1. Introduction

In addition to providing a half of the genome, sperm also transfer essential transcripts and proteins to the oocyte. Any defects in sperm proteins derived from either genome or epigenome vitally affect male fertility and early embryonic development (Dada et al. 2012). The prevalence of male infertility among the American infertile couples was found to be 17% in 2010 according to the Society for Assisted Reproductive Technology data (SART). Male infertility is also a concern in cattle reproduction as well because thousands of cows can be inseminated using cryopreserved sperm from a single bull. Since male fertility has such a paramount influence on genetic improvement of the herd, much greater considerations of male selection, their management, and replacement are needed (Rahman et al. 2017). Using high quality sperm and proper fertilization techniques are critical for maintaining the conception rates with artificial insemination (AI) in the field (Kwon et al. 2015b).

Sperm proteins can be classified by their physiological functions or their cellular locations. Locations of several sperm proteins were demonstrated to be acrosomal, mitochondrial, nuclear matrix, cytoskeletal (i.e., tubulins and actins) and membrane proteins (i.e., aquaporins). Most of the sperm proteins were structural proteins such as ODF2 and tubulin which are located in the flagellum play important roles in sperm physiology (Hoyer-Fender et al. 1998; Donkor et al. 2004). On the other hand, some other sperm proteins such as kinases and superoxide dismutase (SOD) are synthesized in the cytosol and have enzymatic functions. Although the significance of sperm in cattle reproduction has been obvious, sperm proteins and molecular mechanisms of uncompensable infertility in Angus breed are vastly undefined. Because the bulls were similar in their genotypes, epigenetics (such as posttranslational modifications of proteins that
influence gene expression without any changes in DNA through environmental factors such as nutrition, management, and climate) may play important roles in male fertility. Therefore, the purpose of this study was to uncover sperm proteome of Angus bulls to identify possible protein markers affecting male fertility. To accomplish our goal, we used a quantitative proteomics methods 2D-DIGE and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (Choi et al. 2008) and bioinformatics. This pioneering and comprehensive proteomics study of Angus bull sperm is significant because the results are hypothesis generators and potential fertility markers to determine and measure semen quality and bull fertility.

2. Materials and Methods

2.1. Experimental Design

The frozen semen samples and the fertility data from four Angus bulls with different fertility index and satisfactory semen quality were obtained from Alta Genetics, Inc. (Watertown, WI, USA). Cryopreserved semen samples were washed three times to remove the cryoprotectants, and then the proteins were extracted for 2D-DIGE analysis. In addition, MALDI-TOF/TOF analysis was used to identify proteins. Further, bioinformatics and pathway analyses were carried out to identify the protein networks and pathways. All chemicals were purchased from Sigma-Aldrich Chemicals, St. Louis, MO, USA except those stated.

2.2. Determination of Bull Fertility and Isolation of Sperm

Fertility of the bulls was tested through artificial insemination (AI) of 1,265 cows on seven farms. Frozen semen samples from four bulls were distributed to seven herds, and cows were bred in standing heat. The pregnancy diagnoses were performed by rectal palpation on day 40 post insemination. The breeding numbers and the conception rates of the four bulls are presented (Figure 1). Sperm were isolated from four Angus bulls with different fertility using percoll gradient according to Feugang et al. (2009). Sperm pellets were washed with PBS (Gibco, Grand Island, NY, USA) three times, centrifuged at 500 g and aliquoted as $10 \times 10^6$ spermatozoa per tube. The pellets were kept at -80°C prior to shipping, and shipped on dry ice to Appliedbiomics (Hayward, CA, USA) for 2D-DIGE and mass spectrometry.

2.3. Isolation of Proteins from Sperm and CyDye Labeling

Sperm pellets were resuspended in 120 µl of 2-D cell lysis buffer (30 mM Tris-HCl, pH 8.8, 7 M urea, 2 M thiourea, and 4% CHAPS) supplemented with protease inhibitor cocktail (Roche San Francisco, CA, USA) followed by two seconds of sonications (VerTis, Gardiner, NY, USA). Samples were then incubated on a rotator at room temperature (RT) for 30 minutes and centrifuged at 13,000 g at 4°C for 30 minutes. The supernatant was collected, and the protein concentration was measured using Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Proteins were labeled for each sample. Thirty micrograms of proteins were mixed with 0.7-0.9 µl of diluted CyDye (1:5 diluted with DMF from 1 nmol/µl stock) and kept in the dark on ice (~4°C) for 30 minutes. The labeling reaction was stopped by adding 0.7-0.9 µl of 10 mM Lysine to each sample and incubating in the dark on ice for an additional 15 min. The labeled samples were then mixed together, and 130 µl destreak solution (GE Healthcare, Piscataway Township, NJ, USA) and 100 µl of Rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% Pharmalyte, and trace amount of bromophenol blue) were added to the labeling mix for a total volume of 260 µl. The samples were put on a rotator for 15 min.
and centrifuged at 16,060 g for another 15 min. The labeled samples were then loaded onto a strip holder and immersed with a 13 cm IPG strip. Two 2D-DIGE gels were replicated for each experiment, for Bull C vs. Bull A and similarly for Bull D vs. Bull B. Since the protein expression patterns from two high and two low fertility bulls were compared in the same gel, only two gels were used.

2.4. Isoelectric Focusing (IEF) and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Image Scan and Data Analysis

Once the labeled samples were loaded, IEF (pH3-10 linear) was run according to the protocol provided by Amersham BioSciences (GE Healthcare, Piscataway Township, NJ, USA). The IPG strips were then incubated in the freshly made equilibration buffer-1 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue, and 10 mg/ml Dithiothreitol; DTT) with gentle shaking for 15 min. Then the strips were rinsed in the fresh equilibration buffer-2 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue, and 45 mg/ml DTT) with gentle shaking for 10 min. Next, the IPG strips were rinsed in the SDS-gel running buffer prior to transferring into 12% SDS-gels. The SDS-gels were run at 15°C until the dye ran out of the gels. Gel images were scanned immediately following the SDS-PAGE using typhoon TRIO (Amersham BioSciences, GE Healthcare, Piscataway Township, NJ, USA). The scanned images were then analyzed by Image Quant software (version 6.0, Amersham BioSciences, GE Healthcare, Piscataway Township, NJ, USA), followed by in-gel analysis using DeCyder software version 6.0 (Amersham BioSciences, GE Healthcare, Piscataway Township, NJ, USA). The fold changes of the protein expression levels were obtained from in-gel DeCyder analysis.

2.5. Identification of Differentially Expressed Proteins Using Mass Spectrometry

Protein spots of interest were excised from preparative gels (~600 µg of protein) by using an Etten spot picker (Amersham Biosciences, GE Healthcare, Piscataway Township, NJ, USA) and digested with trypsin (Promega, Madison, WI USA). The trypsin-digested peptides were extracted out and de-salted using C-18 ziptip (Millipore, Billerica, MA, USA). Then, the desalted peptides were used for MALDITOF protein identification (MALDI-TOF/TOF mass spectrophotometer, ABI-4700 from Applied Biosystems, Inc, Foster City, CA, USA). Using the Mascot search engine (Matrix Science, Boston, MA, USA), protein databases of national center for biotechnology (NCBI)/SwissProt (http://www.ncbi.nlm.nih.gov/) and (www.uniprot.org) were searched for >95% matches of high-quality mass spectra. For the differentially expressed protein data, a ratio of protein expressions from relatively lower fertility bulls over relatively higher fertility bulls was calculated as C/A and D/B. Subsequently, an average of these two ratios was taken for each protein using the equation \([(C/A + D/B)/2]\) to find the mean, for more accuracy. Eighty proteins were identified to be different in spermatozoa from the four bulls based on their fertility index.

2.6. Bioinformatics and Pathway Analysis of the Differentially Expressed Proteins

Functional gene annotation clustering of the differentially expressed proteins was performed using DAVID bioinformatics database (http://david.abcc.ncifcrf.gov) to reveal their molecular functions in biological processes. The pathway analysis was completed using ingenuity pathway, IPA (http://www.ingenuity.com) to determine a functional interactome between the differentially expressed proteins and bull fertility. The GenInfo Identifier (GI) accession numbers of 80 proteins were imported into the IPA software prior to data analysis. Then, the unmapped proteins were determined and manually converted into their human counterparts using ENSEMBL database (http://www.ensembl.org) with their identity (%). Afterwards, the pathway analysis was performed using IPA with the proteins that were mapped automatically and manually. The proteins that would be further analyzed using bioinformatics tolls were selected according to IPA interactome results.

2.7. Sperm Proteins as Markers Across Breeds

Through harnessing the power of comparative biology, we performed a comprehensive literature
search on proteins of bovine sperm to uncover potential fertility markers that can be used in selection of breeding bulls. The proteins were included in the following groups according to their molecular physiology: Chromatin proteins, seminal plasma proteins, acrosome proteins, ATP synthesis proteins, capacitation proteins, cytoskeletal proteins, and other proteins.

3. Results

3.1. Protein Analysis by 2D-DIGE

We detected approximately 2,000 protein spots in each of the 2D gels developed using sperm from four bulls with varying fertility per gel, Bull C/Bull A and Bull D/Bull B, respectively (Figure 2a and b). Among all the data obtained from the two 2D gels, 80 of the differentially expressed protein spots were detected. Overall about 4% of all protein spots were differentially expressed between relative high (A and B) and low fertility bulls (C and D), and the expression levels of these proteins ranged from -4.65 to 8.2.

3.2. Differentially Expressed Proteins in Sperm from Bulls with Varying Fertility

We found total of 80 proteins that were differentially expressed in the sperm from the four bulls having varying fertility (Table 1). These expressed proteins exhibited various levels of fold differences among the bulls. While most of these proteins corresponded to known proteins, others were similar to known proteins, or to predicted proteins or hypothetical proteins.

3.3. Bioinformatics and Pathway Analysis of the Differentially Expressed Proteins

According to the IPA output, 47 canonical pathways and networks were identified (Table 2 and 3). Twelve of these canonical pathways were...

![Figure 2](image-url)
| Top ranked protein name (species)                                      | Ratio bull C/bull A | Ratio bull D/bull B | Average ratio | Accession no. | Protein MW | Protein PI | Pep. count | Protein score | Protein score C.I. % | Total ion score | Total ion C.I. % |
|------------------------------------------------------------------------|---------------------|---------------------|----------------|---------------|------------|------------|------------|---------------|-------------------|----------------|-----------------|
| PREDICTED: similar to testis specific 10 isoform 1 [Bos taurus]       | 1.71                | 8.95                | 5.33           | gi|194671321    | 81188      | 5.65       | 13         | 164           | 100               | 63             | 99.8            |
| Outer dense fiber of sperm tails 2 [Bos taurus]                        | 1.64                | 14.8                | 8.21           | gi|84000345     | 75450.6    | 7.52       | 13         | 235           | 100               | 166            | 100             |
| PREDICTED: similar to EF-hand domain (C-terminal) containing 1 isoform 1 [Bos taurus] | 1.11                | 1.37                | 1.24           | gi|76650703     | 73984.5    | 5.78       | 26         | 473           | 100               | 272            | 100             |
| Outer dense fiber of sperm tails 2 [Bos taurus]                        | 1.46                | 1.56                | 1.51           | gi|84000345     | 75450.6    | 7.52       | 27         | 554           | 100               | 397            | 100             |
| Outer dense fiber of sperm tails 2 [Bos taurus]                        | 1.62                | 1.49                | 1.55           | gi|84000345     | 75450.6    | 7.52       | 29         | 697           | 100               | 514            | 100             |
| PREDICTED: similar to outer dense fiber of sperm tails 2 isoform 10 [canis familiaris] | 2.03                | 2.07                | 2.05           | gi|33187222     | 23457.4    | 8.26       | 2          | 73            | 96.6              | 56             | 98.9            |
| Tubulin, beta 2C [homo sapiens]                                        | 1.89                | 1.86                | 1.87           | gi|23958133     | 49808      | 4.83       | 20         | 543           | 100               | 324            | 100             |
| Tubulin, beta, 2 [homo sapiens]                                        | 2.09                | 1.93                | 2.01           | gi|5174735      | 49799      | 4.79       | 22         | 585           | 100               | 330            | 100             |
| PREDICTED: similar to outer dense fiber of sperm tails 2 isoform 10 [canis familiaris] | 1.18                | 1.42                | 1.3            | gi|73967892     | 52075.9    | 5.81       | 26         | 536           | 100               | 348            | 100             |
| PREDICTED: similar to outer dense fiber of sperm tails 2 isoform 10 [canis familiaris] | 1.3                 | 1.62                | 1.46           | gi|73967892     | 52075.9    | 5.81       | 23         | 604           | 100               | 434            | 100             |
| Tubulin, alpha 1a [mus musculus]                                       | 2.79                | 1.51                | 2.15           | gi|6678465      | 49927.6    | 4.97       | 15         | 688           | 100               | 520            | 100             |
| Enolase 1 [Bos taurus]                                                 | 1.03                | -1.3                | 0.35           | gi|87196501     | 47296.4    | 6.37       | 21         | 666           | 100               | 458            | 100             |
| Hypothetical protein LOC511761 [bos taurus]                            | 1.69                | 1.49                | 1.59           | gi|115495817    | 30193.3    | 6.21       | 15         | 427           | 100               | 279            | 100             |
| PREDICTED: similar to tubulin alpha-3 chain (alpha-tubulin 3) [canis familiaris] | 3.2                 | 1.83                | 2.51           | gi|73996007     | 49913.6    | 4.97       | 15         | 577           | 100               | 418            | 100             |
| Tubulin, alpha 1a [mus musculus]                                       | 1.97                | 2.53                | 2.25           | gi|6678465      | 49927.6    | 4.97       | 14         | 442           | 100               | 314            | 100             |
| Top ranked protein name (species) | Ratio bull C/bull A | Ratio bull D/bull B | Average ratio | Accession no. | Protein MW | Protein Pl | Pep. count | Protein score | Protein score C. I. % | Total ion score | Total ion C. I. % |
|---------------------------------|---------------------|---------------------|----------------|---------------|------------|------------|------------|---------------|----------------------|----------------|------------------|
| short-chain acyl-CoA dehydrogenase [bos taurus] | 1.68 | 1.96 | 1.82 | gi|77735757 | 44523.9 | 8.82 | 15 | 352 | 100 | 220 | 100 |
| Lactate dehydrogenase a-like 6B [bos taurus] | 2.25 | 1.41 | 1.83 | gi|78369344 | 41565.8 | 8.91 | 17 | 646 | 100 | 483 | 100 |
| Phosphoglycerate kinase 2 [bos taurus] | 1.64 | 1.68 | 1.66 | gi|174840786 | 44729.3 | 8.51 | 19 | 482 | 100 | 332 | 100 |
| mCG20287 [mus musculus] | 2.63 | 1.98 | 2.3 | gi|148676266 | 39342.1 | 5.14 | 13 | 435 | 100 | 261 | 100 |
| mCG20287 [Mus musculus] | 2.71 | 2.3 | 2.5 | gi|148676266 | 39342.1 | 5.14 | 11 | 376 | 100 | 231 | 100 |
| TUBB2C protein [homo sapiens] | 3.72 | 2.45 | 2.5 | gi|142598 | 25858.4 | 4.95 | 13 | 299 | 100 | 147 | 100 |
| hCG1992406 | 3.29 | 2.29 | 2.79 | gi|119576011 | 42187 | 7.03 | 265 | 100 | 194 | 100 |
| Chain D, cytochrome Bc1 complex from bovine Hypothetical protein LOC510569 [bos taurus] | 1.64 | 1.48 | 1.56 | gi|4139395 | 27269.5 | 6.49 | 13 | 301 | 100 | 179 | 100 |
| Hypothetical protein TP0959 [treponema pallidum] | -2.93 | -2.41 | -2.67 | gi|156120505 | 17804.6 | 5.94 | 4 | 77 | 98.5 | 39 | 45.3 |
| Hypothetical protein LOC510569 [bos taurus] | 2.03 | 1.82 | 1.92 | gi|15639943 | 13894 | 8.89 | 7 | 74 | 64.7 |
| PREDICTED: similar to ENSANGP00000002667 [bos taurus] | 3.01 | 1.77 | 2.39 | gi|194674718 | 19864.8 | 5.62 | 5 | 310 | 100 | 253 | 100 |
| mCG20287 [mus musculus] | 2.35 | 1.52 | 1.93 | gi|148676266 | 39342.1 | 5.14 | 14 | 426 | 100 | 310 | 100 |
| Unnamed protein product [mus musculus] | 2.56 | 2.45 | 2.5 | gi|26355849 | 32237.9 | 5.56 | 9 | 323 | 100 | 223 | 100 |
| Phosphatidylethanolamine-binding protein 4 [bos taurus] | 3.15 | 1.32 | 2.23 | gi|77735827 | 25129.6 | 5.87 | 4 | 60 | 35.7 | 23 | 0 |
| Hypothetical protein LOC510569 [bos taurus] | -3.19 | -2.92 | -3.05 | gi|156120505 | 17804.6 | 5.94 | 6 | 309 | 100 | 234 | 100 |
| Manganous superoxide dismutase; MnSOD [bos taurus] | -2.91 | -6.4 | -4.65 | gi|7555818 | 24574.6 | 8.7 | 6 | 313 | 100 | 254 | 100 |
| PREDICTED: hypothetical protein LOC736248 isoform 2 [pan troglodytes] | 4.37 | 4.23 | 4.3 | gi|114585016 | 43100.4 | 5.07 | 10 | 460 | 100 | 342 | 100 |
| TUBB2C protein [homo sapiens] | 2.13 | 1.86 | 1.99 | gi|14124960 | 25858.4 | 4.95 | 13 | 473 | 100 | 303 | 100 |
| Top ranked protein name (species) | Ratio bull C/bull A | Ratio bull D/bull B | Average ratio | Accession no. | Protein MW | Protein PI | Protein Pep. count | Protein score | Protein score C. I. % | Total ion score | Total ion C. I. % |
|----------------------------------|--------------------|--------------------|--------------|--------------|------------|------------|-------------------|--------------|---------------------|----------------|-------------------|
| TUBB2C protein [homo sapiens]    | 2.2                | 2.29               | 2.24         | gi|14124960 | 25858.4    | 4.95       | 10                | 258           | 100                | 140            | 100               |
| PREDICTED: similar to ENSANG P00000002667 [bos taurus] | 2.08               | 1.35               | 1.71         | gi|194674718 | 19864.8    | 5.62       | 6                 | 276           | 100                | 215            | 100               |
| PREDICTED: similar to ENSANG P00000002667 [bos taurus] | 2.18               | 1.52               | 1.85         | gi|194674718 | 19864.8    | 5.62       | 6                 | 286           | 100                | 222            | 100               |
| ACP1 protein [bos taurus] heat shock protein, alpha-crystallin-related, B9 [bos taurus] | -1.91              | -1.97              | -1.94        | gi|148744160 | 18156.9    | 6.71       | 12                | 282           | 100                | 142            | 100               |
| ACP1 protein [bos taurus] heat shock protein, alpha-crystallin-related, B9 [bos taurus] | 1.94                | 2.15               | 2.04         | gi|94966950 | 16773.2    | 8.22       | 8                 | 476           | 100                | 353            | 100               |
| ACP1 protein [bos taurus] heat shock protein, alpha-crystallin-related, B9 [bos taurus] | -1.67              | -1.32              | -1.49        | gi|73954519 | 26598.9    | 5.08       | 6                 | 101           | 100                | 53             | 99.2              |
| Acrosomal vesicle protein 1 [bos taurus] isoform 3 [Ca acrosomal vesicle protein-1] | 1.24                | -1.63              | -0.19        | gi|115495399 | 28934.4    | 4.53       | 7                 | 113           | 100                | 55             | 99.6              |
| Alpha enolase [bos taurus] | -1.4                | -1.37              | -1.38        | gi|4927286 | 47247.3    | 6.44       | 8                 | 154           | 100                | 105            | 100               |
| Chain B, refined 1.8 angstroms resolution crystal structure Of porcine epsilon-trypsin | -2.07               | -2.09              | -2.08        | gi|996927 | 8813.5    | 6.67       | 2                 | 119           | 100                | 96             | 100               |
| Chain B, refined 1.8 angstroms resolution crystal structure Of porcine epsilon-trypsin | -2.14               | -2.88              | -2.51        | gi|996927 | 8813.5    | 6.67       | 2                 | 104           | 100                | 79             | 100               |
| Outer dense fiber of sperm tails 2 [bos taurus] | 1.07                | 1.46               | 1.26         | gi|84000345 | 75450.6    | 7.52       | 20                | 373           | 100                | 282            | 100               |
| PREDICTED: heat shock 60kDa protein 1 (chaperonin) [bos taurus] | 1.13                | 1.06               | 1.09         | gi|119888228 | 74984.7    | 9.05       | 23                | 1060          | 100                | 865            | 100               |
| Plasma glutamate carboxypeptidase precursor [bos taurus] | 1.07                | 2.41               | 1.74         | gi|115495837 | 51646.3    | 5.55       | 11                | 453           | 100                | 389            | 100               |
| Chain A, the refined three-dimensional structure of cat muscle (M1) pyruvate kinase, at a resolution | 1.6                 | 2.37               | 1.98         | gi|157833510 | 57877.9    | 7.23       | 19                | 178           | 100                | 64             | 99.9              |
Table 1. Continued

| Top ranked protein name (species) | Ratio bull C/bull A | Ratio bull D/bull B | Average ratio | Accession no. | Protein MW | Protein PI | Pep. count | Protein score | Protein score C. I. % | Total ion score | Total ion C. I. % |
|----------------------------------|---------------------|---------------------|--------------|--------------|------------|------------|------------|--------------|-------------------|----------------|-----------------|
| Glyceraldehyde-3-phosphate dehydrogenase, spermatogenic [bos taurus] | 1.1 | 1.89 | 1.49 | gi|110626121 | 43260.3 | 8.32 | 19 | 596 | 100 | 422 | 100 |
| Tektin 3 [bos taurus] | 1.04 | 2.73 | 1.88 | gi|149773556 | 56645.5 | 6.42 | 28 | 661 | 100 | 414 | 100 |
| Tektin 3 [bos taurus] | 1.04 | -4.64 | -1.8 | gi|149773556 | 56645.5 | 6.42 | 26 | 571 | 100 | 363 | 100 |
| Tubulin, alpha 1a [mus musculus] Chain A, cytochrome Bc1 complex from bovine 5'-nucleotidase, cytosolic IB [bos taurus] actin-like 7A [bos taurus] | 2.5 | 2.51 | 2.5 | gi|6678465 | 49927.6 | 4.97 | 14 | 452 | 100 | 317 | 100 |
| PREDICTED: similar to actin-related protein T1 (ARP-T1) [bos taurus] actin-related protein T2 [bos taurus] WBP2 N-terminal like [bos taurus] | 1.29 | 2.46 | 1.87 | gi|4139392 | 49181.3 | 5.46 | 25 | 845 | 100 | 588 | 100 |
| 5'-nucleotidase, cytosolic IB [bos taurus] actin-like 7A [bos taurus] | 1.42 | 1.35 | 1.38 | gi|84370143 | 63970.7 | 8.8 | 20 | 416 | 100 | 261 | 100 |
| PREDICTED: similar to actin-related protein T1 (ARP-T1) [bos taurus] actin-related protein T2 [bos taurus] WBP2 N-terminal like [bos taurus] | 1.56 | 1.91 | 1.73 | gi|61878077 | 42103.3 | 5.39 | 14 | 306 | 100 | 202 | 100 |
| actin-related protein T2 [bos taurus] WBP2 N-terminal like [bos taurus] | 2.35 | -1.11 | 0.62 | gi|84000199 | 41886.4 | 5.48 | 20 | 470 | 100 | 281 | 100 |
| PREDICTED: similar to sp32 [bos taurus] PREDICTED: similar to sp32 [bos taurus] PREDICTED: similar to sp32 [bos taurus] | -7.14 | 3.07 | -2.03 | gi|194666681 | 61196.7 | 5.11 | 9 | 362 | 100 | 301 | 100 |
| Tyrosine 3-monoxygenase/tryptophan 3-monoxygenase activation protein, zeta polypeptide [homo sapien] 3-oxoacid CoA transferase 2 [bos taurus] | -14.1 | 7.21 | -3.44 | gi|194666681 | 61196.7 | 5.11 | 9 | 422 | 100 | 354 | 100 |
| PREDICTED: similar to sp32 [bos taurus] PREDICTED: similar to sp32 [bos taurus] PREDICTED: similar to voltage-dependent anion channel 2 [equus caballus] | -4.52 | 2.73 | -0.89 | gi|194666681 | 61196.7 | 5.11 | 11 | 509 | 100 | 454 | 100 |
| Tyrosine 3-monoxygenase/tryptophan 3-monoxygenase activation protein, zeta polypeptide [homo sapien] 3-oxoacid CoA transferase 2 [bos taurus] | -2.33 | -1.07 | -1.7 | gi|149689995 | 31524.5 | 7.46 | 6 | 96 | 100 | 63 | 99.9 |
| Tyrosine 3-monoxygenase/tryptophan 3-monoxygenase activation protein, zeta polypeptide [homo sapien] 3-oxoacid CoA transferase 2 [bos taurus] | 3.7 | 1.3 | 2.5 | gi|68085578 | 27695.8 | 4.73 | 8 | 130 | 100 | 40 | 65.6 |
| Tyrosine 3-monoxygenase/tryptophan 3-monoxygenase activation protein, zeta polypeptide [homo sapien] 3-oxoacid CoA transferase 2 [bos taurus] | -56.7 | 1.1 | -27.8 | gi|148223655 | 55973.1 | 7.15 | 3 | 76 | 98.4 | 45 | 75.5 |
| Tyrosine 3-monoxygenase/tryptophan 3-monoxygenase activation protein, zeta polypeptide [homo sapien] 3-oxoacid CoA transferase 2 [bos taurus] | 1.27 | -6.7 | -2.71 | gi|194676234 | 280818.2 | 4.87 | 5 | 102 | 100 | 91 | 100 |
### Top ranked protein name (species)

| Chain A, 12-Bromodo decanoic acid binds inside the Calyx of bovine beta-lactoglobulin Seminal vesicle secretory protein 109 [bos taurus] | Ratio bull C/bull A | Ratio bull D/bull B | Average ratio | Accession no. | Protein MW | Protein PI | Pep. count | Protein score | Protein C. I. % | Total ion score | Total ion C. I. % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.85 | -4.32 | -1.23 | gi|6980895 | 18355.4 | 4.76 | 12 | 612 | 100 | 463 | 100 |
| 1.55 | -3.42 | -0.93 | gi|47564036 | 15470.2 | 4.91 | 8 | 557 | 100 | 457 | 100 |
| 1.34 | -2.87 | -0.76 | gi|20663779 | 12787.8 | 5.08 | 9 | 610 | 100 | 516 | 100 |
| -1.6 | -1.95 | -1.77 | gi|27806061 | 17378.8 | 5.57 | 4 | 223 | 100 | 197 | 100 |
| 3.37 | -1.1 | 1.13 | gi|157279923 | 18086.8 | 5.87 | 2 | 127 | 100 | 107 | 100 |
| -1.23 | -7.94 | -4.58 | gi|194223000 | 39011.2 | 5.56 | 6 | 50 | 0 | 0 | 0 |
| 3.03 | -2.32 | 0.35 | gi|28849949 | 16129.7 | 4.9 | 6 | 175 | 100 | 117 | 100 |
| -3.18 | -1.1 | -2.14 | gi|194666681 | 61196.7 | 5.11 | 2 | 142 | 100 | 131 | 100 |
| 2.63 | -1.49 | 0.57 | gi|194666681 | 61196.7 | 5.11 | 4 | 95 | 100 | 81 | 100 |
| -1.32 | -5.99 | -3.65 | gi|194223000 | 39011.2 | 5.56 | 6 | 50 | 0 | 0 | 0 |

### Protein score

| Accession no. | Protein score | Protein score C. I. % | Total ion score | Total ion C. I. % |
|---|---|---|---|---|
| gi|6980895 | 18355.4 | 4.76 | 12 | 612 | 100 | 463 | 100 |
| gi|47564036 | 15470.2 | 4.91 | 8 | 557 | 100 | 457 | 100 |
| gi|20663779 | 12787.8 | 5.08 | 9 | 610 | 100 | 516 | 100 |
| gi|27806061 | 17378.8 | 5.57 | 4 | 223 | 100 | 197 | 100 |
| gi|157279923 | 18086.8 | 5.87 | 2 | 127 | 100 | 107 | 100 |
| gi|194223000 | 39011.2 | 5.56 | 6 | 50 | 0 | 0 | 0 |
| gi|28849949 | 16129.7 | 4.9 | 6 | 175 | 100 | 117 | 100 |
| gi|194666681 | 61196.7 | 5.11 | 2 | 142 | 100 | 131 | 100 |
| gi|194666681 | 61196.7 | 5.11 | 4 | 95 | 100 | 81 | 100 |
| gi|194223000 | 39011.2 | 5.56 | 6 | 50 | 0 | 0 | 0 |

### Protein MW

| Accession no. | Protein MW | Protein PI | Pep. count | Protein score | Protein C. I. % | Total ion score | Total ion C. I. % |
|---|---|---|---|---|---|---|---|
| gi|6980895 | 18355.4 | 4.76 | 12 | 612 | 100 | 463 | 100 |
| gi|47564036 | 15470.2 | 4.91 | 8 | 557 | 100 | 457 | 100 |
| gi|20663779 | 12787.8 | 5.08 | 9 | 610 | 100 | 516 | 100 |
| gi|27806061 | 17378.8 | 5.57 | 4 | 223 | 100 | 197 | 100 |
| gi|157279923 | 18086.8 | 5.87 | 2 | 127 | 100 | 107 | 100 |
| gi|194223000 | 39011.2 | 5.56 | 6 | 50 | 0 | 0 | 0 |
| gi|28849949 | 16129.7 | 4.9 | 6 | 175 | 100 | 117 | 100 |
| gi|194666681 | 61196.7 | 5.11 | 2 | 142 | 100 | 131 | 100 |
| gi|194666681 | 61196.7 | 5.11 | 4 | 95 | 100 | 81 | 100 |
| gi|194223000 | 39011.2 | 5.56 | 6 | 50 | 0 | 0 | 0 |
| Table 1. Continued |
|-------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Top ranked protein name (species) | Ratio bull C/bull A | Ratio bull D/bull B | Average ratio | Accession no. | Protein MW | Protein PI | Pep. count | Protein score | Protein score C. I. % | Total ion score | Total ion C. I. % |
| Sperm acrosome associated 5 [bos taurus] | -1.26 | -2.45 | -1.85 | gi|949666873 | 17568.2 | 5.55 | 4 | 127 | 100 | 91 | 100 |
| PREDICTED: similar to sp32 [bos taurus] | -1.48 | -2.51 | -1.99 | gi|94666681 | 61196.7 | 5.11 | 6 | 563 | 100 | 535 | 100 |
| Protease, serine, 2 [rattus norvegicus] | 1.63 | 1.27 | 1.45 | gi|6981420 | 25942.7 | 4.71 | 1 | 68 | 87.2 | 61 | 99.6 |
| Thioredoxin domain containing 3 (spermatozoa) [bos taurus] | -1.34 | -1.49 | -1.42 | gi|114053199 | 46889.1 | 4.94 | 17 | 269 | 100 | 134 | 100 |
| Acrosomal vesicle protein 1 [bos taurus] | 1.27 | -2.08 | -1.41 | gi|115495399 | 28934.4 | 4.53 | 6 | 100 | 100 | 60 | 99.9 |

| Table 2. Ingenuity Canonical Pathways using IPA. The IPA results of canonical pathways were obtained using the 2D-DIGE data and sorted by the significance level of the canonical pathways. A total of 47 canonical pathways with their p-value and related proteins were represented here |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Ingenuity canonical pathways | log (p-value) | Ratio | Molecules |
| Glycolysis/gluconeogenesis | 4.96E00 | 4.55E-02 | PGK2,ENO1,GAPDH,LDHAL6B |
| 14-3-3-mediated Signaling | 3.05E00 | 2.46E-02 | TUBB2C,YWHAZ,TUBA3C/TUBA3D |
| Propanoate metabolism | 2.43E00 | 3.51E-02 | LDHAL6B,ACADS |
| Purine metabolism | 2.05E00 | 1.11E-02 | NT5C1B,HSPD1,PDE6D |
| Mitochondrial dysfunction | 1.72E00 | 1.47E-02 | SOD2,CYC1 |
| Aldosterone signaling in epithelial cells | 1.59E00 | 1.23E-02 | HSPB9,HSPD1 |
| Germ cell-sertoli sell junction signaling | 1.58E00 | 1.24E-02 | TUBB2C,TUBA3C/TUBA3D |
| Phenylalanine, tyrosine and tryptophan biosynthesis | 1.57E00 | 5.88E-02 | ENO1 |
| Gap junction signaling | 1.56E00 | 1.99E-02 | TUBB2C,TUBA3C/TUBA3D |
| Riboflavin metabolism | 1.50E00 | 5E-02 | ACP1 (includes EG:11431) |
| Sertoli cell-sertoli cell junction signaling | 1.48E00 | 1.05E-02 | TUBB2C,TUBA3C/TUBA3D |
| Breast cancer regulation by stathmin1 | 1.42E00 | 9.95E-03 | TUBB2C,TUBA3C/TUBA3D |
| Protein ubiquitination pathway | 1.19E00 | 7.41E-03 | HSPB9,HSPD1 |
| Galactose metabolism | 1.18E00 | 2.33E-02 | LALBA |
| Cell cycle: G2/M DNA damage checkpoint regulation | 1.17E00 | 2.04E-02 | YWHAZ |
| Retinol metabolism | 1.17E00 | 2.27E-02 | NT5C1B |
| β-alanine metabolism | 1.14E00 | 2.13E-02 | ACADS |
| Phototransduction pathway | 1.09E00 | 1.79E-02 | PDE6D |
| Cysteine metabolism | 1.09E00 | 1.89E-02 | LDHAL6B |
| Protein kinase a signaling | 1.06E00 | 6.27E-03 | YWHAZ,PDE6D |
| Myc mediated apoptosis signaling | 1.05E00 | 1.64E-02 | YWHAZ |
| Butanoate metabolism | 1.04E00 | 1.67E-02 | ACADS |
| ERK5 signaling | 1.02E00 | 1.59E-02 | YWHAZ |
| Pyruvate metabolism | 9.99E-01 | 1.52E-02 | LDHAL6B |
| Valine, leucine, and isoleucine degradation | 9.99E-01 | 1.52E-02 | ACADS |
| PDGF signaling | 9.69E-01 | 1.37E-02 | ACP1 (includes EG:11431) |
| Aminosugars metabolism | 9.69E-01 | 1.41E-02 | PDE6D |
| TR/RXR activation | 8.96E-01 | 1.12E-02 | ENO1 |
| Axonal guidance signaling | 8.9E-01 | 4.71E-03 | TUBB2C,TUBA3C/TUBA3D |
Table 2. Continued

| Ingenuity canonical pathways                  | log (p-value) | Ratio  | Molecules        |
|---------------------------------------------|--------------|--------|-----------------|
| IGF-1 signaling                             | 8.34E-01     | 9.62E-03 | YWHAZ           |
| Nicotinate and nicotinamide metabolism      | 8.26E-01     | 9.9E-03  | NT5C1B          |
| Type 1 diabetes mellitus Signaling          | 7.95E-01     | 8.55E-03 | HSPD1           |
| PI3K/AKT signaling                          | 7.61E-01     | 7.52E-03 | YWHAZ           |
| p70S6K signaling                            | 7.47E-01     | 7.87E-03 | YWHAZ           |
| Fatty acid metabolism                       | 7.47E-01     | 8.06E-03 | ACADS           |
| Cardiac β-adrenergic signaling              | 7.2E-01      | 7.19E-03 | PDE6D           |
| Relaxin signaling                           | 7.14E-01     | 6.9E-03  | PDE6D           |
| Pyrimidine metabolism                       | 6.94E-01     | 7.09E-03 | NT5C1B          |
| Oxidative phosphorylation                   | 6.86E-01     | 6.94E-03 | CYC1            |
| Acute phase response                        | 6.29E-01     | 5.81E-03 | SOD2            |
| RAR activation                              | 6.2E-01      | 5.65E-03 | NT5C1B          |
| Ephrin receptor signaling                   | 6.15E-01     | 5.1E-03  | ACP1 (includes EG:11431) |
| NRF2-mediated oxidative stress response     | 6E-01        | 5.26E-03 | SOD2            |
| ERK/MAPK signaling                          | 5.92E-01     | 5.05E-03 | YWHAZ           |
| cAMP-mediated signaling                     | 5.38E-01     | 4.65E-03 | PDE6D           |
| Role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis | 4.11E-01 | 3.04E-03 | PRSS1/PRSS3 |
| C-Protein coupled receptor signaling        | 2.48E-01     | 1.92E-03 | PDE6D           |

Table 3. Networks created by IPA analysis. The data obtained from the 2D-DIGE analysis were analyzed using IPA software and sorted by the significance level of the networks. The interactions of the proteomics were generated by overlaying the first and the second networks

| Top functions                                                                 | Score | Focus molecules | Molecules in network |
|-------------------------------------------------------------------------------|-------|-----------------|----------------------|
| Free radical scavenging, cancer, hematological disease                      | 33    | 13              | AARS,ACADS,ACP1 (includes EG:11431),ACPP,Akt,Alpha tubulin,CD3, CENPJ, CYC1,Cytochrome c,EN01,Enolase, ERK1/2,GZMK, HSP, HSPB9, HSPD1,Jnk,LALBA, mannitol, MLXIP,ODF1,PAC52,PEBP4,P13K (complex), PRSS1/PRSS3, SIRT3,SOD2,TA0K2,TPD52 ,TUBA3C/TUBA3D, TUBB1, TUBB2C,Tubulin, YWHAZ |
| Cellular assembly and organization, cellular development, embryonic development | 9     | 3               | ACRV1,ACTL7A,Od2, YBX2 |
| Carbohydrate metabolism, small molecule biochemistry, antigen presentation  | 3     | 1               | A2M,SPACA3           |
| Cell-to-cell signaling and interaction, reproductive system development, and function, cellular development | 3     | 1               | Gstp1 (includes others),WBP2NL |
| Embryonic development, endocrine system development and function, organ development | 3     | 1               | HNF1A,PGCP,TGFB1 (includes EG:21803) |
Top functions & Score & Focus molecules & Molecules in network \\
--- & --- & --- & --- \\
Cell cycle, cellular compromise, 
cellular growth and proliferation & 3 & 1 & ACRBP, NUMA1, progesterone \\
RNA damage and repair, gene 
expression, RNA post- 
transcriptional modification & 3 & 1 & HNRNPDL, L-lactate 
edehydrogenase, LDHAL6B \\
Drug metabolism, lipid 
metabolism, small molecule 
biochemistry & 3 & 1 & 5'-nucleotidase, NT5C1B, RDH \\
Genetic disorder, neurological 
disease, cell morphology & 2 & 1 & CACNA1E, E2F4, E2F, EFHC1, TEX11, V 
oltage Gated Calcium Channel 
ATP, GAPDH, glyceraldehyde-3-
phosphate dehydrogenase (pho-
sphorylating), HSPA2, MAPK3, MI 
TF, NTHL1, SH3BP4, APP, CreM, 
DNMT3A, DNMT3B, FGF 
2, MAPT, PAPOLB, PBX4, PDK1, PG 
K2, Pgg, PSEN1, SP3, UBC \\
Cell death, liver necrosis/cell 
death, cellular movement & 1 & 2 & 3',5'-cyclic-GMP 
phosphodiesterase, 3',5'-cyclic-
nucleotide phosphodiesterase, 
ARL1, ARL2, ARL3, ARL15, 
C9orf25, Ca2+, CDC42, CETN3, G 
RK1, GRLK7, HRAS, KRAS, NRAS, Pd 
e, PDE6 d), PDE6d, PTGIR, RAB13, 
RAB18, RAD23A, RAP1A, RAP2B, 
RHEB, RHOD, RHOB, RND1, RPGR \\
Neurological disease, genetic 
disorder, organismal injury and 
abnormalities & 2 & 1 & 3',5'-cyclic-GMP 
phosphodiesterase, 3',5'-cyclic-
nucleotide phosphodiesterase, 
ARL1, ARL2, ARL3, ARL15, 
C9orf25, Ca2+, CDC42, CETN3, G 
RK1, GRLK7, HRAS, KRAS, NRAS, Pd 
e, PDE6 d), PDE6d, PTGIR, RAB13, 
RAB18, RAD23A, RAP1A, RAP2B, 
RHEB, RHOD, RHOB, RND1, RPGR \\
Cell-to-cell signaling and 
interaction, cellular assembly 
and organization, tissue 
development & 1 & 2 & 3',5'-cyclic-GMP 
phosphodiesterase, 3',5'-cyclic-
nucleotide phosphodiesterase, 
ARL1, ARL2, ARL3, ARL15, 
C9orf25, Ca2+, CDC42, CETN3, G 
RK1, GRLK7, HRAS, KRAS, NRAS, Pd 
e, PDE6 d), PDE6d, PTGIR, RAB13, 
RAB18, RAD23A, RAP1A, RAP2B, 
RHEB, RHOD, RHOB, RND1, RPGR 

Statistically significant by enriching above the threshold as shown (Figure 3a). Based on the DAVID software, glycolysis/gluconeogenesis is the first cluster with 3.17 of enrichment score and enolase 1 was the first significantly expressed protein in this pathway (Figure 3b) the same pathway was also confirmed by IPA as significant (p<0.0001) in the canonical pathway (Figure 3a).

Additionally, free radical scavenging and cellular assembly or organization, cellular development, and embryonic development networks were identified to be first and second networks, respectively (Table 3). The first and second networks had a ratio containing focused molecules over score; 13/33 and 3/9, respectively. Based on the IPA results, most of the proteins in our sperm proteomics data were found to be enzymes with important functions. Their locations and functions were listed in Table 4. The final interactome was created by overlaying the two networks mentioned earlier (Figure 4) by selecting differentially expressed two proteins: outer dense fiber of sperm tails 2 (ODF-2) from the first network and manganese superoxide dismutase (SOD) from the second network. According to 2D-DIGE results, MnSOD was 4.65 times more abundant in spermatozoa from relatively higher
Figure 3. IPA canonical pathways and proteins of cluster 1 by DAVID. (a) this figure shows 12 canonical pathways generated using IPA, which were statistically significant by enriching above the threshold (out of 47). The bars in the graph represent the total molecules involved in these pathways while the ratio (yellow line) shows the proteins given for each pathway in the data, (b) the glycolysis is shown to be the first cluster of functional gene annotation according to DAVID bioinformatics tool. In this figure, the horizontal axis demonstrates the functions of the related proteins that are represented in the vertical axis
Table 4. Protein cellular locations and physiology. The locations and functions of the detected sperm proteins detected were generated using IPA analyses.

| Id       | Symbol     | Entrez gene name                                                      | Location       | Type(s)        |
|----------|------------|-----------------------------------------------------------------------|----------------|----------------|
| 77735757 | ACADS      | acyl-CoA dehydrogenase, C-2 to C-3 short chain enzyme                 | Cytoplasm      | enzyme         |
| 148744160| ACP1       | acid phosphatase 1, soluble                                           | Cytoplasm      | phosphatase    |
| 194666681| ACRBP      | acrosin binding protein                                               | Extracellular   | other          |
| 115459399| ACRV1      | acrosomal vesicle protein 1                                           | Cytoplasm      | other          |
|          | ACTL7A     | actin-like 7A                                                         | Nucleus        | other          |
| 61878077 | ACTRT1     | actin-related protein T1                                               | Cytoplasm      | other          |
| 84000199 | ACTRT2     | actin-related protein T2                                               | unknown        | other          |
| 115495817| C15orf26   | chromosome 15 open reading frame 2                                     | unknown        | other          |
| 76650703 | EFHC1      | EF-hand domain (C-terminal) containing 1                              | Cytoplasm      | other          |
| 4927286  | ENO1       | enolase 1, (alpha)                                                    | Cytoplasm      | transcription regulator |
| 110626121| GAPDHS     | glyceraldehyde-3-phosphate dehydrogenase, spermatogenic               | Cytoplasm      | enzyme         |
| 94966950 | HSPB9      | heat shock protein, alpha-crystallin-related, B9                      | Cytoplasm      | other          |
| 156120505| IZUMO4     | IZUMO family member 4                                                 | unknown        | other          |
| 78369344 | LDHAL6B    | lactate dehydrogenase A-like 6B                                       | Cytoplasm      | enzyme         |
| 84370143 | NT5C1B     | 5'-nucleotidase, cytosolic IB                                         | Cytoplasm      | phosphatase    |
| 84000345 | Odf2       | outer dense fiber of sperm tails 2                                    | Cytoplasm      | other          |
| 27806061 | PDE6D      | phosphodiesterase 6D, cGMP-specific, rod, delta                       | Cytoplasm      | enzyme         |
|          |            |                                                                       |                |                |
| 77735827 | PEBP4      | phosphatidylethanolamine-binding protein 4                            | Cytoplasm      | other          |
| 115495837| PGCP       | plasma glutamate carboxypeptidase                                      | Extracellular   | peptidase      |
|          |            |                                                                       | Space           |                |
| 174840786| PGK2       | phosphoglycerate kinase 2                                             | Cytoplasm      | kinase         |
| 6981420  | PRSS1/PRSS3| protease, serine, 1 (trypsin 1)                                       | Extracellular   | peptidase      |
|          |            |                                                                       | Space           |                |
| 7555818  | SOD2       | superoxide dismutase 2, mitochondrial                                  | Cytoplasm      | enzyme         |
| 157279923| SPACA3     | sperm acrosome associated 3                                           | Cytoplasm      | enzyme         |
| 149773556| TEKT3      | tektin 3                                                              | Cytoplasm      | other          |
| 6678465  | TUBA3C/TUBA3D| tubulin, alpha 3c                                                   | Cytoplasm      | other          |
| 14124960 | TUBB2C     | tubulin, beta 2C                                                      | Cytoplasm      | other          |
| 126723634| WBP2NL     | WBP2 N-terminal like                                                  | Cytoplasm      | other          |
| 68085578 | YWHAZ      | tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide | Cytoplasm      | enzyme         |

Fertility bulls (Bulls A and B) compared to those from their lower fertility counterparts (Bulls C and D) in the experimental population. On the other hand, ODF2 protein was up regulated in spermatozoa from low fertility animals (Bulls C and D) although their expression levels varied in the individual bulls. The average of protein expression ratios obtained from relatively lower fertility bulls (Bulls C and D) over high fertility bulls (Bulls A and B) (Table 1).

3.4. Sperm Proteins as Fertility Markers and Likely Protein-Protein Interaction

In search for potential fertility markers, we have determined sperm proteins that have been identified in this study and through the literature search. The fertility markers are mainly chromatin/nuclear proteins, seminal plasma proteins, proteins in acrosome, proteins which regulate ATP synthesis, capacitation related proteins, and sperm cytoskeletal proteins (Table 5).
Figure 4. The merged interactome by IPA Networks 1 and 2. The final interactome was created by overlaying of these two networks free radical scavenging and cellular assembly or organization, cellular development, and embryonic development networks. The proteins and their interactions between others including as well as their locations in the cell are also included in the figure. The red and green colors of each protein represent up-regulated and down-regulated proteins in spermatozoa from lower fertility bulls (Bull C and D), respectively.
Table 5. Comparison of sperm protein markers reported among different bovine species belonging to different categories of sperm physiology pathways in current literature

| Category of protein | Protein name | Function | Holstein and other bovine bull sperm | Angus bull sperm |
|---------------------|--------------|----------|--------------------------------------|-----------------|
| Sperm chromatin proteins | Protamines (PRM) | Substitute for histones in the sperm chromatin spermatid to sperm development phase of spermatogenesis. They compact sperm DNA into a highly condensed, stable and inactive complex | Holstein (Fortes et al. 2014; Dogan et al. 2015) | No reports |
|                     | HIST1H2BA/TH2B | Testis specific histone variant specifically required to direct the transformation of dissociating nucleosomes to protamine in male germ cells | Holstein (Kutchy et al. 2017) | No reports |
|                     | H3K27ac       | Functions as histone acetyltransferase and regulates transcription via chromatin remodeling. Acetylates all four core histones in nucleosomes | Holstein (Kutchy et al. 2018) | No reports |
|                     | H3K27me3      | Catalytic subunit of the PRC2/EED-EZH2 complex, which methylates 'Lys-9' (H3K9me) and 'Lys-27' (H3K27me) of histone H3, leading to transcriptional repression of the affected target gene | Holstein (Kutchy et al. 2018) | No reports |
| Seminal plasma proteins | Osteopontin | Binds tightly to hydroxyapatite and appears to form an integral part of the mineralized matrix. Probably important to cell-matrix interaction | Holstein (Cancel et al. 1997; Cancel et al. 1999) | No reports |
| Acrosomal proteins | Acrosomal tyrosine-phosphorylated proteins | Role in acrosomal formation of spermatids during spermiogenesis | Japanese Black cattle (Harayama et al. 2010) | No reports |
|                     | IZUMO1        | Fusion of sperm to egg plasma membrane | Japanese Black cattle (Fukuda et al. 2016) | No reports |
| ATP synthesis proteins | Adenylate kinase 1 (AK1) | Catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP. Plays an important role in cellular energy homeostasis and in adenine nucleotide metabolism | Holstein (D’Amours et al. 2012) | No reports |
|                     | Enolase 1 (ENO1) | Multifunctional enzyme, plays part in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance and allergic responses. May also function in the intravascular and pericellular fibrinolytic system due to its ability to serve as a receptor and activator of plasminogen on the cell surface of several cell-types such as leukocytes and neurons. Stimulates immunoglobulin production | Hanwoo (Park et al. 2012) | No reports |
|                     | ATP synthase H+ transporting mitochondrial F1 complex β subunit (ATP5B) | ATP synthesis and hydrolysis of proton transport | Holstein (Peddinti et al. 2008) | No reports |
|                     | Na+/K+-ATPase | Its role is to create the electrochemical gradient of sodium and potassium, providing the energy for active transport of various nutrients | Holstein (Thundathil et al. 2006) | No reports |
4. Discussion

Molecular and cellular attributes of sperm are important for fertilization, egg activation and embryonic development. Some bulls that produce high numbers of spermatozoa with normal morphology exhibit low fertility after hundreds of AI (DeJarnette and Marshall 2005). Despite the importance of male fertility in reproduction for both basic and applied science, there is no sufficient method to determine sperm quality other than conventional semen analysis. Indeed, traditional approaches to estimate male fertility such as evaluating of sperm morphology and motility might not be accurate all the time (Bartoov et al. 1993).

Molecular mechanisms of how male fertility can be fully determined by evaluating the sperm quality still remain a mystery. The objective of this study was to identify differentially expressed proteins in spermatozoa from bulls with different fertility taking advantage of both wet lab and computational biology and bioinformatics approaches.

While proteomics is an important high-throughput method providing a panoramic view of proteomes in the cell, bioinformatics is a powerful approach to predict and discover the functions and interactions of the given proteins. Sperm proteome profiling has been reported in human (Martinez-Heredia et al. 2006; Li et al. 2007), in murine (Cao et al. 2006; Baker et al. 2008a, 2008b), in porcine (van Gestel et al. 2007) and in bovine (Lalancette et al. 2006; Peddinti et al. 2008; D’Amours et al. 2010) and results generated by these researchers provided important insights about identities of diverse proteins. Previously, expressions of nine proteins, including two isoforms of epididymal sperm-binding protein E12 and proteasome subunit α type-6, were shown to be differentially expressed among high and low fertility Holstein bulls. Recently, using sperm from low vs. High fertility bulls, three proteins; enolase (ENO1), voltage dependent anion channel 2 (VDAC2), and ubiquinol-cytochrome-c reductase complex core protein 2 (UQCRC2) were detected to be the fertility markers in bulls (Park et al. 2012; Park et al. 2013; Kwon et al. 2015a). Our group previously showed that the expression of certain proteins in spermatozoa from high-fertility Holstein bulls was implicated in energy metabolism, cell communication, spermatogenesis, and cell motility (Peddinti et al. 2008). However, according to our literature search, sperm proteomics profiling of Angus bulls has been elusive.

In our study reported here, there were more than 80 sperm protein spots that notably differed among the bulls with varying fertility. By comparing the protein spot sequences to the databases, we identified most of these proteins. Our 2D-DIGE results demonstrated that ODF2 was up-regulated in spermatozoa from bulls with different fertility taking advantage of both wet lab and computational biology and bioinformatics approaches.

| Category of protein | Protein name | Function | Holstein and other bovine bull sperm | Angus bull sperm |
|---------------------|--------------|----------|--------------------------------------|-----------------|
| Cytoskeletal proteins | Outer dense protein fiber 2 (ODF2) | A major component of sperm tail outer dense fibers (ODF). ODFs are filamentous structures located on the outside of the axoneme in the midpiece and principal piece of the mammalian sperm tail and may help to maintain the passive elastic structures and elastic recoil of the sperm tail. May have a modulating influence on sperm motility. Functions as a general scaffold protein that is specifically localized at the distal/subdistal appendages of mother centrioles. | Holstein (Petersen et al. 1999; Wang et al. 2014) | Current study |
| Other proteins | Super oxide dismutase (SOD) | Destroys radicals which are normally produced within the cells and which are toxic to biological systems | Holstein (Bansal and Bilaspuri 2008) | Current study |
of odf2 gene have been detected in rat and bull spermatozoa (Brohmann et al. 1997; Schalles et al. 1998) and mutations in odf2 gene are known to result in certain tail abnormalities in spermatozoa (Tarnasky et al. 2010). Previously, ODF2 and Cenexin were shown to be the alternative splice variants of exon 3b of odf2 in mouse testis (Huber et al. 2008). Our results from the 2D-DIGE experiments demonstrated the importance of ODF-2 in sperm motility and ultimately bull fertility and the results are supported by (Cao et al. 2006). Protein variations can be induced by additional posttranslational modifications such as phosphorylation, cleavage, and glycosylation (Flickinger et al. 2001). Indeed, our results showed that many of the differentially expressed protein spots corresponded to ODF2, suggesting the possible posttranslational modifications (PTM) occurring in this protein. For example, it was revealed that a tyrosine phosphorylation in sperm ODF2 took place during capacitation (Mariappa et al. 2010).

The other protein of significant function was SOD based on our 2D-DIGE and bioinformatics results. The SOD is an important antioxidant that dismutase O2− into H2O2, improves cell survival by reducing the level of ROS. It is plausible that this increased expression of SOD reflects a defensive response to protect the spermatozoa against oxidative stress (Mruk et al. 2002; Fujii et al. 2003; Cui et al. 2008; Yoon et al. 2016). The SODs are scavenger antioxidants catalyzing the neutralization reaction of superoxide radicals into H2O2 and oxygen in the cell. Because of the cytoplasmic reduction and environmental changes, spermatozoa become vulnerable to oxidative stress in the course of spermatogenesis (Agarwal and Prabakaran 2005). On the other hand, SOD has also a protective effect against oxidation, enhancing sperm motility (Lindemann et al. 1988; Kobayashi et al. 1991). Likewise, there was an increase in sperm motility and viability and a decrease in the LPO levels when bull spermatozoa were subjected to Mn2+ treatment in presence of oxidative stress (Bansal and Bilaspuri 2008).

Another study showed that the expression of MnSOD in bovine blastocysts increased when the culture media was supplemented with fetal calf serum (FCS), which could improve cryotolerance of these blastocysts (Rizos et al. 2003). In addition, it was suggested that SOD activity in bovine spermatozoa might be a metabolic indicator of membrane integrity. Since the same study revealed a correlation between malondialdehyde production and SOD activity, measuring this enzyme in spermatozoa might predict oxidative stress-induced damage (Beconi et al. 1991). In contrast, a study concluded that male infertility was not related to the SOD activities in both human spermatozoa and seminal plasma where the semen was obtained from men with normozoospermia and oligoasthenozoospermia (Hsieh et al. 2002). However, fertility scores of human patients were not as reliable as those obtained from livestock animals. In another study, the MnSOD activity in human spermatozoa was detected to be negligible. However, compared to human blood plasma, the abundance of total SOD activity in the seminal plasma was 20 times higher. According to the same study, it was concluded that the minimal activity of SOD enzymes in spermatozoa might be the reason of its protection against internal and external superoxide radicals (Peeker et al. 1997).

We established here that MnSOD was up-regulated in spermatozoa from relative higher fertile bulls (Bulls A and B) compared to their low fertile counterparts based on the 2D-DIGE results. Therefore, these differentially expressed proteins could potentially play key roles in spermatozoa and may be involved in male fertility and could be used to predict superior sires (Kwon et al. 2015a, 2015c). We concluded that the abundance of SOD in spermatozoa differs among the bulls with different fertility in a given population. This might be an indicator of excessive oxidative stress caused by cryopreservation or centrifugation in spermatozoa, affecting sperm motility and ultimately male fertility.

Protein based molecular markers provide reliable information about the elite sire as well as its progeny. However, comparing Angus cattle across other cattle breeds especially Holstein for recognizing the protein molecular markers for sire selection, we find no report for Angus. Different categories of sperm associated proteins are chromatin/nuclear proteins, seminal plasma proteins, proteins in acrosome, proteins which regulate ATP synthesis, capacitation related proteins, and sperm cytoskeletal proteins. We have report about the sperm chromatin related proteins as potential markers for selection (Fortes et al. 2014; Dogan et al. 2015; Kutchy et al. 2017; Kutchy et al. 2018) out of all these reported proteins no reports are available for Angus cattle one of the well reputed cattle breed in the USA. Therefore, we realize urgent need of selection of Angus bulls based on protein as markers of selection and hence...
dire need to report the potential protein markers in Angus sperm and related proteins.

In conclusion evaluating semen quality and predicting bull fertility are vital for precision livestock agriculture. With the increasing uses of artificial insemination bull effects on herd is becoming more prevalent. Low heritability of the fertility traits implies that much of the differences in bull fertility are related to environment, management, nutrition and epigenetics. As such, sperm functional genomes such as proteomes reflect sperm fertility, and the differentially expressed proteins in high fertility vs. Low fertility can be harnessed as potent fertility markers in sperm evaluation and marker assisted selection. There is a need for reliable phenotypic data in order to identify such fertility markers. Compared to the dairy cattle, there is a disparity of phenotypic data in beef cattle. To remedy this, beef producers should collect phenotypic data and keep records including the pedigree information. Through comparative biology, sperm fertility proteins identified in dairy bulls can be studied to determine to what extent the protein markers can be used for beef bulls. Sperm protein markers can be combined with other sperm parameters and used as complementary tests in genomic selection. Comprehensive studies aimed at sperm functional genome and epigenome in larger sample sizes during the entire year for multiple years are expected to further fundamental science and technology of bull fertility.

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