RESEARCH REPORT
ANIMAL SCIENCE

Title: Identification of biomarkers of fertility in commercial boars **–#18-195 - InPPA**

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Industry Summary:

Conventional semen analysis currently used to evaluate boar fertility is poorly predictive of true fertility. Poor fertility has high economic impact on livestock production. Therefore, new tools that accurately predict boar fertility are needed. There is good preliminary evidence from 2-D gel electrophoresis that seminal plasma proteins are related to boar fertility. However, gel electrophoresis is limited in its ability to identify large numbers of proteins and typically only identifies proteins in high abundance in the seminal plasma. Shotgun proteomics is a growing scientific tool to identify and quantify proteins in biological samples in large quantities and with high accuracy. Therefore, the purpose of this study was to determine whether shotgun proteomics could differentiate boars with known fertility.

Semen samples from 58 boars with known fertility (farrowing rate and litter size from 50 single-sire matings) were sent to Purdue for shotgun proteomic evaluation. Liquid chromatography-mass spectrometry (LC-MS/MS) methods were used to identify all of the proteins present in the seminal plasma from the boars of 4 fertility phenotypes: high farrowing rate and total born (HFHB; n=9), high farrowing rate with low total born (HFLB; n=10), low farrowing rate and total born (LFLB; n=9), and low farrowing rate with high total born (LFHB; n=4).

Proteins identified were associated with biological functions such as sperm-egg interactions, oxidative stress, function of sperm in the female's reproductive tract, and immune function. This method was able to identify proteins that differentiated between the boars with high fertility (HFHB) and the three subfertility phenotypes. Several proteins were identified that could be tested as a panel to identify subfertile boars at entry into the boar stud. The ability to identify subfertile boars at entry into the boar stud before their true fertility is known could have huge economic impacts on the boar stud industry.

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Scientific Abstract:

There is a need to identify subfertile boars before they enter into the breeding herd. Seminal plasma proteins are essential for normal sperm function and transport and play an important role in fertilization. The objective of this study was to use liquid chromatography tandem mass spectrometry for shotgun proteome analysis to investigate whether differences in boar fertility phenotype can be differentiated by seminal plasma protein expression. Following 50 breedings, boars were categorized into one of four phenotypes: high farrowing rate and total born (HFHB; n=9), high farrowing rate with low total born (HFLB; n=10), low farrowing rate and total born (LFLB; n=9), and low farrowing rate with high total born (LFHB; n=4). There were 436 proteins measured in at least one sample across all animals. There were 245 high confidence proteins and 56 were differentially abundant between the high fertility phenotype (HFHB) and at least one of the three subfertile groups. Findings support that seminal plasma protein profiles are distinct between boars with different fertility phenotypes.

Introduction:

Currently, in the swine industry, artificial insemination is performed with large numbers of sperm pooled from multiple males to compensate for reductions in fertility from individual boars. However, the industry is moving towards identifying boars with the greatest genetic potential for economically important traits and then utilizing these boars to breed more females, and produce more genetically superior offspring. This requires the ability to identify boars with high fertility as fewer sperm cells will be used to inseminate individual females. Currently male fertility in humans and other animals is evaluated using sperm morphology test, motility test, swelling/eosin test and penetration assay. Although these tools provide initial quantitative information on semen, their clinical value in predicting fertility is debated. In swine, in particular, it's been found that boars with excellent semen quality do not necessarily have high fertility (i.e. litter size and farrowing rates). Therefore, new analysis tools and new markers are needed to evaluate male fertility.

Seminal plasma proteins are involved in various molecular mechanisms associated with sperm function; sperm interaction with the female's reproductive tract; and oocyte fertilization. Studies aimed at characterizing boar seminal plasma found the majority of proteins belong to the spermadhesin family and possess heparin-binding ability. Spermadhesins form a protective coat around the sensitive acrosomal region of the sperm head, thus possibly preventing premature acrosome reaction, and therefore affecting fertility. Other proteins identified in seminal plasma that may affect fertility include glutathione peroxidase, heat shock proteins, osteopontin, platelet activating factor acetylhydrolase, protein tyrosine acid phosphatase, and lactostransferrin. Several studies investigated whether seminal plasma proteins correlated with fertility in boars, and found that there may be potential differences in protein levels between sires with different litter sizes and farrowing rates. Glutathione peroxidase was positively associated with fertility in several studies as well as heat shock protein 70. Several proteins, D-PSP-1 and a 60 kDa/6.5 pI unidentified protein, were identified as being negatively correlated with fertility. ***Together, these findings support that seminal plasma proteins may be predictive of male fertility.***

Previous studies relied on 2-D gel electrophoresis to identify proteins in seminal plasma that typically resulted in the identification of approximately 200-250 unique peptides, with only a very limited amount of sequencing or mass spectrometry analysis done for post-hoc identification of proteins. Thus, this approach lacked the robustness of current shot-gun proteomics approach for identification and quantification of proteins that is needed for biomarker discovery. Other limitations of these studies were small sample sizes, low sperm numbers and use of ejaculates that were greater than 80% motile and normal (above industry quality standards) for heterospermic inseminations as the indicator of inherit fertility.

Objectives:

The overall goal of this study was to identify potential biomarkers of boar fertility in seminal plasma. For this project high throughput, shotgun proteomics approach was used to characterize seminal plasma proteins in four groups of boars. Groups of boars were characterized by differing fertility status; high fertility (HH; high litter size and high farrowing rate), low fertility (LL, low litter size and low farrowing rate) or mixed HL (high litter size and low farrowing rate) or LH (low litter size and high farrowing rate) with the objective of meeting the following aims:

1: Determine if seminal plasma protein expression can distinguish between boars of high fertility versus low fertility.

2: Determine if seminal protein expression is specifically related to litter size or farrowing rate in boars.

Methods:

Ejaculates were collected from Duroc boars (9-11 mo. of age) of similar genetics at a commercial boar stud and evaluated for motility and morphology. Approximately 2 mL of whole semen (gel fraction removed) from the first ejaculate that met acceptance criteria for use in the breeding program was packed with ice packs and shipped to Purdue University overnight. Upon arrival to Purdue, semen samples were centrifuged at 4ºC and 2700 g for 20 minutes to pellet sperm. Seminal plasma was removed after centrifugation and stored at -20º C until protein isolation. Of the 385 total ejaculates received, semen samples from 128 boars had at least 50 breedings. As a measure of fertility, an average farrowing rate and total born was calculated and plotted for the 128 boars. From scatter plots, four quadrants were created to divide boars that fell above or below mean farrowing rate and mean total born. Thirty-two boars were selected to study based on extremes for: high farrowing rate and high total born (HFHB; n=9), high farrowing rate and low total born (HFLB; n=10), low farrowing rate and high total born (LFHB; n=4), and low farrowing rate and low total born (LFLB; n=9). In particular, boars identified for study were two standard deviations from the mean for each phenotype (Table 1).

Table 1. Means of Farrowing Rate and Total Born for Each Phenotype

Phenotype	*n*	Avg Farrowing Rate, %	Avg Total Born
HFHB	9	86.33	14.78
HFLB	10	83.31	12.81
LFHB	4	79.81	12.38
LFLB	9	55.33	13.86

All proteomic sample preparation and analysis was completed at the Proteomics Core Facility in the Bindley Bioscience Center at Purdue University. Standard LC-MS/MS methods were used to quantify peptides and proteins in the seminal plasma in a single batch. Standard protein identification and database search protocols were used to identify proteins. InfernoRDN software tools were used to evaluate and visualize proteome data quality using box plot and correlation analysis tools. To identify differentially expressed proteins statistical analysis was performed in R using an in-house script that first extracts the LFQ intensity from MaxQuant file, then applies the log 2 transformation to the LFQ values for the analysis of variance (ANOVA) and t-test to determine differential expression between boar phenotypes. Significance was defined as $P \leq 0.05$. Functional annotation analysis was completed with DAVID Bioinformatics Resources version 6.8 as described in conjunction with Ensembl BioMarts. Descriptions of protein function within this manuscript were obtained from the UniProt Knowledgebase in conjunction with GeneCards.

Results and discussion:

Seminal plasma proteins in the HFHB phenotype showed the highest correlation among the boars within that phenotype (R2 range from 0.94-1.00). The subfertility phenotypes were less correlated among the boars within that phenotype. This suggests that reproductive performance is affected by multiple factors which makes the subfertile phenotypes less similar in protein composition. Boars in the high fertility phenotype had higher abundance of proteins that play a role in sperm capacitation, farrowing rate, oxidation-reduction, and sperm-egg interactions. Additionally, boars in the high fertility phenotype had proteins associated with immune function suggesting they may be more equipped to handle pathogen challenges or the inflammatory response.

Across all boars and phenotypes, 436 seminal plasma proteins were identified in at least one animal. A protein was defined as expressed for a phenotype if at least 3 animals in that phenotype expressed the protein. Therefore, there were 245 total proteins representative of at least one phenotype (Figure 1), with 190 of these expressed in all four of the phenotypes. Of these 190 proteins, 157 were unique and were evaluated to determine their biological function. Biological functions identified included processes such as proteolysis, binding of sperm to the zona pellucida of the egg, oxidation-reduction processes, extracellular exosomes, and immune-related processes, to name a few.
Some of the proteins identified play biological role in protecting sperm from oxidative stress and pathogens. Research has shown that sperm are susceptible to oxidative damage in the female's reproductive tract which can result in pregnancy failure. Superoxide dismutase, glutathione peroxidase and several other proteins were identified that protect sperm from oxidative damage. Many proteins from the complement cascade were identified in the seminal plasma. The complement cascade is part of the immune system which targets pathogens for removal. This is consistent with previous research suggesting that several immunological factors are present in seminal plasma.

Thirty proteins were differentially expressed between the HFHB and LFLB phenotypes, whereas HFLB and LFHB had 26 and 20 proteins different from the HFHB phenotype. Several proteins were commonly different in the lower fertility phenotypes and the high fertility phenotype. Of note, inositol-1-monophosphatase (IMPA1) was less abundant in all three subfertile phenotypes compared with HFHB. Fifty-six proteins were more abundant in the HFHB phenotype compared to at least one of the subfertile phenotypes. These proteins had biological function in proteolysis, oxidation-reduction processes, and platelet degranulation (associated with compromised sperm membranes). Thirteen percent of the proteins different between the high fertility phenotype and the subfertility phenotypes were considered blood microparticles, including hemoglobin. The increase in blood microparticles and some proinfalmmatory cytokines in the low fertility phenotypes may suggest that boars with lower fertility have damage or localized inflammation in their reproductive tracts. For example, the protein A2M (alpha-2-macroglobulin) is a chaperone protein which modulates immune function and has been correlated to increase morphological abnormalities in bulls and rams. Inositol-1-

monophosphatase (IMPA1) was the only protein that was lower in all three subfertile phenotypes. This protein plays roles in sperm maturation, maintenance of osmotic pressure in the semen, and as an antioxidant.

Boars with high farrowing rate but low total born expressed different proteins that have biological function in immune responses and uterine inflammation. Therefore, it is possible that boars in the low total born may not be able to combat the massive immune response in the female's reproductive tract resulting in fewer sperm available for fertilization. Fibronectin was a protein also lower in boars with low total born. This protein plays a role in fertilization and protecting sperm from oxidative damage.

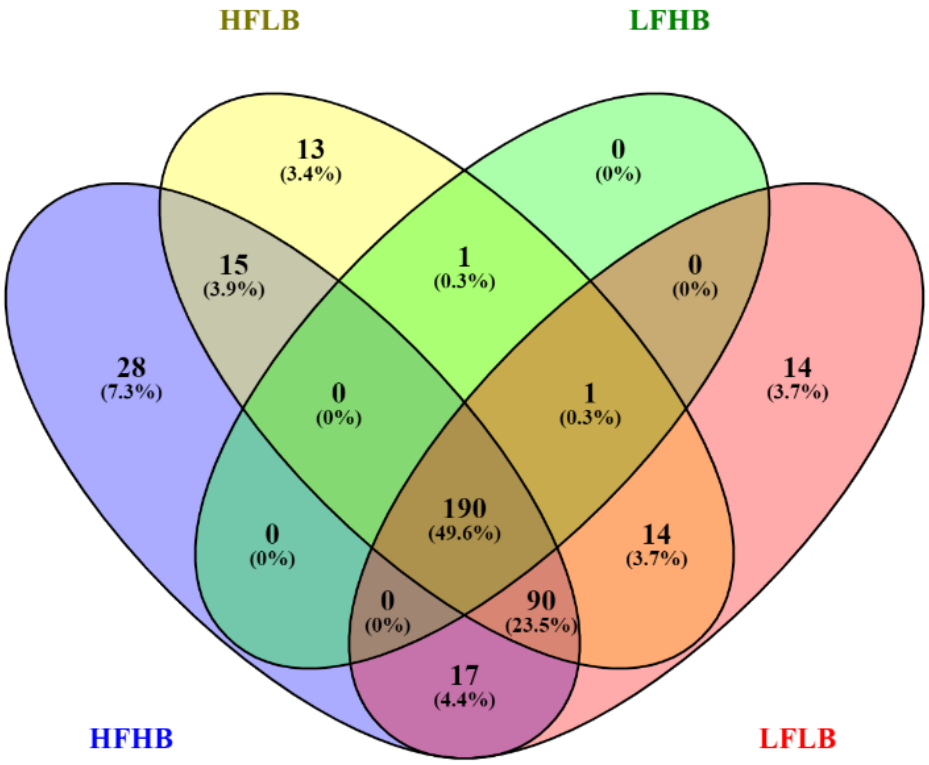

Figure 1. Venn Diagram of 245 high confidence proteins considered expressed by at least one phenotype ($n \geq 3$ boars per phenotype) across all fertility groups. One hundred ninety proteins were commonly expressed across all phenotypes.

Key Findings:

- Shotgun proteomics using LC-MS/MS proteomics approach is capable of differentiating between boars with different fertility phenotypes.
- Proteins associated with fertility and subfertility were identified, including those that are correlated with decreases in total born and not farrowing rate.
- A panel of the proteins identified in this project could be used as a test of subfertility in boars.

Keywords: Shotgun proteomics, boar fertility, seminal plasma

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