at physiological pH (pH 7.3) and epididymal sperm were evaluated at physiological pH (pH 5.8) and at pH 7.3. Epididymal semen from a subset of bulls (n = 3) were cultured for 310 h at 4 °C in three different pH; physiological pH 5.8, pH 7.3 mimicking ejaculated pH, and pH 6.8 which has been reported to be the uterine pH at estrus [18,19]. The remainder of samples were processed for protein analysis.

2.1.2. Spermatozoa culture and analysis

Aliquots of each sample (ejaculated and epididymal) were evaluated immediately at collection, 0 h for ejaculated and 24 h after slaughter for epididymal. Samples were stained with Hoechst 33258 and evaluated for motility and viability (plasma membrane permeability to Hoechst 33258) by a computer-assisted sperm analysis machine (CASA; Hamilton Thorne IVOS II; machine setting are found in Table 1), and mitochondrial membrane potential, by MitoTracker® red staining following manufacture's procedure. Mitochondrial membrane potential was evaluated using a Nikon Fluorescence microscope, and the NIS-Elements software package was used to outline 100 individual spermatozoa and fluorescence intensity was determined.

Samples were diluted (~3 × 10^9 sperm/mL) and cultured in a commercially available media (OPTIXcell, IMV technologies) adjusted to different pH (5.8, 6.8, or 7.3), at 4 °C. Samples were evaluated every 24 h from 24 h after collection (due to transport to the lab a true 0 h was not possible) until 310 h incubation for motility and viability. At each evaluation, 50 μL of each culture was removed and diluted with 150 μL of tris buffer. Samples were then stained with Hoechst 33258 (final concentration 10 ng/mL) for 2 min and evaluated on a CASA for motility and viability.

2.1.3. Protein isolation

Samples were centrifuged (700×*g* for 10 min) to separate spermatozoa and fluids (epididymal fluid or seminal plasma) for protein analysis. Fluids were removed and snap frozen in liquid nitrogen and stored at −80 °C until analyzed. Spermatozoa pellets were then washed with a high ionic solution [20] and vortexed for 1 min to remove proteins loosely attached to the spermatozoa. Samples were then centrifuged (700×*g* for 10 min) to separate spermatozoa from stripped proteins. Stripped proteins were removed, snap frozen in liquid nitrogen and stored at −80 °C until analyzed. This resulted in four types of samples: 1) epididymal fluid, 2) ejaculated fluid, 3) epididymal sperm stripped proteins (epididymal sperm), and 4) ejaculated sperm stripped proteins (ejaculated sperm).

2.1.4. Liquid chromatography mass spectrometry analysis

Protein samples were shipped to the University of Minnesota Mass Spectrometry facility for identification by LCMS/MS. Samples were processed by trypsin digestion, and cleaned by gel purification. Approximately 400 ng of reconstituted peptide were analyzed by capillary LCMS/MS on an Orbitrap Velos mass spectrometer system as previously described [21] with the following modifications: the capillary column diameter was 100 μm, the gradient elution profile was of 8–35% B Solvent over 67 min at 330 nL/min, where A Solvent was 98:2:0.01, H2O:acetonitrile(ACN):formic acid (FA); and B Solvent was 98:2:0.01, ACN:H2O:FA, lock mass was not employed; dynamic exclusion settings were: repeat count = 1,

Table 1
Computer Assisted Sperm Analysis (CASA) system setups and cut-off values used for bull sperm analyses.

CASA setting	Cut-off/Setup
Illumination type	Visible
Illumination led intensity	3287
Illumination xenon intensity	2048
Stage temperature, °C	37
Frame capture speed, Hz	60
Frame count, n	30
Head brightness minimum	200
Head size maximum, μm	50
Head size minimum, μm	4
Progressive STR[1], %	80
Progressive VAP[2], μm/s	50
Slow VAP, μm/s	20
Slow VSL[3], μm/s	30
Static VAP, μm/s	4
Static VSL, μm/s	1

[1-3] STR = Straightness, VAP = Average path velocity, VSL = Straight-line velocity.

exclusion list size was 200, exclusion duration = 12 s, exclusion mass width (high and low) was 15 ppm and early expiration was disabled.

All LCMS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 2.1.0.81). Sequest was set up to search the bovine (taxid 9913) protein sequence database from Uniprot.org with canonical and isoforms included, downloaded on March 6, 2013 and merged with the common lab contaminant protein database (thegpm.org/crap/index, 109 proteins). Sequest was searched with the digestion enzyme trypsin, fragment ion mass tolerance 0.100 Da and precursor tolerance of 50 ppm. Oxidation and di-oxidation of methionine, deamidated of asparagine and glutamine and pyroglutamic acid were set as a variable peptide modifications, N-terminal protein acetylation was set as a variable modification and carbamidomethyl cysteine was set as a fixed modification.

Scaffold (version Scaffold_5.0.0, Proteome Software Inc. Portland, OR) was used to validate LCMS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 99.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 7.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [22]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

Further analysis was conducted using peptides identified as exclusive and unique to each protein. Total spectrum counts for proteins were used for abundance comparisons (proteins found in one sample but not the other or found in both samples) and statistical analysis. Comparisons were made for total spectrum counts between epididymal and ejaculated fluid proteins and between epididymal and ejaculated spermatozoa surface proteins. Significant *P*-values were adjusted for multiple testing using the Benjamini-Hochberg calculation to correct the FDR. For each comparison, proteins that were identified in the samples were entered into DAVID v 6.8 [23,24] using their official gene names to determine the top Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with those proteins. For the significant KEGG pathways within each sample, that had a physiological meaning for the study objective, the proteins contributing to that KEGG that were exclusive for one of the samples were entered into the STRING database [25,26] to determine the network interactions of those proteins.

2.1.5. Statistical analyses

Differences of spermatozoa parameters between ejaculated and epididymal (pH 5.3 and 7.3) at collection were evaluated using the GLIMMIX procedures of SAS (v 9.4) proportions were assumed to have a beta distribution; velocities, Hz, μm and fluorescence intensity data were tested with UNIVARIATE procedure and had a Gaussian distribution. The model used was treatment (ejaculated, epididymis 5.8, epididymis 7.3) as a fixed effect and bull as a random effect. For fluorescence intensity, area measured was included as a covariate. Cultured spermatozoa parameters were evaluated using the GLIMMIX procedure of SAS (v 9.4) and data were assumed to have a beta distribution. The model included the fixed effect of treatment (pH 5.8, 6.8, and 7.3), time of incubation and the interaction. Three random statements were used; the first random statement was used to model the R-side of residuals to analyze the data as repeated measures. The subject was bull with covariate structures selected based on the smaller −2 Res Log Pseudo-Likelihood. For total and progressive motility, the covariate structure was Compound Symmetry (CS); for viability, the covariate structure was Toeplitz (TOEP). Time points 286 and 310 h incubation were removed from progressive motility analysis because of all values equaled zero. When a significant effect ($P \leq 0.05$) or tendency ($P \leq 0.10$) in the F statistics was detected, mean separation was performed using least significant differences (means ± SEM).

2.2. Study II

2.2.1. Experimental design

Semen from 20 yearling (12- to 15-mo old) Angus crosses bulls were collected by electro-ejaculation, diluted (~42×10^6 sperm/mL) and incubated at three different pH (5.8, 6.8, and 7.3). Twenty different yearling (12- to 15-mo old) Angus crosses bulls were slaughtered and testes and epididymides were collected at a commercial slaughter facility. Epididymides were dissected and epididymal semen was collected from the cauda section of the epididymis. Epididymal semen was diluted (~60×10^6 sperm/mL) and incubated at three different pH (5.8, 6.8, and 7.3) and transported back to the laboratory in culture, thus, first evaluation at 24 h incubation. Semen dilution and incubation, and sperm analysis followed the procedures described in section 2.1.2. Samples were evaluated every 24 h, until total motility were below 20%, then no further evaluation was made for total motility, progressive motility, or viability.

2.2.2. Statistical analysis

Ejaculated and epididymal sperm parameters were evaluated separately. Total motility, progressive motility and viability were evaluated using the methods described in section 2.1.5 for repeated measures. For ejaculated sperm data the covariate structure for total and progressive motility was TOEP; and for viability, the covariate structure was First-Order Ante-dependence [ANTE (1)]. The covariate structure for epididymis total motility was TOEP; for progressive motility it was ANTE (1), and for viability, it was Heterogeneous Compound Symmetry (CSH). When a significant effect ($P \leq 0.05$) or tendency ($P \leq 0.10$) in the F statistics was detected, mean separation was performed using least significant differences (means ± SEM).

3. Results

3.1. Spermatozoa culture and analysis

In study I, there was an effect of treatment on the mitochondrial membrane potential ($P < 0.0001$). Ejaculated sperm had decreased ($P \leq 0.0015$) fluorescence intensity compared to both epididymis sperm at pH 5.8 and 7.3 (1.79 ± 0.32, 4.30 ± 0.34 and 3.41 ± 0.32, fluorescence intensity, respectively). Epididymis sperm at pH 5.8 tended ($P = 0.07$) to have greater intensity than epididymis sperm at 7.3. Treatment influenced percentage of total motility, progressive motility, viability, viable linearity, and viable straightness ($P \leq 0.001$; Table 2); however, motile linearity ($P = 0.11$) and motile straightness ($P = 0.56$) did not differ (Table 2). There was also an effect of treatment on average path velocity, curvilinear velocity, straight-line velocity, amplitude of lateral head displacement, and beat cross frequency for both motile and viable sperm ($P \leq 0.01$; Table 2). When epididymal sperm were cultured at pH 5.8, 6.8 and 7.3 there was an effect of pH by time interaction on total motility and progressive motility ($P \leq 0.05$; Figs. 1 and 2); however, the pH by time interaction was not significant for viability ($P = 0.16$; Fig. 3). There was an effect of pH on total motility ($P < 0.0001$) and viability ($P < 0.0001$; Fig. 3), but there was no effect of pH on the percentage of progressive motility ($P = 0.59$). There was also an effect of time on total motility, progressive motility and viability ($P < 0.0001$).

Table 2
The effect of treatment (ejaculated sperm at pH 7.3 and epididymis sperm at pH 5.8 or 7.3) on motility and viability parameter measured by CASA (mean ± SEM).

Variables	Ejaculated pH 7.3	Epididymis pH 5.8	Epididymis pH 7.3
Total motility, %	48.0 ± 4.5[b]	39.4 ± 5.4[b]	71.5 ± 4.0[a]
Progressive motility, %	5.5 ± 1.4[b]	5.0 ± 1.7[b]	16.2 ± 2.5[a]
Viable, %	72.4 ± 4.7[b]	95.8 ± 2.0[a]	94.1 ± 2.0[a]
LIN[1] - motile, %	31.9 ± 1.8	28.0 ± 1.9	29.3 ± 1.7
LIN - viable, %	18.9 ± 1.5[a]	10.9 ± 1.4[b]	21.1 ± 1.5[a]
STR[2] - motile, %	61.9 ± 2.9	57.9 ± 3.6	61.6 ± 2.9
STR - viable, %	37.5 ± 2.8[a]*	23.0 ± 3.0[b]	45.2 ± 2.9[a†]
VAP[3] - motile, μm/s	68.2 ± 8.0[ab]*	47.9 ± 9.8[b]	90.1 ± 8.0[a†]
VAP - viable, μm/s	47.1 ± 8.6[ab]*	19.8 ± 10.5[b†]	70.2 ± 8.6[at]
VCL[4] -motile, μm/s	138.5 ± 15.1[b]	111.5 ± 18.5[b]	206.5 ± 15.1[a]
VCL - viable, μm/s	95.9 ± 16.8[b]*	46.2 ± 20.6[b†]	159.9 ± 16.8[a]
VSL[5] - motile, μm/s	41.0 ± 5.6[ab]*	28.65 ± 6.9[b]	56.3 ± 5.6[a†]
VSL - viable, μm/s	26.9 ± 5.2[b]*	11.8 ± 6.4[b†]	43.8 ± 5.2[a]
ALH[6] - motile, μm	7.9 ± 0.54[b]	6.5 ± 0.67[b]	9.3 ± 0.54[a]
ALH - viable, μm	5.5 ± 0.7[a]	2.7 ± 0.9[b]	7.1 ± 0.7[a]
BCF[7] - motile, Hz	28.4 ± 2.9[b]	39.6 ± 3.7[a]	39.5 ± 2.9[a]
BCF - viable, Hz	16.6 ± 2.1[b]	15.7 ± 2.6[b]	28.5 ± 2.1[a]

[a-b] Values within the same row not sharing a common superscript differ ($P \leq 0.05$).
*,†,‡ Values within the same row not sharing a common superscript differ ($P \leq 0.10$).
[1-7] LIN = Linearity, STR = Straightness, VAP = Average path velocity, VCL = Curvilinear velocity, VSL = Straight-line velocity, ALH = Amplitude of lateral head displacement, BCF = Beat-cross frequency.

In study II, there was an effect of pH, time and pH by time interaction for total motility (Fig. 4), progressive motility (Fig. 5) and viability (Fig. 6) for both ejaculated and epididymal sperm ($P \leq 0.05$). Ejaculated sperm at pH 6.8 and epididymal sperm at pH 7.3 maintained total motility above 20% longer than the other samples, at 216 h of incubation motility decreased below 20% for both ejaculated 6.8 and epididymal 7.3 (17.1% and 18.9%, respectively). The percentage of sperm displaying progressive motility at 216 h was 1.6% and 1.1%, and viability 51.3% and 95.4%, for ejaculated 6.8 and epididymal 7.3, respectively.

3.2. Protein identification

An overall total of 458 unique proteins were detected between all samples (Fig. 7), 178 proteins were detected in ejaculated fluid (seminal plasma) and 298 proteins were identified stripped from ejaculated sperm. In epididymal samples, 311 proteins were identified in epididymal fluid, and 334 proteins were identified stripped from epididymal sperm (Fig. 7). There were 103 proteins detected in the fluids that were present in both ejaculated and epididymal samples, ten proteins had increased abundance in ejaculated fluid (A5D9E8, CLUS, Cytokeratin-9, F1MK08, IPSP, LG3BP, Q58DP6, RNS, SFP1, SPAD1) and 29 had increased abundance in epididymal fluid (A6QLB0, ACTB, ACTC, ACTS, CBPQ, DHSO, ENOA, F16P1, F1N0E5, F1N5M2, G3X6N3, G3X757, HBA, HBB, HEMO, HS90A, K2C8, KAD1, KAP0, PARK7, PEBP1, PRDX5, SPA31, SPA37, TBA8, TBB4A, TBB4B, TBB5, TRFE). There were 221 proteins detected in the sperm samples that were present in both ejaculated and epididymal samples, 12 proteins had increased abundance in ejaculated sperm (B2MG, CLUS, F1MK08, F1MTI7, F1MXP8, Q4R0H2, Q58DP6, RNS, SFP1, SPAD1, Trypsin precursor, Z13) and 109 proteins had increased abundance in epididymal sperm.

3.3. Pathway analysis

There were eight significant KEGG pathways (FDR <0.05) for ejaculated fluid proteins and 24 KEGG pathways for epididymal fluid proteins (Table 3). There were ten significant pathways for ejaculated and 18 for epididymal proteins that were stripped from sperm (Table 4). The metabolic pathway was identified as the most important KEGG pathway for this data set. This was expected as sperm is maintained in a quiescent mode in the epididymis which allows sperm to be stored for several days.

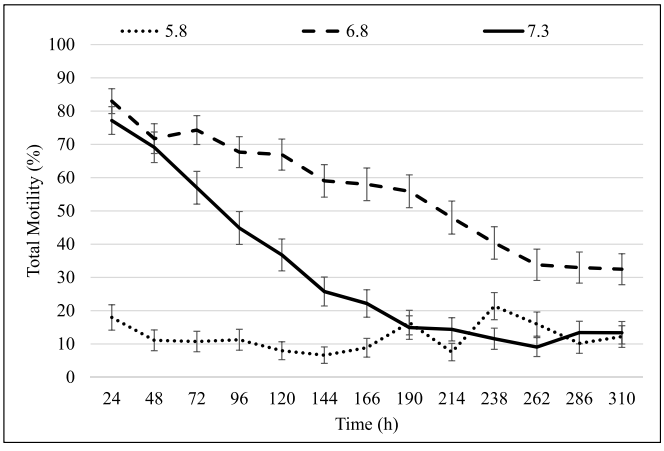

Fig. 1. Percentage of total motility for epididymal sperm culture at pH 5.8, 6.8, and 7.3 (Study I). There was a significant pH, time, and pH by time interaction ($P < 0.0001$).

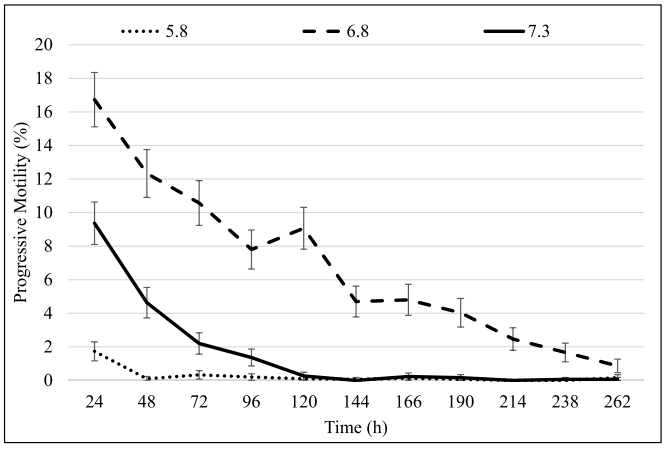

Fig. 2. Percentage of progressive motility for epididymal sperm cultured at pH 5.8, 6.8, and 7.3 (Study I). There was a significant time and pH by time interaction ($P \leq 0.05$); pH was not significant ($P = 0.59$).

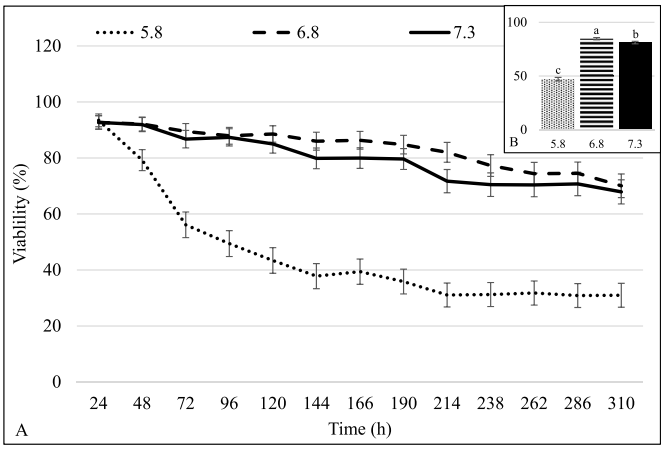

Fig. 3. Percentage of viable epididymal sperm cultured at pH 5.8, 6.8, and 7.3 (Study I). There was not a significant pH by time interaction (A; P = 0.16). There was a significant pH (B) and time effect (P < 0.0001). [a-c] Bars within figure not sharing a common superscript differ (P ≤ 0.05).

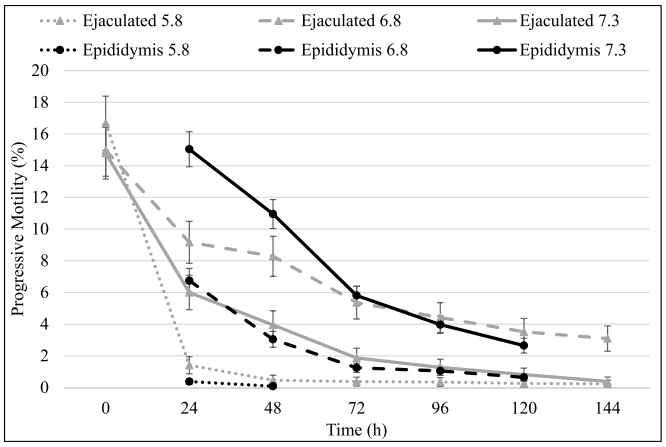

Fig. 5. Percentage of progressive motility for epididymal and ejaculated sperm cultured at pH 5.8, 6.8, and 7.3 (Study II). Samples were considered non-viable when total motility decreased below 20%. There was a significant pH, time and pH by time interaction for both epididymal and ejaculated sperm (P ≤ 0.03).

Metabolic pathway associated proteins in the fluid samples included: 15 proteins that were present in both ejaculated and epididymis samples (AK1, ENO1, FBP1, FH, GALK1, GAPDHS, GLB1, GNS, GPI, LDHA, MDH2, PGAM1, PGAM2, PTGDS, SORD), nine proteins (ATP6AP1, B4GALT4, GAA, GBA, HPSE, NAGLU, NT5E, PLA2G7, PPT1) that were only present in ejaculated fluid and 55 proteins that were only present in epididymis fluid (Fig. 8). The proteins related to the metabolic pathway only present in ejaculated fluid were not highly related and had few connections (PPI enrichment P = 0.22) between proteins; however, the proteins Heparanase (HPSE) and N-acetyl-alpha-glucosaminidase (NAGLU) participate in glycosaminoglycan degradation; two other proteins were detected in this pathway but were not related to the metabolic pathway, they were Beta-galactosidase (GLB1) and N-acetylglucosamine-6-sulfatase (GNS). The proteins Glucosylceramidase (GBA), Lysosomal alpha-glucosidase (GAA), NAGLU, Palmitoyl-protein thioesterase 1 (PPT1), and V-type proton ATPase subunit S1 (ATP6AP1) participate in lysosome pathway. Five other proteins were identified to the lysosome pathway but were not related to the metabolic pathway, they were Lysosomal protective protein (CTSA), Cathepsin B (CTSB), Cathepsin D (CTSD), Legumain (LGMN), and Prosaposin (PSAP).

The proteins present only in epididymis fluid and related to metabolic pathway (Fig. 8) differed from ejaculated fluid and were highly interactive as demonstrated by a complex network. Eleven proteins in this network were related to glycolysis/gluconeogenesis pathway (AKR1A1, ALDH2, ALDH9A1, ALDOA, GALM, GAPDH, LDHB, PGK1, PGM1, PGM2, TPI1), five proteins were related to the oxidative phosphorylation pathway (ATP6V1H, ATP6V1B2, ATP6V1A, ATP5A1, ATP5B), eight were related to the pentose phosphate pathway (ALDOA, G6PD, PGM1, PGM2, PRPS1, TALDO1, TKT, TKTL1), and four proteins were related to fructose and mannose metabolism (AKR1B1, ALDOA, MPI, TPI1).

There were 36 proteins present in both ejaculated and epididymis sperm samples related to the metabolic pathway (AK1, AKR1B1, ALDH2, ALDOA, AOX1, APRT, ATIC, ATP5A1, ATP5B, ATP6V1E2, ATP6V1H, DCXR, ENO1, FBP1, FH, GALK1, GAPDHS, GLB1, GLUL, GNS, GPI, IDH1, ISYNA1, LDHA, MDH2, NME2, PGAM1, PGAM2, PGK1, PGLS, PGM2, PTGDS, QDPR, RPN2, SMS, SORD). Nevertheless, 11 proteins were only present on ejaculated sperm (ATP6AP1, B4GALT4, GAA, GBA, HPSE, NAGLU, NT5E, OAT, PLA2G7, PPT1, SDHA) and 32 were only present on epididymal sperm (Fig. 9). Proteins related to the metabolic pathway only present in ejaculated sperm were not highly related and had few connections

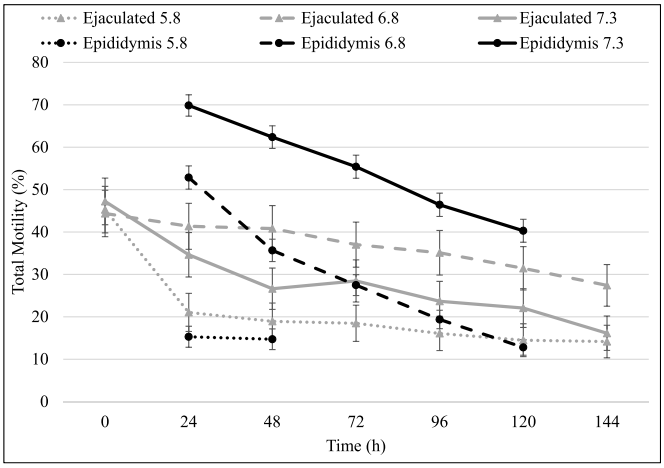

Fig. 4. Percentage of total motility for epididymal and ejaculated sperm cultured at pH 5.8, 6.8, and 7.3 (Study II). Samples were considered non-viable when total motility decreased below 20%. There was a significant pH, time and pH by time interaction for both epididymal and ejaculated sperm (P ≤ 0.04).

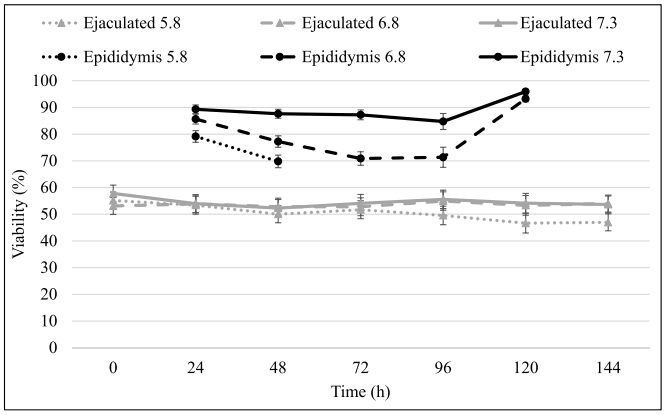

Fig. 6. Percentage of viable for epididymal and ejaculated sperm cultured at pH 5.8, 6.8, and 7.3 (Study II). Samples were considered non-viable when total motility decreased below 20%. There was a significant pH, time and pH by time interaction for both epididymal and ejaculated sperm (P ≤ 0.02).

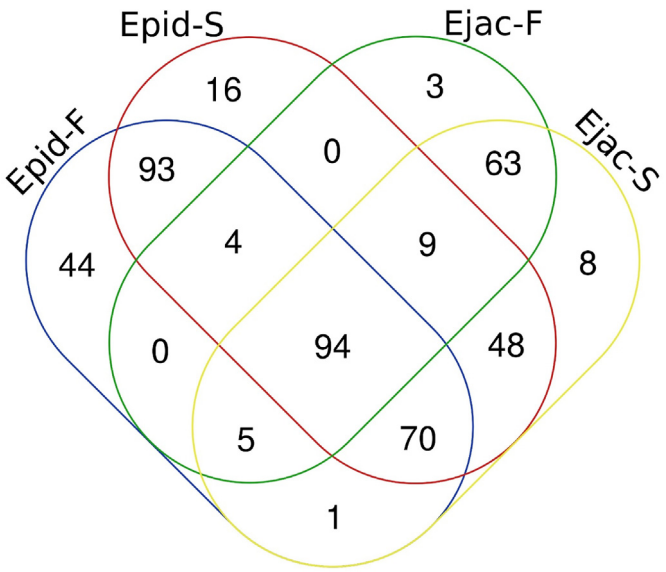

Fig. 7. Venn diagram for the number of proteins detected in epididymal fluid (Epid-F), epididymal sperm (Epid-S), ejaculated fluid (Ejac-F), and ejaculated sperm (Ejac-S). A total of 458 unique proteins were detected by LCMS/MS. Protein identifications were accepted if a minimum of 1 unique peptide was identified to a known protein, and minimum of a 50% confidence in the identity of the protein was achieved. Peptide threshold was set at 95% peptide probability.

(PPI enrichment P = 0.09) between proteins, similarly to proteins only present in ejaculated fluid. The same proteins were detected in the glycosaminoglycan degradation and lysosome pathway between ejaculated fluid and sperm. Similarly to ejaculated fluid (seminal plasma), there were proteins not related to metabolic pathway that were also present in the lysosome pathway [Cathepsin B (CTSB), Cathepsin D (CTSD) and Legumain (LGMN)]; however, glycosaminoglycan degradation was not detected as a significant pathway for sperm proteins. Interestingly, two proteins were detected to be part of the oxidative phosphorylation pathway, Succinate dehydrogenase [ubiquinone] flavoprotein subunit (SDHA) and V-type proton ATPase subunit S1 (ATP6AP1).

The proteins only present in the epididymis sperm samples and related to the metabolic pathway (Fig. 9), different from ejaculated sperm and similarly to those from epididymis fluid, were highly interactive as demonstrated by a complex network. There were seven proteins related to the pentose phosphate pathway (G6PD, PGD, PGM1, PRPS1, TALDO1, TKT, TKTL1), five proteins related to glycolysis/gluconeogenesis pathway (AKR1A1, LDHB, GAPDH, PGM1, TPI1) and two proteins related to the fructose and mannose metabolism pathway (MPI, TPI1) that were present only in epididymis sperm samples compared to the ejaculated sperm samples.

4. Discussion

Efficient transportation of spermatozoa through the female reproductive tract from the site of deposition to the site of fertilization requires that the female be in estrus or under the influence of estrogen [27]. Estrogen may influence fertilization rates through both spermatozoa transport and fertilization efficiency by altering the uterine environment (pH). Uterine pH decreases at the initiation of standing estrus [28] and is also decreased in animals that exhibited standing estrus prior to fixed-time AI compared to animals not exhibiting standing estrus [18,19]. Estrus expression prior to fixed-time insemination increases the number of spermatozoa that reaches the site of fertilization [29] and has a linear relationship with pregnancy success [30]. It is hypothesized that the decrease in pH at onset of estrus would increase sperm longevity and the rise in pH prior to ovulation would increase sperm motility [18,19]. Thus, sperm were incubated at three different pH: physiological pH of the epididymis (5.8), physiological pH upon ejaculation which is similar to the uterine pH at time of ovulation (7.3) and uterine pH at onset of estrus (6.8). In study I, epididymal sperm were able to maintain motility for a longer period of time when they were incubated at pH 6.8 compared to pH 5.8 or 7.3. This is

Table 3
Number of proteins (Count) and level of significance (FDR) for the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway for proteins identified in epididymal and ejaculated fluid.

KEGG	Epididymis fluid		Ejaculated fluid	
	Count	FDR	Count	FDR
bta00010: Glycolysis/Gluconeogenesis	18	5.09E-12	7	0.006655
bta00020: Citrate cycle (TCA cycle)	5	0.047883		
bta00030: Pentose phosphate pathway	10	2.34E-07		
bta00040: Pentose and glucuronate interconversions	6	0.005755		
bta00051: Fructose and mannose metabolism	6	0.010003		
bta00052: Galactose metabolism	7	0.00169	3	0.465539
bta00330: Arginine and proline metabolism	7	0.012378		
bta00480: Glutathione metabolism	8	0.005533		
bta00500: Starch and sucrose metabolism	6	0.009208		
bta00520: Amino sugar and nucleotide sugar metabolism	7	0.010479		
bta00531: Glycosaminoglycan degradation			4	0.041254
bta00620: Pyruvate metabolism	8	6.72E-04	3	0.555899
bta00630: Glyoxylate and dicarboxylate metabolism	5	0.029481		
bta01100: Metabolic pathways	71	5.41E-11	24	0.223982
bta01130: Biosynthesis of antibiotics	39	3.84E-21	10	0.017366
bta01200: Carbon metabolism	25	6.57E-15	7	0.036405
bta01230: Biosynthesis of amino acids	16	4.33E-09		
bta03050: Proteasome	10	3.21E-05		
bta04141: Protein processing in endoplasmic reticulum	13	0.010003	6	0.465539
bta04142: Lysosome	11	0.010479	17	1.64E-10
bta04145: Phagosome	12	0.015453	7	0.154659
bta04610: Complement and coagulation cascades	15	7.21E-08	7	0.007027
bta04612: Antigen processing and presentation	9	0.006383	7	0.007027
bta04614: Renin-angiotensin system			5	0.007027
bta05134: Legionellosis	8	0.006383	5	0.069776

Table 4
Number of proteins (Count) and level of significance (FDR) for the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway for proteins stripped from the sperm and identified in epididymal and ejaculated samples.

KEGG	Epididymis sperm		Ejaculated sperm	
	Count	FDR	Count	FDR
bta00010: Glycolysis/Gluconeogenesis	16	1.2E-09	11	3.8E-05
bta00020: Citrate cycle (TCA cycle)	6	1.1E-02	4	2.5E-01
bta00030: Pentose phosphate pathway	12	1.1E-09	5	4.3E-02
bta00040: Pentose and glucuronate interconversions	6	6.9E-03	4	1.9E-01
bta00051: Fructose and mannose metabolism	6	1.2E-02	4	2.5E-01
bta00480: Glutathione metabolism	10	1.5E-04	6	7.7E-02
bta00620: Pyruvate metabolism	6	2.8E-02	4	3.7E-01
bta00630: Glyoxylate and dicarboxylate metabolism	5	3.8E-02		
bta01100: Metabolic pathways	69	1.3E-09	48	6.1E-04
bta01130: Biosynthesis of antibiotics	35	8.4E-17	21	8.1E-07
bta01200: Carbon metabolism	26	8.3E-16	13	1.2E-04
bta01230: Biosynthesis of amino acids	16	4.8E-09	7	6.0E-02
bta03050: Proteasome	8	2.7E-03	8	1.3E-03
bta04141: Protein processing in endoplasmic reticulum	12	3.8E-02	8	5.1E-01
bta04142: Lysosome	12	5.8E-03	18	1.5E-07
bta04610: Complement and coagulation cascades	15	8.1E-08	8	2.0E-02
bta04612: Antigen processing and presentation	9	7.8E-03	8	2.0E-02
bta04614: Renin-angiotensin system			6	4.8E-03
bta05134: Legionellosis	8	7.8E-03	6	9.1E-02

consistent with the hypothesis that a decrease in uterine pH at the onset of estrus would increase sperm longevity. In study II, this hypothesis held true for ejaculated sperm (pH 6.8 had the greatest longevity); however, epididymal sperm at pH 7.3 had greater longevity (total motility) compared to sperm at pH 6.8. Animals in study I and study II were different, the main differences between the two groups of bulls (age) may have caused the observed differences. In study I, animals were mature bulls (4-y old) with proven fertility and study II animals were 12- to 15-mo old that had just reached puberty and passed a breeding soundness exam.

Previous studies have reported that upon dilution of caudal epididymis fluid, motility was increased [3,4], but when epididymal sperm were incubated in caudal epididymal fluid, motility was inhibited [4]. In the present study, the washing and dilution of caudal epidydimal fluid was sufficient to cause an increase in sperm motility which explains the lack of statistical difference between

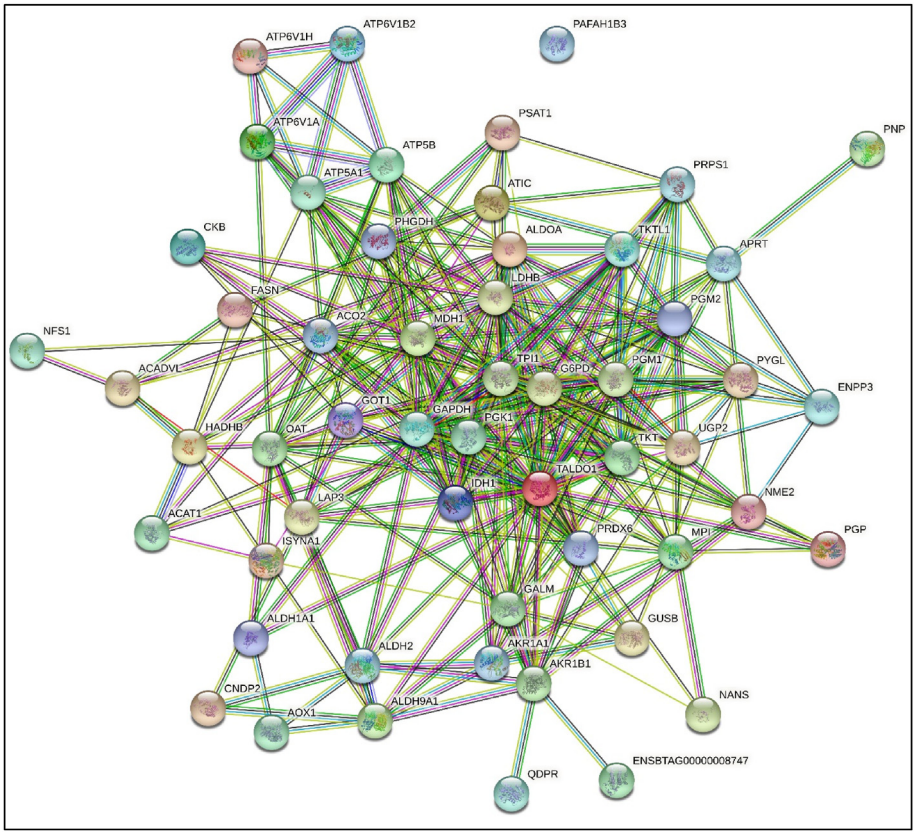

Fig. 8. Protein interaction analyzed by STRING database of proteins present only in the epididymis fluid (PPI enrichment P < 0.0001) from the metabolic pathway (KEGG; Table 3).

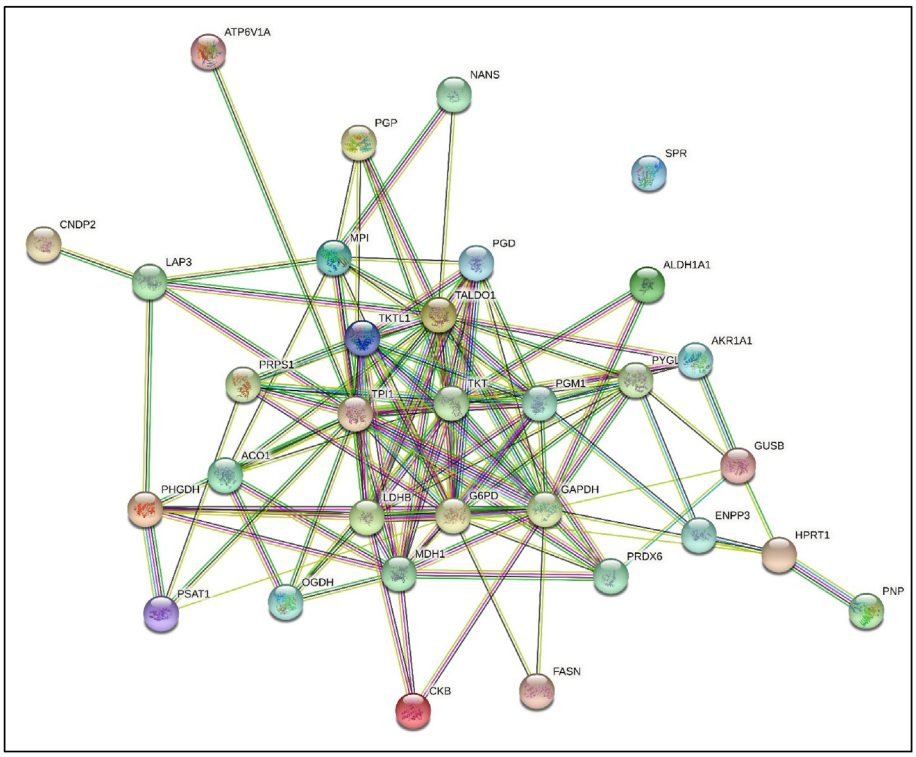

Fig. 9. Protein interaction analyzed by STRING database of proteins present only in the epididymis sperm (PPI enrichment P < 0.0001) from the metabolic pathway (KEGG; Table 4). Sperm proteins were stripped from the sperm with a high ionic solution.

epididymis sperm at pH 5.8 and ejaculated sperm at pH 7.3 in study I and is similar to what has been reported by others [3,4,31]. Interestingly, when the pH of epididymal sperm was adjusted to 7.3 total motility and progressive motility were increased to above ejaculated sperm. This is consistent with the increased mitochondrial membrane potential of epididymal sperm and agrees with the increase in sperm motility reported by others [32].

When comparing sperm viability (study II) between ejaculated and epididymal sperm, even though sperm motility decreased during incubation, epididymal sperm had at least 15% points more viable sperm compared to ejaculated sperm at any time point regardless of media pH (Fig. 6). In the cauda epididymis, sperm are stored for a long period of time. After differentiation and maturation, sperm has a relatively simple metabolism and is highly dependent on its environment [1]. Sullivan and coworkers hypothesized that the increased longevity of epididymal sperm is due to both quiescence and prevention of premature activation of the spermatozoa [2]. In agreement with Sullivan and coworkers [2], the increased viability of epididymal spermatozoa compared to the ejaculated was not only due to the relatively quiescent state it was in, but also due to proteins associated with these spermatozoa, because even after dilution and initiation of motility epididymal sperm had increased viability compared to ejaculated sperm in the present study.

Proteins were identified in ejaculated and epididymal samples in order to investigate which proteins may be involved in increased viability of epididymal sperm. There were 153 proteins identified in epididymis samples only, and 74 were only identified in ejaculated samples. When comparing proteins stripped from the sperm and in the fluids between ejaculated and epididymis samples, the metabolic pathway had the greatest number of proteins. The KEGG metabolic pathway can be subdivided into other pathways, as proteins may have function in multiple pathways [e.g., Fructose-bisphosphate aldolase A (ALDOA) is present in the metabolic pathway, glycolysis/gluconeogenesis pathway, pentose phosphate pathway and, fructose and mannose pathway]. The total number of proteins identified in ejaculated (n = 305) and epididymal sperm (n = 384) suggests that epididymal sperm metabolism and environment are more regulated by proteins than ejaculated sperm, especially, since 153 proteins were present only in epididymal samples compared to 74 proteins present in ejaculated samples only.

Ejaculated bovine sperm can utilize both anaerobic and aerobic methods of energy production to maintain similar levels of motility [33]. Proteins only in the epididymal samples that were involved in the glycolysis/gluconeogenesis pathway (11 and five in fluid and sperm, respectively) and that were associated with oxidative phosphorylation (five proteins identified in fluid) were identified. Two different proteins were present in ejaculated sperm that related to oxidative phosphorylation. The glycolysis/gluconeogenesis pathway had seven and 11 proteins that were present in both ejaculated and epididymis samples, fluid and sperm, respectively. Interestingly, the oxidative phosphorylation pathway was not detected when all proteins from each sample were analyzed by DAVID; however, proteins in this pathway were identified when the metabolic pathway proteins were entered in the STRING database.

Human patients with asthenozoospermia had increased levels of ALDOA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), alcohol dehydrogenase (AKR1A1), L-lactate dehydrogenase B chain (LDHB) in seminal plasma compared to control patients [34]. In this data set, the proteins elevated in seminal plasma of patients with astenozoospermia, ALDOA, GAPDH, and AKR1A1 were only detected in epididymal fluid. Also, AKR1A1, LDHB and GAPDH were only present on epididymal sperm (ALDOA was present in ejaculated sperm but in lower abundance compared to epididymal sperm). Our results suggest that energy production, specifically through glycolysis, in the epididymis is more regulated compared to after ejaculation. Thus, these proteins only detected in epididymal samples may need to be removed or diluted to undetectable levels

to facilitate and promote energy production and, consequently, sperm motility.

It is possible that the increased number of proteins related to glycolysis in epididymal samples is a mechanism to reduce the production of reactive oxygen species (ROS) which is a by-product of oxidative phosphorylation and not produced during glycolysis. Reactive oxygen species are oxygen containing molecules that can be found as free radicals or non-radical oxidants, these molecules remove electrons from specific reactants. The presence of ROS is necessary for normal sperm function; however, the deleterious capacity of ROS is determined by its concentration. The increase in sperm intracellular cyclic adenosine monophosphate (cAMP) is caused by ROS which leads to a cascade of biochemical events that lead to sperm capacitation [35,36]; however, when in elevated concentrations, ROS can causes oxidative stress which leads to lipid peroxidation. Lipid peroxidation has been reported to increase DNA fragmentation, decrease plasma membrane integrity (viability), and reduce motility in bovine sperm [37]. Antioxidant proteins can remove ROS from the media and prevent harmful elevated concentrations of ROS. Aldehyde dehydrogenase (ALDH2) was identified in all samples except for ejaculated fluid, this protein was identified in the metabolic pathway and it has been reported to provide antioxidant properties in the stallion sperm [38,39]. The glutathione metabolism pathway was present in epididymal fluid but not in ejaculated fluid and was present in both ejaculated and epididymal sperm. The glutathione S-transferases proteins have been reported to be antioxidants in stallion [38,39] and have been suggested to be involved in bovine sperm protection [9]. We identified that the protein glutathione S-transferase P (GSTP1) was only present in epididymal fluid and sperm; however, glutathione S-transferase Mu (GSTM1) was present in epididymal fluid and both epididymal and ejaculated sperm. Another pathway that has been reported to have antioxidant properties is the pentose phosphate pathway [40]. Peroxidases (PRDX1, PRDX2, PRDX4, and PRDX6) are important antioxidants that have been reported to protect sperm from oxidative stress, and they were identified in epididymal samples but not ejaculated samples. Additionally, glutathione peroxidases were identified in both ejaculated and epididymal samples (GPX5) or only in ejaculated samples (GPX6) [41].

5. Conclusions

In summary, in the epididymis, sperm energy metabolism appears to be more glycolytic compared to sperm in the ejaculate, based on the greater number of proteins related to this pathway only present in epididymal samples. Sperm also has a greater number of antioxidants available in the epididymis that is likely to be maintaining ROS at low concentrations to inhibit premature sperm activation. This is supported by a greater mitochondrial membrane potential of epididymal sperm compared to ejaculated sperm and the fact that epididymal sperm was able to maintain viability longer than ejaculated when cultured under the same conditions. In addition, when both ejaculated and epididymal sperm were cultured at uterine pH (7.3), epididymal sperm had greater motility. More research is necessary to better understand the specific roles of the proteins only identified in the epididymis with the increase in sperm longevity, regulation of sperm activation, and their possible role in bull fertility and ability to dominate a breeding pasture [42,43].

CRediT authorship contribution statement

Saulo Menegatti Zoca: Data curation, Formal analysis, Investigation, Writing – original draft. **Emmalee J. Northrop-Albrecht:** Investigation, Writing – review & editing. **Julie A. Walker:** Writing – review & editing. **Robert A. Cushman:** Conceptualization, Data curation, Investigation, Formal analysis, Writing – review & editing. **George A. Perry:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Writing – review & editing.

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