Biological Markers of Boar Fertility

by

Natalie Elizabeth Diether

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Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

The ability to identify and remove sub-fertile boars from the breeding herd is critical to improving reproductive efficiency of pork production systems. Use of semen from sires exhibiting sub-fertility can result in decreased sow pregnancy and farrowing rates, as well as smaller litter sizes [1, 2]. Currently, the ability to detect sub-fertile sires has been somewhat limited due to the low correlation between increasing semen quality and increasing relative fertility beyond minimum acceptability standards, as well as the limitations of functional assays [3, 4]. Previous research has identified differences in the relative abundance of seminal plasma proteins between high and low fertility boars using 2-D gel electrophoresis and western blotting [5]. Genomic markers are also promising, due to their usefulness in examining low heritability, complex traits [6]. No existing studies of the associations between single nucleotide polymorphisms (SNPs) and fertility have been completed for boars, although associations have been reported in bulls and stallions [7, 8].

The purpose of this study was therefore to utilize field fertility evaluations of boars in commercial pork production systems to generate a population of boars with known fertility phenotypes. During the evaluation period, blood and semen samples were collected for proteomic and genomic analysis. Proteomic evaluation was completed on boars representing fertility extremes, using a combination of label-free quantitation, isobaric tag for relative and absolute quantification for discovery, and targeted multiple reaction monitoring. A genome-wide association study was completed using Sleuth software, on all boars evaluated. Through this work, proteins and SNPs significantly associated with fertility performance of boars were identified. These markers provide a basis for further validation to identify markers for testing boars during early life. With validation,

these markers will provide a tool to boar stud managers and pork producers to identify potentially sub-fertile sires and improve reproductive efficiency.

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Dedication

For Emma, who has always encouraged me to seek out information I don't already have, and for Dave, who encouraged my love for nature and learning from a very young age.

"The capacity to blunder slightly is the real marvel of DNA. Without this special attribute, we would still be anaerobic bacteria and there would be no music."

- Lewis Thomas, The Lives of a Cell

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List of Abbreviations

AI	Artificial Insemination
ANOVA	Analysis of Variance
AQN	Spermadhesin protein family composed of AQN-1 and AQN-3
AWN	Spermadhesin protein
BA	Piglets born alive
BCA	Bicinchoninic acid assay
CASA	Computer-Assisted Semen Analysis
CD9	Cluster of differentiation antigen 9
CRISP	Cysteine-rich secretory proteins
DAZL	Deleted in azoospermia-like gene
DFI	DNA fragmentation index
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
GPX	Glutathione Peroxidase
GQLS	Generalized quasi-likelihood score
GWAS	Genome-wide association study
h^2	Heritability
iTRAQ	Isobaric Tag for Relative and Absolute Quantitation
ITGB3	Integrin beta-3
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LFQ	Label-free quantitation
MRM	Multiple Reaction Monitoring
mRNA	Messenger ribonucleic acid
MYL4	Myosin, light-chain 4
NADH	Nicotinamide adenine dinucleotide

NV	Non-viable fetuses
OPN	Osteopontin
PR	Pregnancy Rate
PRRS	Porcine Respiratory and Reproductive Syndrome
PSP	Porcine Seminal Plasma Protein
RAB11FIP2	RAB11 Family Interacting Protein 2
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive Oxygen Species
SAS	Statistical Analysis System software
SCR	Sire Conception Rate
SCSA	Sperm Chromatin Structure Assay
SNP	Single Nucleotide Polymorphism
TB	Total piglets born
TCEP	Tris(2-carboxyethyl)phosphine, reducing agent
ZP	Zona Pellucida

Chapter 1: General Introduction

The fertility of breeding males in a pork production system is an important contributor to the reproductive efficiency of the herd [1]. Sows bred using semen from sub-fertile sires may have decreased pregnancy and farrowing rates as well as decreased litter sizes [2, 3]. This is particularly important in an artificial insemination (AI) breeding program where sub-fertility of a single boar can negatively affect reproductive outcomes for many sows [1]. Accurate assessment of male fertility is necessary for AI technology to provide a highly fertile product and superior genetics, and therefore improve reproductive efficiency [4]. To date, the ability to evaluate male fertility has been somewhat limited. There is a low correlation between increasing semen quality and increasing relative fertility beyond the minimum quality standards [5, 6]. Problems can be detected by evaluating semen for abnormal cells or low sperm motility, but is not predictive of relative fertility performance [7, 8]. Development of biological markers to predict boar fertility would therefore be useful to identify and remove sub-fertile sires from the production system early in life.

Seminal plasma proteins and genomic markers are both of particular interest due to their ability to be evaluated earlier in a boar's life compared to the completion of a field fertility testing which requires many breedings. Sperm's vulnerability to external stressors means that seminal plasma proteins have important roles in maintaining sperm cell integrity and function throughout processing and storage for AI, as well as during transit of the female reproductive tract [9-11]. Further understanding of differences in the seminal plasma proteome between high and low fertility boars could help identify differences in underlying physiological functions that contribute to fertility outcomes. Previous research has identified differences in the relative abundance of

seminal plasma protein between high and low fertility boars using 2-D gel electrophoresis and western blotting [12]. Similar work has also been completed in bulls and stallions [13, 14]. Genomic markers are promising for early prediction of fertility, as blood sampling and genotyping could occur in very early life. A review of the literature revealed no previously published work identifying genomic markers associated with boar fertility. However, genome-wide analysis studies examining associations between single nucleotide polymorphisms (SNPs) and fertility have been completed in bulls and stallions [15, 16]. These techniques have identified significant SNPs close to proteins with known functions in the seminal plasma, and have also been used to identify recessive lethal haplotypes [17-19]. Genomic analysis is useful for low heritability, complex traits such as fertility and is a promising technique to identify genes underlying these complex traits [20, 21].

The purpose of this study was therefore to utilize field fertility evaluations of boars in commercial pork production systems to generate a population of boars with known fertility phenotypes. During the evaluation period, blood and semen samples were collected for proteomic and genomic analysis. Proteomic evaluation was completed on boars representing fertility extremes using a combination of label-free quantitation, isobaric tag for relative and absolute quantification for discovery, and targeted multiple reaction monitoring. Genotyping was performed using the Illumina 60K porcine beadchip on all boars with known fertility phenotypes. A genome-wide association study was completed using Sleuth software, on all boars evaluated. Through this work, proteins and SNPs significantly associated with fertility performance of boars were identified. These markers provide a basis for further validation to identify markers for testing boars during early life. With validation, these markers will provide a tool to boar stud managers and pork producers to identify potentially sub-fertile sires and improve reproductive efficiency.

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Chapter 2: Literature Review

2.1 Introduction

The reproductive efficiency of a breeding herd is highly dependent on the reproductive capacity of the breeding male(s) used [1]. The ultimate goal of any artificial insemination (AI) system is to increase the impact of high-value sires on genetic progress and the cost efficiency of these systems is largely determined by the number of sperm used per litter size obtained [2]. In swine production, particularly in an AI breeding program, the reproductive capacity of a single boar affects outcomes for many females. Therefore, accurately measuring and managing male fertility is advantageous [1]. Comparing outcomes (pregnancy rate, farrowing rate and litter size) from single-sire matings to reproductive averages for the boar population allows assessment of reproductive capacity of boars. This relative measurement of fertility is the result of direct boar fertility impacts on sow pregnancy rates and, as a polytocous species, subsequent impacts on litter size [3, 4]. Failure to detect sire subfertility can decrease herd pregnancy and farrowing rates, negatively impacting production efficiency by increasing non-productive days. We know that sperm that reach the site of fertilization and are capable of fertilizing the oocytes, are part of a small, highly selected population within the total ejaculate. Although these impacts are very tangible, detecting male fertility is complex and currently there is no single test available to predict or diagnose sub-fertility [5-8]. With difficulties in prediction of male fertility and a biased focus on female fertility in agriculture production systems, the opportunity to select males of high reproductive capacity may often be missed. In assigning value to sires and setting production goals, female fertility is often accounted for, whereas male fertility is often overlooked [9, 10]. DeJarnette et al. (2004) outlined the role of the artificial insemination industry as follows: to provide a highly fertile product, competent technical support, and superior genetics [11]. To fulfill these goals, accurate assessment

of male fertility is necessary to optimize the genetic contribution of superior AI sires to livestock production. To date, many studies evaluating male fertility are considered "flawed" due to small sample sizes or effects of female fertility on mating outcomes [12]. Selection of terminal sires for superior growth and carcass traits assumes that, if semen characteristics are within the acceptable range, male fertility is adequate although relative fertility performance of these sires can be highly variable [6, 10]. Concurrent selection for high relative fertility, as well as carcass and growth traits, can provide a direct benefit to the AI stud by enhancing production efficiency [10].

2.2 Evaluating Male Fertility

Male fertility encompasses the capacity of the male to produce sperm which are successful in fertilizing oocytes and producing zygotes that can continue to develop through to birth [13]. In swine production, current methods of fertility evaluation for breeding males are limited. Evaluating sperm morphology, motility, and concentration are standard when processing semen for use in artificial insemination. Evaluating motility and morphology is quick and inexpensive and it is easy to train technicians to perform these evaluations [7]. However, there is a low correlation between results for these assessment parameters and relative fertility performance beyond a minimum quality standard [14, 15]. Poor semen quality can identify males with problems contributing to infertility, but above minimum quality standards, improvements in common semen quality measurements are not predictive of higher relative fertility [16, 17].

Semen characteristics affecting fertility can be separated into compensable and uncompensable traits. Compensable traits are those that may be important to sperm transport and function in the female reproductive tract up to the stage of binding with the zona pellucida (ZP) and blocking of

polyspermy, these traits are responsible for differences among males with regards to the number of sperm required at the site of fertilization in order to achieve maximum pregnancy rate [18]. Compensable traits include: poor progressive motility, percentage of cells with intact plasma or acrosomal membranes, and abnormal morphology (including tail abnormalities, cytoplasmic droplets, and minor severity head defects) [18]. As the name would suggest, compensable traits affect the minimal number of sperm required to reach maximum fertility, and as such, these defects can be compensated for by providing higher numbers of sperm at insemination [18]. Conversely, uncompensable traits are those which affect the fertilization and embryogenesis. Typically, these sperm are those with defective chromatin or nuclear vacuoles but that have, at most, subtle deviations from being morphologically normal such that they are still motile and may pass selection processes by the zona pellucida [7, 18]. Since these defects affect functional processes occurring during later stages of fertilization or embryonic development, these uncompensable traits cannot be mitigated by increasing the number of sperm in the inseminate [7]. To predict male fertility, testing must differentiate between compensable and uncompensable traits [1, 7].

One problem with motility assessment is the subjectivity of assessment performed by a technician. The use of a computer assisted semen analysis (CASA) system, which has been optimized to reduce variation, can help to increase the precision of motility assessment. However, even with more in-depth consideration of motility, correlations with relative fertility outcomes are only seen in very low (10-fold lower) insemination doses [19, 20]. This system is useful in examining sperm motility at collection, or following storage, to detect problems indicative of poor ejaculate quality or issues occurring during storage. Beyond this, motility is a poor predictive tool for evaluating fertility of semen [21]. Additionally, sub-populations of sperm within an ejaculate cannot be

captured in conventional semen analysis. These sub-populations can have differing motility and variation in resistance to osmotic stress and only a highly selected group of these cells represent those actually responsible for fertilization [7, 22, 23].

Ensuring that an ejaculate meets threshold values for normal morphology is important in assessing usability of an ejaculate for insemination. Abnormal morphology can indicate deviations during spermatogenesis and sperm maturation, or problems with the accessory sex glands [24]. High percentages of abnormal sperm can also be an indicator of infection of the male reproductive tract or sub-optimal handling during collection and processing for AI, both of which are relevant to the fertility of a single ejaculate, but not necessarily sire fertility over time [24]. Evaluating normal versus abnormal sperm cell morphology as well as severity of any detected abnormalities depends on visual characterization and can be somewhat subjective. In addition, comparison of ejaculates evaluated under different morphological classification systems is difficult as different systems assign defects to different groups [7]. The low correlation between abnormal morphology and relative fertility beyond the minimum cut-off might be in part because of a “tip of the iceberg” effect where, in some cases, disturbances in spermatogenesis (indicated by the abnormal cells) may also be affecting cells that appear normal or near-normal, thus resulting in early embryonic death [18].

An examination of the role of traditional semen evaluation in management of male fertility should also consider that the semen evaluation environment might limit accurate assessment of semen quality. Assessing semen quality immediately post-ejaculation cannot account for the challenging environment experienced by the spermatozoa during transit through the female tract. Currently,

there is no efficient means of predicting the ability of sperm to cope with sperm selection, oxidative stress, and the dynamic biochemical environment of the female tract prior to arrival at the site of fertilization [7, 18]. To bridge this gap, numerous functional assays have been developed, including chromatin evaluation, membrane integrity tests, capacitation or acrosome reaction assays, zona pellucida binding or zona-free hamster egg penetration assays, and evaluation of performance under *in vitro* fertilization conditions [1, 7]. These tests, as subsequently described, have limitations in scope and application. As such, more efficient and accurate means of predicting male fertility would still be advantageous to livestock production.

In conjunction with conventional semen evaluation, functional assays can be utilized to determine sperm cell competence for certain discrete events leading to successful fertilization. Short hypo-osmotic swelling test results were significantly correlated with fertility, but explain only a relatively small proportion of fertility differences between boars [25]. Ability of sperm to tolerate a hypo-osmotic environment is positively correlated with *in vivo* fertility, but can be confounded by breed, incubation temperature and oxidative stress [23]. Acrosome integrity can be measured using staining; however this must be paired with assessments of cell viability, as acrosome integrity does not ensure that cells are live and motile [26].

Another factor that must be considered in male sub fertility is DNA quality; in particular cells that appear morphologically normal but have defective chromatin [18, 27]. Damage to DNA can occur from seminal reactive oxygen species (ROS) or cell apoptosis, as well as pathological conditions such as fever, infection, or heat stress. When these disturbances occur late in spermiogenesis, or during epididymal maturation, they can affect DNA quality without affecting the sperm head [18,

28]. This is problematic, as sperm with morphologically normal (or almost normal) heads may not be selected against by the zona pellucida during sperm/oocyte interactions. However, these cells may be incompetent after fertilization, resulting in abnormal embryonic development or embryonic failure [7, 18]. Reactive oxygen species can cause damage to chromatin cross linking, chromosome deletion, DNA strand breaks or base oxidation [27]. There are four common tests that can be used to measure DNA fragmentation: these are the Single Cell Gel Electrophoresis assay (SCGE, also known as the Comet assay); Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay; sperm chromatin structure assay (SCSA); and the acridine orange test (AOT). These methods can then be used to generate a DNA fragmentation index (DFI) [28]. A limitation of some of these tests is that, with the exception of SCSA, they lack a defined threshold [27]. The most common method used in recent literature is SCSA, which involves exposing sperm to low pH, thereby denaturing the DNA at sites of fragmentation such that the resulting strands can then be stained and evaluated by flow cytometry. Using this technique, four sperm sub-populations with varying levels of DNA fragmentation can be identified [28]. Sperm chromatin structure assays have been used to link boar fertility to chromatin integrity [29]. When DFI of an ejaculate exceeds 5%, samples are more likely to be below the minimum quality standards for motility and morphology [30]. The suggested threshold of DFI for sub-fertility (pregnancy rate and litter size) in boars is a DFI of approximately 8%, which is much lower than for bulls (10-12%) or humans (30%) [28]. Studies on the effect of DFI above threshold values in humans have shown associations with increased spontaneous abortion, perhaps due to fertilization with genetically compromised sperm [28]. The cellular processes underlying normal DNA condensation and subsequent DNA decondensation require an optimal disulfide-thiol balance in the cell nucleus [18]. Additional techniques include staining to identify incomplete histone-

protamine exchange. This can be used to detect ejaculates with problems including a high proportion of diploid spermatozoa resulting from problems during meiosis and reciprocal chromosomal translocations but has not been validated for discriminating between high and low relative fertility individuals [24]. The importance of the quality of DNA contributions to the embryo is clearly demonstrated by these tests of DFI and related effects on fertility. However, staining methods are labour-intensive and require substantial technical expertise and equipment. As such, the use of these current DNA quality assays may be limited in a commercial stud.

Perhaps the biggest limitation of functional assays is their inability to test more than one functional capacity, thus impeding the ability to determine the sperm' capacity to successfully undergo the cascade of events leading to fertilization *in vivo*. To address this limitation, staining assays that concurrently measure multiple functional capacities have been developed [31]. Although these methods for diagnosing infertility may be able to identify specific mechanisms underlying reproductive failure, they require substantial technical expertise and input. Thus, they are limited in their scope, making them more appropriate for diagnosis of underlying causes of sub-fertility rather than routine screening of sires and are more commonly used in diagnosis of human infertility [20]. The results of these functional tests represent only one sample, in time, and the significance of a single poor test result as the cause of consistent subfertility has been debatable [30]. Even in human reproductive medicine, where testing is readily available and cost is less prohibitive than in agriculture, many cases of infertility are still defined as idiopathic [32]. This is the case despite infertility affecting 15% of all couples, 50% of which are attributed to a male factor [32]. As fertilization is a complex process involving many events, and the sperm cell population in a single ejaculate is heterogeneous, it is difficult to reliably predict fertility using in *vitro* testing [14]. In

addition, in agriculture, it can be difficult to eliminate contributions of management, environmental or female factors to reproductive failure, making implementation of functional assays even more complicated.

Conditions under which AI is performed in the commercial pork production environment also complicate assessment of boar fertility. Insemination of high sperm numbers per dose (3 billion viable sperm/AI dose) in commercial pork production systems can mask reduced fertility due to compensable traits, although these could be apparent with lower sperm doses [4, 14, 20]. At higher sperm doses, adequate numbers of normal sperm reach the site of fertilization, despite sperm selection in the uterus [13]. The use of heterospermic semen in North American systems further complicates evaluation of male fertility by eliminating the direct link between a single sire and fertility outcomes. In a pooled semen dose, from sires of unknown relative fertility, it is likely that some sires used in the pool are not contributing to fertilization as efficiently as their counterparts of comparable value and genetic merit [1]. The sperm from higher reproductive capacity animals will outcompete those of sub-fertile individuals and a successful pregnancy will result, but with much less efficient use of high genetic merit individuals. Failure to identify and remove sub-fertile sires from the stud represents a missed opportunity for pork production systems to improve efficiency of utilization of high-value sires for pork production, and efficiency of genetic transfer of valuable traits [1].

2.3 Reproductive Tract of the Boar

When considering the role of the male reproductive tract in fertility, there are many relevant functions to consider. The boar must be anatomically normal, such that natural mating or semen

collection for AI can occur. Testis and epididymis must be capable of producing functional spermatozoa in acceptable quantities, and the accessory sex glands must produce seminal plasma with all the necessary components to support the sperm produced. Thermoregulatory mechanisms of the tract must be functional to maintain the testes within the optimal temperature range for spermatogenesis, and correct endocrine cues must be present, not only for spermatogenesis, but also for reproductive behaviour. Many of these traits can be readily detected through careful observation of the boar entering the stud, and through examination of the ejaculates collected. However, the most difficult of the above-listed traits to examine are functional capacities of the sperm and composition of the seminal fluid.

Reproductive tract structures contributing to the ejaculate in the boar are the testes, epididymis and the three accessory sex glands (vesicular gland, prostate and paired bulbourethral glands). When sperm leave the testis, they are supported by fluid from the rete testes, but this fluid is subsequently resorbed by the epididymis during sperm maturation [33]. Seminal plasma contributions, therefore, start with the epididymis and, as ejaculation takes place, the accessory sex glands contribute to the ejaculate sequentially [6]. Vesicular glands contribute the majority of the volume to the ejaculate, and most of the seminal plasma protein [34]. The first secretions of the ejaculate are those originating from the prostate; their function is to clear the urogenital tract prior to arrival of the sperm [6]. The next fraction of the ejaculate is the sperm-rich fraction, followed by an increasingly dilute ejaculate as vesicular gland contributions increase [6]. Vesicular gland secretions contribute the highest amount of spermadhesins, including AQNs and AWNs as well as PSP-I and -II [35, 36]. Finally, the bulbourethral gland secretes mucin, which absorbs water, becoming the gel

fraction at the end of the ejaculate [37]. These secretions together, with epididymal sperm, comprise the ejaculate.

2.4 Sperm Cell Processes Contributing to Fertility Outcomes

There are many potential factors that affect relative-fertility performance. Poor fertility outcomes can be caused by problems during spermatogenesis or sperm maturation, or they can result from an inability of the sperm to withstand oxidative stress, to undergo capacitation, or to complete the acrosome reaction [1, 18]. Failure of any of these processes can result in sperm that are unable to fertilize an oocyte and produce a viable embryo. Some of these cells may appear morphologically normal, even though they are not functionally competent, making it difficult to discern these differences without extensive functional testing.

Spermatogenesis

As highly specialized sperm develop from spermatogonia, many events can affect sperm functionality. Multiple rounds of mitosis in the basal compartment of the seminiferous tubules, as well as meiosis to transition out of the spermatogonial pool, can affect DNA quality [13]. During the transition from spermatid to spermatozoa, all cellular components must be correctly assembled. Problems during spermatogenesis can often be detected in the semen quality evaluation if morphological abnormalities or low sperm cell numbers are detected. The quality of genetic material present in the sperm cell following spermatogenesis is more difficult to measure. Condensation of DNA, an important mechanism for protecting DNA prior to fertilization, begins during spermiogenesis where histones are transitioned to protamines [38, 39]. This process is completed during the early stages of epididymal maturation, through formation of disulfide

bridges; thereafter, condensed chromatin must remain intact until fertilization. In an AI breeding system, where sperm may be stored for varying intervals before insemination, DNA fragmentation can increase if storage time is prolonged [30].

Sperm Maturation

Sperm maturation, a process that involves plasma membrane remodeling, acquisition of motility, and completion of DNA condensation into chromatin, occurs in the epididymis. These functional changes are androgen-dependent and rely on epididymal lumen contents [40]. The morphology of the epididymis, which is rich in secretory cells, supports secretion of important seminal plasma proteins throughout the epididymal cauda and corpus [41]. Formation of disulfide bridges, which stabilize nuclear chromatin, and mitochondrial capsules in the mid-piece, occurs through protein-thiol oxidation by peroxyl radicals which are present throughout epididymal maturation [42]. Maturation is also important to the sperm cell's ability to regulate intracellular calcium concentrations. This ability allows cells to respond to the calcium gradient in the female reproductive tract as sperm approach an oocyte. Only mature, ejaculated spermatozoa are able to undergo necessary changes in intracellular calcium concentrations occurring during capacitation [26]. Immature cells from the epididymal caput accumulate calcium at a rate 2-4 fold greater than those from the caudal end of the epididymis [43]. Progressive sperm cell motility develops throughout transit of the epididymal cauda and corpus and sperm cell movement becomes increasingly efficient as mitochondrial sheath membrane potential increases [16, 26]. Many proteins associated with signal transduction, translational control, energy metabolism and chromatin are secreted from the epididymis during this time, supporting maturation and maintaining sperm cell viability [44]. In the ram and stallion, the most important proteins of the

epididymal secretions supporting maturation are lactoferrin and clusterin, while other notable proteins include glutathione peroxidase (GPX), and prostaglandin D2 synthase [45]. In the boar, secretion of GPX5 from the epididymis is important throughout maturation. The ability to correctly respond to environmental cues of the female reproductive tract requires correct assembly of signal transduction components during maturation. Without this, sperm are unable to undergo oxidative bursts, which facilitate the acrosome reaction [46]. Achieving full maturation is critical to sperm survival in the female tract and fertilization of the oocyte.

Sperm Capacitation

Following ejaculation, sperm must undergo capacitation in order for fertilization to occur. Capacitation is the series of events that prepare sperm for the acrosome reaction and fertilization [47]. Failure of spermatozoa to achieve capacitation results in an inability to successfully bind and penetrate the oocyte [48]. When freshly ejaculated, sperm generally have a type of motility referred to as "activated", they swim in a straight line with low-amplitude flagellar movement [49]. However, during transit of the uterus and as capacitation progresses, sperm develop "hyperactive" motility, where the amplitude of flagellar movement increases and sperm display a figure-8 type movement (this movement is thought to be advantageous in a more viscous medium such as oviductal secretions, where it generates linear movement) [49]. Discrete sperm cell populations are thought to have different abilities to become hyperactively motile and undergo capacitation in response to secretions from the female reproductive tract. This is one of many potential selection mechanisms occurring in the female tract to ensure that only competent sperm reach the oocyte(s) [50]. The physiological site of capacitation is in the female reproductive tract; however, there are many male factors implicated in successful capacitation of spermatozoa in the correct location.

Capacitation is regulated by calcium and bicarbonate gradients and signal transduction through ROS [46, 51]. As reviewed by Topfer-Petersen et al., (1995), female secretion of heparin and chondroitin sulphate-like glycosaminoglycan side chains are involved in stimulating capacitation [50]. Following ejaculation, intracellular calcium concentrations in sperm increase, allowing ejaculated cells to be much more responsive to *in vitro* capacitation cues and to the calcium gradient which occurs as they transit the female reproductive tract [26]. Increased tyrosine phosphorylation, a hallmark of sperm cell capacitation, is influenced by the sperm cell's generation of ROS, in particular H_20_2, which is produced by activated protein kinases and is important in redox-regulated signal transduction and sperm cell binding [42, 52]. The ability to generate NADH, a regulator of cell redox status, is thought to have an important role in calcium homeostasis throughout this process [43]. Presumably there is an optimal level of oxidative stress for sperm where capacitation can occur but harmful lipid peroxidation is minimized.

Acrosome Reaction and Fertilization

During transit in the female tract, sperm form a reservoir in the oviduct; the epithelial cells of the isthmus are able to select high-quality sperm to form this reserve, and modulate their function (via oviductal fluid) until fertilization [8]. Fertilization is not a single event, but rather a process resulting from a complex series of events. This process requires the sperm acrosome reaction, penetration of the ZP, and de-condensation of chromatin, all of which are critical to generation of a sufficient number of viable embryos. The acrosome reaction is triggered by zona pellucida binding; this binding process induces aggregation of receptor molecules, an important step for initiating exocytosis [50]. The ZP is thought to select against sperm with abnormal head morphology during this time [18]. The ZP is an extracellular matrix composed of three major

glycoproteins, the function of which is to separate the oocyte's plasma membrane from the surrounding cumulus cells, as well as to protect the oocyte and early embryo from physical stress [53, 54]. Acrosome reaction is induced when the sperm head binds to the glycoprotein ZP3 [55]. The acrosome reaction is a signal-induced exocytosis of a proteinase from the sperm acrosome. Acrosomal contents function as a secondary zona binding receptor and also cause local zona pellucida digestion to allow sperm penetration [56]. During this process, calcium channels regulate calcium influx into the spermatozoa, thereby promoting acrosome reaction [50]. Upon fusion with the zona pellucida, acrosomal contents are emptied through pores in the sperm plasma membrane. These contents cause a local breakdown of the ZP and allow the sperm to enter the perivitelline space. Once in the perivitelline space, fusion of the sperm plasma membrane and the oolemma occurs and DNA de-condensation of the sperm head can occur. Although only a single spermatozoon must penetrate the ZP and fertilize each oocyte, many accessory sperm may also fuse with the ZP. This is a good indicator of fertilization capacity of such sperm, as they have fulfilled all the structural and functional requirements to reach the ampulla, and are able to recognize and bind the zona pellucida following transit of the uterus [18, 57]. Following penetration of the oocyte by sperm, sperm DNA integrity is critical to the production of a developmentally competent embryo. Low levels of damage to male pronuclear DNA can be repaired by the enzymatic machinery of the oocyte. However if the damage is extensive, embryo development may fail [27]. Evaluation of *in vitro* embryo development of oocytes fertilized by sires of known *in vivo* fertility has revealed differences in embryonic development that can be detected as early as first cell cleavage. These timing differences may allow embryos generated from high-fertility sires to spend more time in DNA synthesis stages, which may be critical for competent development [13].

The many events required for successful production of a zygote occur in a hostile environment for the sperm. Furthermore, sperm are lost from the female tract due to host female immune response and also physical retrograde loss from the cervix or uterine body. The multitude of molecular events occurring from ejaculation to fertilization, make it important to distinguish failure at fertilization from failure of embryonic development to better understand the mechanisms underlying male fertility [18]. The ability to survive the environmental stressors that sperm face during uterine transport is due in part to the support of seminal plasma proteins and suggests that they are an important consideration in male fertility. However, failure of development may be the result of other factors that must be examined in the context of genotype [1].

2.5 Seminal Plasma Proteins

The haploid sperm cell contains highly compacted and transcriptionally silent DNA that leaves the sperm cell in need of support from external sources to cope with stressors. This vulnerability to damage via environmental stressors is further exacerbated by the sperm cell's lack of cytosolic antioxidant enzymes, high degree of cellular polarity, and high levels of polyunsaturated fatty acids in its membranes [42]. Seminal plasma contains many compounds (Table 2.1) including inorganic ions, sugars, salts, lipids, prostaglandins, and proteins (especially enzymes) which provide the sperm with compounds to maintain sperm viability, to facilitate transport in the female tract, and to achieve optimal sperm function during fertilization [58-60]. The unique seminal plasma proteome required for maintenance of sperm is maintained via the blood-testes and blood-epididymis barriers [44, 61, 62]. As sperm transit the male reproductive tract, dynamic changes in protein composition of the seminal plasma occur [63]. These protein changes begin in the

epididymis where testicular fluid is resorbed and new proteins are secreted. During ejaculation, additional proteins are secreted collectively by the accessory sex glands (vesicular gland, bulbourethral gland, and prostate) [37, 60]. The sperm adsorb these proteins to their surface as they are exposed during ejaculation [34, 47]. The result is a complex and heterogeneous protein repertoire displayed on the sperm cell membrane [64]. Protein content of the ejaculate was reported as variable, with changes occurring based on collection frequency, and season [65]. In the absence of seminal plasma, sperm cell viability is compromised, as sperm undergo premature capacitation or acrosome reaction [66].

The boar ejaculate contains four sequential fractions: the first fraction is the sperm-rich portion, the second is relatively sperm-free, the third is sperm-rich and finally, the gel-fraction is released last [6]. Of these fractions, it is the earliest portion of the first sperm-rich fraction, referred to as the sperm peak fraction, that is considered to contain sperm with the highest probability of fertilizing an oocyte [6]. The protein complement associated with this first sperm-rich fraction confers improved sperm cell motility and storage ability even when added to sperm ejaculated in later fractions [49]. When sperm ejaculated in the first sperm-rich fraction of the ejaculate were co-incubated with seminal plasma from the sperm-free fraction, oocyte penetration rates *in vitro* were significantly lower than those of cells co-incubated with the seminal plasma from the sperm-rich fraction [67]. In natural matings, these cells are exposed to lower amounts of spermadhesins in the seminal plasma, as the majority of the seminal plasma protein is ejaculated after the sperm-rich fraction [49, 60]. Throughout this process, proteins related to peroxidase and antioxidant activity are secreted from all reproductive organs to maintain sperm cell integrity in the presence of reactive oxygen species [65, 68]. This requirement for extracellular support is partly the result

of the sperm cell's own production of reactive oxygen species through its aerobic metabolism following ejaculation, and its transition out of quiescence [27, 42]. Oxidative stress results when there is an imbalance between antioxidant capacity of the seminal plasma and the levels of ROS [27]. It has also been suggested that variation in seminal plasma composition may contribute to variation in sperm DNA fragmentation and ability to withstand prolonged storage times and that this may be a result of variation in susceptibility to acid-induced denaturation [30]. In bulls, there is an unfavorable relationship between ejaculate volume and abnormal spermatozoa, as well as a negative relationship with sperm motility, whereas selecting for sperm cell concentration has positive implications for sperm quality in this species [69]. This relationship suggests that an inappropriate amount of protein per sperm cell may negatively affect sperm. The competency for fertilization acquired between the completion of epididymal maturation and the end of capacitation in the female tract has been suggested to depend in part on the various fluid secretions comprising the ejaculate both in terms of these components absolute and relative amounts [47]. Differences in some seminal plasma protein-encoding mRNAs have also been implicated in seasonal infertility of boars in sub-tropical regions [70].

The protein component of seminal plasma includes proteins, both suspended in the seminal fluid, as well as proteins more closely associated with the sperm cell plasma membrane. Proteins associated with the plasma membrane form a thin protein layer on the sperm head; this layer of proteins is thought to protect and stabilize sperm, preventing premature capacitation [34]. Other proteins forming this layer are associated with the acrosome and are considered to have a role in the cascade of events from sperm capacitation through to oocyte binding [71]. Proteins in the seminal plasma can further be separated into those that can bind heparin and those that cannot.

Heparin-binding ability is an indicator of involvement in capacitation and zona pellucida binding [71]. When seminal plasma proteomes are compared between ungulate species, there is divergence in the seminal plasma protein composition of various species, despite common ancestry. In the case of the boar, the proteome is most similar to the bull (34%) and the buck (39%) [59]. Some of the major proteins in the seminal plasma include osteopontin, glutathione peroxidases, spermadhesins and cysteine-rich secretory proteins. These proteins have many roles in supporting the sperm, which will be further described in subsequent sections.

Osteopontin

Osteopontin (OPN) is a phosphoprotein with numerous cellular functions that is secreted by both the male and female reproductive tract. In the boar, it is secreted from the vesicular gland and ampulla [72]. Osteopontin exists with varying degrees of phosphorylation and glycosylation and has been suggested to have tissue-specific isoforms [73]. At the time of ejaculation, osteopontin, in close association with the sperm cell, is localized on the post-acrosomal region and mid-piece of the sperm and is important for sperm-oocyte interactions [72, 74]. Boars with polymorphisms in the osteopontin gene have significantly different litter sizes (piglets born alive) [75]. Research in water buffalo has shown single nucleotide polymorphisms (SNPs) in the introns of OPN are associated with significant differences in many traits, including gross physiological traits, such as testis size, as well as sperm cell motility and abnormal morphology [76]. In *Bos taurus*, osteopontin is positively correlated with non-return rates, whereas in the pig, there is a negative correlation between osteopontin and litter size as well as farrowing rate [6, 73]. Osteopontin also affects early embryonic development, with increasing concentrations of OPN decreasing zygote cleavage and blastocyst formation in bovine embryos produced in vitro [77]. The mechanistic basis for the different effects of OPN on fertility in various species has not yet been explained. However,

correlation between increased OPN and increased acrosome reaction may be a contributing factor [77].

Glutathione Peroxidase 5 (GPX5)

Aerobic metabolism of sperm, especially during storage for AI, can be detrimental to sperm cell function due to generation of ROS [78]. Glutathione peroxidases (GPX's) are powerful antioxidant enzymes, maintaining a delicate equilibrium of oxygen by-products, by converting hydrogen peroxide into water [79]. They protect sperm plasma membranes from reactive oxygen species, thereby preventing lipid peroxidation and DNA damage [38]. The GPX family contains five distinct protein members, each encoded by a distinct gene. Although many of these GPX enzymes are ubiquitously expressed throughout the body, GPX5 is unique, as it is only detected at high levels in the epididymis and in association with spermatozoa [80]. The secretion of GPX5 has been characterized in the epididymal epithelial cells, with expression limited to the epididymal caput [78, 79]. Glutathione peroxidase 5 also differs from other GPX's in its lack of dependence on selenium, containing only a cysteine residue at its catalytic site [79]. The role of GPX5 as a scavenger of free radicals has been debated, due to its lack of a selenocystine residue, as well as the low level of compatible electron donors present in the male reproductive tract [80]. However, low catalase levels in the male reproductive tract of mammals makes the role of GPX5 in moderating minute changes in ROS important. In the absence of GPX5 secretion from the epididymis, an oxidative response by the epithelial cells increases sperm DNA damage, particularly in older males [81]. Although GPX5 has been detected in the epididymis of many agricultural species, secretion of GPX5 appears to be much lower in the bull and stallion compared to the boar [82]. The role of GPX5 in regulating the level of oxidative stress experienced by sperm

and its ability to respond to small changes in reactive oxygen species suggests that it is an important antioxidant for sperm cell survival during collection, processing for AI, storage, and in the female reproductive tract.

Spermadhesins

Spermadhesins are a family of 12-16 kDa proteins that coat and modify the sperm plasma membrane during ejaculation. They are highly similar in amino acid composition and share 40-60% sequence homology with one another [1, 50, 71, 83]. This family of proteins also share two critical disulfide bridges important for maintaining tertiary protein structure [83]. Of the four groups of zona pellucida-binding proteins identified in the mammal, spermadhesins are members of the low molecular weight group that has been detected in every species examined to date [56]. Spermadhesins characterized in the boar include AWN-1, AQN-1 and AQN-3, as well as porcine seminal plasma proteins I and II (PSP-I and PSP-II). Proteins in this family are noted for their varied glycosylation patterns which contribute to their structural differences, and their different binding abilities [50, 71, 83]. Secreted primarily from the vesicular gland, these proteins become associated with the sperm head, either before or during ejaculation [50]. Some of the spermadhesin proteins are present on the acrosomal cap; these are considered important for zona pellucida binding [84, 85]. These zona-binding proteins meet all the necessary criteria to support their role as primary receptors for binding through carbohydrate recognition [50]. Spermadhesins are also present in seminal plasma exosomes and exhibit a stabilizing effect on sperm capacitation [86]. This stabilizing function is thought to occur through association with the sperm head and stabilization of the zona pellucida binding sites during transit through the female tract and these molecules must then dissociate to allow sperm-zona binding to occur [87]. The ability of some of

these spermadhesins (AWN, AQN-1 and AQN-3) to bind heparin-like glycosaminoglycans promotes capacitation and acrosome reaction [84].

Epididymal sperm are only coated by sufficient quantities of AWN to occupy one third of the sperm head (6 million molecules/sperm). In contrast, an additional 12-50 million molecules of both AQN and PSP-I, as well as a further 50 million molecules of AWN, are associated with ejaculated spermatozoa [47, 50]. After capacitation, 60% of AQNs are lost, and the epididymal levels of AWN are all that remain in association with the sperm cell [47, 50]. Although this family of proteins has many conserved regions, various roles have been postulated for each member of the spermadhesin family, as a function of non-parallel changes in relative amounts of each member associated with the sperm throughout ejaculation and capacitation [47].

The spermadhesins AWN and AQN are capable of binding various molecules, including heparin, zona pellucida components, trypsin-inhibitor and saccharides [35]. Spermadhesins are typically found in their non-glycosylated isoforms in the seminal plasma (versus PSP proteins) [88]. The AWN proteins have been previously identified as AWN-1 and -2; however, AWN-2 varies structurally from AWN-1 only due to the presence of an N-terminus acetyl group [89]. Use of immunohistochemical methods have detected AWN-1 secretion from the rete testis, vesicular gland and prostate, with the highest level of secretion appearing to be from the vesicular gland [35]. Secretions from the rete testis represent the only spermadhesin on epididymal sperm and is thought to be what confers some fertilizing ability on epididymal sperm [47, 50]. Spermadhesin AWN-2 is only secreted by the vesicular gland, while AWN-1 is bound to the sperm cell membrane and is present in the sub-population of sperm binding to the oocyte [47]. Its capacity to bind

oligosaccharides suggests that it is involved in zona pellucida binding during fertilization [85, 90]. When AWN function is impeded, *in vitro* oocyte binding is reduced to 13% of normal levels [35, 89]. Antigens for AWN-1 are detected on the epithelium at the utero-tubule junction and lower isthmus, implicating it in capacitation [91]. Overall, AWN-1 represents only 7-8% of total seminal plasma proteins, likely due to dilution of vesicular fluid with secretions from other accessory sex glands [47]. Of these proteins, AWN and AQN-1 are capable of binding acrosin and soybean trypsin inhibitors, thereby functioning as regulators of capacitation, whereas AQN-3 is not [87]. On ejaculated spermatozoa, AWN covers the entire sperm acrosome, whereas AQN is located solely on the apical part of the acrosome [35, 89]. Secretion of AQN-1 occurs primarily from the vesicular gland, with additional secretions originating from the prostate [35]. AQN-1 is a primary receptor involved in binding carbohydrate components of the zona pellucida [56]. Throughout sperm capacitation, relative amounts of AQN-1 and AQN-3 associated with the sperm increase [47]. Of these three spermadhesins, only AQN-3 can also exist as an isoform, which forms a heterodimer with PSP-I. Unlike sperm-associated AQN-3, this heterodimer is unable to bind the zona pellucida. Instead, deglycosylated AQN-3 from these heterodimers possess the capability to bind serine-proteinase-inhibitors and thus may be important in sperm capacitation [88]. The AQN-3/PSP-I dimer remains in stable amounts in the seminal plasma and associated with sperm throughout ejaculation and capacitation [47]. This activity suggests that the glycosylation moiety mediates the switch in function of these molecules from capacitation factor to primary oocyte binding [88]. Although both of these proteins are capable of binding proteinase inhibitors, their capacity to bind both inhibitor and zona pellucida is different. Therefore, they are proposed to have similar, albeit not overlapping roles during capacitation [87].

Spermadhesins PSP-I and –II are secreted from the prostate and vesicular gland [60, 92]. The PSP spermadhesins differ from the other members of this protein family with regards to their N-terminal sequence, and they are found primarily as part of the non-heparin binding portion of the seminal plasma [83, 92, 93]. These two proteins form both homo- and heterodimers, existing in various forms with differing amino acid sequences and glycosylation [92]. Secreted from the vesicular gland, PSP-I is capable of binding digested zona pellucida protein ZP3, immunoglobulins IgA and IgG, as well as trypsin inhibitor and α-casein. This suggests multiple roles for the protein, although it is not involved in initial oocyte recognition since it does not bind the intact zona pellucida [92]. PSP-I is capable of binding IgG, which may be important in protecting the sperm from the female immune system [92]. Both PSP-I and PSP-II suppress antibody production, with long-lasting effects. In doing so, these proteins may help to protect sperm from naturally occurring immune cells in the female tract [93]. The PSP-I/PSP-II heterodimer binds pig lymphocytes and is able to increase lymphocyte proliferation; this effect is significantly higher in lymphocytes obtained from gilts than those from boars, suggesting a role in modulating uterine immune response following insemination [94]. As reviewed by Schjenken and Robertson, these two opposing effects may be important parts of male-female signalling following insemination. Although an inflammatory response is generated in the uterus by seminal plasma, sperm viability is preserved by the heterodimer adhered to the sperm head [95]. This function of PSP-I is attributed to its ability to bind trypsin inhibitor and therefore prevent premature acrosome reaction [92]. As PSP-I is present in small amounts in the heparin-binding fraction of the seminal plasma when differentially glycosylated, it is believed that the various glycoforms determine which molecules form heterodimers [83]. Assays evaluating the binding capacity of the PSP-I and –II heterodimers have confirmed its ability to bind trypsin inhibitors and zona pellucida proteins, both abilities are

attributed to PSP-II [83]. These proteins appear to occur most commonly as a heterodimer, in which state, heparin-binding ability is lost by both proteins [83].

Spermadhesins are implicated in a variety of roles facilitating sperm cell function. Their varying levels of glycosylation and ability to form dimers enables them to perform several physiological functions as important contributors to sperm cell functional capacity. It is thought that various spermadhesins fulfil discrete functions with regards to gamete interaction and capacitation due to their multi-functionality, and that there are changes in their association with the sperm during their transit of the female tract and subsequent fertilization [50].

CRISP Family of Proteins

Cysteine-rich secretory proteins CRISP1, CRISP2 and CRISP3 are a highly conserved group of proteins containing 16 cysteine residues that are expressed throughout the male reproductive tract [96, 97]. This family of proteins also contain a conserved 12 amino acid region that is important in gamete fusion [98]. Cysteine-rich secretory protein 1 is secreted throughout the epididymis with moderate secretion from the prostate and low secretion from the vesicular gland, wheras CRISP2 is expressed in the testis [44, 97, 99]. Of these proteins, CRISP1 and 2 are more highly conserved between species than CRISP3. The CRISP3 protein of the boar, bull and stallion are similar to one another but distinct from human and chimp [99]. Cysteine-rich secretory protein 1 has an important role in sperm cell quiescence by inhibiting sperm capacitation [99]. In the rat there are two affinity states of CRISP1, with a small amount that is tightly bound to the dorsal region of the sperm head, wheras the majority of the protein molecules are loosely affiliated with the cell and removed during capacitation. Tightly bound CRISP1 is thought to be important in mediating sperm binding to the

zona pellucida [40]. In stallions CRISP3 is secreted by the ampulla and considered important in protecting sperm from the mare's immune response by preventing neutrophil binding and elimination from the reproductive tract [100, 101]. In the boar, CRISP3 is secreted by the prostate and bulbourethral gland. Its biological function is currently unknown, but CRISP3 has been positively correlated with sperm freezability and first cycle conception rate in the horse [97, 99, 102, 103]. Secretion of CRISP3 from the prostate and bulbourethral glands contribute to later portions of the ejaculate in the boar, as well as its various roles in the horse, suggest a physiological role for CRISP3 in protecting phospholipid membranes and inhibiting capacitation of sperm. Although there is limited work on the role of CRISP proteins in boar fertility, part of this may be due to lower levels of CRISP secretion in the reproductive tract of the boar compared to the stallion. Notwithstanding, the importance of this protein in stallions makes it an interesting candidate for further exploration in the boar.

Seminal Plasma Proteomes and Fertility

The role of secreted proteins in the male reproductive tract is complex. Some proteins are associated closely with the sperm membrane, whereas others are mostly present freely soluble in the seminal plasma. Some of these proteins also exist in both states during the various physiological events of sperm storage, ejaculation, or capacitation. These proteins are collectively important in supporting sperm throughout these processes leading to successful fertilization. Proteomic analysis of sperm proteins in bulls have detected 125 proteins that are differentially expressed between high and low fertility sires and are believed to contribute to differences in signalling and metabolic pathways between the two fertility groups [48]. Such differences in a

complex proteome are difficult to capture using electrophoretic techniques compared to modern mass spectrometry methods. Proteomic information can be used to probe underlying causes of infertility that to date have been considered "hidden" due to lack of information on functional deficiencies of sperm [104]. The multitude of roles played by these proteins, as described above, make them excellent candidates for determinants of sub-fertility. Further examination of proteome differences between individuals of known fertility could elucidate important supportive mechanisms for sperm following ejaculation.

2.6 Genomic Prediction

Although highly heritable traits can be easily detected and used as the basis for genetic selection without using genomic methods, more complex and poorly heritable traits such as fertility, feed efficiency, heat tolerance, longevity and immune response are not amenable to traditional selection methods [105]. Genomic analysis provides the additional information necessary to identify genes underlying complex traits [106, 107]. Selection of animals for a trait such as male fertility using phenotypic measurements is limited due to the complexity of the trait and challenges in its measurement [3]. Typically, there is a substantial cost and time associated with collection of accurate phenotypic data. In the case of boar fertility, data on the outcomes of many inseminations are required once the young boar enters the stud, in order to achieve a reliable relative fertility phenotype. Genome-wide association studies (GWAS) using highly multiplexed arrays allows for genotype data to be obtained at a low cost per data point [106]. Most investigations of sperm fertilizing ability rely on *in vitro* trials, due to the high input in terms of labour, time and cost associated with *in vivo* trials [20]. As such, it would be beneficial to identify genomic regions, and eventually individual genes that contribute to heritable differences in sire fertility. Through this

information, a better understanding of the biological pathways affecting male fertility might also be attained [9]. In the stallion, GWAS has been used to detect single nucleotide polymorphisms (SNPs) that are significantly associated with conception rate. The location of these genes has helped to identify candidate genes, some of which are associated with lethal phenotypes in other species [108]. In cattle, SNP information has been used to help identify a recessive lethal mutation that causes spontaneous abortions. This mutation is carried by many sires that can be identified to have lower reproductive capacity [109]. In bulls, SNPs associated with sire conception rate have also been identified, many of which are in close proximity to genes related to the acrosome reaction, chromatin remodelling or meiosis [9]. In stallions, a SNP leading to amino acid substitution in the CRISP3 protein has been linked to lower fertility [110]. Using this genomic information to determine haplotype influences on economically important traits has resulted in identification of haplotypes associated with lower sire conception rates in dairy cows [111].

In swine, there are three contributors to the key reproductive parameter of litter size; ovulation rate, fertilization rate and prenatal survival [3]. Based on the discussion above, one can assume that the boar's contribution to differences in litter size is the result of differences in fertilizing capacity or in the boar's genetic contribution to embryo viability. However, the heritability of sire effect is low (h^2 of 0.004-0.05) compared to the maternal effect [3]. Controlled experiments, where environmental and sow effects are accounted for, do detect an effect of the boar on litter size correlated with embryo survival rates [112]. Differences in embryo development from high and low fertility sires; are correlated with longer duration of DNA replication in the high fertility sires and these differences are apparent prior to expression of the embryonic genome [13]. Partial fertilization, where only some of the oocytes in the oviduct are fertilized, can occur and leads to

decreased litter size and may be related to the fertile lifespan and functionality of sperm [113]. The low heritability of these estimates may reflect, in part, the difficulty in determining the most accurate measurement of the sire's contribution to litter size (total piglets born vs. those born alive) combined with numerous sow and management effects contributing to this outcome. Sow effects, as well as insemination timing, can affect fertilization rates and embryo development as a result of aging of the oocyte [113]. As the number of sperm per AI dose is lowered, influences of other factors such as sire effect on these traits should be carefully considered [2]. However, the low heritability estimates make this trait suitable for better predictions with GWAS than a more highly heritable trait. Successful studies in groups of horses and cattle using SNPs correlated with sire conception rate, suggest that using GWAS on markers related to pregnancy rate is also warranted [9]. Investigation of SNPs associated with sire reproductive capacity, as well as identification of adjacent genes that may be in linkage disequilibrium with the SNPs, can be combined with value considerations when selection decisions are made at the stud level.

2.7 Conclusions

Physiological mechanisms underlying male fertility are complex and difficult to measure using currently available tests. Although many methods to diagnose capacity for single events required for spermatozoa transit of the female tract and fusion with the oocyte are available at the clinical level, they are limited in their scope and not practical in most agricultural production settings [1]. Semen quality evaluations, while important for processing semen doses for AI and detecting problems with sperm cell motility or morphology, are poorly correlated with relative fertility above the minimum semen quality standards [14, 17]. Due to limitations of currently available methods, there is an opportunity to increase the efficiency of identification of sub-fertile sires by utilizing

molecular markers [14]. The current artificial insemination protocols in pork production systems utilize semen doses far in excess of that required for successful fertilization by high-fertility sires, thereby compensating for low-fertility boars [4]. The result of this compensation is the inability to identify and use high fertility sires of superior genetic merit more efficiently to benefit pork production systems. More efficient use of these high-merit boars has implications for improving production efficiency overall and increasing genetic progress by decreasing genetic lag. In addition, more efficient utilization of boars ultimately results in fewer sires needed to provide semen for breeding the same number of sows. This has the effect of freeing up room in the production system for non-reproductive culling and overall improvements to the boar group through an increased ability to remove less-desirable animals. Overall, improved identification of sub-fertile sires will provide an additional tool available to those involved in culling and selection decisions to help improve genetic contributions of valuable sires to pork production systems.

Incorporation of additional tests to health status monitoring of sires has been suggested for easily included metrics such as normal sperm morphology [24]. This could also be extended to early life blood testing for SNPs associated with low relative fertility performance or screening of seminal plasma proteins in boars after their entry into the stud [24]. If reproductive efficiency is to be improved through use of lower sperm numbers and reproductive technologies such as post-cervical insemination, it will be even more important to identify sub-fertile boars. Without this, improvements to the impact of high genetic merit boars will be limited [6]. Furthermore, to capitalize on more advanced sperm technologies such as sex-sorting or cryopreservation in the future, low sperm doses must be used for efficient utilization of these technologies [114]. Boars that give rise to higher pregnancy rates are also more likely to sire larger litters; therefore, overall

improvements in reproductive efficiency can be made through removal of sires with low pregnancy rates [112].

Presently, limited information is available on molecular mechanisms underlying sperm defects or whether any individual proteins are representative of fertility defects [104]. It is important to examine the species under consideration directly, as some markers and mechanisms may not affect different species in the same way [104]. As the physiological mechanisms underlying male fertility are not entirely understood, a GWAS approach may better explain the genetic basis of male fertility than candidate gene approaches [107]. Genomic and proteomic tools provide useful information for marker-assisted selection of animals. Marker-assisted selection is useful for traits with low heritability as well as those that can only be measured later in life, both of which are true of fertility [105]. By examining the fertility of boars under rigorous field fertility testing and establishing known fertility phenotypes for individual sires, we can use this information to examine the seminal plasma proteome as well as genomic markers of male fertility. Identifying proteomic differences in ejaculates between low- and high-fertility boars can be used to better understand species-specific effects of supportive proteins in the seminal plasma. Understanding the role of these proteins can lead to the use of protein markers of sub-fertility, and also to increased efficiency of reproductive technology [17]. Genomic markers associated with fertility phenotype may provide information that can be used even earlier in sire selection, before an ejaculate can be collected. Identification of genomic markers associated with sub-fertility could enable screening of boars of high genetic merit very early in their lives.

To address some of the missing pieces of information with regards to molecular markers of boar fertility, both proteomic and genomic markers of boar fertility were assessed in the current study. To establish a population of boars with known fertility phenotypes, data were collected on a

minimum of 30 breedings per boar from boars of four breeds. Incorporation of data from different production systems, seasons, and breeds allowed for evaluation of molecular markers, keeping in mind effects commonly considered “fixed effects”. Using a subset of 28 boars representing the highest and lowest fertility individuals with regards to pregnancy rates and litter size, proteomic evaluation was conducted using iTRAQ analysis and LC MS/MS. Of the boars evaluated for fertility performance, 549 individuals were genotyped and markers for pregnancy rate, litter size, and incidence of non-viable fetuses were examined. Results from these studies identified both the seminal plasma proteome and genomic markers for relative fertility that can be used for further development of marker-assisted selection of boars with respect to fertility.

Figures

Table 2.1 Major components of boar seminal plasma.

Component	Major Species	Literature
Carbohydrates	Inositol, Fructose, Glucose, Galactose, Glycerol	[115]
Lipids	Lecithin, Triglycerides, Polyunsaturated fatty acids, Phospholipid, Cholesterol,	[116, 117]
Secreted Proteins	Spermadhesins, Antioxidants, Fibronectin	[59]
Inorganic Ions and Salts	Manganese, Cadmium, Chromium, Calcium, Iron, Zinc, Selenium, Magnesium Sodium Bicarbonate	[60, 118-120]

2.8 Literature Cited

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Chapter 3: Field Fertility Evaluation

3.1 Introduction

It has long been known that boars differ significantly in their contribution to pregnancy rate and litter size [1]. Fertility of a single male affects many females, especially in an artificial insemination (AI) environment. Maintaining reproductive efficiency of the breeding herd requires accurate prediction of fertility of a semen sample and ability to avoid sub-fertile semen [2]. Accurate predictors of a boar's relative fertility performance would therefore be advantageous in order to identify and remove boars of poor reproductive capacity and optimize the use of proven high-fertility boars [3]. However, modest correlations between semen quality traits and fertility performance suggest that we still lack the ability to predict fertility using current methods [4]. Genetically determined capacity for oocyte fertilization or contribution to embryonic viability can both influence a boar's contribution to differences in litter size [5]. Positive correlations between fertilization rate and litter size suggest that selection of sires with high conception rates could improve reproductive efficiency both by limiting the number of sows that return-to-estrus but also through increased piglets sired per successful mating [1].

Measuring fertility can be a challenge for many reasons, including inadequate sample size and variability in female fertility in the cohort to which a sire is mated during testing [6]. To date, few studies have investigated the genetic component of boar fertility, with most of the studies carried out prior to recent advancements in genomic technology [1]. Furthermore, The use of pooled semen from several boars in commercial pork production AI systems complicates fertility evaluation by breaking the direct link between a sire and its reproductive outcomes [7]. Using AI doses with relatively high sperm concentrations further contributes to difficulties in evaluating

male fertility by providing sperm greatly in excess of the number required, thus masking the existence of any sub-fertile boars within the pool [8]. Highly fertile males are able to maintain their fertility performance at much lower sperm concentration per insemination dose than sub-fertile individuals [7, 8]. In that regard, evaluating the outcome of 50 matings/boar at semen doses of 1.5 billion sperm/AI dose could be used to detect differences in sire fertility [8].

To evaluate relative fertility and to establish a population of boars of known fertility for further genomic and proteomic evaluations, boars representing four breeds and four production systems were evaluated for field fertility performance. Effects of porcine reproductive and respiratory syndrome (PRRS) on the accuracy of boar fertility estimates were also assessed for a production system in which PRRS breaks occurred intermittently throughout the testing period. Finally, effects of factors such as average parity of sows bred, time point in the stud, boar breed, and production system on fertility measurements were also evaluated.

The purpose of these fertility evaluations was to quantify fertility performance of individual boars in commercial production systems. By using insemination using low sperm concentrations, fertility of the boars could be evaluated and a population of commercial boars of known fertility could be identified. By this approach, individuals of high and low relative fertility could be used for further analyses examining genomic and proteomic markers associated with fertility.

3.2 Materials and Methods

Boars from four production systems were evaluated for relative fertility performance using reduced semen doses (2 billion viable sperm/AI dose). Upon entry into the boar stud and completion of isolation and training, boars of comparable age were used for single sire mating to

assess fertility. The goal upon entry into the stud was for each boar to be mated to at least 50 sows for evaluation. Boars were monitored for any signs of injury or illness that might affect reproductive parameters.

Semen was collected from boars once weekly using the standard gloved-hand technique. Gel fractions were excluded from collection, but the sperm-rich components and subsequent semen fractions were collected. Where possible, motility and sperm cell concentration was assessed using computer-assisted semen analysis system (CASA, SpermVision, Minitube International, Verona, WI). For locations without a CASA, ejaculates were visually evaluated for motility and sperm cell concentration was determined via photometry (SpermaCue, Minitube International, Verona, WI). Sperm morphology was assessed visually by trained technicians. Only boars with commercially acceptable semen parameters were retained for fertility evaluation (ie. greater than 70% motile sperm and less than 15% with abnormal morphology). Semen from such evaluated collections was then processed to yield a 2 billion sperm AI dose using a long-term extender. The extender used differed by boar stud, with two using synthetic extenders and two using a biologically based product. In some cases, it was not possible for boars to be used for 50 breedings during the test period, however only boars bred to a minimum of 30 sows were considered for relative fertility comparisons. Of the 647 boars evaluated, 603 attained this minimum cut-off.

For each breeding, signs of return-to-estrus at 18 to 21 days later were monitored and if no return to estrus was seen, pregnancy was verified at approximately days 25-30 of gestation using transcutaneous ultrasound. These sows were subsequently followed through to farrowing where litter size measurements (total born, born alive and non-viable foetuses) were recorded.

The outcomes of these breedings (pregnancy and litter size) were evaluated in SAS, using a mixed model approach, to assess the effect of various factors on boar fertility evaluation (SAS 9.4, SAS Institute Inc., Cary, NC).

Production Systems

Boars from four production systems were evaluated for fertility and sampled for proteomics and genomics testing. Effects of production system on fertility outcomes was considered, since there were many management differences between systems and boars were completely nested with regards to the system in which they were used.

System 1:

This commercial system is a 45,000 sow system with 12 production barns and an internal boar stud. Of the individual phenotypes analyzed in the study, 108 boars were from this system (approximately 18%). Boars evaluated from this stud were Duroc. Artificial insemination doses from this stud were processed using the synthetic, long-term extender APX2 (Minitube International, Verona, WI).

System 2:

This production system is a 40,000 sow commercial system with an internal boar stud. Semen doses from this system were processed and extended using Gedil (IMV Technologies, L'Aigle, France), a biologically based semen extender. Some doses (Summer 2013) were extended using Duragen (Magapor, Zaragoza, Spain), another long-term biological extender. This system represents 333 of the boars in the dataset (approximately 55%). The boars in this system were Hampshire and were used for terminal crosses. This production system was uniquely challenged by PRRS breaks in some of their sow barns during boar testing. As such, evaluation of PRRS

breaks in the sow barns on the ability to reliably measure fertility phenotype of boars was evaluated for this particular production system.

System 3:

Boars from this external stud were used for breeding in a 3,500 sow commercial system. Semen doses were extended using a long-term, synthetic extender (ReproQuest Preserv Xtra, Fitchburg, WI). Two breeds of boars were evaluated from this stud, 27 boars of Duroc influence and 69 boars of Pietrain breeding (96 total, approximately 16% of the total dataset).

System 4:

Boars for this system were managed alongside boars from System 2 in the same stud but were differentiated for fertility evaluations as they are used for breeding maternal lines. Semen doses from this system were processed and extended using Gedil, a biologically based semen extender. Some doses were extended using Duragen, another long-term biological extender over the summer of 2013. A total of 66 boars (approximately 11% of the total dataset) were evaluated from this stud. These boars were Landrace boars that had been selected more for reproductive performance of their daughters rather than carcass traits.

PRRS:

In one of the production systems (System 2), porcine reproductive and respiratory syndrome (PRRS) symptom breaks occurred intermittently throughout the data collection period. Symptom breaks were reported when large numbers of abortions, or litters with large numbers of mummified foetuses, were detected in the sow barn. The effect of PRRS breaks on the ability to accurately assess fertility was examined. In order to assess the severity of the effect that PRRS breaks had on the reliability of boar fertility phenotypes, the percentage of breedings occurring during PRRS

break periods was considered. In order to capture all breedings potentially affected by PRRS, any sow that was bred within 20 days after the start of the PRRS break, as well as any sow that was pregnant during the break, was considered to potentially be affected. As PRRS is endemic to System 2, and since break dates only indicate the date at which symptoms were detected, a decision to include all animals (from weaned sows to those in the farrowing room) as potentially PRRS affected, as this was the most conservative way to account for any effects of PRRS on the accuracy of boar fertility measurements. Boars were allocated into five groups, based on percentage of trial breedings affected by PRRS disease breaks (Table 3.1).

Using a mixed procedure, the effect of PRRS category on pregnancy rate, litter size (born alive) and mummified foetuses was evaluated. Using the total data set, effects of production system, PRRS group, parity group and boar breed were examined using mixed models in SAS.

3.3 Results

PRRS

Using data from the production system affected by PRRS, the effect of PRRS disease breaks on boar fertility measurement was evaluated. A total of 2993 breedings were potentially affected by PRRS disease breaks. A PRRS category (percentage of breedings, where gestation or breeding period coincided with PRRS symptoms being detected in the herd) was calculated for each boar tested in production system 2 (Table 3.1) and effects of PRRS were evaluated. Degree of PRRS affected matings (PRRS category) significantly affected pregnancy rate, non-viable foetuses in the litter, and piglets born alive. Pregnancy rate was significantly affected by PRRS category (Table 3.2), but only the low group was significantly different from boars with no PRRS affected breedings. There was also a tendency ($p=0.058$) for boars with a high number of breedings

occurring during PRRS break periods to be different from boars with no affected breedings. The inability to detect a significant difference between High and unaffected boars could partly be due to sample size (n=22) in this group. As other categories have numerically lower pregnancy rates than the unaffected boars, but small sample sizes (n=12 and n=6 for intermediate and very low boars respectively), it's likely that the small number of boars in both of these categories makes it difficult to accurately detect the effects of the PRRS percentages on reproductive capacity evaluation for these individuals. The large contribution of management effects to differences in pregnancy rates could additionally make it difficult to assess this effect on small groups of boars.

Litter size was also significantly affected by PRRS category (Table 3.3). The effects of PRRS on litter size (piglets born alive) were much easier to interpret than pregnancy rate. Boars for which a high or intermediate percentage of breedings during their evaluation period were potentially affected by PRRS had significantly lower litter sizes than the other groups. Although Low and Very low groups were significantly different from one another, unaffected boars were not statistically different from either the Low or Very Low PRRS groups.

There was also a significant effect of PRRS category on average number of non-viable foetuses born per litter for boars evaluated in this system (Table 3.4). Very low and unaffected boars had a smaller number of non-viable foetuses born per litter than the other groups. Interestingly, the highest number of non-viable foetuses was detected for boars with an intermediate number of matings affected by PRRS, wheras High and Low PRRS category boars were significantly lower.

All Boars

Since the boars evaluated were used for breeding in four production systems, many random and fixed effects on reproductive parameters had to be considered prior to genome-wide association

studies. The random effects of production system, group, and boar breed on fertility were evaluated. Production system significantly affected pregnancy rate (Table 3.5), as well as litter size (piglets born alive). Only System 1 was significantly different for pregnancy rate achieved from the other production systems.

Boar breed significantly affected litter size both for total number of piglets born and piglets born alive (Table 3.7). Although a significant effect of breed on litter sizes was detected, it is important to note that for all breeds except Duroc, breed was perfectly nested within production system. As such, effects of breed are confounded with management strategies (Table 3.6). For example, the system that uses all the Pietrain boars represented in the trial also has evaluated some Duroc individuals in the past. It is noted that the litter size parameters of the Pietrain and Duroc breeds are not significantly different from one another for either parameter. Likewise, the Hampshire and Landrace boars come from the same integrated production system, that is, the maternal boars are used for breeding of replacement females for the terminal herd and therefore, there may be some similarities in how these populations are managed. The Hampshire and Landrace boars are notably not statistically different from one another in terms of litter parameters. This is the case despite the selection of maternal lines for pigs with increased litter size more so than the terminal male lines.

Boar group, which was examined to identify any temporal effect on fertility, significantly affected all reproductive parameters. When the entire data set is considered, parity group was not significant on any reproductive parameters.

Due to the potential for PRRS affected boars to cofound the data, comparisons were also run in SAS using only the data from System 2 from animals that were unaffected by PRRS or had very low incidence of PRRS in their breedings (1-24%) was included in the analysis. As boars with a very low percentage of PRRS breedings were not significantly different from unaffected boars in

System 2 for any reproductive measure assessed (Tables 3.2-4), they were able to be included in this subsequent analysis.

Breed was significant for both total piglets born (p=0.009) and piglets born alive (p=.0016). When PRRS-affected matings are removed from the analysis of total born the effects are subtle, however, overall, this removes one statistically different group from the total born (Table 3.7 and 3.8). The 0.09 increase in the estimated mean for the Hampshire boars eliminates their statistical difference from the Durocs. However, the Pietrain boars still have significantly larger litters than the Hampshire and Landrace boars included in this trial.

Following removal of PRRS-affected data, there were significant differences (p=0.0061) in the average number of non-viable foetuses born per litter for boars of different breeds (Table 3.9). Notably, when PRRS data were removed, the breed with the largest total born also had the highest incidence of non-viable fetuses born.

Analysis of production system effects including the PRRS-affected phenotypes detected significant differences between systems in both pregnancy rate (Table 3.2) and piglets born alive (Table 3.3). Removing significantly affected PRRS groups resulted in the same trend, with System 1 achieving a significantly lower pregnancy rate than the other systems. The mean pregnancy rate for System 2 did increase by 0.24% when boars for which 25% or more of the test breedings potentially affected by PRRS were removed from consideration.

Removal of PRRS-affected phenotypes increased mean litter size in System 2 to 12.18 piglets (Table 3.11), interestingly, making them statistically similar to System 3, the maternal line breeding program for the same integrated system.

3.4 Discussion

To date, 647 boars have been selected for field fertility evaluation at low sperm doses. Of those, 603 (93.2%) completed the minimum number of single-sire matings required for evaluation. When PRRS-affected data were omitted, a total of 537 boars could be compared (83.0%). Significant effects on litter size were detected for production system and breed. Only the former had a significant effect on pregnancy rate. When the effects of PRRS on fertility were evaluated for the production system in which PRRS is endemic, PRRS breaks significantly affected all reproductive parameters measured. This is not surprising, as one of the symptoms of PRRS is an increase in mummified foetuses, while abortions can also occur during PRRS infection.

Effective predictors of boar reproductive capacity, and exclusion of sub-fertile boars would allow for increased selection pressure at the nucleus level, and increased production efficiency at the production level through greater progress on high-value traits [3].

Litter size is influenced by ovulation rate, fertilization rate and embryonic survival and, of these, both fertilization and prenatal survival can be influenced by sire [5]. Soede et al. (1995) reported that partial fertilization, where only a portion of oocytes in the oviduct are fertilized, occurs in approximately 37% of all litters, which could explain how paternity can influence litter size even when successful pregnancy occurs [9]. One paternal effect on embryonic development is a faster than optimal transit through certain stages of mitosis. As demonstrated in low-fertility bulls, this type of effect represents one way that embryonic survival may be affected in a polytocous species [10]. Differences in either embryonic development or oocyte fertilization could contribute to breed-specific differences in litter size observed in this work. The modern maternal line cross has been selected for high ovulation rate via selection on litter size and it is therefore reasonable to

assume that ovulation rate is not limiting litter size in the production systems evaluated [11]. The nesting of some breeds in only one production system make it impossible to assess the effects of breed versus production system. The significant influence of production system on pregnancy rates could largely be due to differences in management practices during semen collection and processing or during estrus detection and artificial insemination contributing to reproductive success in the different systems.

Figures

PRRS Group

Table 3.12. PRRS status groups. All sows in the affected barn at the time of the break, from those in the pre-conception period to those at the end of gestation were considered potentially affected.

Group	Affected Matings 4(%)	Number of Boars	Percentage of Population (%)
High	75-100	22	6.6
Intermediate	50-74	12	3.6
Low	25-49	32	9.6
Very Low	1-24	6	1.8
Unaffected	No affected breedings	261	78.4

Table 3.13. Summary of PRRS effects on boar pregnancy rate. All boars were bred in the same production system and were from the same genetic line. Boar effect nested within PRRS category on pregnancy rate was also significant and accounted for as a random variable. Statistically significant differences ($p \leq 0.05$) are denoted with a superscript.

PRRS Category	D30 Pregnancy Rate (%)	Standard Error (%)
High	89.28^{ab}	1.58
Intermediate	89.53^{ab}	2.15
Low	87.67^{a}	1.31
Very Low	90.78^{ab}	3.03
Unaffected	92.41^{b}	0.46

Table 3.14. Summary of PRRS effects on litter size. All boars were bred in the same production system and were from the same genetic line. Statistically significant differences ($p \leq 0.05$) are denoted with a superscript.

PRRS Category	Born Alive	Standard Error
High	10.77^{a}	0.24
Intermediate	10.54^{a}	0.33
Low	11.44^{b}	0.20
Very low	12.01^{c}	0.46
Unaffected	12.18^{bc}	0.070

Table 3.15. Summary of PRRS effects on non-viable foetuses born. All boars were bred into the same production system and from the same genetic line. Data was analyzed using a mixed model in SAS and statistically significant differences ($p \leq 0.05$) are denoted with a superscript.

PRRS Category	Non-Viable	Standard Error
High	0.78[a]	0.068
Intermediate	1.44[c]	0.091
Low	0.85[a]	0.056
Very low	0.42[b]	0.13
Unaffected	0.35b	0.020

Production Systems

Table 3.16. Pregnancy rates achieved in the different production systems over the trial period evaluated. Effects were evaluated in SAS using a mixed model where breed, group and parity group were adjusted for and statistically significant differences ($p \leq 0.05$) are denoted with a superscript.

System	Pregnancy Rate (PR) %	Standard Error
1	86.92[b]	1.35%
2	91.66[a]	0.75%
3	93.31[a]	1.28%
4	91.26[a]	1.40%

Table 3.17. Effect of production system on litter size. Effects were analyzed in a mixed model in SAS where the effects of breed, group and parity were included. Statistically significant differences ($p \leq 0.05$) are denoted with a superscript.

System	Born Alive (BA)	Standard Error
1	12.61[ab]	0.21
2	11.94[c]	0.11
3	12.66[a]	0.20
4	12.03[bc]	0.22

Breed Effects

Table 3.18. Breed effects on litter sizes born. Breed effect was evaluated in SAS using a mixed model where group, parity and production system were adjusted for. Statistically significant differences ($p \leq 0.05$) are denoted with a superscript.

Breed	Total Born (TB)	SEM	Born Alive (BA)	SEM
Duroc	13.81^{ac}	0.17	12.58^{b}	0.21
Hampshire	13.24^{b}	0.15	11.94^{a}	0.11
Landrace	13.36^{ab}	0.22	12.03^{a}	0.20
Pietrain	14.23^{c}	0.22	12.74^{b}	0.22

Analysis of effects with PRRS affected breedings removed

Table 3.19. Effect of breed on litter size with more than 25% of matings resulting in potentially PRRS affected litters removed from the dataset. Breed effect was evaluated in SAS using a mixed model where group, parity and production system were adjusted for. Superscripts indicate statistically significant groups at $p \leq 0.05$.

Breed	Total Born (TB)	SEM	Born Alive (BA)	SEM
Duroc	13.80^{ab}	0.18	12.58^{b}	0.13
Hampshire	13.33^{a}	0.18	12.19^{a}	0.09
Landrace	13.35^{a}	0.23	12.02^{a}	0.16
Pietrain	14.25^{b}	0.23	12.72^{b}	0.17

Table 3.20. Effect of breed on number of non-viable foetuses born per litter. Boars with 25% or more matings potentially affected by PRRS were removed from the analysis. Statistical analysis was completed using a mixed model in SAS, superscripts indicate statistically significant groups at $p \leq 0.05$.

System	Non-Viable (NV)	Standard Error
Duroc	0.32^{a}	0.034
Hampshire	0.35^{ab}	0.023
Landrace	0.42^{bc}	0.037
Pietrain	0.49^{c}	0.043

Table 3.10. System differences in average pregnancy rate when PRRS affected data was removed from analysis (25% or more of matings occurring to sows during an active PRRS break). Statistical analysis using a mixed model in SAS was performed controlling for breed, group and parity group of sows.

System	Pregnancy Rate (PR) %	Standard Error
1	86.87[b]	1.28%
2	92.29[a]	0.77%
3	93.26[a]	1.22%
4	91.22[a]	1.35%

Table 3.11. Litter size (born alive) for each production system when PRRS affected data was removed from analysis (25% or more of matings occurring to sows during an active PRRS break). Statistical analysis using a mixed model in SAS was performed controlling for the effects of breed, group and parity.

System	Born Alive (BA)	Standard Error
1	12.61[a]	0.15
2	12.18[b]	0.08
3	12.64[a]	0.14
4	12.02[b]	0.16

3.5 Literature Cited

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Chapter 4: Proteomics and Genomics Analysis

4.1 Introduction

By completion of spermiogenesis, sperm are transcriptionally and translationally silent due to the tight packaging of DNA into chromatin. This, as well as their lack of cytosol, makes them largely dependent on extracellular factors for functional support and mitigation of environmental stressors [1]. Seminal plasma, uterine and oviductal secretions, and extenders used in an AI environment, all contributed to preserving sperm viability and supporting their function. Of particular interest are seminal plasma proteins. Secreted proteins make up the majority of the seminal plasma proteome, and have important functions to support fertility. Their ability to bind a variety of molecules as well as their roles as hydrolases and proteases are important for sperm cell function in the female reproductive tract and the supportive role of these proteins has been described in many species [2-7].

Despite the literature available on the postulated roles of specific proteins and the results of *in vitro* experiments, identification of seminal plasma proteins that can be considered reliable markers of a sire's fertility is still elusive. During ejaculation, proteins are secreted with fluid from the accessory sex glands and, in natural mating, these proteins provide support for sperm during their introduction to the female reproductive tract. However, the role of these proteins as fertility markers may be different in an environment where semen is collected, extended, and stored for use in AI, than it is in a natural service environment. Furthermore, in species where electroejaculation is used for semen quality evaluation, the proteome may not be representative of a natural ejaculate, due to the increased abundance of low molecular weight proteins [8]. In pig breeding, where semen collection for AI is performed without the use of electroejaculation, the

difficulty in identifying which proteins may predict relative fertility is primarily a result of breeding management practices, rather than a function of the collection method. The use of a high concentration of sperm per AI dose (typically 3 billion viable cells/dose) and heterospermic (pooled) inseminations mask the presence of poor fertility sires in the breeding pool and breaks the link between breeding outcomes and a single sire [9, 10]. Challenging boars via single sire inseminations with lower sperm concentrations can reveal differences in fertility phenotype; however, the time required to perform these breedings and evaluate fertility performance makes such testing costly and inefficient [9].

Work to date has identified the sperm-peak fraction occurring early during ejaculation as the fraction containing the highest fertility spermatozoa [5]. Experiments conducted using co-incubation of both spermatozoa and seminal plasma representing different fractions of the ejaculate show an effect of the seminal plasma on sperm cell fertility [11, 12]. Osteopontin was negatively correlated with litter size and farrowing rate and polymorphisms in the osteopontin gene have been significantly associated with litter size in boars [5, 13]. The antioxidant protein GPX5, secreted from the epididymis is important in maintaining sperm cell function, while spermadhesin proteins have been associated with fertility [3, 5]. Existing literature concerning associations between various seminal plasma proteins and fertility have utilized blotting and gel electrophoretic techniques as the basis for assessing relative abundance. Availability of mass spectrometry technologies such as isobaric tag for relative and isobaric quantitation (iTRAQ) and liquid chromatography-mass spectrometry (LC-MS) facilitate absolute quantification of protein abundance, and will lead to a better understanding of optimal amounts of important functional proteins in seminal plasma.

Genome-wide association studies (GWAS) offer an opportunity to identify regions of the genome associated with complex traits, as well as genes in close proximity that could underlie phenotypic differences in fertility. To date, most of the fertility phenotypes identified have used *in vitro* fertilization techniques, due to the cost associated with *in vivo* fertility evaluation. A search of the existing literature did not reveal any previously conducted genome-wide association studies for boar fertility. Studies in stallions and bulls have successfully identified single nucleotide polymorphisms associated with fertility, including some in close proximity to recessive lethal mutations and genes for seminal plasma proteins [14-17]. The use of genomic information has successfully identified haplotypes associated with lower sire conception in dairy sires, suggesting a promising future for genomic markers as indicators of sire fertility [18].

Increased knowledge of differences in seminal plasma proteomes and genotypes between high- and low-fertility boars could lead to development of markers for early fertility screening of young boars prior to their entry into the stud. Potential advantages of these markers lie not only in their ability to enable early screening, but also in their ability to be used even when environmental factors may affect reliability of field fertility measurements. The objective of this project was, therefore, to use boars of known fertility to explore seminal plasma proteome and genomic differences between high and low fertility individuals using spectrometry-based quantification methods and single nucleotide polymorphism (SNP) analysis.

4.2 Materials and Methods

Semen Collection and Sample Processing:

Boar semen was collected using gloved hand technique and the total ejaculate was collected with the exception of gel fractions. Following collection, raw semen was centrifuged at 1300g for 10

minutes to separate sperm from seminal plasma. Seminal plasma was then removed from the sperm pack by pipette and transferred to a new conical tube. During aspiration of the seminal plasma, care was taken to avoid aspirating any sperm. Samples were then frozen at -20 °C. Prior to proteomic analysis, samples were thawed, mixed, and a 1 mL aliquot was removed and re-frozen for total protein analysis.

Seminal plasma for analysis was collected from each boar at three time-points, as a part of the boar's regular semen collection schedule for AI breeding during the fertility assessment period described in Chapter 2 of this thesis. Semen was only collected once per week while boars were on trial, in order to prevent collection frequency effects on sperm cell concentration or seminal plasma components.

Boar Selection for Proteomics:

From the boars evaluated in field fertility trials, a subset of boars representing the high fertility group (n=13) and a subset of boars representing the low fertility group (n=15) were selected for proteomic evaluation. To account for any environmental factors affecting specific groups of boars, each group was evaluated separately to identify the top and bottom 10% with respect to fertility. In order to be considered a high-fertility animal, a boar needed to meet the minimum standard of achieving a 95% pregnancy rate at day 30 in sows bred during the evaluation period. Boars for which the difference between day 30 pregnancy rate and farrowing rate was greater than or equal to 7% were eliminated to minimize the effects of other health and management considerations. Boars that had health issues or semen quality concerns during the trial period were also eliminated from consideration.

Label Free Quantitation (LFQ):

Pilot proteomic analysis was carried out using liquid chromatography-mass spectrometry (LC-MS) through the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University in New Haven, Connecticut. Raw seminal plasma was diluted 50:50 with RIPA buffer and sonicated. Following precipitation using a chloroform/methanol extraction method, protein digestion was performed in 8 M urea/0.4 M ammonium bicarbonate. Digestion was performed by sequential incubations with DTT, IAN, Lys-C and trypsin [19, 20]. Once digested, 0.2 ng of protein was loaded onto each column and samples were run through LC-MS in randomized order with three blanks following each run [21]. Samples from each boar were run in triplicate.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed using a Waters nanoAcquity UPLC system. Trapping was conducted at a rate of 15ul/minute in 0.1% (v/v) formic acid in H_20 for 1 minute. Sample separation was then carried out using a linear gradient of buffer over 90 minutes at a rate of 300 nl/minute. Mass spectra acquisition was conducted using a maximum injection time of 900 ms and five data-dependant MS/MS acquisitions. Peaks targeted for MS/MS fragmentation by collision-induced dissociation were isolated with a 2Da window, and dynamic exclusion was activated where former target ions were excluded for 60 seconds.

Chromatographic alignment, feature extraction, data filtering, and statistical analysis were carried out on the raw data using Nonlinear Dynamics Progenesis LCMS. 8830 features were identified for analysis. Normalized abundance, maximum fold change, and ANOVA value for each feature were then calculated.

Isobaric Tag for Relative and Absolute Quantitation:

A proteomic discovery of boar seminal plasma proteins was performed using iTRAQ to identify proteins that could be exploited in further analysis. Of the 30 boars selected for proteomic evaluation, 15 boars were identified as representing the two extremes for fertility and were run on two iTRAQ 8 channel sets. The highest fertility boar in the cohort was run on both sets to assess technical variation between runs. Mass spectrometric analysis was completed using an AB SCIEX Triple TOF ® 5600 mass spectrometer and AB SCIEX ProteinPilot software. Proteins were precipitated using chloroform/methanol and the pellet was resuspended in 0.5 M Triethylammonium bicarbonate/0.2% SDS [20]. Proteins were reduced using 50 mM tris-(2-carboxyethyl) phosphine (TCEP) and alkylated with 200 mM methyl methanethiosulfonate (MMTS) and digested overnight with trypsin at 37°C. For both chromatographic runs, 100 ng of protein was loaded into the channel in a buffer of 70% (v/v) formic acid/0.1% (v/v) TFA. Data from iTRAQ runs were filtered to only include peptides with no mis-cleavages, which were identified with high confidence (>90%), and which had 2 or more iTRAQ reagent ratios. Peak area distributions within iTRAQ runs were normalized using cyclic loess [22]. To compare results between iTRAQ runs, medians across runs were adjusted using the Log2 scaled area (where zeros were replaced with 0.01). To examine consistency between runs, an independent analysis of each run was used, with fertility group, spectra, and sample included in the model. Combined set analysis was employed to detect differences between high and low fertility individuals. Protein-level differences were evaluated using median spectrum area of peptides to calculate protein area. Proteins detected in only one set were analyzed using a Students *t*-test while proteins observed in both sets were compared using a linear model with the set effect as a covariate. Within iTRAQ sets, these filtered peptides were then normalized using cyclic loess [22]. Fold-change analysis

was then performed on each protein. Results for normalized proteins were analyzed both between and within iTRAQ sets using t-tests and a linear mixed model. Non-normalized results were also examined to identify any low abundance proteins with previously identified roles in fertility for which variation was observed, but not detectable due to normalization. All spectra were searched using the Mascot algorithm version 2.2.0 [23].

Multiple Reaction Monitoring:

Targeted proteomics using MRM on the 28 boars from the proteomics cohort were completed following iTRAQ discovery. Protein digestion was completed as described previously for the pilot run [24]. Peptides of interest were determined through consideration of discovery results as well as previous work completed by Novak et al., (2010). Heavy peptides were synthesized for the proteins of interest, and submitted with their lighter homologs to LC-MS to determine transition and retention time for each peptide [24]. To control for variation between ejaculates, the three samples from each boar were pooled and run in duplicate during the targeted analysis.

Total Protein Analysis:

Total protein in the seminal plasma for each of the 28 boars selected for proteomics was evaluated using a bicinchoninic acid assay (BCA assay) [25]. Aliquots from the same seminal plasma samples evaluated via LC-MS were thawed, re-suspended, and analyzed for total protein concentration at dilutions of 1:50 and 1:100 in ddH_20.

Osteopontin ELISA:

The presence of osteopontin (OPN) was not detected in either the LC/MS pilot run or iTRAQ discovery, despite being reported as present in boar seminal plasma in previous literature [5]. An ELISA was conducted to see if osteopontin could be detected in the seminal plasma samples prior

to digestion. Osteopontin ELISA was performed on aliquots of seminal plasma from the same ejaculates used in targeted proteomics. Aliquots of the three ejaculates from each boar were pooled, and an ELISA was performed using a porcine osteopontin kit (My Bio Source, catalog # MBS2023027), following its standard protocol. Sow's milk, which is known to be abundant in OPN, was used as a positive control.

Genomic Analysis:

Blood samples were collected from each boar during field fertility evaluation, and submitted for genotyping to Delta Genomics in Edmonton Alberta. Subsequent DNA extractions were completed using the Qiagen QIAsymphony SP automated extraction system and a QIAsymphony DNA Mini Kit. Genotyping was then completed using Illumina PorcineSNP60_B and the Infinium HD Ultra Assay. Scanning of the beadchips was completed using the Illumina HiScanSQ.

Genome-wide Association Study:

Genome-wide association analysis was performed using generalized quasi-likelihood scores (GQLS) in the Sleuth software package [26]. The GQLS method uses a logistic-regression model to determine differences in allele frequencies for different phenotypes; this model treats phenotypic values as a covariate and allele frequency as the response [26]. GQLS links the mean value of the allele frequency to phenotypic traits and is useful for populations with differing degrees of relatedness and population stratification [26]. However, as kinship and inbreeding information for these populations was not known, animals were assumed not to be highly related. To account for differences between populations, breed and production system were included as random effects in the linear model for each trait. Only SNP's reaching significance at a p-value of less than 0.01 after Bonferonni adjustment were considered.

4.3 Results

Pilot LFQ:

Using the 8830 peptide features detected using LC-MS, a total of 182 proteins were successfully identified at $p < 0.05$ using Progenesis data extraction (Nonlinear Dynamics, LLC, Durham, NC) and the Mascot search algorithm [23]. A list of identified proteins is provided in Appendix 1.

Total Protein Analysis:

Based on ejaculate data from the stud and BCA total protein results, protein to sperm cell ratios were calculated for 30 boars. For each boar and ejaculate collected, a ratio was generated, and the three ratios were run as a repeated measures analysis in SAS using a mixed model (Table 4.1-3). There was a trend towards a lower protein to sperm cell ratio in high fertility boars ($p=0.09$). There was also a trend for protein to sperm cell ratio to be lower at the third collection than the first, suggesting a change over time ($p=0.07$).

When all three collections were included in a multiple linear regression, there was a significant negative linear relationship between pregnancy rate and protein to sperm cell ratio ($p=0.003$), with an r-squared value of 0.41. The low correlation coefficient may have been due in part to outliers, which were not removed in part due to the small sample size and to account for the effect of biological variation on this relationship.

iTRAQ:

Using iTRAQ, 1318 sequences were observed, and a total of 198 proteins were identified in at least one of the samples evaluated using the Mascot search algorithm. Of these, 64 proteins

overlapped between both sets (Figure 4.1a). Following filtering, 135 peptide modifications and 45 proteins shared between both sets remained (Table 4.4).

Normalization via cyclic loess allowed spectrum levels between runs and iTRAQ wells to be compared (Figure 4.2-3).

When iTRAQ results were considered separately, normalized results identified 16 proteins which were either significantly different or showed a trend to differ between high- and low-fertility boars (Table 4.5). Two proteins were detected which had large differences in fold-change between the two sets (Figure 4.4-4.6). The protein PSP-II is consistently lower for high fertility boars, whereas F6X6N8_horse, a member of the zymogen granule protein 16 homolog b cluster, had a high degree of fold change but was not significant in the normalized results. It was not possible to determine if these values were outliers, due to the small sample size and extreme levels of F6X6N8 in some individuals. Of the 16 proteins identified, seven had a consistent pattern in fold-change direction between high and low fertility individuals on both iTRAQ sets. When both sets of normalized iTRAQ results were included in a linear model, four proteins that were detected in both sets were also significantly different (Table 4.6). These four proteins include three members of the spermadhesin family: AQN-1, PSP-II and AQN-3. The other significantly different protein was lysosomal alpha-mannosidase (Figure 4.7). Three additional proteins tended to be different between high and low boars; these proteins were beta-2 microglobin, cathepsin B, and an uncharacterized protein, which is part of the Zinc-alpha-2-glycoprotein cluster.

As previous work suggests, high fertility boars may have lower expression of many of the most commonly abundant seminal plasma proteins. When non-normalized results for the iTRAQ runs were evaluated separately for potential variation, twelve proteins were found to differ or tended to differ between high and low boars in one of the two sets (Table 4.7). Of these, five showed a

consistent fold-change direction in high versus low fertility animals in both sets. When comparing these to the normalized results, three proteins were significantly different in both the normalized and non-normalized results; these were Beta-2 Microglobin, Lysosomal alpha-mannosidase, and AQN-3. The spermadhesin PSP-I was significantly lower in high fertility boars in set one. However, this change was much smaller in set 2 and, following normalization, this difference did not reach statistical significance. This is likely due to high variation in expression of this protein, relative to the small number of individuals evaluated. As PSP-I is highly abundant in the seminal plasma (238 and 264 spectra detected for set 1 and 2 respectively) and has functions directly related to sperm cell fertility, it was still included in subsequent targeted approaches. Another protein previously implicated in boar fertility, GPX5, was inconsistent in its direction of change for high versus low fertility boars between the two sets, even when normalized.

When the non-normalized results from both sets were analyzed together using a linear mixed model, two proteins differed significantly between high and low fertility boars. These two proteins, AQN-1 and Lysosomal alpha-mannosidase remained significantly different between the fertility groups after normalization. Of the three proteins which exhibited a tendency to be different, Plectin-1 and Clusterin were not statistically different in the normalized data set. Cathepsin B showed a trend following normalization, though the extent of the Log_2 fold change was less once data was normalized.

Multiple Reaction Monitoring:

Based on the iTRAQ results, six proteins from the high- and low-fertility groups were compared. Of these, four proteins had statistically significant differences and one protein exhibited a trend towards a statistically significant difference between the fertility groups. Of the significantly different proteins, GPX5 was more highly expressed in high-fertility individuals

whereas PSP-I, PSP-II and AQN-3 had lower expression in the seminal plasma of high fertility boars than their low fertility counterparts. The expression of AQN-1 tended to be lower in the seminal plasma of high fertility boars as well. Lysosomal alpha-mannosidase (identified as F1SEY1 in subsequent figures and tables), which was the most significantly different in the iTRAQ analysis, was not significantly different in the MRM analysis. This might be explained by the high degree of variability in the protein areas (Figure 4.7) from the iTRAQ results. Of the proteins evaluated, PSP-II exhibits the most significant p-value as well as the lowest expression in high fertility boars (Figure 4.8).

Osteopontin ELISA:

Osteopontin ELISA was performed for all boars in the proteomics cohort. No values above the lowest point on the standard curve (0.78 ng/mL) were detected. Due to minute values on the bottom end of the standard curve, no significant differences could be evaluated.

GWAS:

SNP genotyping on the Illumina 60K chip was completed for 475 boars. Of the boars genotyped, 438 boars had reliable phenotypes for use in further evaluation through GWAS.

Results for a total of 54706 SNPs with known locations were submitted to Sleuth for evaluation on all three traits (pregnancy rate, piglets born alive, and non-viable fetuses) using a minor allele frequency threshold of 0.01 and call rate threshold of 0.9. Of the SNPs submitted, a total of 12508 were excluded, leaving 42198 for consideration in the association analysis. Significant SNPs for Pregnancy Rate (PR), Born Alive (BA), and Non-viable fetuses (NV) were detected following a genome-wise Bonferroni adjustment ($p < 0.01$). Significant SNPs for piglets born alive were detected on all autosomes (2808 total), with the greatest number of significant SNPs present on chromosomes 1, 13, and 6 (440, 244, and 206 significant SNPs respectively). A total of 453

significant SNPs were associated with non-viable fetuses. These were distributed along all chromosomes, with the greatest number of significant SNPs on chromosome (chr) 1, 14, and 13 (59, 39 and 30 respectively). For pregnancy rate, 39 significant SNPs were detected, with the largest number of significant SNPs found on chr 15, 18, 8, 14 and 16 (5, 5, 4, 4, and 4 significant SNPs on each).

Following GWAS, significant SNPs for each trait were compared to detect those shared between traits. A total of 163 SNPs were shared between BA and NV, with shared SNPs on all autosomes. When significant SNPs for PR were compared to the other two traits, fewer were shared between them. For PR and BA, 5 SNPs were shared, with one SNP on each of chr 1, 6, 12, 14, and 15. For PR and NV, 15 SNPs were shared, these SNPs were located on chr 4, 7, 8, 9, 12, 14 (three SNPs), 15 (three SNPs), 16, and 18 (three SNPs). Finally, only three SNPs were shared between all three reproductive traits, these were located on chr 12, 14 and 15.

Based on information from the Sscrofa10.2 assembly, shared SNPs were evaluated for genes in close proximity. The shared SNP on chromosome 12 (ASGA0053492), is located within an intron of an uncharacterized pseudogene (LOC100624995). This SNP is also located in close proximity downstream from ITGB3 and MYL4, and just upstream of an uncharacterized protein coding gene LOC100516640 which is a Ca^{2+} binding protein. The SNP shared among all three traits on chromosome 14 (INRA0047711) is intergenic, with its most closely located gene, RAB11 family interacting protein 2 (RAB11FIP2) found 62kb downstream. RAB11FIP2 is involved in aquaporin and endocytosis pathways. The last common SNP for all three traits, is located on chromosome 15 (DRGA0015434). This SNP is also intergenic, located approximately 300kb upstream from the modeled LOC100623954, a potassium voltage-gated channel subfamily H member 7-like gene.

Finally, significant SNPs for all traits were compared to the location of seminal plasma proteins shown to be related to fertility through MRM. No shared SNPs were found to be located near the genes coding for the spermadhesin proteins or GPX5 on the genome. One SNP that was significant only in GWAS for piglets born alive may be located 105 KB upstream from the spermadhesin proteins (MARC0058375) at 43560194 on chromosome 6. However, this could not be confirmed in the porcine 10.2 assembly dbSNP.

4.4 Discussion

The results of this study have identified four proteins with significantly different abundance between high- and low-fertility boars. These proteins are promising candidates for development of boar fertility performance markers in commercial pork production, and provide a basis for additional experiments. Validation of these markers could be performed by exposing sperm to various levels of individual seminal plasma proteins, and evaluating their functional competencies through in vitro methods, or by an *in vivo* approach through insemination of sows with semen doses processed to contain varying levels of the seminal plasma proteins implicated in fertility. These experiments could be used to ascertain the optimal level of these proteins, as well as the thresholds above which fertility is compromised.

As these proteins all have physiological functions relating to sperm cell function, they also contribute to an increased understanding of the physiological mechanisms underlying male reproductive failure. The results of our final MRM analysis, showing a lower abundance of the spermadhesins in high-fertility animals, is consistent with previous work [5]. The role of the porcine seminal plasma proteins I and II include stabilization of the sperm cell membrane and prevention of premature capacitation [27, 28]. The functions of AQN-1 include zona pellucida binding, although it has also been implicated in preventing capacitation [29, 30]. The last of the

spermadhesins tested, AQN-3, acts as a sperm motility inhibitor [31]. As these spermadhesin proteins are bound to the acrosomal cap of the sperm cell as well as free in the seminal plasma, the influence these proteins may have in fertility outcomes could depend on which of their roles is most affected by changes in their relative abundance in the seminal plasma. The function of seminal plasma proteins PSP-I and -II in stabilizing sperm would also suggest that an over-abundance of these proteins could impede the ability of these cells to respond to environmental cues in the oviduct. This decreased ability to respond to cues and undergo capacitation could decrease fertilization rate. Glutathione peroxidase 5 has previously been suggested to positively correlate with fertility in boars, which is supported by our results of significantly higher GPX5 abundance in seminal plasma from high-fertility boars [5]. The role of GPX5 as scavenger of reactive oxygen species protects sperm from oxidative stress. This protein is able to react to minute changes in oxidative stress, decreasing the risk of sperm cell DNA fragmentation and lipid peroxidation [3, 32]. Increased levels of this epididymis-specific glutathione peroxidase could therefore be advantageous to long-term protection from oxidative stress.

The GQLS analysis identified many SNPs associated with all fertility outcomes, as well as three shared among all three fertility traits. The high number of SNPs detected even after correction using a Bonferroni adjustment, could be due to many factors, including the small sample size of boars and the low heritability of reproductive traits, in particular the heritability of a boar's litter size and number of non-viable fetuses born. The population structure of the boars evaluated might also have led to the high number of significant SNPS. There is likely some correlation with the relatedness of boars in various production systems; however, pedigree information was not available, potentially resulting in inflated positive results [26]. Lastly, the allele frequency of the population was not known, so this limited the ability to control for differences in the analysis.

The proteomic results suggest that there is an optimal level of protection from premature capacitation conferred to sperm by the spermadhesins, beyond which over-abundance of spermadhesins is detrimental to cell function. Unlike spermadhesin quantity, increased GPX5 levels in high-fertility boars appear to be beneficial to the cells through increased antioxidant capacity. In the future it would be informative to utilize controlled experiments involving semen processing that create varying levels of spermadhesin and GPX5 abundance in order to determine the minimum or maximum cut-off levels that predict decreased sperm cell function and therefore poor fertility. Further understanding of the optimal levels of these proteins could also be used in development of semen extenders. Additional work, involving development of a panel of markers for the identification of boars with low reproductive capacities, could benefit commercial pork production by decreasing the number of sub-fertile individuals included in the breeding pool. Through better utilization of sires with known fertility and high genetic merit, efficiency of genetic progress in pork production systems can be further improved.

The genome-wide association analysis linking boars of known fertility to SNPs associated with each trait on the porcine 60K chip has not previously been described; therefore, comparison with previous findings was not possible. Of the SNPs significantly associated with all three reproductive phenotypes, the shared SNP on chromosome 12 (ASGA0053492) is interesting due to its location upstream from Integrin Beta-3 (ITGB3) and Myosin light chain 4 (MYL4). Integrin Beta-3 (ITGB3) has been found to be up-regulated during immune challenge, as it is important for endothelial function, but has also been reported to be expressed by the endometrium during embryo implantation in the pig [33, 34]. Myosin light chain 4 (MYL4), is important for cardiac muscle contraction. Further exploration of protein differences in this area could implicate differences in embryonic development or competency as a result of variable sire fertility. The limitations of the

work outlined in this study make it difficult to determine whether or not the SNPs associated with the traits are biologically significant. For the lower heritability traits examined, it is difficult separate true associations from inflated positive results, due to population structure and small sample size. Further work building on this project and incorporating pedigree information could help to elucidate which SNPs are the most reliably associated with boar fertility.

Figures

BCA Analysis:

Table 4.21. Averages of total seminal plasma protein at each collection point for boars of high and low fertility. Protein was assessed using a BCA assay.

Fertility Group	Ejaculate Total Protein (ug/ml)			
	Collection 1	Collection 2	Collection 3	Average
High	3.38×10^4	3.38×10^4	3.02×10^4	3.26×10^4
Low	4.02×10^4	3.32×10^4	3.78×10^4	3.71×10^4

Table 4.22. Sperm cell concentration averages at each collection point for boars of high and low fertility. Sperm cell concentration was calculated using spectrometry (SpermaCue).

Fertility Group	Sperm Cell Concentration (sperm/ml)			
	Collection 1	Collection 2	Collection 3	Average
High	256.57	268.57	261.33	258.93
Low	211.6	207.07	247.13	222.27

Table 4.23. Protein to sperm cell ratio averages for boars of high and low fertility at each collection point. Ratios were calculated using SpermaCue values and BCA assay results and a ratio generated for each boar.

Fertility Group	Protein:Sperm Cell (pg/cell)			
	Collection 1	Collection 2	Collection 3	Average
High	138.13	141.20	123.20	135.52
Low	196.26	160.60	166.84	174.88

Discovery Proteomics (iTRAQ):

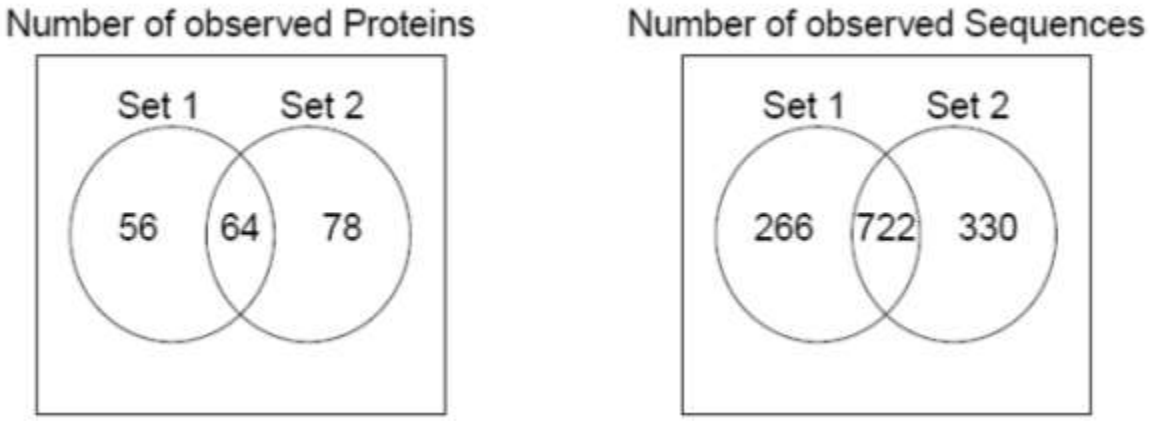

Figure 4.2a and b. Boar seminal plasma protein and peptide (sequences) overlap between iTRAQ runs. Peptide and protein comparisons were made prior to filtering, abundance (areas) were normalized using cyclic loess.

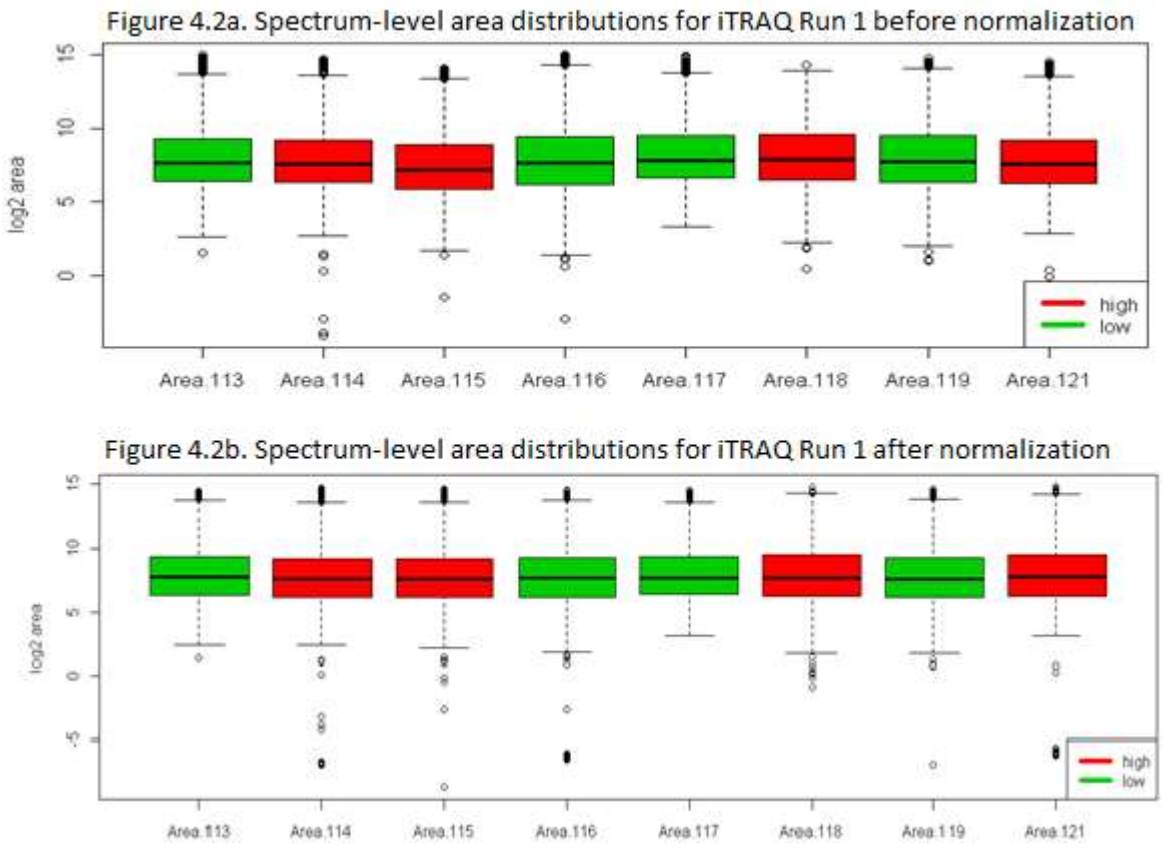

Figure 4.2a and b. Normalization of iTRAQ Run 1. Log2 area of the peptide spectra for each boar are shown before and after cyclic loess normalization. High fertility boars are shown in red and low fertility boars in green

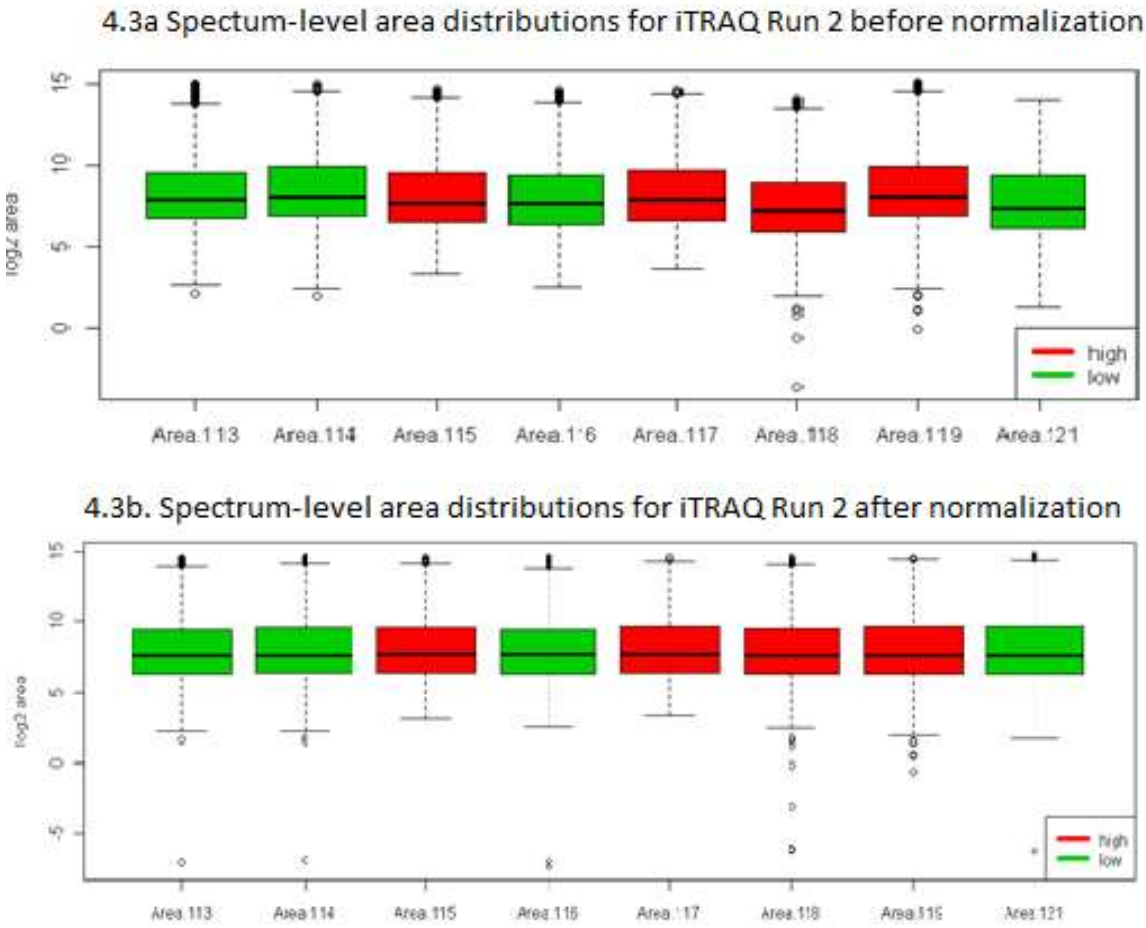

Figure 4.3 a and b. Normalization of iTRAQ run 2. Log2 area of the peptide spectra for each boar are shown using before and after cyclic loess normalization. High fertility boars are shown in red and low fertility boars in green.

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Table 4.24. Summary of iTRAQ discovery results. In total, 135 peptide modifications and 45 proteins were identified in common for both runs.

	Set 1	Set 2	Common (Set 1 and 2)
#. Spectra	692	755	
#. Peptide modifications	196	215	135
#. Proteins	69	71	45

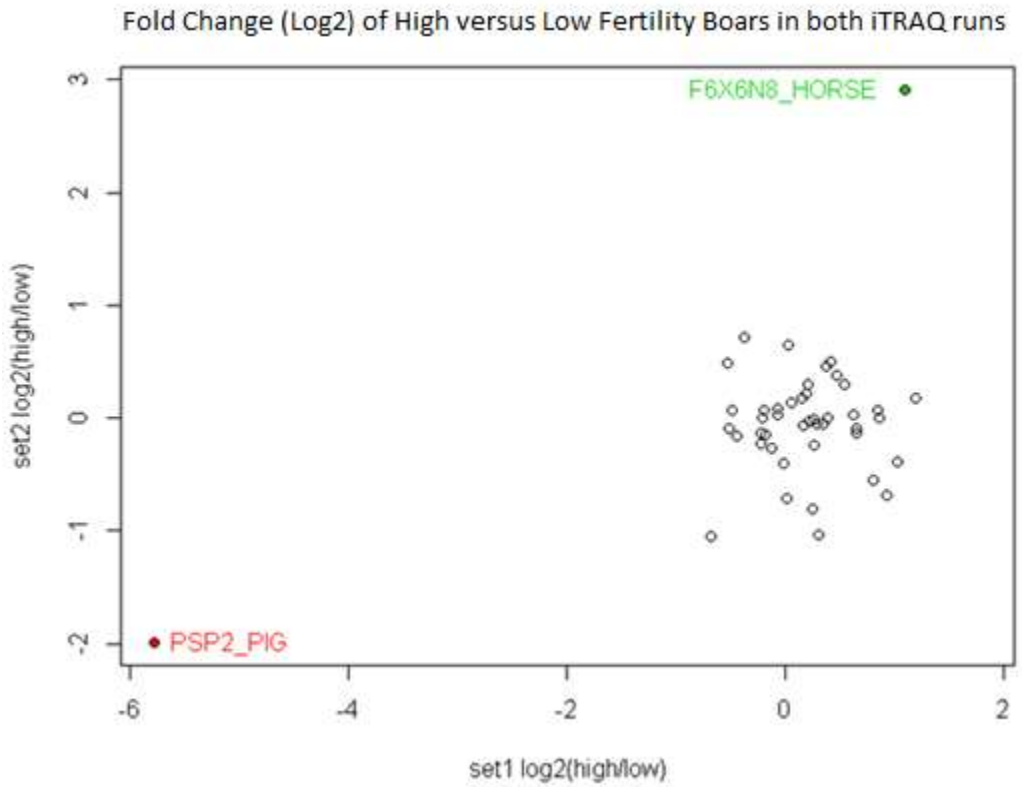

Figure 4.4. Protein expression differences between high and low fertility boars analyzed in iTRAQ. Fold change differences (Log2 area) were calculated for proteins expressed in both iTRAQ runs. Expression data was normalized using Cyclic Loess

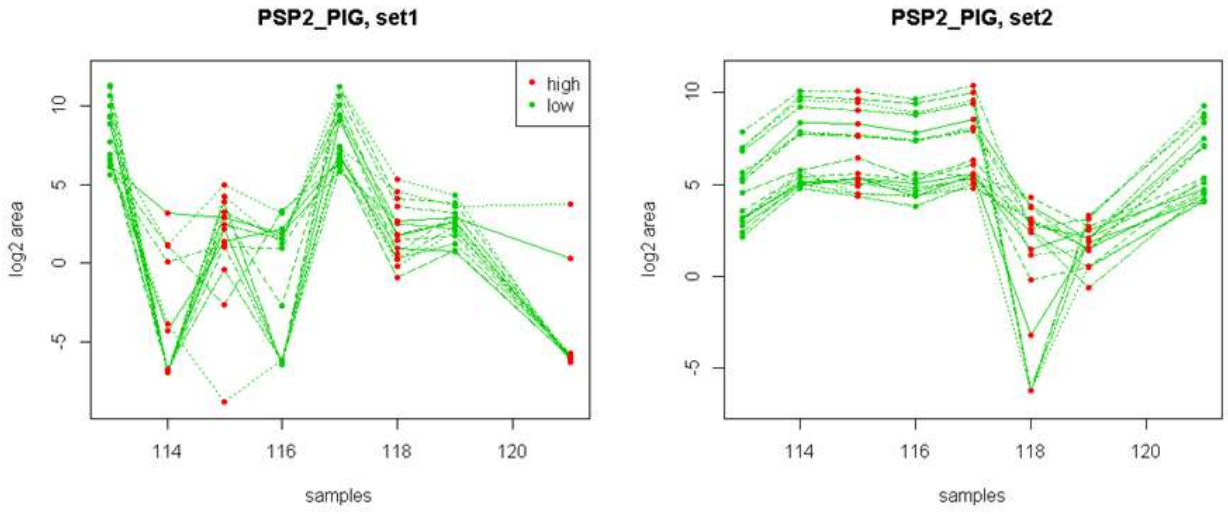

Figure 4.5a and b. Protein abundance (Log2 area) for PSP-II in both iTRAQ runs. Low fertility boars identified with green circles, high fertility boars identified with red circles.

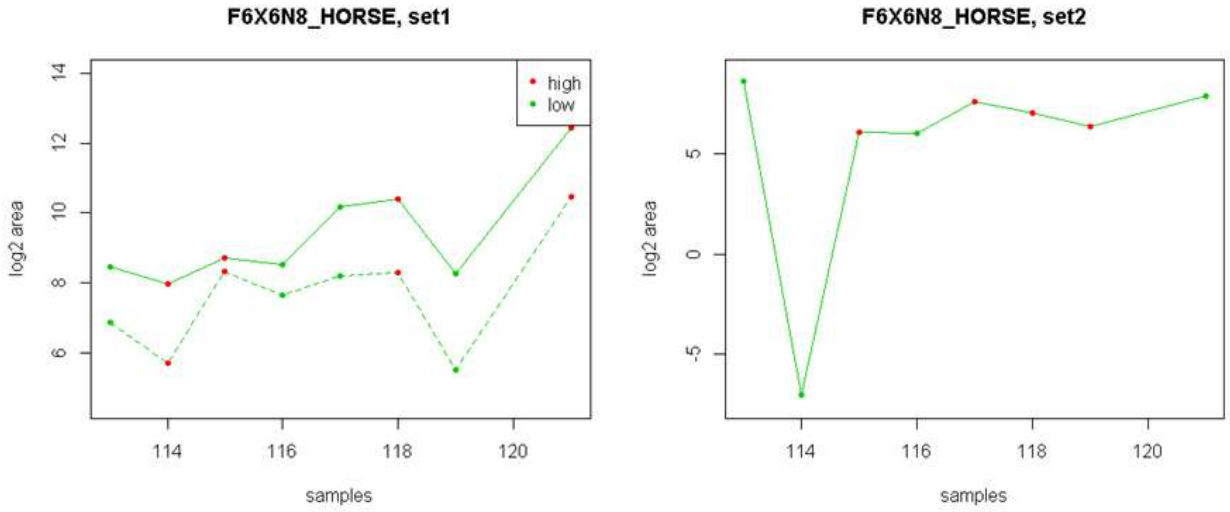

Figure 4.6a and b. Protein abundance (Log2 area) profiles for the uncharactarized protein F6X6N8. Exeptionally low peptide area in chanel 114 of set 2 calls into question the true differences in this protein.

Table 4.5. iTRAQ results for each set considered separately. Spectra areas were normalized using cyclic loess and p-values calculated following normalization. Only proteins achieving a p-value of =<0.10 in at least one of the two iTRAQ sets are shown. Proteins not detected in a given run are denoted with a dash.

Protein ID	Set 1			Set 2		
	Log2 Fold-Change	P-value	Number of Spectra	Log2 Fold-Change	P-value	Number of Spectra
K7EHG8_ORNAN	1.50	**0.04**	1	-	-	-
GPX5_PIG	0.80	0.25	6	-0.54	**0.05**	3
CO3_PIG	-	-	-	-0.48	**0.08**	1
PSP2_PIG	-5.78	**0.10**	15	-1.99	0.31	18
PB1_PIG	0.61	**0.07**	16	0.03	0.86	17
B2MG_PIG	0.37	0.46	1	0.45	**0.003**	1
C4TP28_PIG	0.64	**0.07**	11	-0.13	0.56	14
PSP1_PIG	-0.21	**0.08**	238	0.00	0.98	264
F1RNP2_PIG	0.02	0.94	3	0.65	**0.06**	6
F1RPA7_PIG	0.25	0.68	2	-0.81	**0.03**	1
F1SEY1_PIG	0.41	0.20	1	0.50	**0.03**	1
G5BMM6_HETGA	-0.38	0.41	3	0.72	**0.05**	1
H2NYU0_PONAB	1.79	**0.03**	1	-	-	-
I3L948_PIG	1.02	**0.09**	2	-0.39	0.53	2
I7HJH6_PIG	1.19	**0.04**	24	0.18	0.53	11
Q1HDU1_PIG	0.66	**0.05**	1	-	-	-

Table 4.6. Combined analysis of normalized iTRAQ runs. Results only shown for proteins appearing in both sets and with a combined analysis p-value =< 0.10. Proteins analyzed further with MRM are denoted in bold.

Uniprot ID	Protein	Log2 Fold-change	p-value
tr|F1SEY1	**Lysosomal alpha-mannosidase**	**0.46**	**0.01**
tr|Q4R0H3	**AQN-1**	**-0.50**	**0.04**
sp|P35496|	**PSP-II**	**-3.90**	**0.04**
tr|I7HJH6|	**AQN-3**	**0.64**	**0.05**
sp|Q07717|	Beta-2 Microglobin	0.42	0.09
sp|A1E295|	Cathepsin B	-0.19	0.09
tr|F1RNP2|	Uncharacterized	0.40	0.10

Table 4.7. Non-normalized iTRAQ results for each run analyzed separately. Results for proteins subsequently evaluated with MRM are denoted in bold.

Protein ID	Set 1			Set 2		
	Log2 Fold-change	P-value	Number of Spectra	Log2 Fold-change	P-value	Number of Spectra
K7EHG8_ORNAN	1.16	0.063	1	NA	NA	NA
GPX5_PIG	**0.68**	**0.39**	**6**	**-0.60**	**0.0097**	**3**
B2MG_PIG	0.24	0.61	1	0.40	0.099	1
ICA_PIG	-0.35	0.098	5	0.02	0.97	7
B8Y9T0_BOVIN	-0.63	0.084	2	0.005	0.99	1
PSP1_PIG	**-0.58**	**0.0057**	**238**	**-0.02**	**0.96**	**264**
F1RPA7_PIG	0.13	0.85	2	-0.88	0.0086	1
F1SEY1_PIG	**0.24**	**0.37**	**1**	**0.46**	**0.048**	**1**
F1SS24_PIG	-0.42	0.078	23	-0.18	0.62	29
H2NYU0_PONAB	1.59	0.045	1	NA	NA	NA
I7HJH6_PIG	0.95	0.094	24	0.13	0.75	11
Q4R0H3_PIG	**-1.07**	**0.0062**	**29**	**0.14**	**0.76**	**31**

Table 4.8. Combined analysis of non-normalized results for both iTRAQ runs. Only proteins detected in both sets were included

Uniprot ID	Protein Name	log2FC	p-value
tr|Q4R0H3|	**AQN-1**	**-0.66**	**0.029**
tr|F1SEY1|	**Lysosomal alpha-mannosidase**	**0.35**	**0.037**
RRRRRtr|L5KMA7|	Plectin-1	-0.43	0.067
sp|A1E295|	Cathepsin B	-0.32	0.070
sp|Q29549|	Clusterin	-0.53	0.090

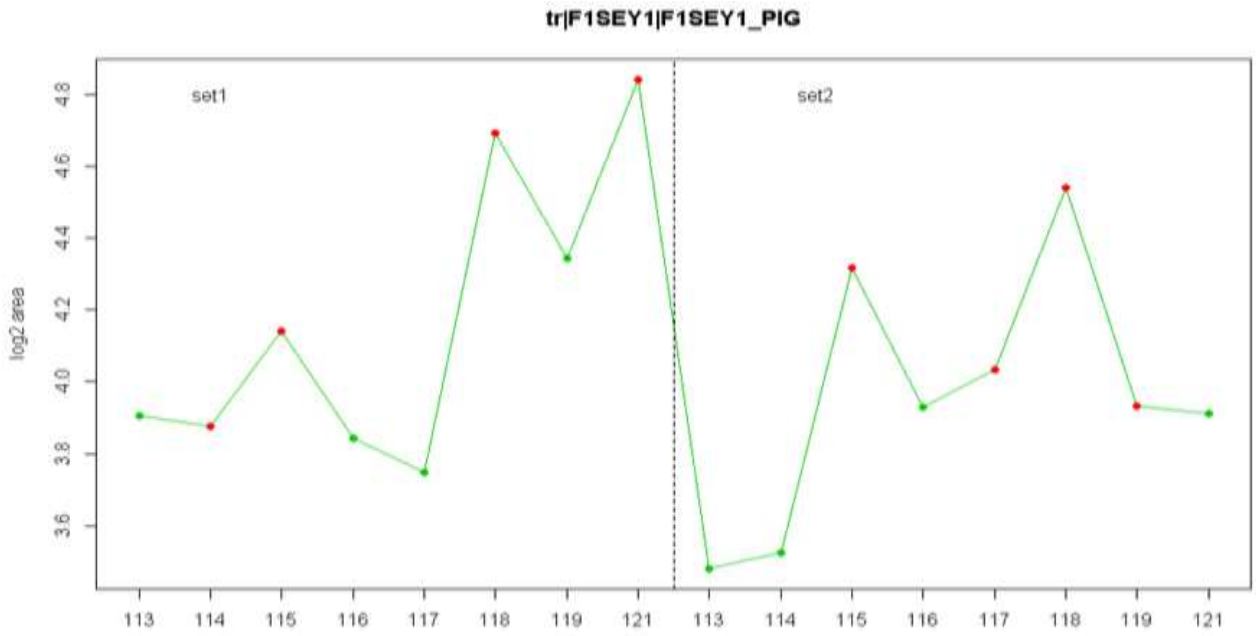

Figure 4.7. Lysosomal alpha-mannosidase (F1SEY1) protein profile of all boars evaluated in the iTRAQ discovery. High fertility boars are represented by red circles and low by green circles. Peptide areas were normalized using cyclic loess.

Targeted Proteomics (MRM):

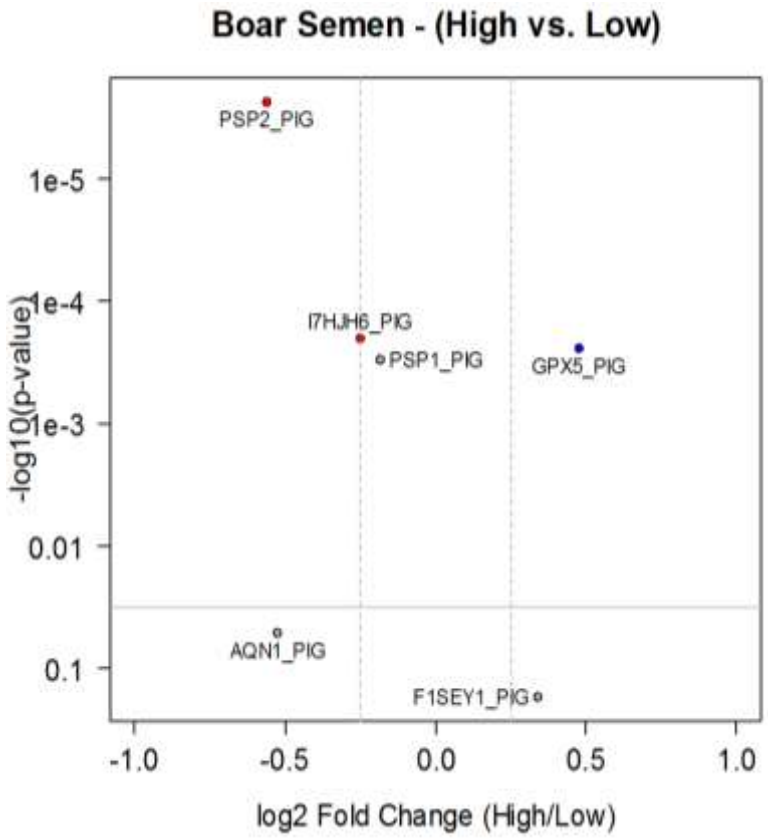

Figure 4.8. Volcano plot of protein fold change and p-value for proteins analyzed using MRM.

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Table 4.9. Results of MRM analysis for proteins compared between high and low fertility boars. Proteins were evaluated using heavy peptides to quantify absolute values for abundance and differences were compared using a linear model. P-values were adjusted in all cases.

Protein	log2 fold-change	SE	T-value	DF	p-value	Adjusted p-value
AQN1	-0.523	0.27	-1.96	753	0.051	0.061
F1SEY1	0.34	0.25	1.37	352	0.17	0.17
GPX5	0.48	0.13	3.69	789	2.40E-05	4.45E-04
AQN3 (I7HJH6)	-0.25	0.07	-3.75	406	2.00E-04	4.45E-04
PSP-I	-0.18	0.05	-3.64	514	2.97E-04	4.45E-04
PSP-II	-0.56	0.12	-4.77	620	2.32E-06	1.39E-05

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Chapter 5: General Discussion

5.1 Introduction

Early identification of sub-fertile males can improve reproductive efficiency and genetic progress of pork production systems; therefore, the goal of these studies was to identify a population of commercial boars of known fertility phenotype and examine possible biological markers of fertility performance. To identify proteins and SNPs with potential to be used as biological markers, proteomic and genomic profiles were examined with the goal of identifying candidate proteins and SNPs.

These experiments identified several promising proteins associated with differences in boar fertility that may help identify sub-fertile sires at a young age. These markers appear to be indicative of differences in the underlying physiology during spermatogenesis, transit through the female reproductive tract, or embryonic development. If sperm are not functionally competent to allow all these events, poor fertility of the boar may generate one of the following outcomes for the sow: a return to estrus, negative pregnancy check, abortion, or a small litter size. However, it is difficult to identify specific traits that underlie these outcomes due to the multitude of mechanisms that can result in failure of fertilization or embryonic development. Regardless of the contributing male mechanism, the outcome is the same. In addition, numerous potential causes of reproductive failure, including those imposed by female or environmental factors, add to the challenge of studying fertility in a large-scale production setting. Furthermore, the predominant focus on the female determinants of fertility has led to a more limited consideration of the role that sires play in fertility outcomes, even though the potential contribution of a superior sire in advancing genetic progress far outweighs the role of a single sow [1]. The lost opportunity for breeding companies and commercial producers to fully realize genetic progress when sub-fertile

males are used has not been quantified, but is likely to be considerable. At the very least, this issue warrants an examination of how the rate of genetic gain might be improved through better quantitation and selection on male fertility traits. When the efficiency of boar utilization is considered, there is a bias toward the consideration of the number of AI doses obtained per ejaculate as being the single most important factor [2]. However, if inseminations using these ejaculates do not result in high pregnancy rates, then the opportunity to improve breeding efficiency is lost. Further to this, utilization of ejaculate parameters requires data to be collected throughout the boar's entire productive lifespan in order to generate a high heritability phenotype with accuracy [3]. For the purpose of efficiently removing sub-fertile individuals from the breeding herd, an early-life predictor of fertility is most desirable. Measurements collected on sexually mature boars following a test period are of limited use because of the time required to obtain these measurements. By the time the data from test matings is collected, the stud has already incurred costs for rearing, housing and maintaining a potentially sub-fertile boar.

Genetic mechanisms underlying male fertility are not yet completely understood [4]. Traditional breeding has made modest progress utilizing ejaculate parameters and reproductive hormone concentrations due to their moderate to high heritability and their relative ease of measurement [3, 5]. The relatively high heritability of these traits compared to other male fertility traits is likely due to simpler mechanisms of genetic control, and the more direct relationship between endocrine signals and spermatogenesis. The ease of accurately measuring phenotypes for these traits also increases precision of calculating heritability. In comparison, identifying factors affecting *in vivo* fertility outcomes is more difficult, and removing unreliable phenotypes from the dataset is critical for understanding the contribution of an individual boar to each measured outcome in the sow. As expected, heritability of a boar's fertilization capacity (as measured through conception rate) is

much greater than the heritability of farrowing rate, due to numerous factors that can cause loss of pregnancy following the first 21 or 30 days of gestation [3]. The low genetic correlation between these traits and production (growth and carcass) traits suggests that inclusion of male fertility traits in boar selection would not be detrimental to traits conferring value to pork producers [2]. Additionally, without including fertility in selection indices, there is the risk of gradual deterioration of male fertility in the breeding herd or, at the very least, failure to capitalize on the performance of high-value, high-fertility sires [2, 3].

5.2 The Female Factor

Field fertility experiments within this study were designed to minimize sow effects on boar fertility phenotypes as much as possible. A review of the existing literature reveals very few experiments designed specifically to elucidate the impact of AI sires on reproductive efficiency in commercial pork production. During the test mating period presented here, the effect of individual females was controlled by balancing the parity of sows bred by each boar, and eliminating sows that exhibited signs of any conditions affecting reproduction. Through this approach, it became possible to detect differences between the highest and lowest fertility boars in the production system. Future work might also separately consider gilt and sow reproductive records and utilize sow productivity indices calculated to include re-breeding variables. The low genetic correlation between the reproductive performance of gilts and multiparous sows, as well as individual sow variability would be easier to account for if a productivity index of the sow was calculated and included in the analysis used to calculate boar phenotypes [6]. The use of farrowing interval in some reproductive studies is also limiting, as it is the outcome of many factors, including optimal body condition at the end of lactation, prompt return to estrus, accurate estrus detection, as well as high conception rates [7]. Separating sire and dam contributions to a reproductive outcome is extremely

difficult in a commercial farm setting, and thoughtfully designed field fertility trials are key to understanding the impact and factors contributing to sire sub-fertility. Further understanding of sire roles in reproductive outcomes could help pork producers make sound breeding decisions for their herd.

5.3 The Sire Role in Fertilization and Embryonic Development

There are many ways that a sire can contribute to poor reproductive efficiency in a herd. When paternity is measured following heterospermic AI, there are often large differences between boars, with some boars being consistent top contributors, while others sire fewer piglets [8]. Studies examining this effect, as early as days 5-6 of embryo development, show differences between boars in both number of embryos fertilized and number of accessory sperm [9]. As reviewed by Ferriera at al [8], there are also relative effects where the ability of some intermediate performers to sire more or less offspring depends on which other sires are included in the pooled AI dose. Lastly, some sires produce very few piglets in the litter, regardless of the other sires included in the pooled AI dose. These results are observed even when the farrowing rates of the boars are comparable, suggesting underlying differences in embryonic development potential among sires [8]. As DNA quality is key to embryonic development, these results can be influenced by events occurring in gametogenesis that lead to service sire effects on litter size as well as pregnancy rate [4]. While some factors affecting sperm DNA quality such as nutrition, health status, and heat stress are well described and can be managed, the intrinsic DNA quality of a boar's sperm may be worth examining in more detail. Whole genome sequencing and identification of haplotypes with a homozygous deficit has been used in dairy bulls to identify sire haplotypes associated with prenatal death. It may be useful to explore the genomes of other livestock species for embryonic-lethal haplotypes [10]. To date, studies examining the molecular mechanisms underlying sperm

cell function and fertility in boars have identified many discrete contributing mechanisms, but there is still much work to be done in linking these to create a more all-encompassing picture of the suite of factors that may influence a boar's fertility performance.

5.4 Proteomic Markers

Proteins identified as significantly different between fertility groups in the current study are all highly abundant in the seminal plasma. Since they comprise such a large proportion of the total protein in the seminal plasma, further elucidation of how changes in spermadhesin secretion contributes to detectable differences in total protein could prove useful in the future. Total protein is easy to measure using a BCA assay, and therefore promising for development of easy-to-use screening tests for field used. Examination of genetic interrelatedness of sperm cell count, semen volume, and sperm concentration across ages, show a considerable of correlation between these traits [5, 11]. The stability of these more heritable traits suggest that if more was understood about proteins in the seminal plasma and their optimal ratio, that measurement of these factors could help identify sub-fertile individuals upon their entry into the boar stud without the need for a test period of single-sire matings. Genetic relationships between semen volume, total sperm, and sperm concentration across boar age classes suggests that stability of these traits could facilitate development of early life seminal plasma proteome screening [5].

The detection of significant differences in spermadhesin proteins between boars of differing fertility supports the importance of previous studies on the spermadhesin family of proteins. The AWN protein is detectable in close association with sperm, even after capacitation, and is capable of binding both beta-galactosides and the zona pellucida; it is thought to be the primary binding protein during fertilization [12, 13]. Cells lining the isthmus and utero-tubal junction of the female

reproductive tract are thought to absorb AWN present in the seminal plasma; a major event leading to capacitation [12]. Higher of expression of AWN is displayed in boars representing the extremes of low relative fertility [14]. Both AWN and AQN proteins have similar binding affinities for glycoproteins, and the ability of AWN and AQN-3 to tightly bind to the sperm surface lend credence to their roles as receptors for the zona pellucida [15]. The more loosely associated fraction of AQN and AWN proteins are thought to function as de-capacitation factors [16]. Recently published studies have identified five proteins in sperm which are correlated with litter size and has identified more AWN and AQN-3 bound to sperm of boars which sire smaller litters [17]. Estimates of total AWN and OPN per AI dose have detected a negative correlation between the abundance of these proteins and total born. While of limited use on its own, *in vitro* zona pellucida binding ability and semen quality assessment results are correlated with litter size, which supports the possibility that differences in the proteome may underlie litter size differences [18]. The protein binding function of PSP-I suggests multiple possible functions [19]. Porcine seminal plasma protein II is capable of forming a heterodimer with specific isoforms of PSP-I and this heterodimer is very loosely associated with sperm, is non-heparin binding, and is not thought to have a major role in oocyte recognition and binding [20]. Porcine seminal plasma protein I (PSP-I) has a strong negative correlation with total number of piglets born [14]. When examined using cytochemical staining, heparin-binding protein distribution of capacitated and non-capacitated sperm differ significantly, likely as a result of protein redistribution occurring during the capacitation process. In addition, much of the heparin-binding protein associated with sperm is quite loosely bound to their plasma membrane [21]. Consideration of heparin-binding protein distribution has been suggested as providing an easy way to identify the proportion of sperm in each physiological state present in a given ejaculate [21]. Of the spermadhesins identified in our MRM experiments as

being significantly different between fertility groups, AQN-1 and AQN-3 are both capable of binding heparin. Their ability to bind heparin and the significant difference in AQN-3 between high and low fertility boars supports the importance of heparin-binding proteins and their role in successful fertilization. The complex nature of these proteins, in terms of their distribution and degree of binding to the plasma membrane, also illustrates the importance of further investigation into the different roles they may play in supporting sperm cell physiology as a function of their location and degree of association with the sperm cell plasma membrane.

Differences detected in GPX5 between high and low fertility boars also supports previous findings. Reactive oxygen species (ROS) are key to sperm maturation as well as for the signalling pathways required for sperm capacitation. However, when present at high levels, ROS can cause oxidative damage to sperm, thereby affecting DNA quality and sperm cell function [22]. Glutathione Peroxidase 5 differs from other glutathione peroxidases as it is selenium independent and is secreted only by the epididymis [23]. In previous studies, GPX5 tended to be positively correlated with pregnancy rate [14]. When GPX5 secretion is low, resilience under oxidative stress may be compromised due to sub-optimal levels of this important antioxidant protein.

Previous work on boars representing extremes in fertility has also detected significant differences in osteopontin (OPN) between seminal plasma of high- and low-fertility individuals, with a negative correlation of OPN with total number of piglets born [14]. In bulls, OPN is positively correlated with fertility, although this seemingly opposite effect of OPN on fertility in these two species is not yet well understood [24]. In our experiments, OPN was not detected in seminal plasma. The lack of detectable levels of OPN in our results compared to previous studies could be due to the different proteomic methods used, as previous studies have utilized western blotting and 2-D gel electrophoresis. The collection of whole ejaculates and isolation of the seminal plasma

differs from the isolation of individual fractions of the ejaculate performed by Novak et al. (2010). Additionally, the different methods used for isolating proteins from other seminal plasma components could have decreased our ability to detect OPN [14]. Use of ELISA on raw seminal plasma from the total ejaculate also did not reveal detectable differences between OPN in boars of differing fertility. It is possible that when the whole ejaculate is considered, OPN levels are diluted by more abundant proteins, and effects of this protein becomes masked.

The MRM phase of this project was performed with pooled seminal plasma from three ejaculates per boar. Although this approach controlled for variation in individual ejaculates, it eliminated our ability to measure repeatability of spermadhesin expression in individual boars across time-points. Information from individual ejaculates may improve our understanding of how the ratio of protein to sperm may be related to the secretion of specific proteins. Understanding which specific proteins cause detectable changes in overall seminal plasma protein may help in the development of on-farm testing protocols for young boars. The ability to consider environmental factors at each time point more precisely would also further expand our knowledge of how seminal plasma protein affects fertility outcomes [2]. While experimental design considerations were used in an attempt to mitigate any sow parity effects on boar fertility phenotype, inclusion of gilt reproductive records could decrease accuracy of the phenotype, due to large differences between gilts and sows in terms of reproduction.

5.5 Genomic Markers

The genome-wide association study identified many SNPs that were significantly associated with the fertility traits examined. Due to the low heritability of the sire effect on litter size and the number of non-viable fetuses, progress on the paternal influence of this trait may be relatively

slow, even with genomic selection tools [3]. Focusing on optimizing sire contribution to fertility through high pregnancy rates may be the best use of genomic selection for improving genetic progress. The GQLS approach to performing GWAS is powerful in its ability to analyze data from large inbred pedigrees or from multiple pedigree. However, in this study, a lack of pedigree data in combination with small sample size and low heritability phenotypes, may have falsely inflated the number of SNPs identified as being significantly associated with the traits examined [25]. Additional work to determine the allele frequencies in the population, as well as the use of boar pedigree information, could help in verifying SNPs that are truly associated with the phenotypes evaluated.

The lower genetic diversity of pigs compared to cattle or sheep allows for a smaller population size and lower number of informative markers being needed in whole-genome studies [26]. These factors suggest promise for additional exploration of genomic markers of sire relative fertility. Through additional studies using genome-wide association, a list of markers for these traits could be further validated for incorporation into boar selection decisions.

Existing literature available concerning the existence of SNPs associated with boar fertility is minimal. Previous work has found candidate markers for sperm quality traits in boars, including CD9 (Cluster of differentiation antigen 9) on chr 5, DAZL (deleted in azoospermia-like) on chr 13, and the heat shock protein 70.2 gene that is currently unplaced on the Sscrofa 10.2 genome build [27-29]. Even more literature is available on female fertility, in particular candidate genes influencing the maternal contribution to litter size [30]. In dairy cattle, 8 SNP markers associated with sire conception rate (SCR), some with locations close to genes with known functions in reproductive traits, have been identified. Additionally, high-density panels have also been used to identify regions associated with non-compensatory fertility traits in Holstein bulls [31, 32].

Additional genotyping of boars with fertility phenotypes could increase the ability to identify true variants, particularly for low heritability traits.

5.6 Conclusions and Future Directions

Proteomic markers discovered in this study support previous work comparing seminal plasma proteins from boars of differing fertility [14]. Detectable differences in these proteins, even when the whole ejaculate was evaluated, make them excellent candidates for use in screening of boars before their entry in the stud. The use of protein and peptide signatures as biomarkers is an exciting emerging field of study. However, even in human medicine, few markers have successfully reached the confidence threshold to be considered useful clinical biomarkers. This is where MS technology holds promise for improving the detection and validation of biomarkers compared to techniques that only generate relative expression values [33]. Further studies in which sperm are exposed to specific exogenous concentrations of the various proteins could help to determine minimum or maximum thresholds, outside of which fertility is compromised. Additional research could also be conducted to examine the relative contribution of these significant proteins to total protein levels in the seminal plasma, and thereby determine the maximum or minimum protein to sperm cell ratio at which optimal fertility is still attained. Screening for sub-fertility could be conducted on pubescent boars prior to their use for AI using this information. However, this would still require boars to be raised until an age that ejaculates could be evaluated for markers of sub-fertility rather than allowing for detection in earlier life.

The multitude of SNPs associated with each of the major fertility traits analyzed makes it difficult to identify markers for further exploration. Evaluation of additional boars could allow for better detection of markers associated with the low heritability traits, piglets born alive, and non-viable

fetuses. However, the smaller number of markers associated with pregnancy rate might make it a better candidate for further exploration. The use of marker-assisted selection for improving breeding herd efficiency is promising, but identification of causal variants generating these fertility differences might be even more useful. This could be more challenging in the pig than in species such as cattle due to the known larger linkage disequilibrium (LD) in pigs than other livestock species [26]. This increased LD necessitates much more extensive interrogation of candidate genes to identify true variants. However, increasing availability and affordability of sequencing technologies could facilitate whole-genome sequencing and more in-depth examination of variations that could underlie relative fertility in boars. Once markers are identified, a selection index for sire profitability could be generated that includes fertility. This could help to simultaneously advance genetic progress on meat and carcass traits while improving breeding herd efficiency and profitability for pork producers. Marker testing in an individual's early life using genomics could then allow for efficient identification and removal of sub-fertile sires before the cost of raising them is incurred.

Breeding herd efficiency is key to improving the economic and environmental sustainability of pork production systems. Decreasing non-productive days resulting from failed breeding is an opportunity to improve profitability of commercial farrowing operations. At a stud level, removal of sub-fertile individuals will help facilitate genetic progress by decreasing the number of sows failing to become pregnant and decreasing the number of boars required in the stud. The use of boars that maintain their reproductive performance, even at lower sperm doses, will allow boars of sub-optimal fertility or lower genetic value can be removed from the herd. Through the use of these strategies, improvements in herd reproductive efficiency will complement genetic strategies

to improve highly valuable carcass and meat quality traits, allowing selection of animals with the most optimal composite of traits to be used by pork producers.

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Appendix 1: Proteins Identified during Pilot LFQ

Accession	Peptide count	Anova p-value	Max fold change	Description
gi|41688298	37	9.14E-06	2.071703	lactotransferrin [Sus scrofa]
gi|262072808	22	4.33E-05	4.140309	hexosaminidase B (beta polypeptide) [Sus scrofa]
gi|47523636	17	0.000662	1.650858	complement factor H precursor [Sus scrofa]
gi|62078603	1	0.004018	1.51362	actin-related protein T2 [Rattus norvegicus]
gi|350585024	2	1.29E-05	15.98961	PREDICTED: carboxylesterase 5A [Sus scrofa]
gi|1127023	1	4.67E-06	72.7155	pancreatic GP2 [Homo sapiens]
gi|26351687	1	0.064226	2.80142	unnamed protein product [Mus musculus]
gi|164318	27	1.63E-08	4.060335	albumin, partial [Sus scrofa]
gi|390473699	1	0.018504	1.578004	PREDICTED: LOW QUALITY PROTEIN: transforming acidic coiled-coil-containing protein 1 [Callithrix jacchus]
gi|345319902	1	0.207095	1.231279	PREDICTED: peptidyl-prolyl cis-trans isomerase-like 2-like, partial [Ornithorhynchus anatinus]
gi|395519723	1	0.000175	7.317595	PREDICTED: low-density lipoprotein receptor-related protein 2 [Sarcophilus harrisii]
gi|344291343	1	0.055385	1.35728	PREDICTED: sodium-independent sulfate anion transporter-like [Loxodonta africana]
gi|311246884	8	2.91E-09	13.29069	PREDICTED: epididymal-specific lipocalin-5 [Sus scrofa]
gi|149050099	1	0.066577	1.379958	rCG37045 [Rattus norvegicus]
gi|194226056	1	3.70E-08	46.33576	PREDICTED: lipocalin-like 1 protein-like [Equus caballus]
gi|12855135	2	0.006393	1.155431	unnamed protein product [Mus musculus]
gi|47523752	1	0.467451	1.340654	solute carrier family 15 member 1 [Sus scrofa]
gi|73973155	1	0.014246	44.93237	PREDICTED: zinc finger protein 451 isoform 2 [Canis lupus familiaris]
gi|296475625	1	0.013186	13.83758	Gag-like [Bos taurus]
gi|62901559	1	0.000153	3.110844	RecName: Full=Hemoglobin subunit beta; AltName: Full=Beta-globin; AltName: Full=Hemoglobin beta chain
gi|17380350	6	1.26E-05	215.7552	RecName: Full=Epididymis-specific alpha-mannosidase; AltName: Full=Mannosidase alpha class 2B member 2; Flags: Precursor
gi|47523184	5	0.032463	1.554162	seminal plasma protein pB1 precursor [Sus scrofa]
gi|162676	6	3.40E-09	35.30316	alkaline phosphatase precursor (EC 3.1.3.2) [Bos taurus]
gi|335284102	5	5.90E-08	10.92574	PREDICTED: zinc-alpha-2-glycoprotein-like [Sus scrofa]
gi|228509	5	0.005247	1.709762	sperm-associated acrosin inhibitor
gi|172072653	6	5.40E-07	22.28165	lactadherin precursor [Sus scrofa]
gi|47523090	4	3.68E-07	19.64627	epididymal secretory glutathione peroxidase precursor [Sus scrofa]

gi|114082	5	0.112228	1.111695	RecName: Full=Carbohydrate-binding protein AQN-1; AltName: Full=Spermadhesin AQN-1; AltName: Full=Zona pellucida-binding protein AQN-1
gi|47523496	5	5.09E-06	7.861068	epididymal secretory protein E1 precursor [Sus scrofa]
gi|2724046	6	8.04E-06	9.503108	beta-actin [Mustela putorius furo]
gi|147906534	5	0.004721	1.550915	cathepsin B precursor [Sus scrofa]
gi|149056486	4	0.000286	2.342029	rCG54015 [Rattus norvegicus]
gi|335307492	4	1.92E-07	21.93682	PREDICTED: ribonuclease-like protein 9-like [Sus scrofa]
gi|194036808	4	0.000401	4.451961	PREDICTED: protein CREG1-like isoform 2 [Sus scrofa]
gi|335306030	5	5.80E-08	56.69846	PREDICTED: A-kinase anchor protein 4-like [Sus scrofa]
gi|350589072	8	6.02E-07	7.137645	PREDICTED: sulfhydryl oxidase 1-like isoform 1 [Sus scrofa]
gi|335304955	4	1.31E-07	100.8537	PREDICTED: kunitz-type protease inhibitor 4-like [Sus scrofa]
gi|301771256	3	0.026016	2.009646	PREDICTED: LOW QUALITY PROTEIN: voltage-dependent P/Q-type calcium channel subunit alpha-1A-like [Ailuropoda melanoleuca]
gi|62738431	3	2.77E-07	4.052052	Chain A, Solution Structure Of Porcine Beta-Microseminoprotein
gi|255068706	3	6.16E-06	11.56011	WAP four-disulfide core domain 10A-like [Sus scrofa]
gi|3318759	8	0.102104	1.203803	Chain B, The Crystal Structures Of Two Members Of The Spermadhesin Family Reveal The Folding Of The Cub Domain
gi|773265	3	0.064255	1.471593	adipsin/complement factor D [Sus scrofa]
gi|242253862	7	3.50E-10	29.03372	phosphatidylethanolamine-binding protein 4 precursor [Sus scrofa]
gi|47523638	3	0.00821	1.464198	nexin-1 precursor [Sus scrofa]
gi|335284522	4	6.30E-07	32.67042	PREDICTED: pancreatic secretory granule membrane major glycoprotein GP2 [Sus scrofa]
gi|388890649	7	0.000473	14.07898	ceruloplasmin [Sus scrofa]
gi|350587143	3	0.019728	9.915407	PREDICTED: legumain [Sus scrofa]
gi|395839121	3	2.95E-05	181.9531	PREDICTED: ezrin [Otolemur garnettii]
gi|399038	4	4.67E-06	3.368355	RecName: Full=Angiogenin; AltName: Full=Ribonuclease 5; Short=RNase 5
gi|350591295	4	4.36E-09	7.613333	PREDICTED: protein FAM3D-like isoform 2 [Sus scrofa]
gi|190360575	9	2.05E-08	28.47862	cysteine-rich secretory protein 1 precursor [Sus scrofa]
gi|395836132	1	9.14E-10	Infinity	PREDICTED: F-box only protein 28 [Otolemur garnettii]
gi|47522880	4	1.35E-07	55.6212	gamma-glutamyltranspeptidase 1 [Sus scrofa]
gi|390458914	2	0.460564	1.464785	PREDICTED: ataxin-10 isoform 1 [Callithrix jacchus]
gi|311256979	2	1.06E-06	38.77562	PREDICTED: metalloproteinase inhibitor 1-like [Sus scrofa]

gi|301789059	2	2.01E-08	56.08997	PREDICTED: hypothetical protein LOC100476602 [Ailuropoda melanoleuca]
gi|17933431	2	1.98E-06	4.46303	beta-2-microglobulin protein [Sus scrofa]
gi|297289622	2	1.72E-06	5.883532	PREDICTED: LOW QUALITY PROTEIN: SCO-spondin-like [Macaca mulatta]
gi|348576561	2	0.093865	1.420601	PREDICTED: putative Polycomb group protein ASXL3-like [Cavia porcellus]
gi|7549809	8	4.65E-08	43.69422	plastin-3 isoform 1 [Homo sapiens]
gi|6690797	2	0.000158	4.551196	metalloproteinase tissue inhibitor 1 [Sus scrofa]
gi|145279215	9	1.15E-06	21.15261	angiotensin-converting enzyme isoform 2 precursor [Sus scrofa]
gi|248304	8	0.723548	1.078083	AWN-1=spermadhesin [swine, Peptide, 133 aa]
gi|47523578	4	0.415202	1.526898	outer dense fiber protein 1 [Sus scrofa]
gi|311248892	4	8.91E-05	4.144316	PREDICTED: lysosomal alpha-mannosidase-like isoform 1 [Sus scrofa]
gi|164503	2	3.39E-08	8.457752	immunoglobulin gamma-chain [Sus scrofa]
gi|223036	3	5.21E-06	16.08109	troponin C-like protein
gi|348575954	2	1.44E-06	7.399298	PREDICTED: hypothetical protein LOC100735114 [Cavia porcellus]
gi|281348732	2	0.779176	1.084456	hypothetical protein PANDA_010523 [Ailuropoda melanoleuca]
gi|347300207	2	0.81546	1.097283	nucleobindin-1 precursor [Sus scrofa]
gi|390458429	2	0.002687	24.12318	PREDICTED: LOW QUALITY PROTEIN: outer dense fiber protein 2 [Callithrix jacchus]
gi|290543356	2	0.081846	1.550422	acrosin-binding protein precursor [Cavia porcellus]
gi|178056175	2	7.05E-07	21.15697	basigin precursor [Sus scrofa]
gi|311263676	2	0.019655	1.623008	PREDICTED: dipeptidyl peptidase 1-like [Sus scrofa]
gi|154707874	3	0.943342	1.028438	protein FAM3C precursor [Bos taurus]
gi|311269573	2	0.000123	33.19214	PREDICTED: neprilysin isoform 1 [Sus scrofa]
gi|311270662	2	1.50E-06	63.58727	PREDICTED: phosphatidylethanolamine-binding protein 1-like [Sus scrofa]
gi|146741296	3	0.000111	3.678213	keratin 1 [Sus scrofa]
gi|3318722	10	0.047299	1.234442	Chain E, Leech-Derived Tryptase InhibitorTRYPSIN COMPLEX
gi|345791797	1	0.008611	1.340273	PREDICTED: eukaryotic translation initiation factor 4B isoform 12 [Canis lupus familiaris]
gi|300794828	1	0.286632	1.140315	zinc finger protein 638 [Bos taurus]
gi|74206976	1	0.075278	1.327002	unnamed protein product [Mus musculus]
gi|5771541	3	3.63E-05	107.5461	matrilysin [Sus scrofa]
gi|194206490	1	0.070702	1.209682	PREDICTED: neogenin [Equus caballus]
gi|346986374	3	0.003781	7.933913	neutrophil gelatinase-associated lipocalin precursor [Sus scrofa]
gi|301757697	1	0.124054	1.134654	PREDICTED: serine/threonine-protein kinase DCLK3-like [Ailuropoda melanoleuca]
gi|116325983	1	0.000182	1.997514	voltage-dependent calcium channel gamma-like subunit [Homo sapiens]

gi|126310747	1	0.214079	1.322555	PREDICTED: plasminogen-like isoform 2 [Monodelphis domestica]
gi|72535165	9	0.006052	1.232926	seminal plasma sperm motility inhibitor precursor [Sus scrofa]
gi|301767702	1	0.002868	204.4223	PREDICTED: glutathione S-transferase Mu 5-like isoform 1 [Ailuropoda melanoleuca]
gi|395536838	1	0.158321	1.389091	PREDICTED: dynein heavy chain 9, axonemal-like, partial [Sarcophilus harrisii]
gi|118142832	2	0.107487	19.49097	Hsp90aa1 protein [Mus musculus]
gi|3283021	1	0.579927	3.454255	keratinocyte growth factor [Oryctolagus cuniculus]
gi|311251254	1	0.252246	Infinity	PREDICTED: prostasin-like [Sus scrofa]
gi|350592508	1	4.78E-06	Infinity	PREDICTED: putative phospholipase B-like 2-like isoform 1 [Sus scrofa]
gi|301758294	1	0.522422	1.27687	PREDICTED: DDB1- and CUL4-associated factor 10-like [Ailuropoda melanoleuca]
gi|358415806	1	0.685513	1.180234	PREDICTED: doublecortin domain-containing protein 5-like [Bos taurus]
gi|403293543	1	7.66E-06	6.436663	PREDICTED: cystatin-M [Saimiri boliviensis boliviensis]
gi|149059155	1	1.36E-05	27.95213	rCG44752 [Rattus norvegicus]
gi|18307776	1	0.029563	1.288238	anti-CEA monoclonal antibody Vh region [Mus musculus]
gi|395841056	1	1.77E-07	11.84598	PREDICTED: uncharacterized protein LOC100946522 [Otolemur garnettii]
gi|386195	1	0.409218	1.42292	zona pellucida-binding protein AWN-1=spermadhesin {N-terminal} [swine, seminal plasma, Peptide Partial, 15 aa]
gi|354467375	1	0.373885	1.92152	PREDICTED: prohibitin-2-like [Cricetulus griseus]
gi|297271525	1	0.004042	2.249085	PREDICTED: nucleoredoxin-like [Macaca mulatta]
gi|109484717	1	0.616812	1.135569	PREDICTED: tumor necrosis factor alpha-induced protein 8-like protein 3-like [Rattus norvegicus]
gi|16758582	1	0.000347	7.356632	C-type natriuretic peptide precursor [Rattus norvegicus]
gi|194018710	1	3.55E-06	20.9447	beta-defensin 129 precursor [Sus scrofa]
gi|291400621	1	0.593318	1.260723	PREDICTED: UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 4-like [Oryctolagus cuniculus]
gi|297673154	1	2.66E-05	2.536243	PREDICTED: zinc finger protein 518B [Pongo abelii]
gi|403306382	1	5.49E-09	73.40965	PREDICTED: protein FAM171A2 [Saimiri boliviensis boliviensis]
gi|119613985	1	0.379774	2.11655	hCG1991907 [Homo sapiens]
gi|332265734	1	0.001709	1.405966	PREDICTED: interferon kappa-like [Nomascus leucogenys]
gi|395532392	1	0.260349	1.770335	PREDICTED: potassium voltage-gated channel subfamily H member 4 [Sarcophilus harrisii]
gi|124487275	1	0.993633	1.055649	sucrase-isomaltase, intestinal [Mus musculus]
gi|119595193	1	5.71E-05	10.31819	hCG2042671 [Homo sapiens]

gi|73996384	1	0.355449	1.677501	PREDICTED: sodium channel protein type 8 subunit alpha isoform 2 [Canis lupus familiaris]
gi|311259936	1	2.16E-06	30.68269	PREDICTED: SCAN domain-containing protein 3-like [Sus scrofa]
gi|296219688	1	9.13E-05	8.569342	PREDICTED: pancreatic secretory granule membrane major glycoprotein GP2 [Callithrix jacchus]
gi|74136509	1	3.06E-05	240.2943	DNA nucleotidylexotransferase [Monodelphis domestica]
gi|55162806	1	1.85E-06	79.07546	transporter 1, ATP-binding cassette, sub-family B (MDR-TAP) [Canis lupus familiaris]
gi|58037313	2	0.000143	5.296157	coiled-coil domain-containing protein 18 [Mus musculus]
gi|47522770	9	1.38E-06	14.45054	clusterin precursor [Sus scrofa]
gi|344291361	2	0.000474	1.579967	PREDICTED: metalloproteinase inhibitor 2-like [Loxodonta africana]
gi|355716324	1	0.741973	1.415081	regulator of G-protein signaling 11 [Mustela putorius furo]
gi|251823933	2	0.004151	1.730949	cadherin-1 precursor [Sus scrofa]
gi|338721109	1	5.47E-06	3.730624	PREDICTED: myosin-9 [Equus caballus]
gi|47523020	1	5.93E-07	17.03426	zona pellucida-binding protein 1 precursor [Sus scrofa]
gi|4503155	1	4.06E-05	4.032954	cathepsin L1 isoform 1 preproprotein [Homo sapiens]
gi|47523862	11	5.93E-07	18.17891	epididymal sperm-binding protein 1 precursor [Sus scrofa]
gi|47523086	2	0.000321	19.17229	peroxiredoxin-5, mitochondrial [Sus scrofa]
gi|348573193	1	2.38E-07	31.19356	PREDICTED: LOW QUALITY PROTEIN: sterile alpha motif domain-containing protein 15-like [Cavia porcellus]
gi|9558755	1	0.202952	6.511003	dnaJ homolog subfamily B member 9 precursor [Homo sapiens]
gi|392343172	1	5.27E-12	Infinity	PREDICTED: uncharacterized protein LOC317533 [Rattus norvegicus]
gi|27807209	1	3.07E-06	25.67893	alpha-2-antiplasmin precursor [Bos taurus]
gi|90083242	1	0.689875	1.263897	unnamed protein product [Macaca fascicularis]
gi|351715118	1	0.000874	36.8196	Di-N-acetylchitobiase [Heterocephalus glaber]
gi|47523936	1	0.000606	2.743839	beta-defensin 2 precursor [Sus scrofa]
gi|148228720	1	0.010995	171.8227	carboxypeptidase E precursor [Sus scrofa]
gi|149429452	1	0.009722	10.83241	PREDICTED: 5-hydroxytryptamine receptor 3A-like, partial [Ornithorhynchus anatinus]
gi|350585298	1	0.000927	28.44492	PREDICTED: testis-expressed protein 101-like [Sus scrofa]
gi|311274664	1	1.26E-06	14.23622	PREDICTED: beta-defensin 128-like [Sus scrofa]
gi|335306955	1	0.006129	2.725016	PREDICTED: serine protease inhibitor Kazal-type 13-like [Sus scrofa]
gi|95147682	1	3.43E-07	11.52926	preprobeta-defensin 108-like [Sus scrofa]
gi|2642187	1	0.41779	1.619317	endo-alpha-D-mannosidase [Rattus norvegicus]
gi|149018570	1	0.213969	1.498447	rCG25421 [Rattus norvegicus]

gi|305855190	1	3.00E-05	14.56146	ly6/PLAUR domain-containing protein 4 precursor [Sus scrofa]
gi|347666849	1	0.1207	2.600033	apolipoprotein B, partial [Furipterus horrens]
gi|395515810	1	0.207316	3.255979	PREDICTED: polycystin-1, partial [Sarcophilus harrisii]
gi|9858825	1	1.50E-05	6.745928	quiescent cell proline dipeptidase precursor [Mus musculus]
gi|238999	1	0.001286	26.29782	glutathione S-transferase class Pi isozyme Yd1-1Yd1-1(II-Ha) {EC 2.5.1.18} [dogs, liver cytosol, Peptide Partial, 30 aa]
gi|351697801	1	0.019372	1.892025	Long-chain-fatty-acid--CoA ligase ACSBG1 [Heterocephalus glaber]
gi|37693468	1	0.095327	30.3209	prostaglandin D synthase [Sus scrofa]
gi|47522614	1	0.000987	4.183962	ribonuclease 4 precursor [Sus scrofa]
gi|113205858	1	0.001341	4.2848	cystatin-C precursor [Sus scrofa]
gi|26326057	1	4.32E-07	55.94344	unnamed protein product [Mus musculus]
gi|395751618	1	0.009964	282.0811	PREDICTED: leucine-rich repeat-containing protein 4B [Pongo abelii]
gi|121443	1	0.025811	11.80072	RecName: Full=Glutaredoxin-1; AltName: Full=Thioltransferase-1; Short=TTase-1
gi|403256748	1	0.268682	1.495082	PREDICTED: far upstream element-binding protein 3 [Saimiri boliviensis boliviensis]
gi|395822093	1	0.030084	84.48962	PREDICTED: uncharacterized protein C1orf173 homolog [Otolemur garnettii]
gi|114051854	1	0.003661	419.3558	tubulin alpha-1D chain [Bos taurus]
gi|50978862	1	3.04E-08	67.20256	glyceraldehyde-3-phosphate dehydrogenase [Canis lupus familiaris]
gi|344254346	1	0.618868	1.58304	Hormone-sensitive lipase [Cricetulus griseus]
gi|296193471	1	0.040032	1.710472	PREDICTED: docking protein 3 [Callithrix jacchus]
gi|332219445	1	0.096413	11.19942	PREDICTED: myelin protein zero-like protein 1-like isoform 1 [Nomascus leucogenys]
gi|311248676	1	0.033648	100.1853	PREDICTED: choline transporter-like protein 2-like isoform 2 [Sus scrofa]
gi|395528452	1	0.534043	1.389706	PREDICTED: uncharacterized protein ENSP00000244321 homolog [Sarcophilus harrisii]
gi|311251865	1	0.021861	39.9963	PREDICTED: brain-specific serine protease 4-like [Sus scrofa]
gi|298948	1	5.18E-06	46.53421	macrophage migration inhibitory factor, MIF {N-terminal} [cattle, brain, Peptide Partial, 39 aa]
gi|355713739	1	0.253023	4.470955	protein S [Mustela putorius furo]
gi|147903647	1	0.002965	1.452119	von Willebrand factor D and EGF domain-containing protein precursor [Mus musculus]
gi|395850040	1	0.006324	138.8855	PREDICTED: phosphoglycerate mutase 2 [Otolemur garnettii]
gi|47523176	8	0.015076	1.319124	major seminal plasma glycoprotein PSP-I precursor [Sus scrofa]
gi|73997667	1	0.013011	2.028179	PREDICTED: C-type lectin domain family 9 member A [Canis lupus familiaris]

gi|402895598	1	0.947826	1.126368	PREDICTED: 60S ribosomal protein L36-like [Papio anubis]
gi|194676704	1	0.000857	1.676997	PREDICTED: treslin [Bos taurus]
gi|109096102	1	0.030824	1.775483	PREDICTED: 60S ribosomal protein L31-like [Macaca mulatta]
gi|114555723	2	2.66E-06	8.710068	PREDICTED: zinc finger MYM-type protein 1 isoform 3 [Pan troglodytes]
gi|348561916	1	1.62E-05	13.91424	PREDICTED: brain acid soluble protein 1-like [Cavia porcellus]
gi|311273023	61	0.00023	1.295234	PREDICTED: fibronectin isoform 1 [Sus scrofa]
gi|344290717	2	0.000397	1.800336	PREDICTED: LOW QUALITY PROTEIN: epithelial splicing regulatory protein 2-like [Loxodonta africana]
gi|350582024	1	0.002621	60.04241	PREDICTED: prominin-2 [Sus scrofa]
gi|311273514	10	1.62E-08	17.40539	PREDICTED: brain acid soluble protein 1-like isoform 1 [Sus scrofa]