IDENTIFICATION OF PROTEIN AND LIPID BIOMARKERS OF INFERTILITY IN YOUNG BOARS AND PREPUBERTAL GILTS

by

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To my friends, family, and mentors who always believed in me. Without your undying support, I would not be where I am today.

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ABSTRACT

Reproductive efficiency in sows and boars affects the profitability of swine production systems. However, breeding stock selection is primarily based on progeny performance traits such as feed efficiency, growth rate, carcass characteristics, physical appearance, and structure, especially for terminal sire lines, with less emphasis on reproduction. While maternal sire lines are co-selected for reproductive traits including birth litter size, number weaned, weaning weight, and wean to estrus interval, currently, there is no single test predictive of fertility, and thus subfertile males and sub-fertile or even infertile females enter the swine breeding herds (Oh et al., 2006b; Safranski, 2008). Thus, to maximize economic returns and swine production efficiency there is a need for a biomarker to identify boars and gilts with the greatest reproductive potential before admittance into the breeding herd. The overall aim of the described studies was to determine if biomarkers of fertility of boars and gilts could be identified in biological samples taken prior to or just after animals entering the breeding herds using high throughput omic screening tools resulting from recent advancements in mass spectrometry.

Current semen evaluation techniques only identify boars with fertility issues associated with sperm motility, morphology, and concentration. We know that seminal plasma proteins are essential for proper sperm function and play an important role in fertilization. Therefore, we hypothesized that fertility differences could be reflected in the seminal plasma proteome profiles. A fertility index was created from 110 boars with data on total born and farrowing rate following 50 single-sired matings. Thirty-two of the 110 boars were identified as having extreme phenotypes for total born and farrowing rate and were categorized into one of the following: high farrowing rate and high total born (HFHB; n=9), high farrowing rate with low total born (HFLB; n=10), low

farrowing rate and low total born (LFLB; n=9), and low farrowing rate with high total born (LFHB; n=4). The seminal plasma proteins were isolated and measured using label-free liquid chromatography-tandem mass spectrometry (LC-MS/MS). There were 436 proteins measured in at least one sample across all animals, with 245 proteins considered for analysis (detected in samples of at least n=3 animals/phenotype). Of the 245 proteins, 56 were differentially abundant ($P < 0.05$) between the high fertility phenotype (HFHB) and at least one of the three subfertile groups. Proteins previously associated with fertility such as Porcine seminal protein I (PSP-I) and epididymis-specific alpha-mannosidase (MAN2B2) and free radical detoxification such as superoxide dismutase 1 (SOD1), peroxiredoxin 4 (PRDX4), and glutathione peroxidase 6 (GPX6) were more abundant in HFHB. Subfertile phenotypes had a greater abundance of blood microparticle proteins, biomarkers of inflammation, and lower inositol-1-monophosphatase (IMPA1), which regulates inositol production. Findings supported that seminal plasma protein profiles were distinct between boars with different fertility phenotypes and have the potential to predict boar reproductive performance.

The selection of replacement females for the sow herd is one of the most important facets in sow system management. However, selection of gilts for sow herd replacements is primarily based on how the animal appears such as feet and leg confirmation, the gilt's underline, and parent past performance. This practice resulted in a high degree of variation in sow reproductive performance traits such as pigs per sow per year (PSY) and increased culling rates due to reproductive failure. In female swine, perinatal nutritional environment has been associated with their long-term fertility. The vaginal lipidome of 2 day and 14 day old gilts was found reflective of nutrition source, which suggests that perinatal nutrition affects the composition of reproductive tissues. Thus, it was hypothesized that the vaginal lipidome profiles of gilts at weaning would be

reflective of fertility later in life. The first study aimed to find potential on-farm biomarkers that technicians could use to make selection decisions. Variables chosen as potential biomarkers have potential to influence or predict long-term fertility. Data were prospectively collected from 2146 gilts born on a commercial sow production facility and included birth and weaning weights, vulva length and width at 21 d postnatal (PN), birth and nursing litter size, days nursed, average daily gain from birth to weaning, and age at first estrus. Of the initial animals, 400 (17%) were selected for the sow herd, 353 remained after removing animals culled for non-reproductive reasons. Animals were assigned to 1 of 5 reproductive performance categories based on observation of estrus or pigs per sow per year (PSY) across two farrowings: High Fertility (HF; 23%; n=82; ≥26 PSY), Middle Fertility (MF2; 12%; n=43; 20-25 PSY), Low Fertility (MF3; 15%; n=54; <20 PSY), Infertile-Estrus (IFe; 10%; n= 36; estrus, no pregnancy), and Infertile-No Estrus (IFno; 39%; n=138; no estrus, no pregnancy). Generalized linear model analysis indicated vulva width (P=0.03) was related to PSY, however, it only explained 1.5% of the total variation in PSY. To determine if preweaning variables were predictive of gilt fertility outcome, animals were grouped as those that became pregnant (n=179) or not (n=174). Vulva width tended to be greater in fertile animals versus infertile (P=0.07). Binomial regression analysis revealed a positive relationship between vulva width and gilt fertility; however, this relationship is not strong enough to make sow herd selection decisions.

Because gilts are so phenotypically similar at weaning, we hypothesized that the biomarker predictive of fertility at this stage of selection might need a more sensitive means of detection. Therefore, we evaluated the vaginal lipid profiles from a subset of animals enrolled in the previous study that were the extremes of fertility phenotype: High Fertility (HF; n=28; ≥26 PSY) and Infertile (IF; n=34; no estrus, no pregnancy). Vaginal swabs of the anterior vagina were taken at

21 ± 4 d PN. Lipids were extracted from cellular material collected with swabs and analyzed using multiple reaction monitoring (MRM) profiling for lipidome analysis. Relative abundance of arachidonic acid (ARA, C20:4) and docosahexaenoic acid (DHA, C22:6) were lower (P<0.05) in IF gilts than HF gilts, whereas abundance of the free fatty acids cerotic (C26:0), ximenic (C26:1), and nonadecanoic (C19:0) acids were greater (P<0.05) in IF gilts. Additionally, eicosapentaenoic acid (C20:5), a precursor of prostaglandins, was also higher (P<0.05) in IF gilts.

Previous studies support that higher levels of arachidonic acid in vaginal lipidomes maybe a biomarker of colostrum intake, and thus provides further evidence for a relationship between fertility and the perinatal nutritional environment. The perspective of having a panel of lipids captured with vaginal swabs at weaning that can predict the reproductive efficiency of gilts shows promise and warrants future research in this area. Taken together, the experiments described above demonstrate that detection of infertile and subfertile animals before entering the breeding herd is possible and warrants further development and validation of diagnostic panels capable of doing so.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Currently, there are 6.28 million boars and sows used for breeding in the United States commercial swine industry (USDA, 2020). Reproductive efficiency of the breeding herd is an important economic factor for producers. Historically, breeding stock were typically selected for progeny performance traits such as feed efficiency, growth rate, and carcass characteristics (Oh et al., 2006b; Safranski, 2008) with little emphasis placed on fertility. Selecting replacement animals based on performance traits can result in the inclusion of infertile or subfertile males and females. Subfertility in boars and sows negatively impact farrowing rates and litter sizes and increase culling rates due to poor reproductive performance (Ruiz-Sánchez et al., 2006; Roongsitthichai et al., 2013; Roca et al., 2015). To maximize economic returns, increase farm efficiency, and reduce culling rates, there is a need to identify subfertile and infertile animals prior to their entry into the breeding herd.

There is no single, early test for boar or sow fertility. For boars, their ejaculate's sperm motility and morphology are routinely evaluated prior to use for artificial insemination, as these traits are associated with male fertility (Flowers, 1997; Xu et al., 1998; Ruiz-Sánchez et al., 2006; Flowers, 2008; Knox et al., 2008). If a boar's semen quality falls below established thresholds for motility and morphology, fertility is likely to be compromised and the boar will not be used for breeding programs (Flowers, 1997, 2013). However, subfertility unrelated to sperm motility and morphology are not identified with this approach (Flowers, 1997, 2013), and subfertile boars still included into the breeding herd reduce farrowing rates and total born in a litter.

Sow fertility is evaluated by number of piglets weaned per year, known as pigs per sow per year (PSY). This measure accounts for multiple measures of fertility such as pregnancy rate, farrowing rate and litter size. Despite the existence of highly prolific sows with an average PSY of 26.5, sows are still being removed from the herd at rates of 49% in the U.S primarily for poor reproductive performance (MetaFarms, 2020; PigChamp, 2020). With approximately 6 million breeding females in the U.S. being replaced at approximately 50% each year, this means 3 million young replacement gilts are needed each year. The likelihood that each replacement gilt born will enter into the breeding herd and produce piglets ranges from 38-51% (Roongsitthichai et al., 2013; Li et al., 2018). Thus to replace 3 million sows, 6 million gilts must be raised. Each replacement gilt is a large financial investment for the producer in terms of costs of feed, vaccination and medication, housing, and labor. Moreover, a genetics premium is often added to the cost of each replacement gilt. Economic analyses found that a gilt to fully pay for her investment, she must produce 3-5 litters (Stalder, 2009; Patterson et al., 2010; Gruhot et al., 2017). Currently, the only way to predict fertility or future reproductive performance in gilts are through the use of expected progeny differences (EPD) or the maternal line index (MLI) which are used in genetic selection. However, these both lack the ability to predict whether a female will exhibit estrus which would be a huge economic advantage for the swine industry.

Similar to how semen quality is correlated with fertility in boars, several traits are correlated with fertility in females. Currently, age at first estrus is the parameter most predictive of sow reproductive efficiency (Patterson et al., 2010). More recent work demonstrated that there is a relationship between vulva width at 95-115 days of age and a gilt's ability to achieve puberty by 200 days, providing the earliest known predictor of reproductive performance (Graves et al., 2020). These researchers also found that larger vulva width at 105 days is associated with greater

reproductive success through two parities (Romoser et al., 2020). Work of multiple researchers have established a link between perinatal nutritional environment and fertility in females (Bagnell et al., 2005; Bartol et al., 2006b; Yan et al., 2008; Bagnell et al., 2009; Bartol et al., 2009; Frankshun et al., 2012; Vallet et al., 2015b; Bagnell and Bartol, 2019; George et al., 2019). The amount of colostrum consumed is predictive of long-term fertility of gilts (Vallet et al., 2015b). Work of Bagnell and Bartol demonstrated a relationship between reproductive tract development and colostrum. Work described in chapter four in this dissertation describes the first steps aimed at developing a screening system based on these observations.

Fertility is a complex phenotype that is influenced by multiple factors, some of which are not readily identifiable by producers when selecting males and females to use in a breeding program. Genetic selection has established highly prolific pigs, however, factors such as biotic (e.g. disease), abiotic (e.g. heat stress), and nutrition can affect fertility outcomes (Lawlor et al., 2002; Schinckel et al., 2009). Current knowledge can identify several factors in males and females that are associated with fertility, while a predictor of fertility is lacking in young animals. Therefore, there is a need to identify markers of infertility and subfertility as well as technologies that can accurately do so.

A lack of readily available, on-farm phenotypic markers of fertility in young animals requires investigations to identify biomarkers using more sensitive tools. Recent advancements in sequencing and mass spectrometry are allowing for efficient screening of genes, proteins and metabolites that may differentiate animals based on fertility potential and serve as biomarkers. The field of omics sciences is a rapidly evolving field that queries what *might* happen, what *is* happening, and what *has happened* within a biological system and encompasses genomics, transcriptomics, proteomics, and metabolomics. A multi-omics approach provides a

comprehensive insight as to the how and why of the physiological state of an animal, and how it changes in response to disease, environment, and time. These tools have been used successfully to query for biomarkers of infertility (Rapino et al., 2014; Wahid et al., 2017) and disruptions in pubertal development (Rockett et al., 2004) in humans.

The overall aim of the studies conducted for the dissertation research described was to identify biomarkers predictive of infertility in boars and gilts. In this first chapter, an overview of development of the reproductive system is given for both sexes, and then literature regarding factors and windows of opportunity that affect swine fertility are reviewed. Scientific work regarding the connection between early nutrition, management and reproductive success later in life is also highlighted. Furthermore, works regarding technological advancements in mass spectrometry and the use of omics for biomarker discovery are reviewed to emphasize the potential that these technologies hold to identify a biomarker predictive of infertility in young animals.

1.2 Development of the Male and Female Reproductive Tract

1.2.1 Development of the Male Reproductive Tract

At conception, inheritance of XX or XY chromosomes determines sexual differentiation of the reproductive system in embryos. Regardless of chromosome inheritance, all mammalian embryos undergo a process known as gastrulation where cells organize into three distinct germ layers known as the ectoderm, mesoderm, and endoderm. Gastrulation begins 6.5 days post coitum (DPC) in the mouse (Downs and Davies, 1993) and 11-12 DPC in the pig (Blomberg et al., 2006). The reproductive tract originates from the ectoderm and the mesoderm. The ectoderm gives rise to the nervous and integumentary system as well as the hypothalamus, pituitary, external male and female genitalia, posterior vagina and vestibule. The mesoderm gives rise to connective tissue, and

components of the uterus, cervix, anterior vagina, gonads, secondary sex glands, ductus deferens, and epididymis. The urogenital ridge contains coelomic epithelium that are characterized into three different segments: the pronephros, mesonephros, and the metanephros (Capel, 2000). The mesonephros eventually forms the mesonephric (male) and paramesonephric (female) ducts (Wilson and Bordoni, 2020). Migration of germ cells to the urogenital ridge occurs between 15-28 DPC in swine (Kocer et al., 2009; Hyldig et al., 2011), and leads to the formation of the genital ridge (Piprek et al., 2017). In the pig, germ cells are bipotential, meaning they have the potential to become gametes from either genetic sex until 21 DPC (Cupp and Skinner, 2005). Genes encoded on the Y chromosome, specifically the SRY gene, direct the gonad to differentiate into testis in males (Lovell-Badge and Robertson, 1990; Koopman et al., 1991).

Both mesonephros and paramesonephros ducts are present in early-stage embryos and are later regressed depending on the presence of gender specific signals. Formation of these ducts occurs around the same time as the development of the bipotential gonad. The mesonephros eventually develops into the epididymis and vas deferens in male embryos (Cupp and Skinner, 2005). Sertoli cells in male embryos produce anti-mullerian hormone (AMH), around 26 DPC in the pig (McCoard et al., 2002), which binds to the paramesonephros ducts (Baarends et al., 1994; Allard et al., 2000), and causes their regression while maintaining the mesonephros ducts. The action of AMH was first discovered in rabbits when French endocrinologist, Alfred Jost, removed the bipotential gonad from male fetal rabbits. Upon removal of the gonad, chromosomal XY rabbits developed to be phenotypically female and lacked secondary sex glands such as the prostate (Jost, 1947). Additionally, Jost discovered that exogenous testosterone masculinized the mesonephros ducts in female fetal rabbits, but not the paramesonephros (Jost, 1953). It was through the

culmination of these findings that Jost was able to demonstrate that the regression of the paramesonephros in the male is the result of AMH and not testosterone (Jost, 1953).

Several other genes in addition to *SRY* regulate sexual development in the male. The gene *SOX9* is responsible for inducing the production of AMH in sertoli cells and is upregulated by *SRY*, which functions as a transcription factor (Hayashi et al., 1984; Appert et al., 1998; Sekido et al., 2004). In the male, steroidogenic factor 1 (*SF1)* and *SRY* cooperatively regulate *SOX9* expression by binding to the promoter region known as TES (testis-specific enhancer of *SOX9*) (Sekido and Lovell-Badge, 2008). Transgenic mice with mutations in *SRY* or *SF1* had similar TES activity when compared to wild type mice, however, mice with both *SRY* and *SF1* mutations showed no activity at all indicating they must be expressed together to properly activate the *SOX9* promoter region (Sekido and Lovell-Badge, 2008). Studies in mice found *SF1* is first expressed within the coelomic epithelium at 9.5 DPC (Ikeda et al., 2001) and is important for the development of both male and female gonads at 11.5 DPC (Luo et al., 1994). Additional studies demonstrated that expression of *AMH* in developing embryos is upregulated by the interaction of *SF1*, *GATA* transcription factors, Wilm's Tumor Suppressor Gene 1 (WT1), and *SOX9* (Yomogida et al., 1994; Onodera et al., 1997; De Santa Barbara et al., 1998; Meeks et al., 2003; Bouchard et al., 2019) whereas DAX1 downregulates *AMH* transcriptional activity by repressing WT1 and SF1 (Nachtigal et al., 1998) and GATA-4 and SF1 (Tremblay and Viger, 2001). In the bipotential gonad of mouse embryos, expression of GATA-4 and SFI were found to precede AMH expression, (Giuili et al., 1997; Nachtigal et al., 1998; Tremblay and Viger, 1999). The female gonad of mice was found to only expression *WT1* (Munsterberg and Lovell-Badge, 1991; Viger et al., 1998).

Following germ cell migration, an animal that inherits XY at conception will express *SRY* mRNA in pre-sertoli cells (Rossi et al., 1993; Koubova et al., 2006). The aggregation of pre-sertoli

cells initiates testis determination from the primary sex cords followed by the separation of the seminiferous cords that become interstitial cells and differentiate into leydig cells (Hughes, 2001). *SOX9* is also important for leydig cell differentiation and testis cord development. Homozygous inactivation of *SOX9* in mice embryos caused a complete sex reversal in XY mice and resulted in a lack of leydig cell differentiation and testis cord development (Barrionuevo et al., 2006). Increased proliferation of the gonad and migration of what will become the peritubular myoid (PM) and leydig cells are also dependent on *SRY* expression (Martineau et al., 1997; Capel et al., 1999; Schmahl et al., 2000). This process happens concurrently with sertoli cell differentiation between 26-28 DPC in the pig (McCoard et al., 2002; Wilhelm et al., 2007). Between 27-35 DPC in the pig, sertoli and leydig cells become recognizably distinct cell populations (Anderson, 2009). Testicular descent begins around 60 DPC and gonads enter the scrotum by 90 DPC in the fetal boar (Anderson, 2009).

Leydig cell development and differentiation is also dependent on AMH production. Leydig cells are the testosterone-producing cells of the testes, and testosterone is responsible for the differentiation and maintenance of the mesonephros ducts into the vas deferens, epididymis, seminiferous tubules, and portions of secondary sex glands (Josso, 1970; Morrish and Sinclair, 2002). Additionally, testosterone produced by the testis acts on the hypothalamus to block the formation of the gonadotropin surge center in males. To masculinize the nervous system, testosterone must cross the blood-brain barrier and is converted to estradiol within the brain via aromatase. Alpha-fetoprotein (AFP) is produced by the yolk sack of the embryo and later by the fetal liver (Andrews et al., 1982; Tilghman and Belayew, 1982). AFP prevents masculinization of the female brain by binding to estrogen produced by the placenta. AFP binding of estradiol prevents it from crossing the blood-brain barrier in females, and thus prevents the masculinization

of the brain (McEwen et al., 1975). In female AFP-knockout mice, male behaviors such as mounting were increased and female behaviors such as lordosis decreased (Bakker et al., 2006). Female AFP knockout mice were found to be infertile due to anovulation (Gabant et al., 2002).

Postnatal development of the male reproductive tract is marked by increased sertoli cell proliferation in the boar during the first month after birth followed by final maturation occurring at approximately 3-4 months of age (Erickson, 1964; Tran et al., 1981; Kosco et al., 1989; Swanlund et al., 1995; França et al., 2000). Sertoli cells modulate spermatogenesis, support sperm development, and form the blood-testis barrier, preventing the immune system from attacking developing sperm (Setchell et al., 1969; Kaur et al., 2014). Leydig cells develop and proliferate during the perinatal period and then again between 3 months to the peripubertal period, which is 6 months in the boar (Van Straaten and Wensing, 1978; França et al., 2000). Leydig cells are responsible for testosterone production (Van Straaten and Wensing, 1978; Pinart et al., 1999), which is central to spermatogenesis and development of secondary sexual characteristics of males.

As a boar approaches puberty, which can range from 7 to 9 months of age, the hypothalamus becomes incrementally desensitized to the negative feedback created by testosterone and estradiol. Decreased sensitivity to negative feedback in the prepubertal period enables the initiation of pulsatile waves of gonadotropin releasing hormone (GnRH) production from the hypothalamus. GnRH enters the hypothalamo-hypophyseal portal system, and travels to the anterior pituitary where it stimulates FSH and LH secretion into the blood. FSH and LH bind to receptors on sertoli and leydig cells in the testis to regulate testosterone synthesis needed for spermatogensis (Plant, 2015). Upon LH binding to membrane-bound LH receptors on the leydig cell, cholesterol produced within the smooth endoplasmic reticulum (Ewing et al., 1983) is converted to testosterone (Hall et al., 1969). Binding of LH to its receptors stimulates the production of cAMP, followed by the the

activation of protein kinase A (PKA) (Dufau and Catt, 1979). PKA is responsible for the translocation of cholesterol across the mitochondrial membrane. Once inside, cholesterol is converted into pregnenolone and eventually into testosterone. The ability of leydig cells to synthesize testosterone is directly related to the amount of smooth endoplasmic reticulum (SER) within the leydig cell. Rats that received subcutaneous implants of testosterone-17β-estradiol had reduced levels of SER which resulted in a decrease in conversion of progresterone to testosterone (Wing et al., 1984).

Following binding to its receptor on the sertoli cell, FSH stimulates the production of inhibin (Chowdhury et al., 1978; Steinberger, 1979), and androgen binding protein (ABP) (Tindall et al., 1975; Fritz et al., 1976). Testosterone works in conjunction with FSH to stimulate spermatogenesis by inducing production of ABP. ABP binds testosterone produced by leydig cells to maintain high levels of testosterone necessary for spermatogenesis to occur (Joseph, 1994).

1.2.2 Development of the Female Reproductive Tract

Similar to the male, sexual determination begins at conception based on genetics of the female, where primordial germ cells of XX embryos are predetermined to develop into oogonia in the absence of the *SRY* gene (Lovell-Badge and Robertson, 1990; Koopman et al., 1991). Upon germ cell arrival to the gonad at approximately 10.5 DPC in the mouse, oogonia undergo mitosis and meiosis until 13 DPC (Pepling, 2006), and by 13.5 DPC become arrested in the diplotene stage of meiosis (Mayes and Sirard, 2002; Wassmann et al., 2003; Shoji et al., 2006). The multiplication of germ cells during this stage of development results in the formation of germ line cysts (Saito et al., 2007). Germline cysts are clusters of germ cells that are connected by intercellular bridges and surrounded by a single layer of somatic cells (Saito et al., 2007). In vertebrates, each germ cell

within a cyst will develop into a distinct oocyte surrounded by follicular cells, which are primordial follicles. In the mouse, germline cysts persist until postnatal (PN) day 2 (Pepling, 2006). When germline cysts breakdown, they form and divide into individual follicles that are defined by pre-granulosa cells surrounding a single oocyte. These structures will differentiate to become primordial follicles (Pepling, 2006). Primordial follicles begin to form d 4 PN in mice and in most species, remain dormant until puberty (Pepling, 2006). The formation of these follicles prevents oocytes from going beyond prophase I in meiosis (Anderson, 2009).

In addition to the absence of *SRY*, the determination of the bipotential gonad into ovarian tissue is controlled by the interactions of R-spondin homolog 1 (*RSPO1*), *WNT4*, and β-catenin by way of inhibiting *SOX9* expression, the key component in male gonad differentiation. RSPO1 is responsible for initiating the WNT4/β-catenin pathway (Binnerts et al., 2007). Mutations in *RSPO1* in both mice and humans resulted in complete female-male sex reversal (Parma et al., 2006). Additionally, expression of RSPO1 and SOX9 in 10.5-14.5 DPC mouse gonads were measured and showed that as *RSPO1* increased in the XX gonad and *SOX9* remained unchanged while the XY gonad showed the opposite (Parma et al., 2006). WNT4 activates β-catenin signaling which destabilizes SOX9. Mice bred to stabilize β-catenin in gonadal somatic cells showed a complete male-female sex reversal in XY mice and could not be distinguished from XX wild type when comparing both external genitalia or internal organs (Maatouk et al., 2008).

The process of development of germ cells during embryogenesis in swine is similar to mice, but the timeline is distinct with migration to the genital ridge completed around 28 DPC (Hyldig et al., 2011). On 26 DPC, the fetal ovary has distinct surface epithelium and mesenchyme (Anderson, 2009). Porcine oocytes become arrested in prophase I of meiosis around 35 DPC (Monk and McLaren, 1981; Anderson, 2009), and like rodents, remain arrested until the LH surge

occurs just prior to ovulation in sexually mature animals. Around 40 DPC in the pig, germ cysts appear, and the ovary is organized into the medulla and cortex (McCoard et al., 2001). Primordial follicles appear around 60 DPC followed by primary and secondary follicles on 70 and 90 DPC, respectively (Anderson, 2009).

In the absence of AMH, the paramesonephros ducts develop into oviducts, uterus, cervix, and cranial vagina while the mesonephros regress (Cupp and Skinner, 2005). The caudal tip of the paramesonephric ducts fuse to form the uterine body, cervix, and anterior vagina and all originate from the mesoderm (Orvis and Behringer, 2007; Sajjad, 2010; Robbins et al., 2015; Roly et al., 2018). The urogenital sinus, originating from the ectoderm, forms a small bud and attaches to the paramesonephric ducts forming the posterior vagina and vestibule (O'Rahilly, 1977). Different layers of uterine tissue, such as the endometrium and myometrium, originate from undifferentiated embryonic mesenchymal tissue (Bal and Getty, 1970; Marion and Gier, 1971).

The development of the female surge center in the fetal brain is important for normal reproductive function later in life. The absence of *SRY* in an XX individual means the bipotential gonad will not differentiate into testosterone producing leydig cells. When testosterone is absent and AFP binds circulating estrogen, no estrogen can reach the brain, and thus allows the hypothalamic surge center to develop. As reviewed by Simerly (2002), the surge center contains neurons that make up the anteroventral periventricular nucleus (AVPV). The function of the AVPV is to regulate GnRH secretion, which is greater in amplitude in females than males (Simerly, 1998; Kauffman et al., 2007). Aromatization of testosterone into estrogen causes apoptosis of cells in the AVPV of males, which leads to diminished amplitudes of release of GnRH in males.

Prior to puberty, both male and female animals maintain the same, tonic pulsatile pattern of GnRH secretion. As the female approaches puberty, the hypothalamic-pituitary-gonadal (HPG)

axis is activated, which initiates the beginning of gonadotropin control of growth and development of the gonad (Strauss et al., 2014; Casteel and Singh, 2020). HPG axis activation is initiated by cues related to gilt body composition, specifically the increasing number of adipocytes secreting the hormone leptin (Robert et al., 1998). Leptin serves as the body's permissive signal to initiate the process of pubertal development. Serum leptin concentrations increase in the mouse (Chehab et al., 1997), gilt (Qian et al., 1999), and heifer (Garcia et al., 2002) as they approach puberty. Mutant mice that lack the leptin receptor failed to express signs of estrus (Bates et al., 2003). Leptin acts directly on kisspeptin-producing neurons in the hypothalamus (Smith et al., 2006; Donato et al., 2011) and its production is heavily influenced by energy balance of the pig (Spurlock et al., 1998; Barb et al., 2001; Barb and Kraeling, 2004). Leptin production is also positively related to estrogen levels (Shimizu et al., 1997). Ovariectomized rats treated with 17β-estradiol benzoate showed increased serum leptin concentration and leptin mRNA expression when compared to ovariectomized rats that received sesame oil (Fungfuang et al., 2013).

Kisspeptin, the neuroendocrine peptide produced by the gene *KISS1*, is responsible for activating the HPG axis (Casteel and Singh, 2020), which stimulates the pulsatile secretion of GnRH (Matsui et al., 2004; Messager et al., 2005; d'Anglemont de Tassigny et al., 2008). Exogenous kisspeptin administered to prepubertal gilts induced activation of the HPG axis as indicated by increased LH and FSH secretion (Lents et al., 2008). Initiation of GnRH pulsatile secretion initiates ovarian follicular development between 60-100 days of age in the gilt (Schwarz et al., 2008). Similar to the male, hypothalamic nuclei secrete GnRH which travels to the anterior pituitary via hypophyseal portal veins (Anderson, 2009). GnRH binds to its receptor on pituitary gonadotroph cells which triggers the cascade of releasing LH and FSH from the anterior pituitary into the blood stream (Casteel and Singh, 2020).

With the onset and completion of puberty, ovarian follicular development and growth occurs in two phases: gonadotropin independent and gonadotropin dependent. Early phases of follicular growth are not regulated by FSH and LH, making this early stage of development gonadotropin independent (Zuckerman and Baker, 1977; Baker and Hunter, 1978). Over time, follicles become responsive to FSH and LH as they begin to express receptors for these two hormones and enter the gonadotropin-dependent phase. Gonadotropin-independent follicular development is characterized by a primordial follicle containing a primary oocyte arrested in prophase I of meiosis that will increase in size, develop a zona pellucida, begin protein synthesis, and cortical granule formation. Folliculogenesis begins with the formation of the primary follicle which is characterized by an oocyte that is surrounded by one layer of granulosa cells that become cuboidal in their morphology (Williams and Erickson, 2012). Granulosa cells continue to proliferate and begin to express FSH receptors in the secondary follicle along with the formation of the basement membrane and zona pellucida (Oktay et al., 1997).

While the entire process of follicular development from primordial to early antral follicles takes 83 days (Foxcroft and Hunter, 1985; Morbeck et al., 1992; Knox, 2005), every 21 days, the gonadotropin dependent growth occurs to create a population of dominant follicles that can be selected for ovulation. When secondary follicles begin expressing FSH receptors the gonadotropin dependent phase of folliculargenesis is initiated, as they can now respond to FSH secretion. During the gonadotropin-dependent stage of growth, FSH activates an intracellular cAMP-kinase signaling cascade in granulosa cells that results in expression of enzymes that convert testosterone produced by theca cells into estradiol (Strauss et al., 2014; Casarini and Crépieux, 2019). When LH binds to its receptor on the theca cell in the ovary, cAMP activation of a kinase occurs which transforms cholesterol into pregnenolone, then progesterone, then androstenedione, and finally to

testosterone (Strauss et al., 2014; Riccetti et al., 2017). FSH stimulation induces granulosa cells to proliferate and differentiate into two cell types: cumulus cells and mural granulosa cells. Cumulus cells are adjacent to the oocyte and the mural cells stay attached to the follicle wall and are responsible for estrogen production. Estrogen production occurs when testosterone produced by theca cells is transported into the mural granulosa cell and converted into estrogen. Cumulus cell differentiation is dependent on signals originating from the oocyte (Diaz et al., 2007a, b). Cumulus and mural granulosa cells are separated by a fluid filled cavity known as the antrum.

Follicles recruited for ovulation are FSH dependent (Knox, 2005). As the follicles increase in size and approach dominance they increase the number of LH receptors they express and become LH dependent (Knox, 2005), thus further increasing estrogen production. The surge center in the hypothalamus becomes increasingly sensitive to estrogen, and GnRH production is further increased leading to more LH secretion from the pituitary and eventually the LH surge which ultimately induces ovulation (Moenter et al., 1991). Just prior to ovulation, the LH surge stimulates cumulus expansion and the resumption of meiosis in the oocyte which results in the extrusion of the first polar body and formation of the secondary oocyte with the completion of meiosis I (Dekel et al., 1981; Clarke and Masui, 1983). The oocyte continues into meiosis II and is arrested at the metaphase stage of the second meiotic division when ovulation occurs (Jones, 2004). Meiosis II is completed during the process of fertilization. Cumulus cell expansion just prior to ovulation is necessary for the release of a fertile ovum. A cascade of proteolytic enzymes such as plasmin and plasminogen activator are stimulated by the LH surge and breaks down the follicular wall (Tsafriri, 1995). Inhibition of these proteins prevents ovulation (Tsafriri, 1995). The first estrus and ovulation typically occur at 150-220 days of age in the gilt (Anderson, 2009; Soede et al., 2011). Age at first estrus can be influenced by nutrition, adipocyte stores, management practices such as

boar exposure, and stress. Upon attainment of puberty the gilt begins a regular estrous cycle that is approximately 21 days in length.

Development of the female reproductive tract continues after birth in pigs. At the time of birth the lumen of the uterus is lined by a single layer of cuboidal epithelial cells that is adjacent to stromal tissue, encased in the myometrium, which is concentric layers of inner circular and outer longitudinal smooth muscle tissue (Bal and Getty, 1970; Marion and Gier, 1971). Organization of the uterine wall begins with the stratification of mesenchymal and endometrial stromal cells followed by differentiation of the myometrium and endometrial gland development (Bartol et al., 1993a; Spencer et al., 2019). Endometrial gland development is initiated in the first weeks after birth in pigs (Massman, 1980) and proceeds with the proliferation and invasion of luminal epithelium into the underlying stromal tissue, creating a network of tubules that extend to the adluminal border of the myometrium (Spencer et al., 1993). Between birth and postnatal (PN) day 3, glandular epithelium of the endometrium rapidly proliferates and differentiates from the luminal epithelium (Tarleton et al., 1999; Masters et al., 2007). Over the first 14 days PN, uterine weight increases which is accompanied by increased endometrial thickness and rapid glandular development (Tarleton et al., 1999). Additionally, porcine endometrial stroma lacks estrogen receptors (ER) at birth, but protein expression of ER within the glandular epithelium and luminal epithelium become evident by PN d 15 (Tarleton et al., 1998; Tarleton et al., 1999).

There is a natural increase in uterine weight at the same rate as growth of body size of the gilt; however, at approximately 60-84 days of age, development of uterine glands and thickness in response to the increase in circulating ovarian estrogen causes the uterus to increase in weight faster than the rate of body growth (Hadek and Getty, 1959; Bal and Getty, 1970; Wu and Dziuk, 1988; Bartol et al., 1993a; Tarleton et al., 1998). Gilts ovariectomized at birth showed no adverse

effects on uterine development the first 2 months following birth. However uterine growth and endometrial thickness between d 60-120 was restricted which suggests that uterine growth becomes ovarian dependent beginning on 60 d PN (Tarleton et al., 1998). Uterine weight, growth rate, and endometrial thickness continue to increase until near puberty (Dyck and Swierstra, 1983). It is estimated that by 120 d PN the uterine wall has reached full maturity and is ready to maintain pregnancy (Spencer et al., 1993; Tarleton et al., 1998).

The developmental processes described above must all occur correctly in order for an animal to have the potential to become fertile. The periods of active and rapid development of the male and female reproductive systems can serve as windows of opportunity or windows of vulnerability to influence fertility. In the subsequent sections, the literature evaluating the causative factors of infertility is reviewed and reflect back to these stages of development to understand how-why genetic mutations and environmental perturbations or exposures affect fertility, and biomarkers of these events can be elucidated.

1.3 Windows of Opportunity that Influence Fertility in Males and Females

Fertility is a complex trait affected by many factors such as genetics, nutrition, metabolic state of the animal, environment, and management practices. Fertility phenotype can be influenced as early as conception through the onset of puberty, and reproductive system development is vulnerable to environmental factors. Periods, or windows of opportunity or vulnerability to influence fertility phenotype have been identified as being during *in-utero* development, perinatal and peripubertal development, as well as when animals are exposed to stressors such as disease challenges or heat stress. As stated in the introduction, there is no single test for fertility potential of boars and gilts prior to entering the breeding herd. Determination of fertility potential as early

as possible would be advantageous to the producer as it would reduce financial loss associated with raising infertile animals and improve farm efficiency. Knowledge of factors that affect fertility and timing of exposure to these factors can enable development of approaches for evaluation of future breeding animals for reproductive potential.

1.3.1 Chromosomal Abnormalities and Fertility

It is estimated that the genetic influence over fertility in swine is only 10-20% with the remainder being influenced by environmental conditions (Goodwin, 2004). The trajectory of fertility development in both humans and animals begins with chromosomal inheritance. In normal development, mammals will either inherit a set of XX or XY chromosomes, however, it is also possible to inherit a single X (Turner syndrome-infertile female), XXX (Triple X syndrome-fertile female), XXY (Klinefelter syndrome-infertile male), or XYY (XYY syndrome-normal male) chromosomal arrangements. Women with Turner syndrome exhibit gonadal failure, have issues with puberty induction, and are estrogen deficient throughout their lifetime (Ogata and Matsuo, 1995; Stochholm et al., 2006). As reviewed by Smyth and Bremner (1998), Klinefelter syndrome patients are characterized by impaired spermatogenesis, inhibin B insufficiency, and hypogonadism. Reproductive symptoms associated with Klinefelter syndrome have been attributed to abnormal Sertoli cell function (Anawalt et al., 1996).

Because of the potential economic loss and loss of efficiency on farm, genotyping boars and gilts that are born on nucleus farms is routinely practiced. More information on genetic flow and selection can be found in the ‘Optimization of Breeding Herds’ section of this literature review. In general, monitoring genotypes at the nucleus level largely prevents problematic defects and traits from entering herds. Currently, not enough is known regarding single nucleotide polymorphisms

(SNP) that negatively affect fertility (Perkel, 2008; Sironen et al., 2010; Fischer et al., 2015; Guo et al., 2017; Bakoev et al., 2020) to use allele frequency as a selection tool for breeding stock. Identifying a biomarker of fertility within the genome would be the most advantageous to the swine industry as this is the earliest timepoint in an animal's life where we could test. However, more research validating SNPs associated with reduced fertility is warranted before implementation at the commercial level.

An interesting study of note though was conducted by Rezai et al. (2020). During routine karyotyping of 5,481 commercial boars, they discovered 39 cases of mosaic translocation. Chromosomal trisomy (three copies of a chromosome, or region of chromosome) is the most common mosaic translocation and can occur during meiosis of germ cells, or in somatic cells (Campbell et al., 2015). As noted above for humans, trisomy of sex chromosomes may be related to infertility or subfertility. Rezaei et al. (2020) were the first group to evaluate boar fertility based on mosaic rearrangements within somatic cells. They found a high frequency of trisomies in somatic cells, however there was no negative impact of mosaic translocations identified on reproduction. Litter size average was not different between animals that carried mosaic translocation versus noncarriers (Rezaei et al., 2020).

1.3.2 Intrauterine Environment and Developmental Programming

Since heritability of fertility is fairly low, understanding environmental factors that affect reproductive potential is important. One of the most sensitive timepoints of development in an animal's life is during prenatal period when environmental and xenobiotic stressors can affect the normal development of the reproductive tract. Intrauterine exposures have been known to affect fertility of both boars and gilts later in life. A concept commonly referred to as the 'Barker

hypothesis', also referred to as developmental programming hypothesis, states that exposures during early development can affect physiology and metabolism for the rest of an individual's lifetime (Barker and Clark, 1997; Barker, 2002; De Boo and Harding, 2006). Multiple studies in humans and animals have shown that adverse intrauterine environments can lead to increased risk for disease in adulthood (Frankel et al., 1996; Rich-Edwards et al., 1997; Leon et al., 1998). Decreased growth rate associated with adverse uterine environments is known as intrauterine growth restriction (IUGR) and is a serious concern for domestic livestock species (Foxcroft et al., 2006; Wu et al., 2006; Ji et al., 2017). IUGR reduces neonatal survival (Omtvedt et al., 1971), postnatal growth, and impairs long-term health (Foxcroft et al., 2009; Johnson et al., 2015; Johnson et al., 2020), and linked to low fertility in humans (Sharma et al., 2016). Causes of IUGR can range from uterine crowding to environmental stressors such as temperature or disease (Redmer et al., 2004; Wu et al., 2004; Boddicker et al., 2014; Johnson et al., 2020).

In cattle, twinning of fetuses of different sexes can also influence fertility of the twin *in utero.* Freemartins are heifers born twin to bull calves, and become masculinized by exposure to androgens produced by the fetal male, and ultimately results in infertility of the female (Padula, 2005; Szczerbal et al., 2019). Although most commonly described in cattle, a study on a commercial swine facility that evaluated incidence of sex development disorders in piglets, found freemartinism the most common development disorder, however, it is still very rare among the population (Szczerbal et al., 2019). Interestingly, authors concluded that this may be a consequence of high litter size.

As reviewed in the previous section regarding reproductive tract development, there are major developmental milestones of the testes from days 26-90 of gestation in the boar making it a window of opportunity for fertility to be influenced (Pelliniemi, 1975; Pelliniemi et al., 1979; Van

Vorstenbosch et al., 1982; França et al., 2000; Anderson, 2009). In a study that evaluated boar fertility following *in utero* heat stress (IUHS) events during gestation, Lugar et al. (2018) revealed that boars that are heat stressed *in utero* have reduced sperm production over their lifetime, reduced testicle size at puberty, and increased number of sperm abnormalities. Another study evaluating prenatal stress in boars stimulated the endocrine stress response by injecting sows with adrenocorticotrophin (ACTH) once per week from d 42-77 of gestation (Schenck et al., 2006). Boars born to sows that received ACTH had a smaller anogenital distance when compared to offspring from sows roughly handled for 10 minutes once per week from d 42-77 of gestation or control sows (Schenck et al., 2006). IUHS also has effects on female reproduction. Much like the boar, there is a critical period of gonadal development during d 30-60 of gestation where stress could reduce the number of potential oocytes formed in the prenatal gilt (Black and Erickson, 1968). Gilts born to first parity, IUHS sows showed a numerical reduction in total born and number born alive when compared to gilts born to thermoneutral sows (Safranski et al., 2015).

1.3.3 Perinatal Nutrition and Environment

Meeting proper nutritional requirements of the boar and gilt is imperative for proper reproductive tract development and function. Although oogenesis happens in-utero in the pig, the majority of uterine differentiation and development occurs postnatally (Bartol et al., 1993a). As previously reviewed in the 'Development of the female reproductive tract' section, the formation of uterine glands continues through the first weeks postnatal in the swine (Ogasawara et al., 1983; Bigsby and Cunha, 1985; Bartol et al., 1988; Tarleton et al., 1998). Therefore, there is a window of opportunity to influence the development of the female reproductive tract during this time. Multiple studies have connected early perinatal environment with long-term fertility in swine. The

connection is believed to be due to exposure to factors in colostrum, the first milk produced by the mammary glands. Additionally, the timing of colostrum availability is coincident with the period prior to tight junction closure between enterocytes lining the neonates' gastrointestinal tract. Prior to closure of tight junctions, which occurs 24-36 hours after birth, larger molecules can pass between cells and gain access to circulation (Bourne et al., 1978; Drew et al., 1990).

Insufficient colostrum consumption the first 24 h postnatal impairs future reproductive performance later in life (Vallet et al., 2015b). It is well documented that the size of a litter a gilt is born into and weaned from is negatively related to lifetime reproductive performance (Robison, 1981; Van der Steen, 1985; Bartol et al., 2006a; Bagnell et al., 2009; Bartol et al., 2009). Therefore, females born into larger litters tend to have a lower reproductive performance than those from smaller litters. Litter size likely influences birth weight and access to intake of colostrum. Gilts born into smaller litters at birth tend to be heavier and consume more colostrum (de Passille and Rushen, 1989; Milligan et al., 2002; Le Dividich et al., 2005). These animals also display estrus earlier in life and have better lactation performance which indicates that low colostrum consumption is associated with impaired reproductive performance in sows (Vallet et al., 2015b; George et al., 2019). The relationship of litter size to future productivity is also likely influenced by nutritional environment throughout lactation. This has been demonstrated by Flowers (2009a) who cross-fostered piglets at 24 hours to create a nursing litter size less than seven or greater than ten from litters that all had more than ten animals *in utero*. Sows raised in small litters remained in the breeding herd longer as opposed to sows raised in large litters. Another study conducted by Flowers (2015b), reported that more of the progeny from small litter remained in the sow herd after 6 parities than gilts who were raised in the litters of 12 piglets (Flowers, 2015b). This data

suggests that reduced competition for milk throughout lactation results in increased pre-weaning growth and positively effects reproductive development into adulthood.

Colostrum is the only source of nutrition for the newborn piglet for the first 24 hours of life and plays an important role in piglet survival. Colostrum plays a multi-faceted role in a piglet's life including supplying energy for thermogenesis, passive immunity from the dam, bioactive factors that regulate the growth of the piglet, in addition to promoting reproductive tract development. (Devillers et al., 2004; Langley-Evans, 2009; Devillers et al., 2011; Vallet et al., 2015b). Devillers et al. (2011) determined a colostrum intake threshold of at least 200 g was needed for survival to weaning where preweaning mortality spiked from 7.1% to 43.4% when colostrum intake was below 200 g. In another study evaluating the effects of induction on preweaning survival, Mills et al. (2021b) observed that the largest factors influencing preweaning survival was birth weight and colostrum intake which is consistent with other works (Quesnel et al., 2012a; Amdi et al., 2013; Ferrari et al., 2014; Feldpausch et al., 2019). Piglets born under one kilogram in the study had a preweaning mortality rate of 49% and consumed 214 g of colostrum on average which is just above the minimum amount recommended by Devillers et al. (2011).

Colostrum also plays an important role in maintaining thermogenesis for the piglet. Upon birth, piglets experience a dramatic decrease in environmental temperature, which can affect survival (Kammersgaard et al., 2011; Vande Pol et al., 2020). The change in body temperature can be exacerbated by evaporative cooling that occurs with the drying of the animal, which can result in a further drop in core body temperature (Vande Pol et al., 2020). When piglets are born, they lack brown adipose tissue and rely on glycogen stores in the liver and muscle to maintain thermogenesis (Quesnel et al., 2015). Piglets also have a limited ability to oxidize fatty acids for metabolism which also causes them to rely on liver and muscle glycogen stores (Mersmann et al.,

1973). Glycogen stores are depleted around 12-17 h PN when piglets do not have access to colostrum (Theil et al., 2011).

Colostrum also provides passive immunity to the newborn. Because of the epitheliochorial structure of the sow's placenta, piglets are born completely void of an active immune system and therefore must acquire passive immunity from the dam until their own immune system develops. At birth, newborns have low proteolytic activity in the stomach and intestine which enables maintenance of antibody structure during transit through GI tract (Day and Schultz, 2014). Maternal antibodies in colostrum, specifically IgG, provide systemic immunity for the piglet while IgA stimulates mucosal immunity and protects the piglet from pathogens overwhelming the gut (Quesnel et al., 2015). Newborns also have specialized receptors expressed on their enterocytes known as neonatal Fc receptors (FcnR) that mediate the transfer of immunoglobulins (Day and Schultz, 2014). Immunoglobulins can be transferred directly into the blood or taken into the lacteals and passed from the lymphatic system to the blood (Day and Schultz, 2014).

Immunoglobulins from the dam's serum are transferred to the mammary gland just prior to parturition which gives colostrum its characteristic high immunoglobulin profile (Klobasa et al., 1987). Previous work has shown that we can increase the concentration of immunoglobulins by supplementing the sow prior to parturition with conjugated linoleic acid (Bontempo et al., 2004), shark-liver oil (Mitre et al., 2005), phytogenic (essential oils) feed additives (Wang et al., 2008), fermented liquid feed (Demeckova et al., 2003), and mannan oligosaccharides (O'quinn et al., 2001). Sows are also vaccinated prior to parturition to provide additional protection to the neonate during suckling (Matías et al., 2017).

Beyond the function of nutrition, thermogenesis, and passive immunity, colostrum contains other bioactive factors such as relaxin (RLX) that affect the development of the animal. Maternal

programming of neonatal development by bioactive components in milk is termed the lactocrine hypothesis (Bagnell and Bartol, 2019). Relaxin (RLX) is a hormone released during pregnancy that softens and increases the size of the cervix and facilitates the birthing process (Sherwood, 2004). Levels of RLX in colostrum are higher than circulating in the sow at the time of parturition (Bartol et al., 2009). Relaxin receptors are expressed by somatic cells, including those that line the reproductive tract of neonatal pigs (Bagnell et al., 2009) and acts as a growth promoter of uterine and cervical tissues of porcine neonates (Yan et al., 2006b; Yan et al., 2008; Bagnell et al., 2009; Frankshun et al., 2011). Yan et al. (2006a) studied the effect of RLX on uterine gene expression where piglets were injected with RLX. Uterine growth occurred on d 2 and d 14 when piglets were administered RLX on d 0 and d 12, respectively (Yan et al., 2006a). In another study, ESR1 and VEGF expression within the uterus increased when piglets were administered RLX for 48 hours after birth (Yan et al., 2008). It has also been shown that piglets administered RLX orally via milk replacer every six hours for 2 days after birth also increased ESR1, VEGF, and uterine growth (Chen et al., 2011). Piglets who were given milk replacer as opposed to nursed controls had reduced endometrial gland development and thickness (Miller et al., 2013). High levels of RLX present within colostrum coupled with the uterotrophic effects of RLX suggest a tie between colostrum intake and reproductive tract development (Bartol et al., 2013).

1.3.4 Seasonal Infertility

Boars

Reproduction can be negatively impacted due to elevated ambient temperatures that alter much of normal physiology of animals. Pigs normally maintain their normal body temperature

around 38°C, however when environmental temperatures increase above their upper critical limit, disruption of the core body temperature occurs and the animals experience heat stress (Huynh et al., 2005; Patience et al., 2005). Sperm are particularly thermal sensitive, which has resulted in the evolution of many thermoregulatory systems in the body to moderate the temperature in the testis, such as the pampiniform plexus which uses countercurrent heat exchange to regulate the temperature of the testis.

In boars, heat stress (HS) alters sperm morphology and production. Wettemann et al. (1976) observed a significant reduction in sperm reserves in HS boars compared to thermoneutral boars. Malmgren and Larsson (1989) investigated the effects of localized HS on boar testes. Localized HS was accomplished by covering the scrotum in an insulation device that was sutured to the skin. After 100 hours of HS, histological analysis revealed minimal damage to the testes immediately after the stressor was removed. However, the testes had severe damage appear ten days later within the seminiferous tubules. It took approximately 40 days in total for the seminiferous tubules and sperm morphology to appear normal again, which is consistent with the timeline of spermatogenesis in the boar.

Heat stress likely impacts spermatogenesis via oxidative stress which ultimately causes apoptosis in sperm cells (Ikeda et al., 1999). Ikeda et al. (1999) demonstrated in rats that HS stimulates increased peroxide levels in germ cells, with antioxidants having the ability to prevent this apoptosis during HS. The cells most impacted by HS are spermatocytes (Rockett et al., 2001; Zhu and Setchell, 2004). The increased susceptibility of spermatocytes may stem from malfunctions in the blood-testis barrier, which protects the developing sperm cells from the immune system (Setchell et al., 1969; Dym and Fawcett, 1970). HS causes a disruption in the tight junction that make up the blood-testis barrier between sertoli cells in the seminiferous tubules (Cai

et al., 2011). In experiments conducted by Cai et al. (2011), mRNA concentrations of occludin and zona-occludins were significantly reduced shortly after HS. Occludin and zona-occludins are the major proteins that form tight junction complexes. Additionally, they demonstrated that the permeability of tight junctions is increased due to the downregulation of these proteins (Cai et al., 2011). This study suggests that HS disrupts the tight junctions in the seminiferous tubules exposing the developing spermatocytes to immunological destruction. Taken together, HS induced apoptosis reduces sperm output and is accompanied with an increase in sperm abnormalities such as abnormal acrosomes, presence of proximal and distal droplets, and tail abnormalities, ultimately reducing boar fertility. It is hypothesized that morphological abnormalities are caused by oxidative damage to the sperm in the epididymis during maturation (Banks et al., 2005). Morphological alterations are also delayed from the onset of heat stress to presence in ejaculated semen (McNitt and First, 1970; Wettemann et al., 1976; Wettemann et al., 1979; Malmgren, 1989; Malmgren and Larsson, 1989). Following a heat stress event, normal sperm morphology was reduced beginning two weeks after the event with acceptable semen parameters returning to the ejaculate 5-7 weeks later (McNitt and First, 1970; Wettemann et al., 1976; Wettemann et al., 1979; Malmgren, 1989). Sperm motility was also reduced following the heat stress event. While the majority of sperm parameters are significantly impaired due to HS, the volume of collected semen and gel weight are not influenced by HS in boars, suggesting that that the accessory sex glands are not particularly vulnerable to HS (McNitt and First, 1970; Wettemann et al., 1976; Wettemann et al., 1979; Malmgren, 1989). However, because the seminal plasma is a complex fluid of proteins and growth factors, assuming HS does not impact accessory sex glands based on ejaculate volume alone is not enough to confirm HS does not impact the secondary sex glands. Therefore, further research

evaluating the seminal plasma for biomarkers of stress before, during, and after a HS event is warranted.

Gilts

High ambient temperatures can potentially delay puberty onset in gilts (Peltoniemi and Virolainen, 2006; Iida and Koketsu, 2013). Puberty delay due to high ambient temperatures is likely due to a reduced secretion of gonadotropins or responsiveness of the ovary to gonadotropins. Feed restriction in gilts disrupts the HPG axis (Tokach et al., 1992) and ultimately puberty onset. Decreased feed intake is one of the most common responses to heat stress. Flowers et al. (1989) explored the effects of elevated ambient temperature on puberty and observed related physiological changes in gilts. Gilts placed in environmentally controlled chambers with high ambient temperatures at 140 d of age reached puberty later than control gilts. Additionally, HS gilts had a greater incidence of cystic follicles following injection of pregnant mare serum gonadotropin (PMSG) when compared to control gilts. Sows exposed to high ambient temperatures have reduced follicular growth rate, increased silent estrus, and ovulation failure (Lucy et al., 2001; Bertoldo et al., 2012). Impaired ovarian function is likely due to the physiologic response to HS which is the shunting of blood from visceral organs to the skin to dissipate heat (Wolfenson et al., 1981; Redmer et al., 2004; Wu et al., 2004; Boddicker et al., 2014; Johnson et al., 2020). A study that evaluated the effects of high ambient temperatures on reproductive performance in gilts, found heat stressed gilts had half the number of viable embryos 8-16 d post-breeding when compared to thermoneutral gilts (Omtvedt et al., 1971). Therefore, delayed puberty onset in gilts during high ambient temperatures can likely be attributed to a lack of blood flow to the developing gonads coupled with feed restriction that disrupts the HPG axis.

1.3.5 Disease Challenges

With the vast majority of the swine industry utilizing artificial insemination (AI), it is important to understand how diseases transmitted during insemination can affect fertility (Gerrits et al., 2005a). Disease spread via contaminated semen used in AI is costly to the boar stud and the sow herd as its impact is immense due to one boar having the potential to inseminate approximately 676 sows over the course of a single year (PigChamp, 2020). Contamination can be introduced from the boar's reproductive tract due to an infection or during collection, semen processing, and storage. The presence of pathogens can result in reduced fertility in boars via reduced sperm production and increased sperm abnormalities while embryonic death, fetal death, and uterine infection occur within the female.

Bacterial populations within a boar's ejaculate are normal and typically reside at a concentration of 10^4 to 10^5 per mL (Sone, 1990) with most being non-pathogenic. However, if certain bacterial populations grow to a high concentration they can have spermicidal effects (Althouse et al., 2000). It is also important to note that transmission of pathogenic bacteria can still occur by AI, even if extended semen contains antimicrobials because of resistance or environmental conditions that would change the antimicrobial activity (Maes et al., 2008).

Some common bacterial pathogens found in boar semen include brucellosis, chlamydia, leptospirosis, enterococci, staphylococci, and pseudomonas. Swine brucellosis is of venereal origin and readily infects sows that are inseminated with contaminated semen (Lord et al., 1997). Early abortions beginning at 17 d DPC and infertility are typical signs of brucellosis in swine. Infected boars can exhibit low libido coupled with infection of the accessory sex glands. However, boar fertility may not be affected by brucellosis (Vandeplassche et al., 1967). Porcine chlamydia can cause inflammation of the boar's urogenital tract, and has been detected in semen of boars

using PCR testing (Kauffold et al., 2006). As reviewed by Kauffold et al. (2006), chlamydia has a relatively high prevalence ranging from 16.3-61.7% depending on the sow herd. In boar studs serological tests revealed approximately 36.9% of boars are positive for chlamydia (Kauffold et al., 2006). In females, chlamydia causes abortion (Daniels et al., 1994) and poor reproductive performance (Eggemann et al., 2000). In a study conducted to investigate the prevalence of chlamydial infection in German sow herds, sows with antibodies for chlamydia were more likely to have issues returning to estrus and a higher incidence of mastitis and agalactia (Eggemann et al., 2000). Leptospirosis mainly causes abortion, stillborn piglets, and infertility in sows and boars (Boqvist et al., 2002; Ramos et al., 2006). Leptospires may persist for extended intervals in genital tracts of sows and boars, and be excreted in urine and genital tract fluids for up one year (Ellis et al., 1986; Ramos et al., 2006).

Several viruses have been recovered from infected semen such as classical swine fever, Porcine circovirus, pseudorabies, and Porcine reproductive and respiratory syndrome virus (PRRSv) and have been reported in relation to infertility or reduced reproductive performance in swine (Guerin and Pozzi, 2005). Boars inoculated with classical swine fever virus have been shown to shed the virus in semen for up to 53 d post-infection (Choi and Chae, 2003). Sows inseminated with semen containing classical swine fever experienced seroconversion and embryonic death (De Smit et al., 1999).

Although rare, Porcine circovirus has been linked to reproductive failure, late term abortions, and stillbirths. Shedding of Porcine circovirus in semen can occur starting as early as day 5 post-infection and last until d 47 (Larochelle et al., 2000) with the seminal plasma playing host to the virus (Hamel et al., 2000; Kim et al., 2003). Embryonic death as a result of Porcine circovirus is

likely due to the virus's ability to replicate within zona pellucida-free embryos (Mateusen et al., 2004; Mateusen et al., 2007a).

Clinical signs following PRRSv infection vary widely, ranging from reproductive failure and death in breeding animals, mortality in suckling pigs, to respiratory disease in nursery and grow-finishing pigs. The virus can be shed in semen, even in the absence of viremia and in the presence of neutralizing antibodies (Christopher-Hennings et al., 1995; Christopher-Hennings et al., 2001). This virus most likely reaches the tissues of the reproductive tract and semen by migration of infected monocytes and macrophages (Prieto et al., 1997). The duration of shedding in semen samples of experimentally infected boars varies widely, ranging from 2 d (Shin et al., 1997) to 92 d (Christopher-Hennings et al., 1995) after infection. This marked variability may be due to various factors, including individual boar variation, the type of virus strain, and the technique used for detection of the virus. Semen shedding has also been demonstrated after vaccination with a modified live vaccine virus (Christopher-Hennings et al., 1997). Alterations in seminal quality following PRRSv infection include reduced motility, an increase in the percentage of abnormal acrosomes, and an increase in morphologically abnormal sperm, especially those with abnormal heads (Feitsma et al., 1992).

Pseudorabies is a herpes virus that has viral replication in the genital tract. Boars inoculated with the virus within the testicle showed testicular degeneration and an increase in abnormal sperm morphology (Hall Jr et al., 1984). It is also important to note that the virus can be isolated from urine or preputial membranes (Medveczky and Szabo, 1981) which increases its risk of being spread not only from the boar, but during the collection process by the technician. Sows inseminated with contaminated semen show seroconversion, and may suffer from vaginitis, endometritis, and embryonic death.

Early embryonic death may result from direct invasion of the embryo by the pathogen, and/or by induced uterine epithelial changes (Wrathall and Mengeling, 1979). The zona pellucida, surrounding the developing embryo until d 6-7 after conception, forms a barrier to many pathogens, including pseudorabies, Porcine circovirus, and PRRSV (Mateusen et al., 2007b). However, after hatching, blastocyst-stage embryos may become susceptible to the infection, which creates another window of opportunity and vulnerability for development.

1.4 Optimization of the Breeding Herd and Current Selection Practices

1.4.1 Genetic Selection

Phenotype, or the performance of an animal, is based on genotype by environment interactions. Genotype is determined by the genes inherited from the dam and the sire and determines the genetic potential of an individual animal for a particular trait. Whereas as environmental exposures, to include nutrition, housing, and disease determine whether progeny meet their genetic potential. Heritability describes the amount of variation in a trait explained by genetic factors (Wray and Visscher, 2008). Knowledge of the heritability of a trait is used to calculate the estimated Breeding Value (EBV) of an animal. EBV is a prediction of an animal's genetic merit for a particular trait relative to other animals in the same population. Genetic selection of both male and female animals for breeding programs is based on their EBV. In swine, heritability estimates of economically important traits include both reproductive and carcass factors and are used to calculate EBV. Backfat heritability varies from 40-70%, whereas most reproductive traits like ovulation rate, number born alive, and embryo survival rate are 25% or less (Table 1.1). Carcass and growth traits such as average daily gain, backfat thickness, and muscle

depth are negatively correlated to ejaculate volume, concentration, and motility of sperm (Smital et al., 2005; Oh et al., 2006b; Safranski, 2008), making genetic improvement for reproduction challenging. Making genetic progress involves one of three systems: a terminal system where all animals are marketed and replacement females are purchased, a rotation system where there is a rotation of boar breeds with each generation, or a rotaterminal system where both the terminal and rotational system are combined to gain the advantages of both (Whittemore and Kyriazakis, 2008). Crossbreeding maximizes hybrid vigor which ultimately improves reproductive traits. For terminal systems, replacement gilts are key to their success and therefore, require the careful consideration of maternal and terminal sire lines. Terminal lines are used in a breeding system for their growth performance and carcass characteristics whereas maternal lines are selected for traits such as preweaning progeny growth performance, litter size, and number of pigs weaned. Briefly, all genetic progress and gene flow in the swine industry begins with the nucleus farm (Figure 2) which are farms that produce purebred animals that are genotyped and provide semen to the multiplier for crossbreeding and to production barns for meat production (Towers, 2015; Lopes, 2016). The multiplier farm produces crossbred animals from purebred lines to produce an F1 generation to be sold to production (meat-producing) farms (Lopes, 2016).

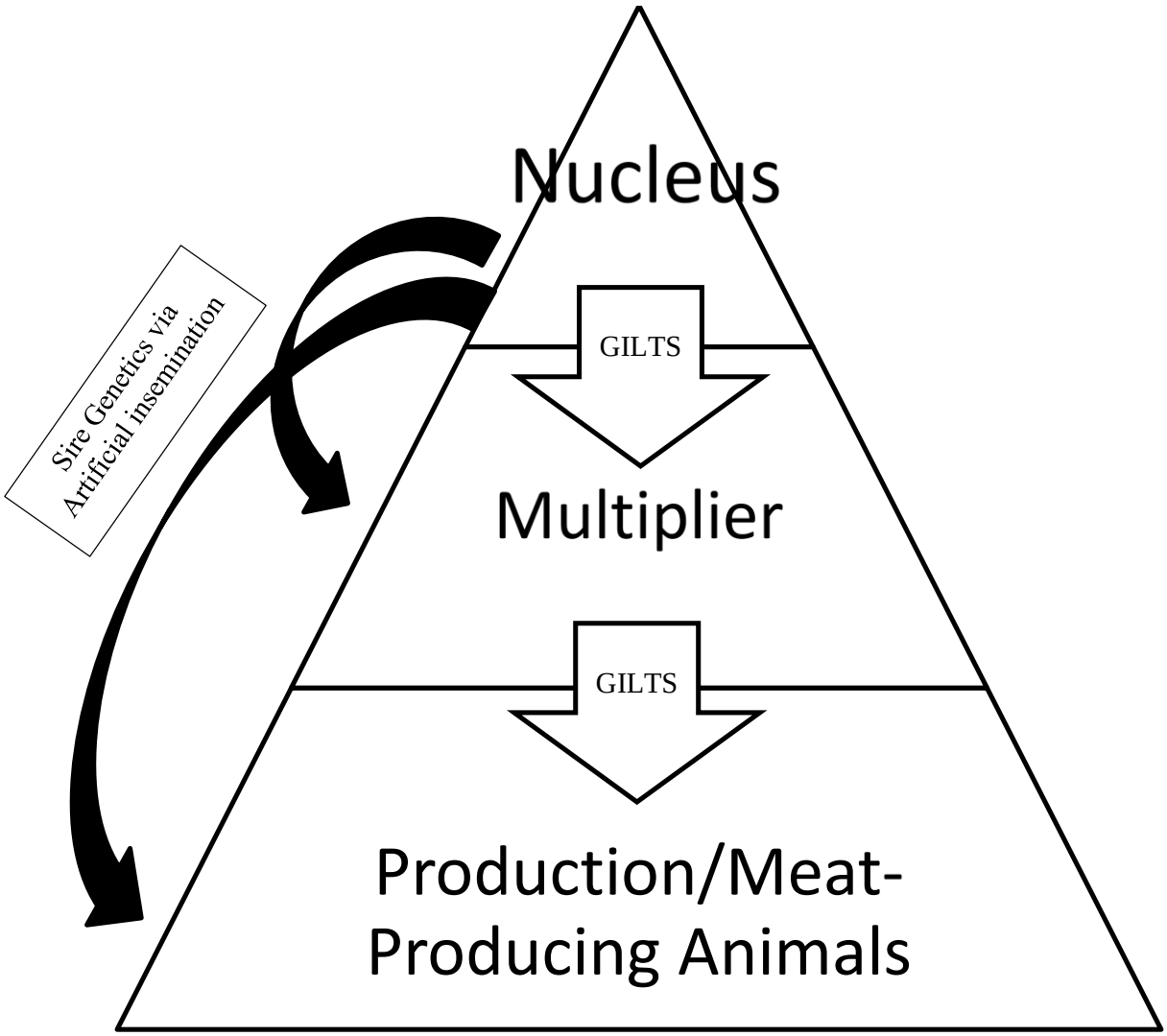

Figure 1.1 Flow of genetics within the Commercial Swine Industry

Adapted from Lopes (2016) and Towers (2015)

Table 1.1 Heritability estimates of economically important traits for swine

Trait[1]	**Heritability estimate**
Wean-to-estrus interval	0.05-0.10
Number born alive	0.10-0.20
Sow Longevity	0.10-0.20
Ovulation rate	0.10-0.25
Embryo survival	0.10-0.25
Average daily gain	0.30-0.60
Loin muscle area	0.40-0.60
Backfat depth	0.40-0.70

[1]Adapted from Whittemore and Kyriazakis (2008)

1.4.2 Boar Management and Selection

Boars are selected into the breeding herd based on progeny growth traits as well as sperm motility, morphology, and concentration (Whittemore and Kyriazakis, 2008). For genetic selection, boars can be selected from either terminal or maternal lines. Terminal genetic lines are specifically bred for carcass quality, growth rate, and efficiency traits whereas maternal genetic lines focus more on litter size produced and less on growth and carcass traits. Before boars are delivered to the stud, they first undergo a rigorous selection process where they are evaluated based on their Estimated Breeding Value (EBV) index. The EBV index is based on phenotypic progeny traits such as growth rate, feed efficiency, or carcass quality (Robinson and Buhr, 2005). EBVs are helpful as they quantify the genetic merit of an animal by accounting for variation in phenotype. The EBV is calculated by multiplying the heritability of a particular trait by how much an individual's performance deviates from the mean. As more progeny performance records become available, EBVs become more accurate (Chu et al., 2019). The challenge with genetic

improvement using EBVs is that progeny growth characteristics are negatively correlated to sperm quality. A higher average daily gain and muscle depth is negatively correlated to sperm motility and concentration (Oh et al., 2006b; Wolf, 2009).

Boars with high EBV index are further screened for suitable conformation, lack of reproductive defects, and are genotyped before final selection into the boar stud for semen collection to be used in artificial insemination. When a boar arrives at the boar stud at approximately 6-8 months of age, he is housed in an isolation facility for about 60 days (Knox et al., 2008). Isolation before entering the breeding herd prevents the spread of potential diseases, allows for an acclimation period, and gives technicians time to train the boar for semen collection. Once out of isolation, trained boars are routinely collected to provide ejaculates approximately 1-2 times per week. Semen samples are evaluated for concentration, volume, motility, and morphology, prior to packaging for artificial insemination (Knox et al., 2008). There is a high correlation between fertility of an ejaculate and motility and morphology of the sperm in the ejaculate and, therefore, are parameters used to evaluate individual ejaculates (Flowers, 1997; Xu et al., 1998; Ruiz-Sánchez et al., 2006; Flowers, 2008; Knox et al., 2008). Acceptable parameters for semen quality are 70-80% motile sperm and 70% normal morphology. If an ejaculate falls below established thresholds for motility and morphology it will not be used for artificial insemination (Flowers, 1997, 2013).

Boar selection is rigorous because one boar provides the genetic material to produce thousands of animals each year. For example, the average boar produces approximately 80 billion sperm cells in a single ejaculate. Conventional artificial insemination uses two insemination doses of 2.8-3 billion sperm cells. Therefore, a single ejaculate produces ~26 doses of semen (Knox et al., 2008) that are used to artificially inseminate thirteen sows per week. Over the course of a year,

this equates to 1352 doses of semen used to inseminate 676 females. On commercial operations in the US, the average total born alive is 13.5 pigs per litter (PigChamp, 2020), therefore, an average litter size of 13 pigs results in 9,126 piglets born alive from a single boar in a year. Additionally, the use of post-cervical artificial insemination (PCAI) is increasing in production practices due to its advantage in reducing the time required for insemination and potential for reducing cost of semen doses (Ketchem et al., 2017). In a study conducted by Swine Management Services LLC, breeding records from over 100,000 sows were evaluated to compare the differences between PCAI and conventional AI. PCAI showed an increase litter size of approximately 0.31 piglets (Ketchem et al., 2017). Another advantage of PCAI is that the number of sperm cells is reduced to 1.8-2 billion per dose, ultimately improving boar utilization. Thus, when PCAI is used, a boar can potentially produce 14,040 piglets per year.

Although stringent boar selection parameters are in place, subfertile animals are included in the boar stud. Genetic selection for increased fertility of boars has been slow or non-existent in the industry. Subfertility is a term that describes reduced or delayed ability to conceive, which is different from infertility which is the inability to conceive (Gnoth et al., 2005). It is much more common for a subfertile male to enter the boar breeding herd than an infertile male as there no test predictive of subfertility. In swine, subfertility is defined by reduced litter sizes and farrowing rates. Reduced farrowing rates and number born per litter results in a loss of production efficiency for the farm and has negative economic consequences for producers (Roca et al., 2015). Currently, the only way to identify a subfertile boar is by evaluating its impact on production following 50 or more single-sire matings (Clark et al., 1989). However, most boar studs pool ejaculates from multiple boars to make insemination doses, making identification of subfertile boars impossible without paternity testing of offspring. Typically ejaculate pools contain semen from approximately

6 boars. Paternity of piglets is not performed to know if boars used in the pool are more fertile than others. Therefore, the practice of pooling masks subfertile boars (Dziuk, 1996; Ferreira et al., 2015), yet their inclusion in insemination doses can reduce the fertility potential of the entire dose of semen. Thus, there is a need to distinguish subfertile boars prior to entering the breeding herd, before they can impact farm efficiency.

1.4.3 Gilt Development and Selection

Proper gilt development and management is imperative for the long-term reproductive success of the sow herd. Genetic selection of replacement gilts begins with genetics of the dam where sows are selected based on the maternal objective which includes back fat, growth, percent lean, and sow productivity. (Stalder et al., 2003; Stalder, 2009; Stalder et al., 2019). One way sow productivity can be evaluated is by pigs weaned per sow per year (PSY). The PSY encompasses traits such as number born alive, number weaned, ovulation rate, farrowing rate, preweaning mortality, and wean-to-service interval. Genetics are responsible for 10-20% of the variation evident in reproductive efficiency within a single genetic line (Goodwin, 2004) with heritability for reproduction and longevity within the breeding herd being moderate at best (Knauer et al., 2010). A study of factors influencing sow retention, evaluated data from six maternal lines encompassing 2293 sows and found average age at puberty, age at first farrowing, and lactation feed intake had the greatest influence on longevity in the herd (Knauer et al., 2010). Only about 10-20% of sow longevity is considered heritable (Table 1.1), and thus research into other factors that affect these variables is needed.

Commercial producers can control environmental factors such as management practices that influence reproductive development that included nutrition, herd health, housing, heat

detection, and estrus stimulation, and account for the remaining 80-90% of the variation seen in reproduction on farm (Goodwin, 2004). After gilts are born, on-farm selection occurs at multiple timepoints: weaning, start of boar exposure at approximately 140-160 days of age, and again when gilts reach puberty around 200 days of age (Patterson et al., 2010; Patterson and Foxcroft, 2019). At weaning, replacement gilts are evaluated for overall health, weight and size, structural soundness, obvious defects, and number of teats on their underline (Patterson and Foxcroft, 2019). If gilts are selected, they are moved into an on-site nursery or transported to an off-site nursery from the farrowing rooms. As gilts age, they are moved to a grow-finish facility which accommodates their larger body size. Gilts selected for the breeding herd are re-evaluated by the producer for any unsoundness, poor growth performance, or sickness (Patterson and Foxcroft, 2019).

At approximately 140-160 days of age, selected gilts are moved into the gilt development unit (GDU). The GDU is a term that describes the facility where gilts grow to pubertal age and receive boar exposure to induce puberty. Gilts begin receiving boar exposure at ~160 days of age to stimulate the onset of puberty as well as to identify gilts that have achieved puberty as determined by the expression of estrus (Patterson et al., 2002; Patterson et al., 2010; Patterson et al., 2016; Patterson and Foxcroft, 2019). Boar exposure to prepubertal gilts has been established as an effective method of inducing puberty (Pearce and Hughes, 1985; Van Lunen and Aherne, 1987). It is optimal for gilts to receive direct contact boar exposure for puberty induction. A study that compared gilt exposure to boar via direct contact versus daily fence line exposure beginning at 160 d of age, found direct contact resulted in a reduced interval from first boar exposure to first estrus by 10 days (Patterson et al., 2002). In an effective boar exposure program, ~75% of gilts will exhibit estrus naturally within 40 days of the start of boar exposure (Patterson et al., 2003;

Amaral Filha et al., 2009; Kummer et al., 2009; Patterson et al., 2016). Gilts that attain puberty later are typically less fertile and will accumulate more non-productive days (Patterson et al., 2010). Additionally, gilts that are older at their first mating, greater than 250 days of age, have up to 6 less piglets born alive over her lifetime and are removed from the herd almost an entire parity earlier (Saito et al., 2011). Gilts that fail to show evidence of estrus, are treated with P.G. 600® which contains two gonadotropins, equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG). Gilts that do not express estrus in response to P.G.600® are culled (Patterson and Foxcroft, 2019). A study that validated the effects of P.G.600® on puberty induction, found out of a gilt pool of 4,489 animals, 2,654 (59.1%) exhibited standing estrus through boar exposure alone. Of the remaining 1835 gilts, 821 (44.7%) exhibited estrus after the administration of P.G.600® coupled with boar exposure (Patterson et al., 2016). Thus, 22.6% of the gilts never exhibited estrus, even with P.G.600® administration (Patterson et al., 2016), and this may suggest that there is a subpopulation of gilts that have issues related to activating their HPG axis. Once estrus is observed, gilts are typically bred on their second or third estrus. It is ideal to breed gilts on their second or third estrus as it improves ovulation rate and thus litter size (MacPherson et al., 1977; Walker et al., 1989; Young et al., 1990b; Beltranena et al., 1991; Gaughan et al., 1997; Levis, 2000; Grigoriadis et al., 2001). Gilts bred at second estrus versus first estrus showed an increase in the number of total piglets born alive at first farrowing from 0.4-1.3 piglets per litter (Levis, 2000) and increased total lifetime born by 1.3 piglets after four litters (Young et al., 1990a).

Even with rigorous selection parameters and gilt rearing practices in place, approximately 53% of potential replacement gilts at birth are no longer part of the herd by the time they reach puberty and are removed due to poor growth, poor structure, mortality, lameness/injury, or reproductive failure. Mortality during the preweaning period is currently 15.4% (PigChamp, 2021)

and is highly correlated to low birth weight and low colostrum intake. Selection for increased total born has resulted in a greater variation in birth weight within litters, increasing low birthweight piglets (Roehe, 1999; Opschoor et al., 2010). Piglets with birth weight under 1.0 kg have a 40% risk of preweaning mortality (Roehe and Kalm, 2000) and consume less colostrum (Devillers et al., 2011; Mills et al., 2021b). In 2015, the mortality rate was reported to be 3.6% and 4.1% in the nursery and grower-finisher phases of production, respectively (USDA, 2016b). The majority of postweaning deaths were attributed to respiratory disease (USDA, 2016a). The rest of the removals that add up to the 53% were due to reproductive failure and then soundness issues (Knox et al., 2015). In the United States, almost 65% of gilts enrolled in the PIGCHAMP® research database were removed due to reproductive failure that included failure to exhibit standing estrus, conception failure, and failure to farrow (Lucia Jr et al., 2000). A study evaluating cull patterns of sows and gilts in Southwest China found 56.3% of gilts were culled due to failure to show estrus at greater than 9 months of age and 11.21% were culled for lameness (Wang et al., 2019b).

The cost to raise a replacement gilt from birth to breeding is estimated to be approximately $216, including costs for vaccination, feed, labor, housing, genetics, etc. It is also important to note that there are additional costs depending on which cross system is utilized for genetic selection. The current strategy to manage gilts that do not cycle is to send them to a cull market. At the 2021 current market value, a gilt that weighs 300 pounds will only make $165 resulting in a net loss of $51 per animal that does not cycle. Currently, there are no clear predictors of infertility for gilts. Therefore, the ability to identify gilts that will not cycle prior to entering the breeding herd would be extremely valuable to the GDU.

1.5 Significance of Biomarkers

A biomarker is a physiological or morphological indicator of a phenotypic or disease state that can be measured accurately and reproducibly (Strimbu and Tavel, 2010). Although extensive work has been done to identify biomarkers associated with fertility, there is no single test predictive of lifetime reproductive performance of swine. To maintain and improve production efficiency to meet increasing global protein demands, efficiency at every level of animal production—including reproduction, must be improved. Identifying biomarkers of fertility would optimize breeding herd selection and reduce on-farm variation with subfertile boars comprising 7-13% of the breeding population (Minton, 2013) and over half of replacement gilts produced never making it to or achieving puberty (Knox et al., 2015).

The advent of omics tools for measuring DNA (genomic), RNA (transcriptomic), proteins (proteomic), and metabolites (metabolomic) at the global scale has enabled a rapid means for high throughput screening for biomarkers (Hu et al., 2011). For example, genomic research in livestock aims to understand how genetic variation effects economically important traits like feed efficiency and reproductive performance (Keel et al., 2018). Genome-wide association study (GWAS) analysis is done to screen for single nucleotide polymorphism (SNP) in the genome of the animals that exhibit variation in traits. A SNP is a variation in single nucleotide in a DNA sequence among individuals and are the most common variation in the genome (Perkel, 2008), and just over 26 million SNPs have been identified in the porcine genome (Keel et al., 2018). Analysis of the distribution of SNP in a population can be used to determine how genetic variation is related to disease susceptibility or phenotypic trait. SNPs can also ultimately result in changes related to protein abundance or function and cellular metabolism creating a need to explore both the proteome and metabolome of individuals which could act as potential biomarkers indicative of

fertility. The literature below summarizes current markers associated with fertility in swine ranging from morphological measurements through to the metabolome.

1.5.1 Morphological Biomarkers of Boar Fertility

As briefly discussed in the optimization of breeding herd section, semen quality in terms of sperm concentration, morphology, and motility is used to evaluate fertility potential of boars. AI is utilized by 90% of the swine industry (Gerrits et al., 2005b) and its outcome is largely influenced by semen quality parameters due to the reduction in number of sperm cells per dose when compared to a single, whole ejaculate. Sperm concentration, especially in each dose of semen for artificial insemination, is highly influential in determining the number of piglets born per litter with the optimal concentration for AI being 3 billion cells (Flowers, 2002). In a study evaluating the relationship between motility and fertility, Flowers (1997) collected 12 mature boars once per week for 26 weeks and motility evaluations were conducted on each ejaculate and a portion was processed and utilized for in vitro fertilization with the rest of the ejaculate used to inseminate five sows. When motility was below 60%, the reproductive performance of inseminated sows was reduced by a piglet per litter, and also decreased farrowing rate (Flowers, 1997). Sperm penetration rate in vitro was also reduced when motility fell below 60%. However, Flowers concluded that motility could not be used as a predictive measure alone, but in conjunction with morphology and concentration. In another study evaluating effective predictors of relative boar fertility, nine Genex Large White boars were collected twice per week and their ejaculates were evaluated for cytoplasmic droplets and motility on days 3, 7, 10 from first collection day relative to fertility index, as defined by pregnancy rate, farrowing rate, and total born (Ruiz-Sánchez et al., 2006). The presence of cytoplasmic droplets was negatively correlated (r= -0.34), whereas motility on d

3 (r=0.38), 7 (r=0.46), and 10 (r=0.40) was positively correlated to fertility index (Ruiz-Sánchez et al., 2006). Xu et al. (1998) also found sperm morphology at time of collection was predictive of 52% of the variation in fertility index (r^2=0.59). It is also important to note that both Xu et al. (1998) and Ruiz-Sánchez et al. (2006) used suboptimal (less than 3 billion) sperm cell numbers for each dose to measure these outcomes.

Although analysis of sperm morphometrics is highly effective for preventing the inclusion of infertile boars in the breeding herd, it cannot predict or detect the presence of subfertile boars that lack gross differences in semen quality. Therefore, other morphological features in boars have been analyzed for their relationship to fertility. Larger testicular size is related to ejaculates with higher sperm concentration. Ytournel et al. (2014) developed a subjective scale that ranged from 1 (very small) to 5 (very big) and scored testicular size upon arrival to the boar stud during isolation. Sperm concentration and number of doses produced increased with testes score, but motility and percentage of spermatozoa alive were unaffected by testes size. These results were consistent with other studies that evaluated testicular size and sperm production (Rathje et al., 1995; Huang and Johnson, 1996). Therefore, testicular size could have the potential to predict which boars can produce more doses of semen per ejaculate. In the same study, number born alive and litter size were shown to be positively related to testicle size which suggests potential of testicular size to serve an indirect biomarker for fertility (Ytournel et al., 2014). In another study evaluating the relationship of testes size to semen and boar production traits, Jacyno et al. (2015) measured the testes size (length and width) using a vernier caliper of 120 boars at 180 days of age and volume of each testis was calculated based on the equation described by (Young et al., 1986). Testes volume was positively correlated with sperm concentration (r=0.42), total sperm in semen (r=0.36) and sperm with progressive motility (r=0.40). Additionally, it is interesting to note that Jacyno et

al. (2015) observed a small relationship (backfat thickness r= -0.06; leanness r=0.14) between testes volume and body composition which is consistent with previous work (Schinckel et al., 1983).

Birth weight of boars is also related to testicular size and development. A subset of low birth weight (0.8-1.0 kg) and high birth weight (2.0-2.2 kg) boars were castrated at 8 days PN (Almeida et al., 2013). Low birth weight boars exhibited a decrease in testicular volume, weight, and sertoli cell number when compared to high birth weight boars. However, it is important to note that low testicular size does not affect sperm quality, only the number of sperm a boar is capable of producing per ejaculate (Huang and Johnson, 1996).

1.5.2 Genetic Markers of Fertility in Boars

The emergence of high throughput analysis in the field of omics allow for the physiological exploration into the central dogma of biology like never before. Evaluating the genome and the transcriptome can allow us to peek into the future as it gives information on what can happen. The proteome is reflective of the genome and transcriptome in real time, thus giving a current physiological perspective. Lastly, the metabolome is the result of all biochemical reactions within the cell and is indicative of past environmental-gene interactions. Through the combination of different omics techniques, a single test for fertility prediction is becoming more attainable.

In boars, SNPs that have been identified and associated with boar fertility hold the potential to act as selection tools for fertility prediction in the future. A comprehensive study screened 16 Landrace and 16 Duroc boars for 14 SNPs previously identified as being related to boar fertility (Tremoen et al., 2019). Both breeds had fertility phenotypes characterized by high (n=4) and low (n=4) breeding values for total born for both male (n=8) and female (n=8) fertility. SNPs on genes

of interest include: Steroid 5α-reductase (SRD5A), Phospholipase C zeta (PLCz), Cyclooxygenase isoenzyme type 2 (COX-2), β-actin (ACTB), Catsper (CATSPER), Androgen receptor (AR), Sperm protamine 1 (PRM1), Estrogen receptor 1 (ESR1), Estrogen receptor 2 (ESR2), Growth differentiation factor 9 (GDF9), Zona pellucida glycoprotein 3 (ZP3), Cluster of differentiation antigen 9 (CD9), Bone morphogenic protein 15 (BMP15), Bone morphogenic protein receptor 1B (BMPR1B), and Von Willenbran factor (VWF). Overall, SNPs identified as significantly associated with total born only explain 0.27-1.18% of the genetic variation seen in total born with genes BMPR1, COX-2, PLCz, VWF, and ZP3 suggested as playing a role in determining total born. In Landrace boars, two SNPs in BMPR1 and one SNP in COX-2 were significantly associated with the number of total born and two SNPs in PLCz, one SNP in VMF, and one SNP in ZP3 were associated with total born in Durocs (Tremoen et al., 2019). Although this information is valuable, the amount of variation seen in total born is not greatly explained by the presence of these SNPs and therefore further investigation into what is causing this variation is warranted.

Other studies evaluated the association between estrogen receptor 1 (ESR1) and 2 (ESR2) as candidates for boar fertility and sperm quality (Gunawan et al., 2011; Gunawan et al., 2012). ESR polymorphisms in men have been shown to contribute to lower sperm count (Guarducci et al., 2006) azoospermia, and oligozoospermia (Suzuki et al., 2002). In a trial that enrolled 300 boars from both Pietrain and Pietran x Hampshire crosses, two SNPs in exon 1 (coding) and intron 1 (non-coding) of ESR1 were associated with sperm motility and cytoplasmic droplets. Heterozygous individuals for the exon 1 SNP exhibited decreased motility (85.0% vs 83.5%) and increased plasma droplets (5.3% vs 6.9%) when compared to homozygous ESR1 individuals with no SNP (Gunawan et al., 2011). The intron 1 SNP, showed a difference in non-return rate data at 42 days after insemination where homozygous wild type boars had the lowest non-return rate

(0.1%), heterozygous individuals having the highest (2.5%), and homozygous individuals with the SNP being intermediate between the two (0.2%) (Gunawan et al., 2011). Another experiment from the same research group evaluated the association of ESR2 with boar sperm quality and fertility traits in a follow-up study to provide a comprehensive role of ESRs and their realtionship to boar fertility (Gunawan et al., 2012). Semen samples from 203 Pietran and 100 Pietrain x Hampshire boars were used in this study and a polymorphism of the coding region of ESR2 in exon 5 was used to genotype the purebred and crossbred lines. The SNP for ESR2 had an additive effect on motility where percent motility linearly decreased from 85.4% to 83.8% to 81.6%, respectively, in the AA to AG to GG genotypes for the exon 5 SNP in purebred animals. Crossbred animals showed a decrease in sperm concentration homozygous for GG SNP, decreased percent motility when the SNP is present, and increased plasma droplets (Gunawan et al., 2012). Taken together, the presence of SNPs within ESRs could be utilized as a detection method for subfertile boars in the future, however, analysis of how much of the variation seen in reproductive performance explained by the presence of these SNPs is required to make further selection decisions.

Exploration of mRNA expression profiles in spermatogonial stem cells, leydig cells, and sertoli cells revealed that different splice variants exist within the pig steroidogenic acute regulatory protein (StAR) gene (Zhang et al., 2018). As discussed in the development of the male reproductive tract section, sertoli cells modulate spermatogenesis (Setchell et al., 1969; Kaur et al., 2014) and induce the differentiation of leydig cells within the testes by way of AMH production (Hayashi et al., 1984; Appert et al., 1998; Sekido et al., 2004). Additionally, the production of testosterone in leydig cells is essential for proper male reproductive tract development (Josso, 1970; Morrish and Sinclair, 2002) and masculinization of the male brain (Andrews et al., 1982; Tilghman and Belayew, 1982). The StAR gene has been identified as essential for testosterone production and

male fertility with its primary role being regulation of testosterone synthesis (Manna et al., 2009; Miller and Bose, 2011). Among 263 testis samples from 15-day-old Yorkshire male piglets, three splice variants of StAR were identified with StAR-a being highly expressed in the leydig cells (Zhang et al., 2018). Additionally, five base pair duplicated deletion identified at intron 5 in the StAR gene was associated with lower testes weight and size (Zhang et al., 2018). Authors concluded that due to its high expression levels, StAR-a may influence testosterone production. They also concluded that the five base pair deletion identified on StAR could potentially be used as a marker predictive of fertility which would be advantageous since these piglets would be identified before weaning.

In another study evaluating SNPs, Fischer et al. (2015) observed 57 nonsynonymous SNPs present among genes with high expression in the testis. Seven genes identified with polymorphisms, Progesterone Receptor (PGR), Fraser Extracellular Matrix Complex Subunit 1 (FRAS1), Transcription Factor 4 (TCF4), Adenosine Deaminase TRNA Specific 1 (ADAT1), Sperm Associated Antigen 6 (SPAG6), Piwi Like RNA-Mediated Gene Silencing 2 (PIWIL2), and Dynein Axonemal Heavy Chain 8 (DNAH8), were related to reproduction based on functional annotation analysis (Fischer et al., 2015). In a study that enrolled 340 Pietrain and Pietrain-Hampshire cross boars, a SNP in intron 6 of CD9, an integral membrane protein associated with sperm-egg fusion, was significantly decreased motility (85.2% vs 83.6%), increased proximal droplets (6.86% vs 5.33%), and increased percentage of abnormal sperm (7.44% vs 6.12%) (Kaewmala et al., 2011). However, it is important to note that only additive effects on motility, proximal droplets, and number of abnormal sperm were significant with no dominance effect observed. Within the same study, animals with extreme phenotypes for sperm concentration and motility were selected for mRNA and protein expression (n=6). Western blotting and qPCR

revealed that CD9 expression was higher in reproductive tissues and spermatozoa collected from boars with high sperm concentration and motility (n=3) than boars with low concentration and motility (n=3). A higher rate of CD9 localization in leydig cells, sertoli cells, and spermatozoa in the high versus low boars was found and suggests CD9 plays a role in spermatogenesis. Based off the mRNA and protein expression profiles, CD9 may also play a crucial role during sperm development making it a potential candidate gene for selection of good sperm quality in boars (Kaewmala et al., 2011).

Transcriptomic analysis found Solute carrier family 9, subfamily A, member 3, regulator 1 (SLC9A3R1) differed between boars with sperm with different motility and capacitation status, in a commercial herd (Kim et al., 2019a). Three high litter size (13.97 piglets) and three low litter size (10.80 piglets) Yorkshire boars were enrolled in this study. Hyperactivation, measured capacitation status via computer-assisted sperm analysis, of sperm differed between high and low litter size boars. Hyperactivation was positively correlated with boar litter size (r=0.468) while the expression of SLC9A3R1, a gene important in sperm ion channel regulation, was negatively related (r= -0.523) with boar litter size. When hyperactivation and SLC9A3R1 levels were combined, they were found to have high sensitivity, specificity, and overall accuracy (90%) for predicting male fertility. Therefore, measuring SLC9A3R1 expression in spermatozoa may be a more accurate marker for evaluating male fertility and infertility than conventionally used motility parameters and capacitation status (Kim et al., 2019a).

Taken together, evaluating the swine genome for SNPs provides the swine industry with potential fertility prediction methods employable prior to puberty which would result in the greatest economic benefit for the producer. However, SNPs do not explain the majority—or even

one-third—of the variation seen on farm in male fertility and therefore further validation and functional assays are required to prove efficacy of these methods for fertility prediction.

1.5.3 Protein Markers of Fertility in Boars

In addition to SNPs, protein markers associated with fertility have been identified within spermatozoa from boars with different fertility phenotypes. Proper function and maturation of the male gamete is essential for fertilization to occur therefore evaluating the proteome of spermatozoa for fertility biomarkers is appealing. The Kwon group conducted a series of studies aimed at identifying sperm surface proteins that differentiated between boars based on fertility. For these studies, protein was isolated from sperm collected from boars that produced high (n=3; 12.3 piglets) and low (n=3; 10.2 piglets) litter sizes. Boars that produced high litter size had sperm with greater abundance of l-amino acid oxidase, mitochondrial malate dehydrogenase 2, NAD (MDH2), cytosolic 5'-nucleotidase 1B, lysozyme-like protein 4, and calmodulin (CALM). Equatorin (EQTN), spermadhesin (AWN), triosephosphate isomerase (TPI), Ras-related protein Rab-2A (RAB2A), spermadhesin (AQN3), and NADH dehydrogenase [ubiquinone] iron-sulfur protein 2 (NDUFS2) were more abundant in sperm of boars that produced low-litter size (Kwon et al., 2015a). These differences in sperm proteins maybe important biomarkers as there was no difference between in vitro capacitation capability in sperm of these Landrace boars that produced high and low litter size (Kwon et al., 2015a). The 8 proteins differentially abundant between boars that produced high and low litter size were further analyzed for relationships with litter size. UQCRC2 was positively correlated to litter size. UQCRC2 is a component of ubiquinol-cytochrome c oxidoreductase complex that is part of the mitochondrial electron transport chain involved in oxidative phosphorylation and the production of ATP. Low litter size was associated

with high abundance of EQTN, UQRC1, TUBB, RAB2A, SPMI, AQN-3, and SPRN. Following correlation analysis the ratio of UQRC2 to UQRC1 and RAB2A were identified as the most accurate predictors of litter size (Kwon et al., 2015b). A follow-up study from the same research group confirmed that fertility-related proteins in *capacitated* spermatozoa more accurately predict male fertility with levels of RAB2A (r=-0.691) and UQCRC1 (r=-0.807) negatively correlated and levels of UQCRC2 (r=0.822) positively correlated with average litter size (Rahman et al., 2017). The relationship likely indicates the importance of the balance between energy generation and minimization of oxidative stress in capacitated sperm. The mRNA expression level of sperm adhesin proteins Porcine seminal protein-I and II (PSP-I/PSP-II) in spermatozoa was found to be negatively correlated with fertility, as determined by litter size. The correlation was r =-0.72 and -0.46, respectively, but when combined a predictive value of 94.1% for litter size was found (Kang et al., 2019).

Seminal plasma proteins have also been screened for potential biomarkers of fertility. Seminal plasma proteins are essential for proper sperm function as they provide a protective and nutritive environment for sperm to survive within the female reproductive tract. Using two-dimensional (2D) gel proteome approaches, differences in the seminal plasma proteome have been found between subfertile and high fertility boars. Flowers (2001) used 2D proteomes and reported variation in the concentrations of two seminal plasma proteins (26 kDa/pI 6.2 and 55 kDa/pI 4.5) in ejaculates from 400 boars in a commercial stud with 84,448 sow records on farrowing rate and number born alive. Farrowing rate and number born alive increased as the relative concentrations of these proteins increased. Both of these seminal plasma proteins have also been associated with fertility in Holstein bulls (Cancel et al., 1997). The seminal plasma protein 26 kDa/5.9 pI was identified as glutathione peroxidase-5 (GPX5) and independently found to have a positive

relationship with boar fertility (Novak et al., 2010a). In a study comparing fertility of heterospermic and homospermic inseminations, high fertility ejaculates (n=3) had a higher concentration of three seminal plasma proteins (25 kDa/5.9 pI, 55.1 kDa/4.8 pI, and 70.1 kDa/5.2 pI) when compared to low fertility ejaculates (n=3) with concentrations of 25 kDa/5.9 pI being the best protein to rank boars based on fertility (Flowers et al., 2016).

More recent studies have used liquid chromatography with tandem mass spectrometry (LC-MS/MS) to analyze the relationship between seminal plasma proteins and boar fertility. Pérez-Patiño et al. (2018) compared proteomes between two different groups of high (n=7) and low (n=6) farrowing rate boars and boars that produced small (n=6) versus large (n=7) litter sizes with high and low phenotypes determined by their deviation from the mean for each trait. Eleven proteins were found differentially abundant between boars with high versus low farrowing rate which included furin (FURIN), aldose reductase (AKR1B1), ubiquitin-like modifier-activating enzyme 1 (UBA1), peptidyl-propyl *cis-trans* isomerase (PIN1), sperm adhesion molecule1 (SPAM1), bleomycin hydrolase (BLMH), sphingomyelin phosphodiesterase acid like 3A (SMPDL3A), keratin type I cytoskeletal 17 (KRT17), keratin type I cytoskeletal 10 (KRT10), tetratricopeptide repeat protein 23 (TTC23), and angiotensinogen (AGT). Four proteins were differentially abundant between boars producing small versus large litter sizes which included nexin-1 (PN-1), thrombospondin-1 (THBS1), desmocollin-1 (DSC1), and catalase (CAT). Taken together, these data suggest that boars with different fertility phenotypes can be distinguished by seminal plasma proteomes and have the potential to be identified through their proteome. It is important to note that these phenotypes are not extreme, but rather high performing boars versus boars that are not performing as well when compared to the rest of the herd. One caveat to this study is that the

sample sizes are relatively small and therefore the study should be repeated with a larger population to support these claims.

1.5.4 Metabolomic Markers of Fertility in Boars

Exploration and characterization of the metabolome is a relatively new area of research, particularly regarding fertility prediction. The metabolome is representative of the sum of all biochemical reactions within the body and is highly susceptible to changes in environment (Watson, 2006; Aldana et al., 2020). With fertility being a complex trait influenced by up to 90% environmental factors, there is potential in evaluating the metabolome of sperm and seminal plasma (Goodwin, 2004).

In boars, scientists are beginning to characterize the seminal plasma metabolome and identify differentially expressed metabolites within seminal plasma. Mateo-Otero et al. (2020) recovered metabolites from different ejaculate portions from 8 boars of different breeds (Large White n=5; Pietrain n=3). Using H Nuclear Magnetic Resonance (H NMR), 19 different metabolites were identified within the seminal plasma. Principal component analysis revealed metabolomic variation between the ejaculate fractions. An abundance plot representing relative levels of metabolites comparing each ejaculate portion showed that glutamic acid was highly expressed in the first 10 mL, and linearly decreased. All other metabolites which included adenosine triphosphate, alanine, choline, citrate, creatinine, glutamine, glycine, glycerophosphocholine, lactate, and valine increased in relative abundance from sperm rich to post-sperm rich fraction. All of these metabolites are related to or involved in energy production and lipid metabolism in conjunction with being previously associated with fertility. Further

research in this area is warranted, as there was a high degree of correlation between metabolites, and changes in these relationships may be indicative of factors related to fertility in boars.

The lipidome, a component of the metabolome which encompasses all the lipids within a tissue, holds potential for fertility biomarkers as well. Studies of humans have found a relationship between accumulation of ultralong-chain fatty acids (ULCFAs) and fertility. ULCFA are normally found in the skin, retina, testis, and brain (Sassa and Kihara, 2014). Fatty acids longer than 22 carbons are exclusively broken down in the peroxisome (Steinberg et al., 2006). Impairment of ULCFA degradation pathways is associated with several diseases related to impaired peroxisomal function and biogenesis (Sassa and Kihara, 2014). Elevated levels of cerotic acid, a fatty acid that contains 26 carbons, is found in individuals with adrenoleukodystrophy (ALD). ALD is an X-linked recessive condition characterized by buildup of VLCFA in tissues due to a mutation in the *ABCD1* gene (Lu et al., 1997; Moser et al., 2007). ABCD1 functions to transport VLCFA into peroxisomes. In addition to the development of cognitive impairment, males with ALD are infertile. Accumulation of saturated fatty acids within Sertoli cells is hypothesized to be a cause of infertility in men with ALD (Powers and Schaumburg, 1981; Moser et al., 2007). Therefore, it may be worthwhile to survey the lipidome of the seminal plasma as well as the sperm plasma membrane as some of the variation seen in fertility could be explained by differences in lipid composition.

1.5.5 Phenotypic and Morphological Markers of Fertility in Gilts

As discussed in the optimization of breeding herds section above, replacement gilts are selected early in life based on morphometric traits such as foot and leg conformation, teat number, and weaning weight. However, these selection practices do not have the ability to predict lifetime

reproductive performance or longevity. The most predictive and reliable indicator of reproductive performance is age at first estrus (Patterson et al., 2010; Rohrer et al., 2017). In a study of 431 prepubertal gilts, animals that achieved puberty by 150-180 days of age displayed increased retention rate to their third parity (57.8%) within the breeding herd, compared to gilts that achieved puberty after 180 days of age (47.4%) (Patterson et al., 2010). In this same study, 26% of gilts failed to show estrus from boar exposure alone and required the administration of P.G. 600. Of the gilts that received P.G. 600, 20% did not respond, i.e. never showed estrus, which suggests the presence of a subpopulation of truly infertile gilts. Based on research findings like these, it is recommended that gilts that attain puberty within 35-40 days of starting boar exposure should be selected into the breeding herd as they become the most productive animals (Li et al., 2018) and have an increased probability of having their first litter (Tart et al., 2013). Gilts who attain puberty later tend to have an increased wean-to-estrus interval after their first litter (Knauer et al., 2011) and are more likely to be culled due to pregnancy failure (Koketsu et al., 2017).

A relationship between vulva width and age at first estrus and sow productivity has been explored as a biomarker of fertility in swine. In a study aiming to identify a relationship between age at prepubertal ovarian development and age at first estrus, 155 crossbred gilts had their vulvas measured on postnatal days 75, 85, 95, 105, and 115. Vulva width at d 115 was inversely related to age at first estrus (r=-0.28; P=0.01) (Graves et al., 2020). However, vulva width at 115 d only explained 8% of the variation in reproductive performance and therefore should not be used alone when making selection decisions (Graves et al., 2020). Because of this observed relationship, a follow-up study of 731 gilts was conducted to explore this relationship further. Romoser et al. (2020) observed that a smaller vulva width, <27 mm, at 105 days of age showed a reduced inclusion rate into the final breeding herd (66% vs 77%) and animals produced approximately 2.4

less pigs through their second parity (Romoser et al., 2020). Although vulva width shows promise in predicting reproductive performance, it does not explain enough of the variation in reproductive performance on farm to utilize as a selection tool on its own.

Another potential biomarker of fertility in gilts is birth weight which is likely influenced by litter size and access to intake of colostrum which is imperative for piglet growth and survival. Gilts born into smaller litters at birth tend to be heavier and consume more colostrum (de Passille and Rushen, 1989; Milligan et al., 2002; Le Dividich et al., 2005) and it has been well documented that litter size is negatively related to a gilt's lifetime reproductive performance (Robison, 1981; Van der Steen, 1985; Bartol et al., 2006a; Bagnell et al., 2009; Bartol et al., 2009). Gilts with a birth weight under 1.1 kg, on average, have compromised growth, survival, and longevity (Magnabosco et al., 2015; Magnabosco et al., 2016). It has also been determined that a smaller body size limits the size of reproductive tract and thus the female's reproductive capacity (Flowers, 2015a). Therefore, heavier gilts at birth are likely going to be the most productive and are recommended to be selected into the breeding herd. The relationship between litter size and future productivity has been demonstrated by Flowers (2009a) who performed strategic cross-fostering at 24 hours after birth to create lactational litter sizes of <7 or >10 from litters that all had ≥10 animals at birth. Following 6 parities, 35% of the sows raised in small litters remained in the herd as opposed to 17% of the those raised in large litters. It was also observed that sows who received boar exposure at 140 days of age and were from a small litter had 13.5 more pigs after 6 parities than large litter sows who also received boar exposure at 140 days. In another study conducted by Flowers (2015b), piglets were nursed in litters of 6 or 12. More piglets out of the smaller litters remained in the sow herd after 6 parities than animals who were raised in the litters of 12 (Flowers, 2015b). This data suggests that reduced competition at the udder for milk throughout lactation

results in increased pre-weaning growth and positive effects on reproductive development into adulthood.

It is possible to quantitatively estimate colostrum intake based on immmunocrit, the measure of total immunoglobulins present in serum. In a study conducted by Vallet et al. (2015b) immunocrit ratio at 24 h had a 41% predictive ability for age at first estrus with higher immunocrit levels being associated with an earlier age at puberty. However, immunocrit has not been established as a biomarker of fertility which could be due, in part, to the labor associated with blood sampling of piglets and variation seen in immunocrit ratio across studies (Peters et al., 2016).

1.5.6 Genomic Markers of Fertility in Gilts

Because our current process of selecting breeding females is inefficient, efforts have been made at the molecular level to understand the critical physiology controlling female fertility as well as ways to improve replacement gilt selection. As of today, there have been a total of 306 SNPs identified as being related fertility, with 218 SNP related to total number born, and 88 SNPs related to number born alive (Bakoev et al., 2020). Many of these have been associated with chromosomal regions in candidate genes for fertility already identified.

A genome-wide association study (GWAS) conducted by Nonneman et al. (2014) evaluated the genome of 91 gilts that failed to display estrus by 240 days and 127 pubertal littermates using the Illumina Porcine SNP60 Beadchip. Twelve SNPs were significantly related to delayed age at first estrus with the most significant region associated with delayed puberty on chromosome 4 surrounding the nescient helix loop helix 2 transcription factor (NHLH2). Delayed puberty and decreased reproductive longevity have been associated with the targeted deletion of NHLH2 in mice (Johnson et al., 2004), and has been associated with processes related to regulating

energy expenditure, body mass, voluntary physical activity, mating behavior and reproductive longevity.

Other studies have evaluated the relationship between polymorphisms and reproductive traits. Wang et al. (2015) performed a GWAS on DNA extracted from ear tissue collected from 22 Tongcheng pigs which are an indigenous Chinese breed known for large litter sizes. The genome of the Tongcheng was then compared to seven wild Chinese boars, 22 Chinese domestic breeds, and 25 European domestic breeds. The allele frequency for the synonymous substitution in ESR1 was greater than 0.5 in the Tongcheng, Meishan, Jinhua, Neijiang, and Large white pigs. The SNP in ESR1 was not present in Landrace, Durocs, or European wild boars. Another study evaluating SNPs and their relationship to litter size showed a relationship between ESR1 and retinol-binding protein 4 (RBP4) (Mencik et al., 2019). Genotyping in 101 sows of the Topigs 20 line (Landrace x Large White) revealed that total born and number born alive across all parities were greater in sows who were homozygous (AA) for the ESR1 SNP. However, in first parity sows, homozygous individuals for the RBP4 SNP had a higher total number born. When sows reached their second parity, ESR1 was more influential in regard to number weaned and number born alive. Therefore, knowing how the alleles of ESR1 and RBP4 affect the breeding herd creates potential for genetic progress in pig production.

Although GWAS has a lot of promise for fertility detection methods, especially for detection prior to puberty, it is important to note that the statistical methods used to analyze GWAS data influences associations and their significance. Currently, there is no single “best” method to conduct GWAS data analysis and therefore, the best method for analyzing the genome for associations needs to be confirmed before validation for selection decisions can take place (Guo et al., 2017; Wen et al., 2017).

1.5.7 Metabolomic Markers of Fertility in Gilts

Circulating hormones levels have been used as potential biomarkers of fertility in females. As reviewed by Visser et al. (2012) levels of anti-Mullerian hormone (AMH) in women is used as a biomarker to detect early ovarian failure and is indicative of ovarian reserve in cattle (Hirayama et al., 2012; Batista et al., 2014), sheep (Torres-Rovira et al., 2014), and mice (Kevenaar et al., 2006). In a study that evaluated the response of serum estradiol (E2) levels after administration of human chorionic gonadotropin (hCG) in women seeking IVF as a conception method, a >10% decrease in estradiol (E2) post-hCG was indicative of reduced ovarian reserve in women (Kondapalli et al., 2012). Both E2 and AMH have been evaluated for their ability to predict fertility in gilts. Steel et al. (2018) demonstrated that gilts with higher basal E2 levels at 60 days of age have a greater probability of stillbirths in the first three parities. Higher levels of E2 on days 0, 2, and 4 after P.G. 600 administration were negatively associated with number of pigs born alive per litter. They also observed that gilts with shorter gestation lengths had a rapid decrease in AMH concentration two days after receiving P.G. 600 (Steel et al., 2018). To explore how these two hormones correlate with age at first estrus, our current best predictor of fertility, Am-In et al. (2020) evaluated AMH and E2 levels in 30 of the youngest gilts achieving puberty (169.3 ± 0.6 days), 30 of the oldest gilts (195.2 ± 0.9 days), and 18 gilts that never achieved estrus. At 90 days of age, serum AMH is negatively related to age at first estrus (r= -0.81; *P*=0.005) and was highest in the youngest gilts category (12.5 ± 1.1 ng/mL). Levels of AMH decreased with gilts who achieved puberty later and those that never achieved puberty with serum levels of 9.8 ± 1.2 and 7.6 ± 1.2 ng/mL, respectively. Additionally, estrogen levels at 90 days of age were lower in gilts that never achieved estrus (65.2 ± 6.2 pmol/L) when compared to both the oldest (89.2 ± 8.3 pmol/L) and youngest group (95.4 ± 11.2 pmol/L) (Am-In et al., 2020). Authors concluded that circulating

AMH could act as a predictor of puberty onset; however, further validation and testing across multiple genetic lines to confirm its efficacy is needed before commercial implementation.

High levels of lipid intermediates within non-adipose tissues, also known as lipotoxicity, can also be used as indicators of infertility. Lipotoxicity is associated with mitochondrial and peroxisomal dysfunction and negatively impacts fertility (Minge et al., 2008; Jungheim et al., 2010; Wu et al., 2010; Luzzo et al., 2012). Individuals with Zellweger syndrome, a condition that negatively impacts fertility, have mutations in genes that encode for peroxin (PEX) proteins and lack intact peroxisomes that can properly metabolize prostaglandins (Diczfalusy et al., 1991). Therefore, Zellweger patients can be and are characterized by elevated long chain fatty acids in non-adipose tissue. In other species, elevated levels of prostaglandin intermediates, more specifically eicosanoids, have been associated with a decrease in receptivity in cows (Belaz et al., 2016). Cows that were less receptive to pregnancy had a greater abundance of PC(40:6) and PC(40:7) in their uterine lipidome (Belaz et al., 2016). A similar trend in mice has been identified as well where perigonadal tissue from reproductively old females had a higher abundance of PC(40:6) and PC(40:7) (Dipali et al., 2019).

However, the reproductive lipidome of gilts and how it relates to fertility has been largely unexplored with the exception of preliminary work conducted by Casey et al. (2018b). As discussed in the morphologic biomarkers section above, colostrum intake is 41% predictive of age at first estrus (Vallet et al., 2015b), where age at first estrus is the most predictive marker of female fertility (Patterson et al., 2010). Casey et al. (2018b) captured lipids with vaginal swabs taken from d 14 postnatal (PN) piglets that distinguished gilts fed colostrum versus milk replacer. Gilts that received colostrum had a higher abundance of a glycerolipid containing arachidonic acid (C20:4)

which suggested a potential biomarker for colostrum intake. Therefore, the evaluation of the vaginal lipidome of gilts as it relates to true fertility has potential in identifying fertility biomarkers.

1.6 Using Emerging Technologies in Mass Spectrometry to Hunt for Fertility Biomarkers

1.6.1 Mass Spectrometer Function and Design

Biomarker discovery studies performed in Chapters 3 and 4 employed mass spectrometry. Mass spectrometry is a technology that measures mass-to-charge (m/z) ratio of ions (particles, atom or molecules with a net electrical charge) in a purified or complex mixture (De Hoffmann, 2000). There are three main components to a mass spectrometer which include an ion source, a mass analyzer, and an ion detector. Particle or molecule ionization is typically through a high-pressure system or by striking the sample with a beam of electrons from a laser. An extraction system of mass spectrometers then removes ions from the sample to send them through mass analyzer and then into the detector. The resulting data are presented as a plot of intensity, which represents relative abundance of the ion, versus m/z ratio, and is referred to as a spectrum. Spectral data is used to calculate abundance or relative abundance of the molecule. The mass-to-charge ratio measurements are utilized to calculate the exact molecular weight of the sample components, identify unknown compounds, quantify known compounds and determine molecular structure and chemical properties of unknowns (De Hoffmann, 2000).

The ionization source generates gas phase charged molecules that are introduced to the mass analyzer. There are several methods for generating gas phase ions including electrospray ionization (ESI) (Fenn et al., 1989; Fenn et al., 1990), matrix-assisted laser desorption (MALDI) (Caprioli et al., 1997), electron ionization (EI) (Kaufman et al., 1965), and chemical ionization (CI) (Munson and Field, 1966). ESI and MALDI are used for proteomic analysis. CI and EI utilize thermal

vaporization which decreases molecule stability, thus decreasing sensitivity and exploratory capabilities of the mass spectrometer that are needed for proteome analysis (Siuzdak, 2004). ESI operates by applying high amounts of pressure coupled with positive voltage to a highly purified liquid sample that contains acetonitrile or methanol to enhance evaporation (Fenn et al., 1989; Fenn et al., 1990). For ESI to be successful, samples must be free of compounds such as detergents and salts as they affect the rate of evaporation, and thus, decrease sensitivity of the machine. Additionally, ESI allows multiple charging which is a technique to increase resolution when analyzing molecules such as full proteins. Resolution of the mass spectrometer is the ability of the instrument to distinguish between ions with different m/z ratios even if their peaks are in close proximity to one another. MALDI is another ionization technique that utilizes a matrix compound that contains organic acids that are UV-light absorbing (Caprioli et al., 1997). When combined with the matrix, the acidic environment facilitates protonation of sample molecules when struck by a nitrogen laser. Although MALDI is very sensitive and more tolerant to contaminants, ESI in comparison is more ideal for biomarker discovery as it creates a higher resolution while analyzing large, unknown molecules due to its multiple charging capabilities.

The mass analyzer component separates ions based on their mass-to-charge ratios. To increase sensitivity of the machine, a second mass analyzer can be added to analyze both precursor ion (first MS) and product ion (second MS) (i.e. tandem mass spectrometry) (Dass, 2007). The product ion is produced after the precursor ion is fragmented with high energy. Ion fragmentation is usually completed in a triple quadrupole MS (QQQ). The quadrupole reduces noise and enables the capturing of more structural information based on the fragmentation pattern (Yang et al., 2002). Quadrupoles separate ions by alternating the current between DC and AC voltages and can specify ion path for ions approaching the ion detector (Dass, 2007). Alternating between DC and AC

allows for the selection of ions of interest passing through the machine which increases the specificity of the machine. The triple quadrupole separates fragmentation features through three stages: where the first (Q1) and third (Q3) quadrupoles act as a mass filters and the second (Q2) as a radio frequency-only quadrupole (Dass, 2007).

Data in tandem mass spectrometry can be acquired through four different scan modes depending on the settings placed on the triple quadrupole. These scan modes include the product ion scan, precursor ion scan, constant neutral loss scan, and selected reaction monitoring/multiple reaction monitoring The product ion scan is useful when identifying chemical structure of a specific molecule (Dass, 2007). Data obtained from the product ion scan is specific to only those product ions that are formed exclusively from a mass-selected precursor ion. The precursor ion scan provides all possible spectra produced by precursor ions that might fragment into a common product ion which can be later utilized for distinguishing between closely related compounds within a biological mixture. A third possible scan mode, constant neutral loss scan, evaluates all precursor ions that undergo neutral loss. Neutral loss occurs during ionization when precursor ions fragment into a charged and uncharged fragment (Martin et al., 2005). Specific peptides can be characterized by the neutral loss of amino acid residues which can make constant neutral loss scan mode attractive to the field of proteomics (Roepstorff and Fohlman, 1984; Cordero et al., 1993; Paizs and Suhai, 2004). Lastly, selected reaction monitoring (SRM), adjusts the mass analyzers to scan for one or more selected precursor-product ion pairs of the analyte of interest (Dass, 2007). When more than one reaction is being monitored, this is referred to as multiple reaction monitoring (MRM). Both SRM and MRM are useful for quantifying molecules in complex mixtures (Dass, 2007).

1.6.2 Shotgun Proteomics as a Method for Biomarker Discovery

Proteomic analysis aims to characterize all the proteins within a sample, thus giving a view of the current physiologic state of bodily tissues and fluids (Cox and Mann, 2007). Proteome analysis can be approached two different ways depending on desired analysis outcomes: top-down and bottom-up (shotgun sequencing). Top-down sequencing is for targeted analysis where the proteoform, the precise composition and form of a protein (Smith and Kelleher, 2013), is identified. Top-down sequencing is utilized when the specific form of the protein needs identification to answer a research question. Bottom-up or shotgun sequencing refers to the characterization of proteins by analyzing peptides (Zhang et al., 2013). In bottom-up sequencing, whole proteins are digested by a proteolytic enzyme prior to ionization. After the ion detector reads the m/z of peptides, software such as MaxQuant interprets the peaks and builds the protein based on peptides present for identification (Tyanova et al., 2016) with its own search engine known as Andromeda (Cox et al., 2011). Andromeda utilizes a probabilistic scoring method to determine which proteins are present within the sample.

After peptide sequences are determined, a process known as sequence alignment is done to compare the results of the MS to a species-specific protein database. Alignment computes the similarity between peptide sequences identified by the MS to a protein database to help determine the functionality or structure of proteins within samples (Gollery, 2005). In 2020, the *Sus scrofa* protein database from Uniprot contained 49,792 proteins (Bateman et al., 2020). Because the process of protein extraction and identification is not perfect, there are certain quality control steps put in place to increase the accuracy of protein identification. Constraints are placed on the minimum number of amino acids within a peptide sequence for protein identification, number of

missed cleavages allowed during the digestion process (Deng et al., 2018), and false discovery rate to reduce the number of false positive identifications (Korthauer et al., 2019).

Advancements in liquid chromatography-tandem mass spectrometry (LC-MS/MS) based proteomics enables the ready identification of hundreds of proteins in complex samples across magnitudes of sizes traditionally excluded from 2D gel proteome analysis (Brewis and Gadella, 2009). More specifically, reverse phase chromatography separates peptides based on hydrophobicity and is the most common separation method in proteomics as it prevents the loss of specific proteins that occurs in 2-D electrophoresis (Brewis and Gadella, 2009). However, the quality and robustness of data collected in proteomic experiments depends on sample preparation and how well biological samples are fractionated. In general, the greater the fractions and simpler the fractions of the sample the more widespread proteome coverage (Matthiesen, 2020). For example, as described by Matthiesen (2020), prior to proteome analysis samples can be fractioned by protein size or cellular component or protein solubility, or in the case of liquid chromatography, hydrophobicity. Therefore, adding liquid chromatography to tandem mass spectrometry increases the potential for identifying fertility biomarkers by increasing the machine's sensitivity and resolution. One challenge in using LC-MS/MS techniques for proteomics is that it is not inherently quantitative due to differences in molecules' size and chemical nature (Bantscheff et al., 2007). Therefore, it is necessary to compare each peptide between experiments to achieve accuracy when quantifying proteins (Bantscheff et al., 2007).

Quantitation is achieved through several ways, which can include the use of stable isotopes (Ong et al., 2002) or without isotopes. While using stable isotopes is very accurate and has been used for over 20 years in the field, it is costly, time-consuming, and only a limited number of experiments or sample types can be compared (Ong et al., 2002; Bantscheff et al., 2007). Label-

Free Quantitation (LFQ) enables relative quantification of proteins between samples without the use of isotopes. It is a technique that is compatible with any experimental system and can compare an unlimited number of samples and detect more proteins than isotope labeling. These advantages make LFQ desirable for biomarker discovery as it increases exploratory potential in an experiment (Bantscheff et al., 2007; Kumar et al., 2020).

Following protein quantitation and identification, software tools can be used to evaluate and visualize proteome data quality using both box plot and correlation analysis tools. Samples with excellent data quality will be more similar within phenotypes or treatments. For example, if comparing a healthy population versus a diseased population, individuals within the healthy population should be highly correlated to each other with a low amount of variation. Following quality control checkpoints, differential expression is determined using the LFQ intensity obtained from MaxQuant. A log 2 transformation is placed on the LFQ values to normalize the data and then an analysis of variance (ANOVA) and t-test determines differential expression. Differentially expressed proteins can be analyzed for biological function through bioinformatic tools such as the Database for Annotation, Visualization, and Integrated Discovery (DAVID) for biological interpretation of MS output (Huang et al., 2009). DAVID provides a wide-ranging set of functional annotation tools that identify enriched biological gene ontology groups. In addition to DAVID, Ensemble Biomarts can be used to help identify proteins that are not yet annotated in the swine proteome, but are annotated in humans (Kinsella et al., 2011). The process of comparing unannotated swine proteins to annotated human proteins increases the number of identifiable proteins within a sample. Another online tool utilized for biological interpretation is PANTHER. PANTHER is part of the gene ontology (GO) consortium and therefore is more up to date on GO data, allows users to incorporate genome data, and allows for ortholog prediction with the inclusion

of phylogenetic trees (Mi et al., 2013). However, PANTHER does not compare data outside of its own database, which can limit the potential of pathway analysis identification.

1.6.3 Multiple Reaction Monitoring Profiling as a Method for Biomarker Discovery

The lipidome represents the sum of biochemical processes within the cell related to lipids and is highly susceptible to changes in physiological, pathological, and environmental conditions (Watson, 2006; Aldana et al., 2020). Therefore, evaluating the lipidome is an attractive approach for biomarker discovery reflecting differences in phenotype. The use of multiple reaction monitoring-profiling (MRM profiling) methods of lipids and small molecules for biomarker discovery based on chemical functionalgroups was developed by researchers at Purdue University (Ferreira et al., 2016; Cordeiro et al., 2017; Xie et al., 2019; Xie et al., 2021). The basis of this approach is that although most of the estimated 75,000 estimated metabolites in a cell have not been annotated, their chemical classes are well known.

MRM profiling is typically completed in a two-step process starting with a discovery phase and followed by a screening phase. The discovery phase evaluates the composition of molecules in pooled samples to identify different fragmentation features related to chemical classes present in the sample. Once functional groups are identified pooled representative samples, individual samples are screened in the second step the second step of MRM profiling organized into methods of closely related functional groups. The screening stage of MRM profiling interrogates each individual sample for targeted chemical functional groups and classes identified within the discovery phase and can be accomplished in a matter of a few minutes.

MRM profiling combines the precursor and neutral loss scan to identify lipid classes (Yannell et al., 2018). The precursor ion can be detected when a specific lipid class fragments and

gives off a charge. Detection of this ion can help identify the lipid class—the neutral loss ion results when a fragment has no charge. The neutral loss fragment shows loss of mass as opposed to a charge and is characteristic of certain classes of lipids, chemical functionalities, and metabolites. Combining neutral loss and precursor ion increases the ability to identify molecules by chemical class and mass.

Once molecular profiles are determined within samples, data are analyzed using univariate and multivariate statistical approaches as well as for potential biomarkers that distinguish between phenotypic groups or treatments. MetaboAnalyst is a user-friendly metabolomics data analysis interface written in R that can perform basic statistical analysis, hierarchical cluster analysis, biomarker analysis, and pathway analysis (Pang et al., 2020). After determining relative ion intensity following both the discovery and screening phase, data are uploaded to MetaboAnalyst to determine if groups can be distinguished through univariate (fold change, t-test), multivariate (principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA)), and clustering analysis (heatmap).

Principal component analysis (PCA) is an unsupervised method that seeks to best explain the variation in the data without assigning or assuming treatment of each group. If there are relatively large differences in lipid profiles between phenotypes or treatments, there will be clear separation between groups in PCA. If there is a high degree of similarity between samples or treatment groups, PLS-DA can be used as an alternative method to explain variance and can handle high levels of noise (Wold et al., 2001; Brereton and Lloyd, 2014; Lualdi and Fasano, 2019). PLS-DA is a supervised method, which means the model "knows" the treatment or phenotype and uses multivariate regression to predict where a sample might belong based on its lipidome profile. PLS-DA is more sensitive in its separation of groups and therefore, should be run and reported in tandem

with PCA to obtain an accurate conclusion from the data (Gromski et al., 2015). Another way to visualize data is to perform hierarchical cluster analysis where the similarity of samples can be evaluated (Eisen et al., 1998; Boccard et al., 2010; Caesar et al., 2018). In MetaboAnalyst, one can select the number of top distinguishing lipids based on their p-value determined in the t-test at the start of data analysis.

In addition to statistical analysis, MetaboAnalyst has biomarker analysis capabilities through classical receiver operator characteristic (ROC) curve analysis. ROC curve analysis evaluates the performance of a single feature such as a metabolite or a biomarker using area under the curve (AUC) and summarizes the sensitivity and specificity of a single metabolite or lipid. AUC of ROC analysis is a measure of the usefulness of a test, in terms of sensitivity and specificity, where an AUC closer to 1.0 indicates a more useful test (Faraggi et al., 2003).

An advantage of MRM profiling is that it is a quick, and cost-effective identification method and is the most sensitive in MS. By evaluating one parent ion, one product ion, and one mass unit increases mass detection resolution and sensitivity. MRM profiling is particularly excellent at distinguishing between healthy and diseased samples or between two distinct treatment groups. Biomarkers for polycystic ovary syndrome (PCOS) have been identified in follicular fluid using MRM profiling (Cordeiro et al., 2017). In a previous study, follicular fluid of women with PCOS were compared to control individuals which resulted in the identification of a lipidome signature that was comprised of differences in phosphatidylglycerol and sphingolipids (Cordeiro et al., 2015). With this information, Cordeiro et al. (2017) successfully identified over 70% of the samples in accordance to the clinical diagnosis using the selected biomarkers.

Exploratory work conducted by Casey et al. (2018a) revealed that lipids captured with vaginal swabs taken from d 14 PN piglets could be distinguished and measured using MRM

profiling between gilts fed colostrum versus milk replacer. The group successfully detected differences in the vaginal lipidome of gilts who suckled colostrum and those that were fed milk replacer the first 48 h postnatal. Gilts that received colostrum had a higher abundance of a glycerolipid containing arachidonic acid (C20:4) which suggested a potential biomarker for colostrum intake. Harlow et al. (2019a) further explored the efficacy of using MRM profiling in a follow-up study and determined that the vaginal lipidome of gilts on d 2 PN is reflective and altered by nutrition and perinatal environment. Gilts were assigned to one of four treatments: suckled, suckled plus fat supplement, bottle-fed with milk-replacer, and bottle-fed plus fat supplement. Receiver operating characteristic (ROC) curve analysis identified 18 lipids that distinguished between colostrum versus milk replacer with the most excellent candidate markers including triacylglycerols, phosphatidylethanolamine lipids, phosphatidylcholine lipids, phosphatidylglycerol lipids, and an acylcarnitine. The lipidome of piglets supplemented with and without fat was distinguished by 15 MRMs where fat-supplemented piglets had a higher relative ion intensity for cholesterol ester. These findings indicate that perinatal nutrition affects tissue composition and therefore, may affect reproductive tract development and fertility. However, this work also highlights the need to identify biomarkers of colostrum intake specifically related to fertility.

1.7 Summary

The inclusion of subfertile and infertile animals into the breeding program decreases economic returns and farm efficiency. Currently, there is no single way to predict fertility in boars and gilts, and thus more research is warranted in the area of biomarker discovery as it relates to infertility in swine. It has also been demonstrated that early developmental periods can impact the

developmental trajectory of the animal, therefore, impacting reproductive success. Therefore, the purposes of these experiments are to identify biomarkers predictive of subfertility and infertility in swine and a link between perinatal nutrition and long-term fertility.

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CHAPTER 2. SHOTGUN PROTEOME ANALYSIS OF SEMINAL PLASMA DIFFERENTIATE BOARS BY REPRODUCTIVE PERFORAMNCE.

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2.1 Abstract

There is a need to identify subfertile boars before they enter into the breeding herd. Seminal plasma proteins are essential for normal sperm function and transport and play an important role in fertilization. The objective of this study was to use liquid chromatography tandem mass spectrometry for shotgun proteome analysis to investigate whether differences in boar fertility phenotype can be differentiated by seminal plasma protein abundance. Following 50 single-sire breedings, boars were categorized into one of four phenotypes: high farrowing rate and total born (HFHB; n=9), high farrowing rate with low total born (HFLB; n=10), low farrowing rate and low total born (LFLB; n=9), and low farrowing rate with high total born (LFHB; n=4) that were distinct ($p<0.05$) from each other by these variables. There were 506 proteins measured in at least one sample across all animals. There were 245 high confidence proteins and 56 were differentially abundant between the high fertility phenotype (HFHB) and at least one of the three subfertile groups. Findings support that seminal plasma protein profiles are distinct between boars with different fertility phenotypes.

2.2 Introduction

Boars used for breeding stock are selected for progeny performance traits such as feed efficiency, growth rate, and carcass characteristics with little emphasis placed on male fertility (Oh et al., 2006b; Safranski, 2008). Boars with suboptimal reproductive performance contribute to variability in total piglets born per litter and farrowing rates of sows on commercial farms (Ruiz-Sánchez et al., 2006; Roca et al., 2015). There is thus a need to identify subfertile boars before they enter the breeding program.

Currently there is no single test of a boar's fertility. However, several semen traits have been correlated with fertility such as sperm motility and morphology (Flowers, 1997; Xu et al., 1998; Ruiz-Sánchez et al., 2006; Flowers, 2008; Knox et al., 2008). When an ejaculate falls below established thresholds for motility and morphology, fertility is compromised (Flowers, 1997, 2013). However, ejaculates that meet thresholds indicative of good quality can still result in subfertility due to reasons which are not associated with sperm motility or morphology (Flowers, 1997, 2013). Inclusion of subfertile boars in current breeding programs results in a loss of production efficiency for the farm and has negative economic consequences for producers (Roca et al., 2015).

Seminal plasma proteins are essential for normal sperm function and fertility, as they provide nutritive support and a protective environment for sperm. Analysis of seminal plasma proteins using 2D gel proteome approaches found significant differences between subfertile and high fertility boars (Flowers, 2001, 2009b; Flowers et al., 2013; Flowers et al., 2016), suggesting that seminal plasma proteomes reflect reproductive performance of boars. However, due in part to the labor-intensive nature of protein identification, 2D gel proteome analysis studies were limited in their ability to reveal proteins reflective of fertility. Recent advances in liquid chromatography

tandem mass spectrometry (LC-MS/MS) based proteomics enables ready identification of hundreds of proteins in complex samples across magnitudes of sizes traditionally excluded from 2D gel proteome analysis (Brewis and Gadella, 2009). Therefore, it is likely that LC-MS/MS has a greater potential for identifying fertility biomarkers in seminal plasma. Others recently tested the efficacy of using LC-MS/MS analysis of seminal plasma proteome to identify biomarkers indicative of extremes for litter size or farrowing rate using pooled samples that were collected quarterly over a year (Pérez-Patiño et al., 2018). They identified 11 proteins differentially abundant for farrowing rate and four proteins for litter size (Pérez-Patiño et al., 2018). However, in order for biomarkers to be useful to the swine industry they need to identify sub-fertile boars before animals enter the breeding program. Therefore, the objective of this study was to use shotgun LC-MS/MS proteome analysis to investigate whether differences in seminal plasma protein abundance patterns differentiated boars by fertility phenotype at their first acceptable ejaculate. Further, we investigated protein profiles of four distinct fertility phenotypes, by analyzing proteome profiles of 32 commercial boars with similar genetics assigned to one of four fertility phenotypes based on percent farrowing rate and total born across 50 individual artificial inseminations of sows.

2.3 Materials and Methods

2.3.1 Animals

Ejaculates were collected from Duroc boars (9-11 mo. of age) of similar genetics at a commercial boar stud and evaluated for motility and morphology using standard semen collection protocols. Briefly, boars were moved into a collection crate where preputial fluid was evacuated using a double gloved hand. The sheath area and extended penis were cleaned and dried with a

paper towel. The outer glove was removed and using the clean gloved hand the penis was placed into the artificial cervix (AC). Once the boar was fully extended and locked the AC was put into the clamp on the BoarMatic. The gel fraction and pre-sperm faction were collected into an inner bag that was detached and discarded prior to collecting the sperm-rich and post-sperm fractions, hands free in the collection mug. Following collection, the filter was removed from the collection bag. The bag was closed by using a band and sent to the laboratory via pneumatic tube. Sperm motility and morphology estimates needed to be at least 75% motile and normal in order for boars to enter the breeding program. Approximately 2 mL of whole semen (gel fraction removed) from the first ejaculate that met acceptance criteria for use in the breeding program was packed with ice packs and shipped to Purdue University overnight. Upon arrival to Purdue, semen samples were centrifuged at 4ºC and 2700 g for 20 minutes to pellet sperm. Seminal plasma was removed after centrifugation and stored at -20º C until protein isolation.

Of the 385 total ejaculates received, semen samples from 110 boars had data on farrowing rate and litter born of at least 50 breedings. Fifty single-sire breedings were used to select boars for this study, as data from 50 breedings is needed to reduce female influence on boar fertility phenotype (Clark et al., 1989). All breedings were done as a single, fixed-time insemination using post-cervical artificial insemination (PCAI). All ejaculates were cooled and used by day 3 following collection for PCAI. A total of three sow farms were used to collect boar fertility data. As a measure of fertility, an average farrowing rate and total born was calculated and plotted for 128 boars (Figure 2.1). From scatter plots, four quadrants were created to divide boars that fell above or below mean farrowing rate and mean total born. Thirty-two out of the 110 boars with both semen samples and fertility data were selected to study based on extremes for: high farrowing rate and high total born (HFHB; n=9), high farrowing rate and low total born (HFLB; n=10), low

farrowing rate and high total born (LFHB; n=4), and low farrowing rate and low total born (LFLB; n=9). Boars identified for study were two standard deviations from the mean for each phenotype (Table 2.1). A fertility index was created to identify differences between fertility phenotype means by multiplying farrowing rate and total born within fertility phenotype. Post-hoc Tukey's test confirmed four distinct phenotypes (Table 2.1).

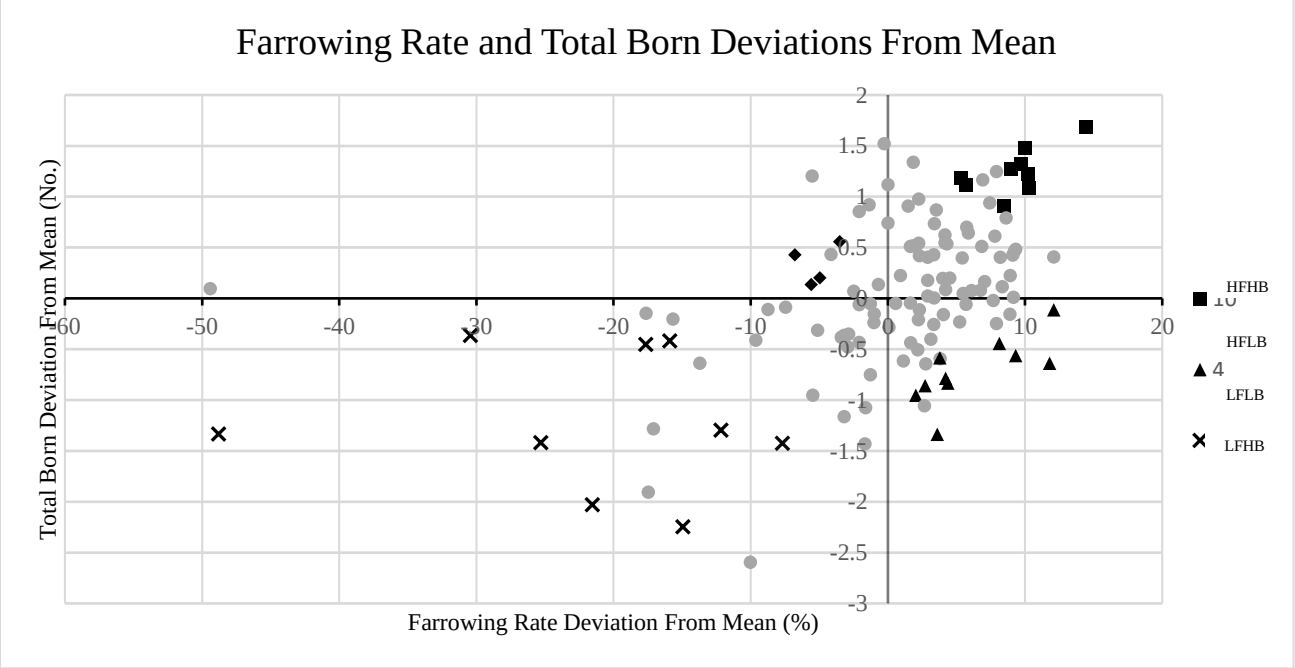

Figure 2.1 Farrowing rate and total born data used to create boar fertility index.

Four quadrants were created from scatterplots of the 128 boars with fertility data in order to divide and identify boars who fell above or below mean Farrowing Rate and mean Total Born. Thirty-two out of 110 boars with both semen samples and fertility data from 50 single sire matings were selected to the study from the quadrants based on extremes for Farrowing Rate and Total Born. Boars were categorized into one of four phenotypes: high farrowing rate and high total born (HFHB; n=9), high farrowing rate and low total born (HFLB; n=10), low farrowing rate and high total born (LFHB; n=4), and low farrowing rate and low total born (LFLB; n=9).

Table 2.1 Means and ranges of farrowing rate and total born for each phenotype and Tukey's post-hoc comparisons for total born, farrowing rate, and fertility index

Parameters	HFHB		HFLB		LFHB		LFLB		Overall Effect	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	SE	*p*-value
Total Born	14.78[a]	14.43-15.21	12.81[b]	12.19-13.41	13.86[c]	13.66-14.08	12.38[b]	11.28-13.16	0.433	<0.001
Farrowing Rate, %	86.33[a]	82.40-91.54	83.31[a]	79.13-89.19	71.89[b]	70.33-73.58	55.33[c]	28.30-69.42	7.070	<0.001
Fertility Index (FRxTB)	1276[a]	1212-1392	1068[b]	984-1196	996[b]	977-1036	682[c]	345-840	95.82	<0.001

Thirty-two boars with fertility data and semen samples were enrolled in the study. The mean and range for each parameter is given in the table above. An ANOVA was conducted to determine if variable was different by phenotype (*p*-value), followed by post-hoc Tukey's comparisons, with differences between each phenotype indicated by differences in superscript letters (a, b, c). A fertility index was created by multiplying the average farrowing rate and total born (FRxTB) for each boar enrolled in the study.

2.3.2 Proteomic Analysis

Protein extraction and LC-MS sample preparation

All proteomic sample preparation and analysis was completed at the Proteomics Facility in the Bindley Bioscience Center at Purdue University. Seminal plasma samples were thawed on ice then vortexed and centrifuged at 14,000 rpm for 15 min at 4º C. After centrifugation, the supernatant was removed, and 4 volume of cold acetone (-20º C) was added to precipitate proteins by incubating overnight at -20º C. Proteins were isolated by centrifugation at 14,000 rpm for 15 min at 4º C, the supernatant was removed, and the pellet was resolubilized in 20 µl of 8 M urea. Samples were incubated for 1 hour at room temperature until the pellet was completely dissolved and centrifuged for 15 min at 14,000 rpm. A bicinchoninic acid (BCA) assay using BSA as a standard was used to determine protein concentration. One hundred µg of protein from each sample were incubated in 10 mM of dithiothreitol (DTT) for 45 min at 50º C, and then cysteine alkylated by incubating for 45 min at RT in the dark in presence of 20 mM iodoacetamide (IAA); followed by an additional 20 min incubation with 5 mM DTT at 37˚C. A trypsin digestion was completed overnight using sequencing grade trypsin and Lys-C mix form Promega at a 1:25 (w/w) enzyme-to-protein ratio at 37˚ C. Peptides were cleaned with C18 micro spin columns (The Nest Group Inc., Southborough, MA) according to the manufacturer's protocol and eluted using 80% acetonitrile containing 0.1% formic acid (FA). Samples were vacuum dried and re-suspended in 3% acetonitrile and 0.1% FA. Peptide concentration was determined by BCA assay using BSA as a standard and adjusted to 0.2 µg/µL with the soluble and insoluble fractions being mixed together. Five µL was used for LC-MS/MS analysis.

LC-MS/MS data acquisition

Standard LC-MS/MS data acquisition protocols were used as previously described (Harlow et al., 2018). Briefly, reverse-phase LC-ESI-MS/MS analysis of samples was performed using the Dionex UltiMate 3000 RSLC nano System in conjunction with the Q Exactive High Field (HF) Hybrid Quadrupole Orbitrap MS and a Nano-electrospray Flex ion source (Thermo Fisher Scientific, Waltham, MA). Peptides were loaded into a trap column packed with PepMap C18 medium and then separated on a reverse phase column packed with PepMap C18 silica (Thermo Fisher Scientific). All LC-MS/MS measurements were taken in the positive ion mode using 120 min LC gradient. Mass spectrometer operation was done using the standard data-dependent mode with a Top20 MS/MS scan method. To avoid repeated scanning of identical peptides, dynamic exclusion was set at 15 s. Calibration of the instrument was done at the beginning of the batch run using calibration mix solution (Thermo Fisher Scientific). All samples were run in the same batch. Instrument performance was monitored routinely by utilizing an *E. coli* digest purchased from Sigma (St. Louis, MO).

Data and statistical analysis

Standard protein identification and database search protocols were used (Harlow et al., 2018). Raw LC-MS/MS data were analyzed using MaxQuant software (v.1.6.0.16) with peptides being identified with its integrated Andromeda search engine (Huang et al., 2009; Kinsella et al., 2011; Consortium, 2018). Protein identification was completed by comparing the results of the MS/MS spectra against the *Sus scrofa* protein database downloaded from Uniprot on August 21, 2018 containing 40,707 protein sequences. For the database searches, a minimum of 6 amino acids was required for peptide identification. The number of missed cleavages was limited to two for the

database search with the enzyme specificity for trypsin and Lys-C. Methionine oxidation (M) and carbamidomethylation of cysteine (C) were defined as a variable modification and a fixed modification for database searches, respectively. False discovery rate (FDR) was set at 0.01 for both proteins and peptides. Proteins were quantified using 'unique plus razor peptides. Peptide quantitation was performed using label free quantitation (LFQ) intensities were used to determine relative protein abundances across all samples. All proteins matching to reverse database were filtered out. Proteins were considered as true identification if they match to at least two peptides (unique, razor or both), and have LFQ $\neq 0$ and a minimum of $\geq$2 MS/MS (spectral) counts. Proteins identified by only shared peptides were reported as a protein group.

Bioinformatic analysis

InfernoRDN software tools were used to evaluate and visualize proteome data quality using box plot and correlation analysis tools. To identify differentially abundant proteins statistical analysis was performed in R using an in-house script that first extracts the LFQ intensity from MaxQuant file, then applies the log 2 transformation to the LFQ values for the analysis of variance (ANOVA) and t-test to determine differential abundance between boar phenotypes. Significance was defined as $P \leq 0.05$. Functional annotation analysis was completed with DAVID Bioinformatics Resources version 6.8 as described in conjunction with Ensembl BioMarts (Huang et al., 2008; Kinsella et al., 2011; Consortium, 2018). Descriptions of protein function within this manuscript were obtained from the UniProt Knowledgebase in conjunction with GeneCards (Stelzer et al., 2016a; Consortium, 2018).

2.4 Results

2.4.1 Characterization of Seminal Plasma Proteome

Seminal plasma proteomes of the HFHB phenotype showed the highest correlation among samples. Across all phenotypes 506 seminal plasma (SP) proteins were identified with 436 of them being classified into protein groups in at least one animal. A protein was defined as being present within a group when at least 3 animals within a phenotype had LFQ values for that protein. After placing this selection criterion on the data, there were 245 total proteins considered representative of at least one phenotype, with 190 of those being commonly present across all phenotypes (Figure 2.2).

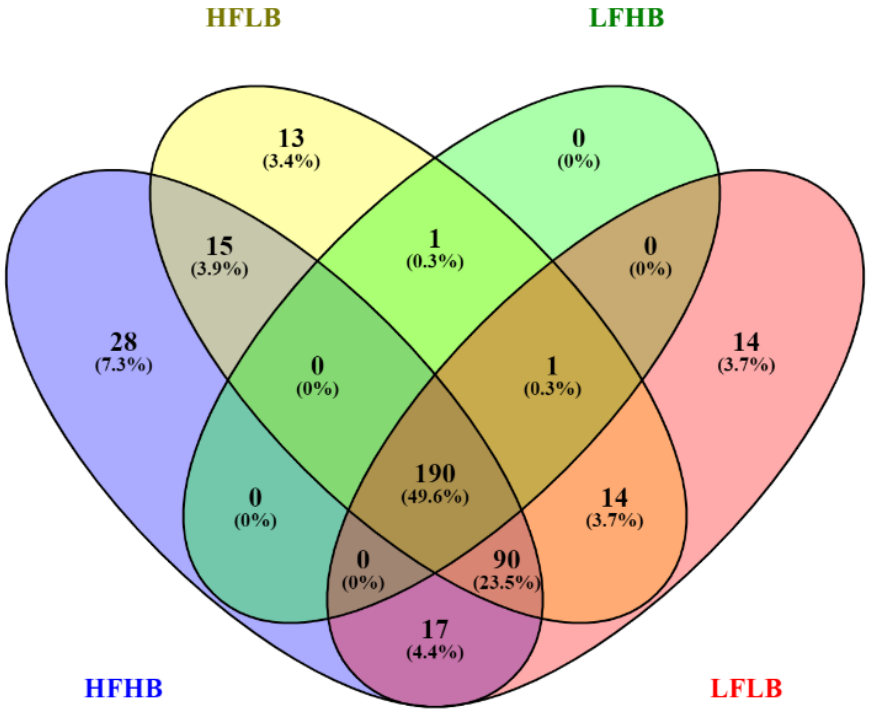

Figure 2.2 Venn Diagram of 245 proteins considered expressed by at least one phenotype (n ≥ 3 boars per phenotype) across all fertility groups.

One hundred ninety proteins were commonly expressed across all phenotypes.

When the 190 common proteins were submitted for functional annotation analysis in DAVID using *Sus scrofa* Uniprot protein accession IDs, 116 IDS were returned. To maintain the robustness of the data set, gene names of the 190 common proteins were submitted to BioMart and converted to human equivalent Ensembl gene IDs. BioMart successfully converted 185 total proteins with 157 of those being unique. Proteins not converted to human ENSEMBL gene IDs were pig-specific and were hand annotated or were unidentified proteins. Functional annotation analysis of the 157 human ENSEMBL IDs grouped 92 percent into biological process (BP) gene ontology (GO) terms, 98 percent into cellular component (CC) GO terms, 88 percent into molecular function (MF) GO terms, and 49 percent of proteins into KEGG pathways.

Approximately 15 percent of proteins enriched BP GO term *proteolysis* and included complement factor I (CFI), lactotransferrin (LTF), dipeptidyl peptidase 7 (DPP7), and ADAM metallopeptidase 2, 21, and 28 (Table 2.2). Proteins categorized as *binding of sperm to zona pellucida* included heat shock protein 70 (Hsp70), acrosin (ACR), and zona pellucida binding protein (ZPBP). Within the cellular compartment (CC) GO term, 71 % of proteins were characterized as extracellular exosome (P=4.1E^{-55}) and 50 % as extracellular space (P=3.2E^{-46}). Proteins within the extracellular exosome and extracellular space included antimicrobial as well as immune-related proteins, such as LTF, complement 3 (C3), CFI, complement factor D (CFD), and H (CFH). The *lysosomal* KEGG pathways was enriched with 14 % of the proteins commonly present across the phenotypes (P=9.9E^{-19}). Some of lysosomal proteins identified were cathepsin B, C, D, and V, aspartylglucosaminidase (AGA), tripeptidyl peptidase, glucosylceramidase beta (GBA), and galactosidase beta 1 (GLB1). The KEGG pathway *other glycan degradation* was enriched with 6 % of common proteins and included mannosidase alpha class 2B member 1 and 2

(MAN2B1; MAN2B2), hexosaminidase subunit beta (HEXB), and neuraminidase 1 (NEU1) (P=6.8E^{-12}). In addition, 4% of proteins enriched the KEGG pathway *complement and coagulation cascades* (P=9.9E^{-4}).

Table 2.2 Representative DAVID functional annotation categories enriched by proteins common across all phenotypes

Gene Ontology/KEGG Pathways	%	P-value	Gene Symbol
Biological Process: proteolysis	15.0	1.7E-10	AGA, C3, PRSS22, PARK7, GGH, CF1, NAALAD2, CFD, ADAM21, CTSD, MMP7, CTSB, TPP1, MMP2, PRSS8, CTSC, CTSA, MME, LTF, DPP7, CPQ, ADAM2, ADAM28
Biological Process: binding of sperm to zona pellucida	5.23	1.3E-08	ARSA, GLIPR1L1, ACR, ZPBP, CRISP1, ADAM2, Hsp70, HSPA1L, ADAM21
Biological Process: response to reactive oxygen species	3.92	1.7E-05	GSTP1, SOD3, PRDX5, SOD1, P4HB, GPX5,
Biological Process: single fertilization	3.92	1.3E-04	ACR, ADAM2, PARK7, ADAM21, MFGE8, RNASE10
Cellular Component: extracellular exosome	71.2	4.1E-55	LTF, ACPP, AGA, HSP70, HSPA5, CP, HEXB, PRSS8, ITIH4, GDI2, CTSA, ATP6AP1, ENO1, ACTB, GSTP1, CD59, AKR1B1, PEBP1, TF, EZR, CRISP3, GGT1, ARSA, CST3, TIMP1, CPQ, ASAH1, MAN2B1, NUCB1, GPI, NAGLU, CPE, SOD3, CTSC, HPX, CUTA, EFEMP1, FN1, QPCT, QSOX1, PARK7, CTSD, C3, PRDX5, EGF, MFGE8, GALNS, SOD1, AZGP1, ZG16B, ALP, PIGR, ALB, EDIL3, PGAM2, CTSB, DEFB1, YWHAZ TPP1, B2M, PPIB, EFNA1, GP2, GLB1, PGK2, CST6, BASP1, DPP7, GBA, COL18A1, PROS1,TCN2, P4HB, MME, GM2A, FAM3C, PSAP, CFD, S100A6, NAGA, NEU1,CFI, HEXA, ANG, RNASE4, FCGBP
Cellular Component: extracellular space	50.3	3.2E-46	CFH, FUCA2, LTF, ACPP, RNASET2, GRN, TIMP2, AGA, CP, HEXB, PRSS8, ENO1, ACTB, GSTP1, CD59, AKR1B1, MMP2, TF, EZR, CRISP3, GGT1, ARSA, CST3, TIMP1, CPQ, ASAH1,

			MAN2B1, NUCB1, GPI, CPE, SOD3, CTSC, HPX, EFEMP1, FN1, QSOX1, CTSD, PGF, CRISP1, C3, PRDX5, SPINK2, GOLM1, SERPINE2, ITM2B, CTSV, GGH, MMP7, EGF, MFGE8, SOD1, DEFB1, YWHAZ, B2M, GLB1, CST6, GBA, COL18A1, PROS1, TCN2, GCNT1, PSAP, CFD, CFI, ANG, GPX5, MSMB
KEGG Pathways: Lysosome	13.7	9.9E-19	AGA, HEXB, CTSA, ATP6AP1, ARSA, ASAH1, MAN2B1, NAGLU, CTSC, CTSD, CTSV, GALNS, CTSB, TPP1, GLB1, GBA, GM2A, PSAP, NAGA, NEU1, HEXA
KEGG Pathways: Other glycan degradation	5.88	6.8E-12	FUCA2, MAN2B2, AGA, HEXB, MAN2B1, GLB1, GBA, NEU1, HEXA
KEGG Pathways: Complement and coagulation cascades	3.92	9.9E-04	CFH, CD59, C3, PROS1, CFD, CFI

2.4.2 Subfertile Phenotypes Show Differential Abundance of Seminal Plasma Proteins

Thirty proteins were differentially abundant (P <0.05 and $\log_2$ fold change of at least ±0.5) between LFLB and HFHB, whereas HFLB and LFHB groups had 26 and 20 proteins, respectively, differently abundant from the HFHB group (Figure 2.3). The difference of abundance of several proteins between the high fertility group (HFHB) was similar between some of the subfertile groups. For example, Inositol-1-monophosphatase (IMPA1) was less abundant in all three subfertile phenotypes compared with HFHB. Whereas, ACTB and CTBS were more abundant, and AGA, FOLR2, PPT1 and UBB were less abundant in HFLB and LFHB versus HBLB. FN and MDH1 were less abundant in LFLB and HFLB compared to highest fertility group (HFHB).

Gene Names	Protein Names	Log$_2$ Fold Change LFLB-HFHB	HFLB-HFHB	LFHB-HFHB
A2M	Alpha-2-macroglobulin	1.22	-	-
ACPP	Acid phosphatase, prostate	-	-	-0.51
ACTB	Actin; cytoplasmic 1	-	1.55	2.19
AGA	N(4)-(Beta-N-acetylglucosaminyl)-L-asparaginase	-	-1.69	-2.41
ALDOA	Fructose-bisphosphate aldolase (EC 4.1.2.13)	-	-1.66	-
ALPL	Alkaline phosphatase (EC 3.1.3.1)	0.48	-	-
ASRGL1	asparaginase like 1	-	0.51	-
AWN	Spermadhesin AWN	-	-	0.57
B4GALT1	Beta-1,4-galactosyltransferase 1	-	-	0.85
BSG	CD147	0.61	-	-
BSP1	Seminal plasma protein pB1 (Protein DQH) (pAIF-1)	-	-	0.67
C3	Complement C3	-	-	-1.52
CFI	Complement Factor I	-	-	-6.59
CTBS	chitobiase	-	0.78	0.66
CTSF	Cathepsin F	-	-0.60	-
CTSS	Cathepsin S	0.56	-	-
DEFB112	Beta-defensin	-	0.52	-
DYNLL2	Dynein light chain	1.19	-	-
FAM3D	Family with sequence similarity 3 member D	0.69	-	-
FN	Fibronectin (Fragment)	-1.02	-1.05	-
FOLR2	Folate binding protein	-	-0.68	-0.88
GALNT1	Polypeptide N-acetylgalactosaminyltransferase	-0.50	-	-
GALNT6	Polypeptide N-acetylgalactosaminyltransferase	-0.48	-	-
GOT1	Aspartate aminotransferase; cytoplasmic	-	-1.15	-
GP2	Pancreatic secretory granule membrane major glycoprotein GP2 isoform 1	0.95	-	-
GPX5	Epididymal secretory glutathione peroxidase	0.57	-	-
GPX6	Glutathione peroxidase 6	-1.34	-	-
HBA	Hemoglobin subunit alpha	1.69	-	-
HBB	Hemoglobin subunit beta	1.77	-	-
HPX	Hemopexin (Hyaluronidase)	-	-	-0.78
IMPA1	Inositol-1-monophosphatase	-0.87	-1.06	-1.28

Figure 2.3 Relative log2 fold changes expressed by boars of different phenotypes.

Figure 2.3 Continued

Gene Names	Protein Names	Log$_2$ Fold Change		
		LFLB-HFHB	HFLB-HFHB	LFHB-HFHB
ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4	-	1.52	-
LOC106504547	serpin A3-8	-	-0.70	-
MAN2B2	Epididymis-specific alpha-mannosidase	-	-	-1.44
MDH1	Malate dehydrogenase; cytoplasmic (EC 1.1.1.37) (Cytosolic malate dehydrogenase)	-0.62	-0.59	-
MFGE8	Milk fat globule-EGF factor 8 protein	0.55	-	-
MIF	Macrophage migration inhibitory factor (Fragment)	0.52	-	-
MPZL1	Myelin protein zero like	-	-	-0.76
MSMB	Beta-microseminoprotein	-	0.55	-
MSMB PSP94	Beta-microseminoprotein (Prostate secreted seminal plasma protein)	-	1.27	-
NAALAD2	N-acetylated alpha-linked acidic dipeptidase 2	0.48	-	-
PEBP4	Phosphatidylethanolamine-binding protein 4	0.51	-	-
PLS3	plastin 3	-	0.95	-
PPA21239	Carboxylic ester hydrolase	-	1.45	-
PPT1	palmitoyl-protein thioesterase 1	-	-0.64	-0.57
PRDX4	Peroxiredoxin 4	-	-1.24	-
PRDX6	Peroxiredoxin-6	-0.70	-	-
PROM2	Prominin 2	0.58	-	-
PRSS8	Serine protease 8	-	-	-0.62
PSAP	Saposin-B-Val	0.67	-	-
PSP-I	Porcine seminal protein I	-0.60	-	-
QSOX1	Sulfhydryl oxidase	0.48	-	-
RNASE12	Ribonuclease A family member 12	-0.55	-	-
SIL1	SIL1 nucleotide exchange factor	-	1.24	-
SMCT1	Monocarboxylic acid transporter 1	-	0.64	-
SOD1	Superoxide dismutase [Cu-Zn]	-	-	-1.79
SORT1	Sortilin	0.53	-	-
SPINK2	Serine peptidase inhibitor, Kazal type 2	-	-	-0.61
ST6GALNAC1	ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 1	0.97	-	-
TPPP2	tubulin polymerization promoting protein family member 2	-	-0.85	-
UBB	Ubiquitin B	-	-0.85	-1.28
WFDC10AL	WAP four-disulfide core domain 10A-like	0.50	-	-
WGA16	Heparin-binding protein WGA16	-	0.54	-
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	-	-1.11	-

Fifty-six proteins were differentially expressed within each phenotype. All changes are relative to the HFHB Phenotype. Values highlighted in red are upregulated within a phenotype and values highlighted in green are downregulated.

Functional annotation analysis of the 56 proteins differentially abundant from HFHB in at least one of the subfertile phenotypes returned 53 unique DAVID IDs. Fifteen percent of proteins were categorized in the BP GO term *proteolysis* including cathepsin F (CTSF), which was more abundant in HFLB and LFHB phenotypes, and cathepsin S (CTSS), which was less abundant in LFLB (Table 2.3). Complement component 3 (C3) and complement factor I (CFI) were also within the *proteolysis* ontology, and both were less abundant in LFHB relative to the high fertility phenotype. Other categories enriched with proteins differentially abundant from the high fertility group were o*xidation-reduction process* and *platelet degranulation*. Glutathione peroxidase 6 (GPX6), peroxiredoxin 4 and 6 (PRDX4; PRDX6), and superoxide dismutase 1 (SOD1) were all lower in subfertile phenotypes. Interestingly, Alpha-2 macroglobulin (A2M), a platelet degranulation protein associated with compromised sperm membranes was higher in LFLB. Alodolase, fructose-bisphosphate A (ALDOA) was less abundant in HFLB while inter-alpha-trypsin inhibitor heavy chain family member 4 (ITIH4) was more abundant in HFLB.

Table 2.3 DAVID functional analysis of the fifty-six seminal plasma proteins differentially abundant between all subfertile (HFLB, LFHB and LFLB) and high fertility (HFHB) boar phenotypes

Gene Ontology/KEGG Pathways	%	p-value	Gene Symbol
Biological Process: proteolysis	15.1	4.5E-4	NAALAD2, ASRGL1, AGA, CTSF, CTSS, C3, CFI, PRSS8
Biological Process: platelet degranulation	11.3	9.8E-6	ALDOA, A2M, ITIH4, PSAP, QSOX1, SOD1
Biological Process: oxidation-reduction process	11.3	2.4E-2	GPX5, GPX6, PRDX4, PRDX6, QSOX1, SOD1
Cellular Component: extracellular space	52.8	1.6E-17	SIL1, ACPP, ACTB, ALDOA, ALPL, AGA, B4GALT1, CTSF, CTSS, CTSBS, C3, CFI, GPX5, HPX, MIF, MDH1, MSMB, MFGE8, PPT1, PRDX4, PRDX6, PSAP, PRSS8, QSOX1, SPINK2, SOD1, YHAZ, UBB
Cellular Component: extracellular exosome	62.3	4.6E-14	ACPP, ACTB, ALDOA, ALPL, A2M, AGA, BSG, B4GALT1, CTSF, CTBS, C3 CFI, GOT1, GP2, HBB, HPX, IMPA1, ITIH4, MIF, MDH1, MAN2B2, MFGE8, PPT1, PRDX4, PRDX6, PEBP4, PROM2, PSAP, PRSS8, QSOX1, SOD1, YWHAZ, UBB
GO:0072562~blood microparticle	13.2	4.5E-6	ACTB, A2M, C3, HBB, HPX, ITIH4, YWHAZ
KEGG Pathways: Lysosome	11.3	1.8E-4	AGA, CTSF, CTSS, PPT1, PSAP, SORT1
KEGG Pathways: Complement and coagulation cascades	5.7	3.8E-2	A2M, C3, CFI
KEGG Pathways: Metabolic pathways	20.8	3.6E-2	ST6GALNAC1, ALDOA, ALPL, B4GALT1, GOT1, IMPA1, MDH1, PPT1, PRDX6, GALNT1, GALNT6

The majority of proteins were characterized within the Cellular compartment GO categories *extracellular space* and *extracellular exosome* at 53 % and 62 %, respectively. Thirteen percent of the proteins differentially abundant from the high fertility group were categorized in the cellular component GO term *blood microparticle* and included hemoglobin subunit beta (HBB), alpha-2-macroglobulin (A2M), C3, and inter-alpha-trypsin inhibitor heavy chain family member 4 (ITIH4). HBB was higher in LFLB than any other phenotype. Approximately 3 % of differentially abundant proteins enriched the KEGG pathway *complement and coagulation* and included the same proteins found in blood particle (A2MC3 and CFI). Eleven percent of the differentially abundant proteins were categorized as being part of the KEGG pathway *metabolic pathways* and included ALPL, ALDOA, GOT1, PRDX6.

2.4.3 Proteins Differentially Abundant in Fertility Parameters

Six proteins distinguished boars by farrowing rates. Boars with high farrowing rate (HFHB and HFLB) had a higher abundance of Superoxide dismutase (SOD1), Porcine seminal protein I (PSP-1), and Inositol-1-monophosphatase (IMPA1). On the contrary, boars with low farrowing rates (LFLB and LFHB) had higher abundance of hemoglobin subunit beta (HBB)and Golgi Membrane Protein 1 (GOLM1) present. Seven proteins distinguished phenotypes by total born. IMPA1, PSP-1, and folate receptor beta (FOLR2) were higher in boars with greater total born. Whereas, SOD1, Quiescin Sulfhydryl Oxidase 1 (QSOX1), Myelin Protein Zero Like 1 (MPZL1), and Actin Beta (ACTB) were greater in boars characterized as low total born (LFLB, HFLB).

2.5 Discussion

Data from at least 50 single-sire matings enabled categorization of boars into four distinct fertility phenotypes based on sire effect on farrowing rate and total born. Shotgun proteomic analysis of seminal plasma from boars in the extremes of each phenotype suggests that differences in fertility may be reflected in abundance of seminal plasma proteins. Boars in the highest fertility phenotype show the least amount of variation in their seminal plasma proteome profiles. Higher variation in the seminal plasma proteome profile of subfertile boars suggests that decreased farrowing rates and lower total born may be caused by multiple factors.

2.5.1 Swine Seminal Plasma Proteins Common Amongst Phenotypes

Functional annotation analysis of proteins common present in seminal plasma across all phenotypes indicated seminal plasma proteins function to protect sperm from oxidative stress and pathogens, with enrichment of proteins in several antioxidant and immune-related categories. Sperm are susceptible to oxidative damage from fluids within the female reproductive tract. Sperm damaged from oxidative stress were associated with pregnancy failure and embryonic loss (Sanocka et al., 1997; Aitken et al., 2003). The presence of high levels of antioxidant species such as superoxide dismutase, glutathione peroxidase, and catalase in seminal plasma function to protect sperm from oxidative stress during transit through reproductive tracts (Garrido et al., 2004; Bromfield, 2014).

Among the 190 proteins common across all phenotypes, 30% were related to the innate immune system either as an enzyme in activation pathways or components in the complement cascade. The presence of proteins such as C3, CD59 molecule, CFD, CFH, and CFI support previous research which identified immunological factors in seminal plasma (Milardi et al., 2012;

González-Cadavid et al., 2014a). Complement component 3 (C3) is secreted as a zymogen and is activated at the site of infection and induces proinflammatory responses (Janeway, 2001). Lactotransferrin (LTF) is an innate immune response protein with antimicrobial activity commonly found associated with mucosal tissues and biological secretions (Wang et al., 2019a).

2.5.2 Differential Protein Abundance Within Seminal Plasma Distinguishes Boar Fertility Phenotype

Correlation analysis found seminal plasma proteomes more similar among samples categorized as HFHB phenotype, relative to relationship of samples in other phenotypes. The lower level of correlation among subfertile phenotypes suggests that reproductive performance is affected by multiple factors making protein profiles less similar. Boars with high reproductive performance (HFHB) had a higher abundance of porcine seminal protein I (PSP-I) precursor. PSP-I is associated with sperm capacitation, farrowing rate, and immune modulation in the sow during insemination (Caballero et al., 2009; Novak et al., 2010b; Guimarães et al., 2017) and was identified as a potential biomarker of fertility in boars (Druart et al., 2019). The high fertility phenotype (HFHB) had a greater abundance of epididymis-specific alpha-mannosidase (MAN2B2) than in LFHB. MAN2B2 is important for sperm maturation and may play a role in specific sperm-egg interaction due to the fact that mannosidases on the sperm surface act like a receptor for mannose-containing oligosaccharides located on the zona pellucida (Okamura et al., 1992; Okamura et al., 1995; Okamura et al., 1997).

Higher performance boars (HFHB) also had greater abundance of two immune-related proteins, C3 and CFI than the subfertile groups with low farrowing rates (LFLB and LFHB). Complement component 3 (C3) is associated with a microbial stimulated immune response, and CFI codes for

an enzyme responsible for regulating the complement cascade. These differences in seminal plasma proteomes may suggest that boars with better reproductive performance are better equipped to handle pathogenic challenges and modulate inflammatory responses.

Several antioxidant proteins were less abundant in subfertile phenotypes relative to the high fertility group. Superoxide dismutase (SOD1) was lower in LFHB than HFHB and abundance of peroxiredoxin 4 (PRDX4) was less in HFLB than HFHB. Sperm of transgenic PRDX4 knockout mice are more susceptible to cell death due to oxidative damage (Iuchi et al., 2009). Peroxiredoxin 6 (PRDX6) and glutathione peroxidase 6 (GPX6) were both lower in abundance in the LFLB phenotype. Transgenic, human erythropoietin (hEPO) boars have low sperm fertility, conception rates, and litter size when compared to wild type boars (Choi et al., 2012). Investigators noted that hEPO boars lacked PRDX6 when compared to wild type boars (Choi et al., 2012). Glutathione peroxidases are important for protection against free radicals caused by lipid peroxidation and cellular metabolism (Murase et al., 2007; González-Cadavid et al., 2014a). Therefore, a lower abundance of antioxidative enzymes SOD1, PRDX4, PRDX6, and GPX6 across the subfertile phenotypes may have resulted in decreased reproductive performance due to oxidative damage to sperm cells, thus reducing the number of cells capable of fertilization.

The higher abundance of blood related proteins (HBB, HBA, A2M) and the proinflammatory cytokine (MIF) in seminal plasma of boars with low reproductive performance (LFLB) may indicate damage or localized inflammation of the reproductive tract. Macrophage migration inhibitory factor is a product of activated T cells and is rapidly released in the presence of microbial antigens as a response to stress (Thierry and Thierry, 2003). Alpha-2-macroglobulin (A2M), a clusterin chaperone protein, has been associated with damaged sperm membranes (Bailey and Griswold, 1999) and inversely related to sperm quality in peccaries (Santos et al., 2014). An

increase in A2M in bulls and rams correlated to an increase in morphologic abnormalities in sperm (Ibrahim et al., 2001a; Ibrahim et al., 2001b). The greater abundance of Cathepsin S, which functions to degrade antigenic proteins and convert them to peptides for MCH II presentation (Beers et al., 2005), in LFLB than HFHB, suggest a potential for a pathogenic infection associated with subfertile phenotype.

Boars with high farrowing rate but low total born (HFLB) had more abundant acute phase immune response and antimicrobial peptides, inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) and beta-defensin (DEFB112). DEFB112 can modulate the uterine inflammatory response by coating sperm cells and preventing immunorecognition by the female's reproductive tract (Yudin et al., 2005). Therefore, lower abundance of DEFB112 in subfertile groups may have resulted in a greater immune response in the female, thus decreasing the number of viable sperm available within the female's reproductive tract and may provide an explanation for why total born was decreased.

Inositol-1-monophosphatase (IMPA1) was the only protein that was lower in all three subfertile phenotypes compared to HBHF. IMPA1 is important for maintaining inositol homeostasis by dephosphorylating inositol-3-phosphate synthase INO1 (Hirsch and Henry, 1986; Murray and Greenberg, 2000). INO1 is present in all tissues, but is highest in testes (Loewus et al., 1980; Guan et al., 2003). Boar seminal plasma contains very high levels of inositol, and initial roles attributed its function to maintenance of osmotic pressure in seminal vesicle and semen (Mann, 1954). Inositol is an antioxidant that has been shown to alleviate sperm alterations due to ROS excess when dosed in-vitro (Palmieri et al., 2016), and plays a central role as a secondary messenger in follicle stimulating hormone (FSH) signaling which is important for sperm maturation and testosterone production. Spermatazoa readily metabolize phosphatidylinositol, a

product synthesized by the epididymis from inositol, which increases motility and glycolysis (Scott and Dawson, 1968; Voglmayr and Amann, 1973; Voglmayr, 1974). Inositol was shown to be essential for sperm maturation, as *c-ros* knockout mice were infertile due to reduced levels of inositol (Yeung et al., 2004). Boars fed high levels of phytase, the enzyme responsible for releasing inositol from the plant component phytate, showed increased sperm production compared to boars who received normal levels (Stewart et al., 2018). Malate dehydrogenase (MDH1), an essential enzyme for metabolic activity in sperm cells, was also lower in LFLB and HFLB. Low levels of MDH1 in men is associated with infertility (Leventerler et al., 2013). Therefore, screening boars in the future for metabolites such as inositol or decreased metabolic capacity of semen may be a component of tests to identify subfertile boars.

Fibronectin (FN1) was also less abundant in the phenotypes characterized with low total born. Fibronectin has been shown to be important for fertilization and plays a protective role on sperm by reducing oxidative stress produced by sperm metabolism (González-Cadavid et al., 2014a). In bull seminal plasma, fibronectin interacts with a number of integrins and binds to osteopontin which is an important marker of fertility (Killian et al., 1993; Cancel et al., 1997; Moura et al., 2006). Fibronectin is also inversely related to midpiece and tail defects in bulls (González-Cadavid et al., 2014a).

2.5.3 Proteins Influencing Farrowing Rate and Total Born as Individual Traits

Functional annotation analysis of proteins present in boars with high farrowing rates showed a greater quantity of proteins associated with free radical protection (SOD1 and IMPA1) and proteins correlated with high fertility (PSP-1). Boars with low farrowing rates had a greater abundance of HBB and Golgi Membrane Protein 1 (GOLM1). The presence of blood

microparticles within seminal plasma suggests that these boars may be experiencing inflammation within the reproductive tract which can potentially impair the survival and production of mature spermatozoa. Moreover, increased expression of GOLM1 was found increased in response to viral infection, and in seminal plasma (Kladney et al., 2000; Kladney et al., 2002) of men who were experiencing high levels of oxidative stress (Intasqui et al., 2015). Thus, the higher abundance of GOLM1 and HBB maybe indicative of responses to viral or oxidative stress, and inflammation and maybe related to the lower farrowing rate observed in these phenotypes.

Boars with high total born also had a greater abundance of PSP-1, IMPA1, and Folate Receptor Beta (FOLR2). Folate Receptor Beta has a high affinity for and binds folate. While it is not clear what the function of FOLR2 is in boar seminal plasma, it has been shown that boars supplemented folic acid during heat stress mitigates DNA damage to spermatozoa by protecting it from ROS (Peña Jr et al., 2019). Low levels of seminal plasma folate in men are associated with decreased sperm count (Wallock et al., 2001). This suggests that both total born and farrowing rate could be greatly influenced by the seminal plasma's ability to protect spermatozoa from reactive oxygen species (ROS). Boars with phenotypes associated with low total born had a greater abundance of Actin Beta (ACTB) and Myelin Protein Zero Like 1 (MPZL1). It is not clear what the function of ACTB is in swine seminal plasma at this time, but it is in high abundance within the seminal plasma proteome (Sakaue et al., 2010). Other research demonstrated in boars of normal fertility that the sperm-rich fraction seminal plasma of the boar's ejaculate has lower amounts of MPZL1 (Perez-Patino et al., 2016). MPZL1 is a receptor for Concanavalin A (ConA), which upon binding activates the immune system, recruits lymphocytes and elicits cytokine production (Dwyer and Johnson, 1981). Down-stream signaling can also result in programmed cell death. The high

amount of MPZL1 in low total born boars may thus indicate an underlying issue that is not yet known.

2.5.4 Comparison of Our Findings to Previous Literature

Perez-Patiño and others also investigated whether seminal plasma proteomes reflected differences in fertility status of boars (Pérez-Patiño et al., 2018). The Perez- Patiño study compared proteomes between two different groups of high and low farrowing rate boars and boars that produced small versus large litter sizes. Using SWATH technology for proteome analysis Perez-Patino reported identifying 679 proteins, which corresponded to 543 protein groups across the 26 samples they analyzed. They identified approximately 25% more proteins using SWATH, than we did across 32 samples using shotgun LC-MS/MS. Approximately 142 of the 190 (75%) proteins commonly abundant across all phenotypes in our study overlapped with what Perez-Patiño, et al. identified (Pérez-Patiño et al., 2018). Perez- Patiño et al. identified 11 differentially abundant proteins between high versus low farrowing rate, and 4 proteins differentially abundance between boars producing small versus large litter sizes. None of the 15 proteins overlapped with the 56 proteins differentially abundant between the high fertile group and the three subfertile groups in our study. Lack of similarity of findings between Perez-Patiño study and ours may be due to the different approach of categorizing boars by fertility phenotype where we created an index for fertility, and they looked at farrowing rate and litter size as independent traits across two different groups of boars. The differences in fertility data included in our study were also greater for each fertility phenotype compared to the fertility data of the boars they enrolled in their study. Moreover, they pooled seminal plasma across multiple collections for proteome analysis, whereas we used

plasma from the first ejaculate that met industry standards for each boar since we had a different overall objective in this study than Perez-Patiño and others.

2.5.5 Study Limitaitons

This study has several limitations that may have affected data analysis. Sperm were not separated from seminal plasma samples upon collection, and the time it took for semen to reach Purdue University may have allowed for the exchange of proteins between sperm cells and seminal plasma. To limit this potential, semen was shipped in a chilled cooler to slow spermatozoa activity within seminal plasma and therefore modifications to the proteome are expected to be minimal.

Protease inhibitors were not added to semen samples or during sample preparation, and thus there is the potential for some protein degradation. To limit proteolytic activity prior to protein isolation, samples were shipped on ice and seminal plasma samples were immediately frozen following centrifugation. It is also important to note that sample preparation includes the addition of trypsin and Lys-C endopeptidases to cleave proteins into shorter peptide sequences for LC-MS/MS analysis. Peptide identification was done using sequences of at least six amino acids during MaxQuant database search, and proteins were considered as true if at least two peptides (unique, razor or both) matched. Proteins identified by only shared peptides were reported as a protein group

2.6 Conclusions

Shotgun proteomic analysis of seminal plasma samples from the boar's first ejaculate support that differences in protein profiles may prospectively reflect differences in boar fertility phenotype. Seminal plasma proteomes were more similar among samples from highly fertile boars.

The lower level of correlation among and within subfertile phenotype protein profiles suggests that reproductive performance was affected by multiple factors. Only one protein, IMPA1, was commonly different from high performance boars across the three subfertile phenotypes. Overall, it appears that the ability of seminal plasma to protect sperm cells from reactive oxygen species is very important in determining how farrowing rate and total born are influenced. Taken together we envision that as research progresses in this area that abundance of a panel of seminal plasma proteins, rather than a single protein, will be needed to be screened to predict reproductive performance of boars. Among the potential candidates to screen for subfertility would include higher abundance of proteins categorized as blood components and inflammation markers of subfertility, and lower abundance of antioxidant levels and IMPA1, or simply inositol levels. Proteins indicative of higher fertility potential may also be used as a screening tool, and may include PSP-1, MAN2B2, SOD1, PRDX6, and GPX6.

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CHAPTER 3. EVALUATION OF ON-FARM INDICATORS OF ON-FARM GILT REPRODUCTIVE PERFORAMNCE POTENTIAL AT 21 DAYS OF AGE.

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3.1 Abstract

Selection of replacements for the sow herd is one of the most important facets in swine production. Although our current methods of selection are effective, there is still a large amount of variation in sow reproductive performance traits such as pigs per sow per year (PSY). Therefore, the objective of this study was to determine if on-farm phenotypic traits at 21 d postnatal (PN) or perinatal environmental factors could predict sow reproductive performance. Data were prospectively collected from 2146 gilts born on a commercial sow production facility and included birth and weaning weights, vulva length and width at 21 d PN, birth and nursing litter size, days nursed, average daily gain from birth to weaning, and age at first estrus. Of the initial animals, 400 (17%) were selected for the sow herd, 353 remained after removal of animals culled for non-reproductive reasons. Animals were assigned to 1 of 5 reproductive performance categories based on observation of estrus or pigs per sow per year (PSY) across two farrowings: High Fertility (HF; 23%; n=82; ≥26 PSY), Middle Fertility (MF2; 12%; n=43; 20-25 PSY), Low Fertility (MF3; 15%; n=54; <20 PSY), Infertile-Estrus (IFe; 10%; n= 36; estrus, no pregnancy), and Infertile-No Estrus (IFno; 39%; n=138; no estrus, no pregnancy). Generalized linear model analysis indicated vulva width (P=0.03) was related to PSY, however, it only explained 1.5% of the total variation in PSY. To determine if preweaning variables were predictive of gilt fertility outcome, animals were

grouped as those that became pregnant (n=179) or not (n=174). Vulva width tended to be greater in fertile animals versus infertile (P=0.07). Binomial regression analysis revealed a positive relationship between vulva width and gilt fertility, however, this relationship is not strong enough to make sow herd selection decisions.

3.2 Introduction

A gilt's reproductive efficiency has a major effect on economic profitability in the swine industry. A common method to measure reproductive efficiency is pigs weaned per female per year (PSY) (Stalder et al., 2003; Stalder, 2009; Stalder et al., 2019). The 2019 average of PSY was 26.08 for farms in PigCHAMP database and 26.61 in MetaFarms (MetaFarms, 2020; PigChamp, 2020). Another way to measure sow farm reproductive efficiency is sow replacement rate. Despite high yielding sows, the removal rates of animals from sow herds averaged 45% across the US in 2018, with culling primarily due to poor reproductive performance. In addition to a high sow removal rate, 38.5-51.1% of gilts selected as potential replacements are culled due to reproductive failure (Roongsitthichai et al., 2013; Li et al., 2018).

A negative relationship exists between the litter size in which a gilt is raised in and her lifetime reproductive efficiency (Robison, 1981; Van der Steen, 1985; Bartol et al., 2006a; Bagnell et al., 2009; Bartol et al., 2009), and is likely due to animals in smaller litters having heavier birth weights which is positively related to colostrum consumption (Le Dividich et al., 2005; Morton et al., 2019). Gilts that consume greater amounts of colostrum tend to be heavier at birth and gain more weight postnatally (de Passille and Rushen, 1989; Milligan et al., 2002), as well as display signs of estrus earlier and have better lactation performance as sows than their low-colostrum

counterparts (Vallet et al., 2015b). Together demonstrating that low colostrum consumption is associated with impaired reproductive performance in sows (George et al., 2019).

Because of the strong relationship between a gilt's reproductive potential and pre-weaning nutrition and growth, swine operations have implemented perinatal piglet care and fostering programs that minimize competition among littermates and maximize access to suckling. These measures often result in standardization of nursing litter size, so animals have similar growth rates and size at weaning. At weaning, farm managers are tasked with identifying gilts to be reared for replacement breeding females instead of entering the food chain. However, the minimization of differences in size and growth rates of animals at weaning makes selection of females with the greatest reproductive potential challenging (Robison, 1981; Van der Steen, 1985; Serenius and Stalder, 2006; Bagnell et al., 2009; Bartol et al., 2009).

To maximize economic returns there is a need to identify animals with the greatest reproductive potential prior to their entering the breeding herd. An efficient gilt management system has three selection timepoints: in the nursery, start of boar exposure, and when gilts enter puberty (Patterson et al., 2010). Average age at first estrus, which marks puberty, is the most predictive parameter of sow reproductive efficiency (Patterson et al., 2010). However, average age of estrus is 240 d and at this point of production the producer has already invested in an animal that may fail to ever cycle. Vulva width by 95-115 days of age has been associated with a gilt's ability to achieve puberty by 200 days of age (Graves et al., 2020), and vulva width at 105 days was found associated with sow productivity through two parities (Romoser et al., 2020). The objective of this prospective longitudinal study of over 2000 gilts born on a commercial sow production facility was to determine if there was a relationship of preweaning traits such as average

daily gain from birth to weaning, birth and weaning weight, and vulva size at weaning with reproductive efficiency and longevity in sow breeding herd.

3.3 Materials and Methods

3.3.1 Animals

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (1605001416). Gilts (n=2146) born between February 5, 2018-April 24, 2018, on a commercial farm in central Indiana were enrolled in the longitudinal observational study (Figure 3.1). Animals at the commercial facility were maternal lines PIC 1070 (Large White x L019) and C29 (Landrace x PIC 1070) which are specifically bred for preweaning piglet performance and sow lifetime productivity. Between postnatal d 0 and 2 piglets were individually identified by an ear tag and weighed and processed on d 2-3 (200 mg iron, tail docking, Baytril ®, Tylan® 50) as per routine management on the farm. Following individual identification, litter sizes were standardized to 14 ± 1 piglets by farm technicians through cross-fostering between litters of similar aged piglets. Litter (farrowing date, birth litter size, number weaned) and individual piglet (birth weight, weaning weight) information were entered into MetaFarms (MetaFarms, Inc., Burnsville, MN) database for each sow.

Piglets were weaned at 21 ± 4 d, and 1084 gilts from the initial pool of animals were selected as replacements for the farm's onsite nursery (Figure 3.1). Animals not selected as replacement gilts were transferred to an offsite wean to finish facility for market pig production. At the time of weaning, gilts in the on-site nursery were weighed and vulva lengths and widths were measured using digital calipers (Fisherbrand™ Traceable™ Digital Carbon Fiber Calipers, Fisher Scientific Company L.L.C., Pittsburgh, PA). Vulva length was measured from the bottom

most point of the vulva to the top of the vulva (Figure 3.2), and width was measured at the widest part of the vulva (Figure 3.2). Vulva metrics were entered and stored in a research database.

At 25 weeks of age, gilts were moved from the into the onsite gilt development unit (GDU). Gilts in the GDU received daily, full-contact boar exposure to induce puberty and screen for signs of estrus. Gilts were observed for signs of estrus daily beginning at 25 weeks of age. Date of gilt's first and second estrus were recorded. When a second estrus was detected, gilts were moved to a gestation crate and bred using artificial insemination (AI) on their third estrus. If gilts did not show any signs of estrus following three weeks of heat detection they were given a full dose of P.G. 600 (Intervet America, Inc., Millsboro, DE) to induce estrus and were bred on the subsequent heat. Gilts that did not respond to P.G. 600 were culled from the selection pool. Data on reproductive history to include day of first estrus, breeding date, and treatment with P.G. 600 were entered into Metafarms database.

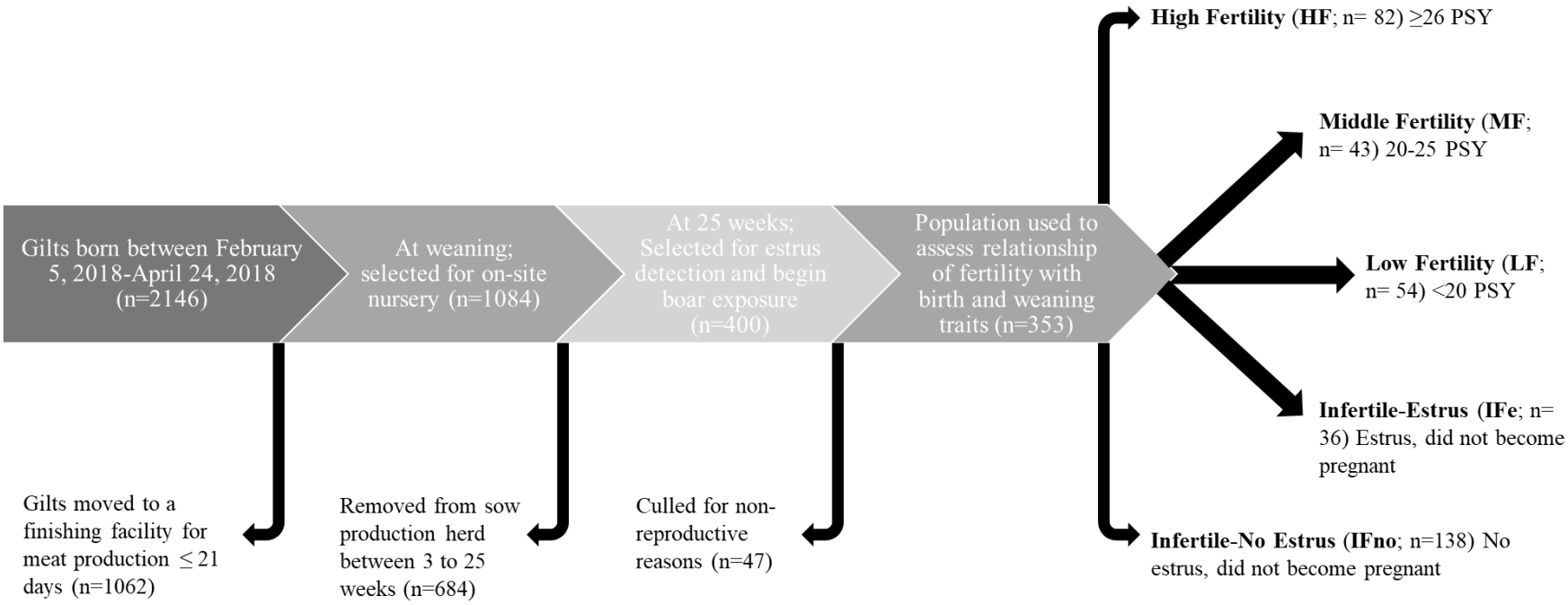

Figure 3.1 Longitudinal study design and fertility phenotype classification

Timeline of the study representing different timepoints of selection and different fertility phenotypes. PSY; Pigs per sow per year.

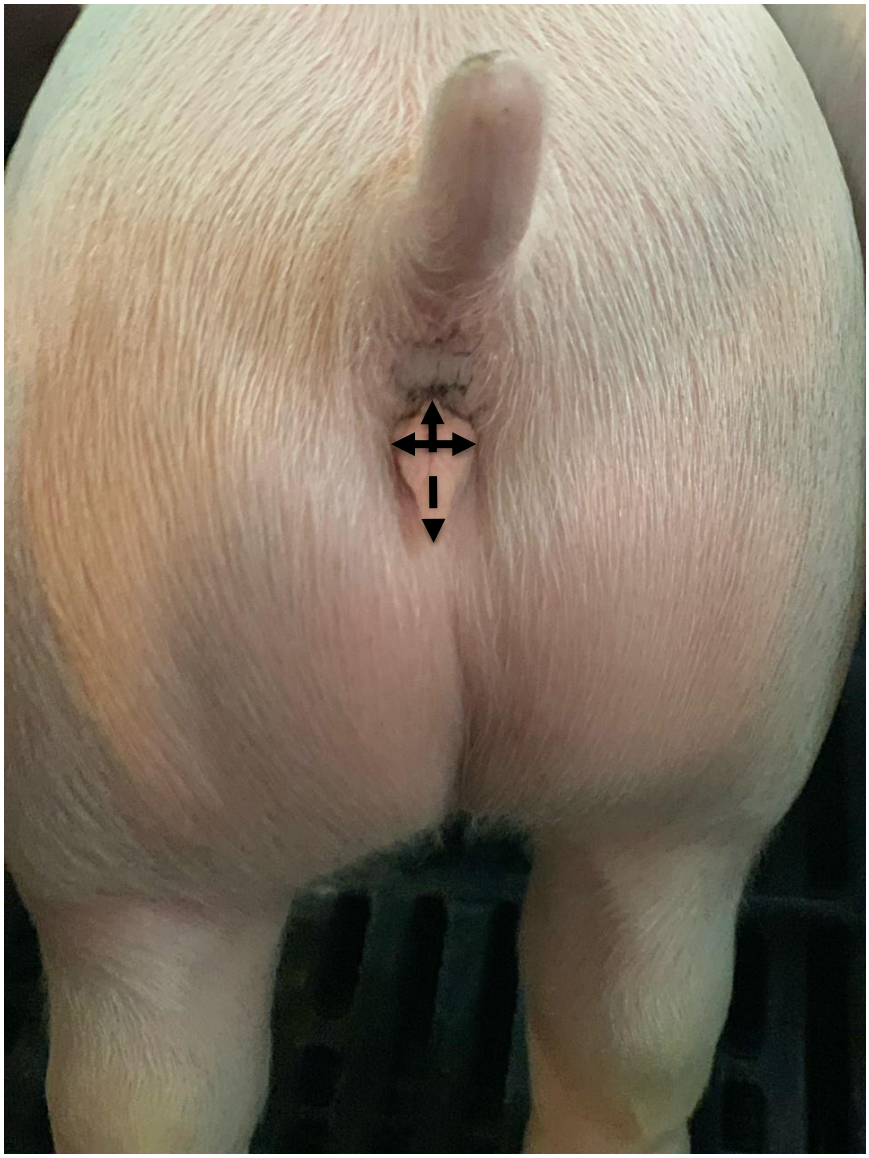

Figure 3.2 Vulva morphometric measurement

Vulva length (dashed line) and width (solid line) were measured at 21 ± 4 days using digital calipers from the very top of the vulva to the bottom of the vulva and at the widest part of the vulva, respectively. Vulva measurements were taken in millimeters. The gilt pictured is 21 days of age.

3.3.2 Categorization of Fertility Groups

On September 22, 2019 performance data from breeding herd animals that had birth weights, weaning weights, and vulva measurements recorded (n=400) were extracted from Metafarms reports which included date of birth, date of first boar exposure, dates of estrus detection, date of mating, herd removal (cull) date, reason for removal, farrowing date and number of piglets born alive. Date of data extraction was selected to allow time for at least two farrowings of all study animals from time of birth. Animals that were culled from the breeding herd for non-reproductive reasons such as lameness, disease, or leg injuries, were removed from data set. The 353 that remained were divided into two main classes based on whether an animal was fertile (Figure 3). If animals were fertile, they were divided into three subclasses based on PSY. High Fertility (HF; n=82) animals were defined as sows that had at least 26 PSY. Middle Fertility (MF; n=43) gilts were characterized as sows that had 20-25 PSY. Sows that had less than 20 PSY were classified as Low Fertility (LF; n=54). PSY was chosen as it is a calculation that encompasses multiple facets of fertility and was calculated by totaling the number of piglets weaned from a sow during her first productive year up to two parities. Infertile animals were divided into two subclasses based on whether they exhibited estrus. Infertile-Estrus (IFe; n= 36) were gilts that showed signs of estrus but did not become pregnant. Gilts that did not show any signs of estrus following boar exposure and P.G. 600 were characterized as Infertile-No Estrus (IFe; n=138).

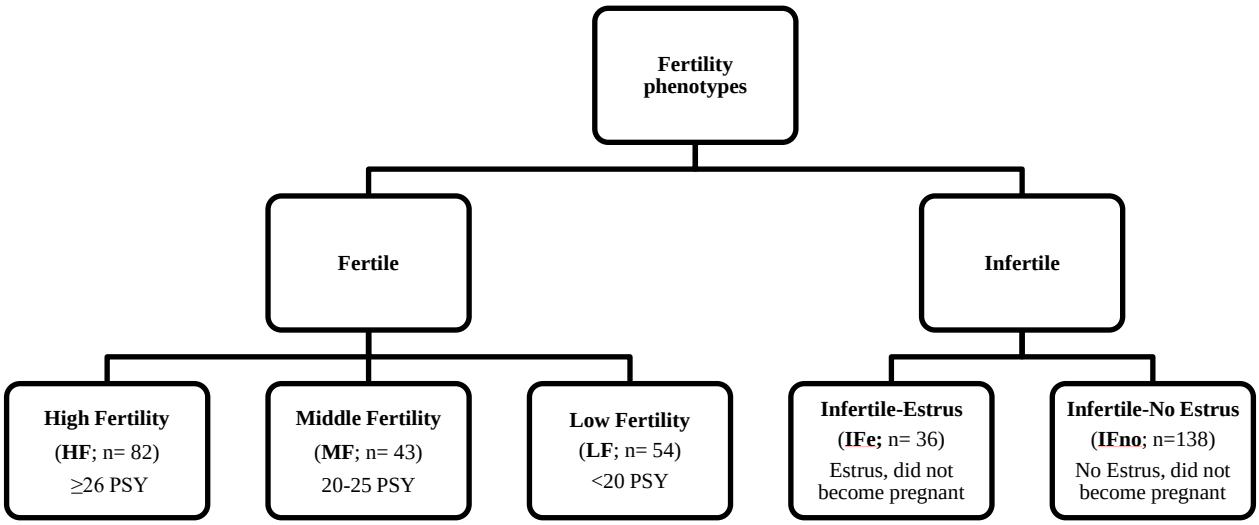

Figure 3.3 Dendrogram of fertility phenotype categories.

The population used to assess the relationship of fertility with birth and weaning traits was divided into two main classes: Fertile or Infertile. Within those classifications, fertile animals were divided into 3 subclasses based on pigs per sow per year (PSY): High Fertility (HF), Middle Fertility (MF), and Low Fertility (LF). Infertile animals were divided into two subclasses based on whether they exhibited estrus: Infertile-Estrus (IFe) and Infertile-No Estrus (IFno).

3.3.3 Statistical Analysis

Analysis of production variables by fertility category was performed using the GLM procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC). All other analyses were completed in R (v 3.5.1). A generalized linear model was used to assess whether birth weight, weaning weight, vulva length, vulva width, birth litter size, days nursed, nursing litter size, average daily gain from birth to weaning, and age at first estrus contributed to the variation in PSY. Predictors of sow reproductive performance were assessed using binomial regression analyses where:

$$\text{Probability}_{\text{Bred}} = (21 \text{ d of age vulva width, mm})*0.12609 - 1.14445$$

A value of $P < 0.05$ was used to determine significance and values of $0.05 \leq P \leq 0.10$ were considered trends.

3.4 Results

Of the 400 that entered the breeding herd with birth and weaning weight data, 82 (23%) were considered highly fertile animals (HF; ≥26 PSY), and 138 (39%) categorized as infertile (IFno) due to failure to show any signs of estrus upon boar exposure by 29 weeks of age, and failure to respond to hormonal induction of estrus. Intermediate phenotypes were characterized as sows that had 20-25 PSY (MF; 12%; n=43), sows that had less than 20 PSY (LF; 15%; n=54), and gilts that showed signs of estrus during boar exposure but did not become pregnant (IFe; 10%; n=36; Figure 3). Gilts that were not selected for the gilt development unit from the initial replacement selection pool (n=684) had lower birth and weaning weights, smaller vulva widths, lower average daily gain (ADG) from birth to weaning, and had more piglets in their birth litter than gilts selected for the final breeding herd at 25 weeks of age (Table 3.1; $P<0.01$).

Table 3.1 Preweaning characteristics of gilts that were culled at 25 weeks of age or selected for the final breeding herd from the initial replacement weaning pool.

Preweaning traits	Gilts culled from initial selection pool[2] (n=684)	Gilts selected for final breeding herd (n=400)	SE	*p*-value
Birth weight, kg	1.698	1.775	0.019	<0.001
Weaning weight, kg	4.979	5.738	0.080	<0.001
Vulva length, mm	13.48	13.03	0.195	0.124
Vulva width, mm	8.810	9.077	0.089	0.010
Birth litter size	13.87	12.99	0.125	<0.001
Days nursed	21.52	21.37	0.110	0.276
Nursing litter size	13.60	13.52	0.045	0.112
ADG[1]	0.152	0.186	0.004	<0.001

[1]Average Daily Gain from birth to weaning
[2]Gilts selected at weaning as potential replacements

Vulva width (Table 3.2; P=0.03) and nursing litter size (Table 3.2; P=0.05), were differentiating factors in PSY. However, all factors included in the statistical model only accounted for 3.4% of the total variation in PSY. A reduced statistical model that only included vulva width and nursing litter size was used to further evaluate whether vulva width or nursing litter size influenced PSY. Vulva width was found to be the only differentiating factor (reduced model A; Table 3.2; P=0.04) which suggests that nursing litter size was slightly correlated to a variable that was removed from the full model. Therefore, a second reduced model that included only vulva width (reduced model B) was used to determine how much variation in PSY was explained by vulva width. Vulva width was found to explain 1.5% of the variation in PSY and was still a differentiating factor (Table 3.2; P=0.03). When all phenotypes were compared, vulva width was

not different (Table 3.3; P=0.35). There were no differences among all phenotypes for any of the other pre-weaning variables from the full model (Table 3.3).

Table 3.2 Independent variables of statistical models and their influence on the variation in PSY

Statistical Model	Independent Variables	Sum of Squares	*p*-value
Full Model[1]	Birth weight, kg	4.64	0.84
	Weaning weight, kg	10.8	0.76
	Vulva length, mm	22.0	0.66
	Vulva width, mm	590	0.03
	Birth litter size	32.7	0.60
	Days nursed	382	0.07
	Nursing litter size	447	0.05
	ADG[2] from birth to weaning	12.6	0.74
	Age at first estrus	338	0.09
Reduced Model A[3]	Vulva width, mm	633	0.04
	Nursing litter size	200	0.26
Reduced Model B[4]	Vulva width, mm	805	0.03

[1]Adjusted $r^2 = 0.034$
[2]Average Daily Gain
[3]Adjusted $r^2 = 0.012$
[4]Adjusted $r^2 = 0.015$

Table 3.3 On-farm characteristic means among all phenotypes and their differentiating phenotype

Parameters	HF[1]	MF[2]	LF[3]	IFe[4]	IFno[5]	SE	*p*-value
Birth weight, kg	1.81	1.81	1.73	1.79	1.75	0.07	0.64
Weaning weight, kg	5.95	5.68	5.88	5.78	5.53	0.24	0.43
Vulva length, mm	12.9	13.2	13.4	13.1	13.2	0.42	0.72
Vulva width, mm	9.31	9.30	9.08	8.74	8.95	0.30	0.35
Birth litter size	13.1	13.0	12.9	13.2	13.0	0.44	0.99
Days nursed	21.5	21.7	21.2	21.1	21.3	0.38	0.51
Nursing litter size	13.6	13.7	13.4	13.7	13.4	0.13	0.23
ADG[6] birth to weaning	0.19	0.18	0.20	0.19	0.18	0.01	0.39
Age at first estrus, days	186	186	186	189	-	1.40	0.42

[1]High Fertility; ≥26 PSY
[2]Middle Fertility; 20-25 PSY
[3]Low Fertility; <20 PSY
[4]Infertile-Estrus; Estrus, did not become pregnant
[5]Infertile-No Estrus; No Estrus, did not become pregnant
[6]Average daily gain

To determine if preweaning variables were predictive of fertility outcome, animals were grouped as those that were fertile (n=179) or infertile (n=174). Vulva width tended to be greater in animals who were fertile (Table 3.4; P=0.07). Fertile animals also tended to come into estrus 3 days earlier than open animals (Table 3.4; P=0.10). Binomial regression analysis revealed a positive relationship between vulva width and probability of becoming pregnant later in life (Figure 3.3), however, the relationship is not strong (Nagelkerke r^2= 0.014) due to the variation in vulva width among phenotypes (Figure 3.4). Binomial regression analysis was also completed using age at first estrus, birth weight, and weaning weight as predictors of pregnancy, however, these parameters were not predictive of pregnancy status.

Table 3.4 Fertile vs. infertile on-farm characteristic means and p-values

On-farm Characteristic	Fertile n=179	Infertile n=174	SE	*p*-value
Birth weight, kg	1.79	1.76	0.05	0.53
Weaning weight, kg	5.87	5.61	0.14	0.14
Vulva length, mm	13.1	13.2	0.20	0.89
Vulva width, mm	9.23	8.89	0.15	0.07
Birth litter size	13.0	13.1	0.21	0.77
Days nursed	21.5	21.2	0.13	0.27
Nursing litter size	13.6	13.5	0.09	0.45
ADG[1] from birth to weaning	0.19	0.18	0.01	0.26
Age at first estrus, days	186	189	1.13	0.10

[1]Average Daily Gain

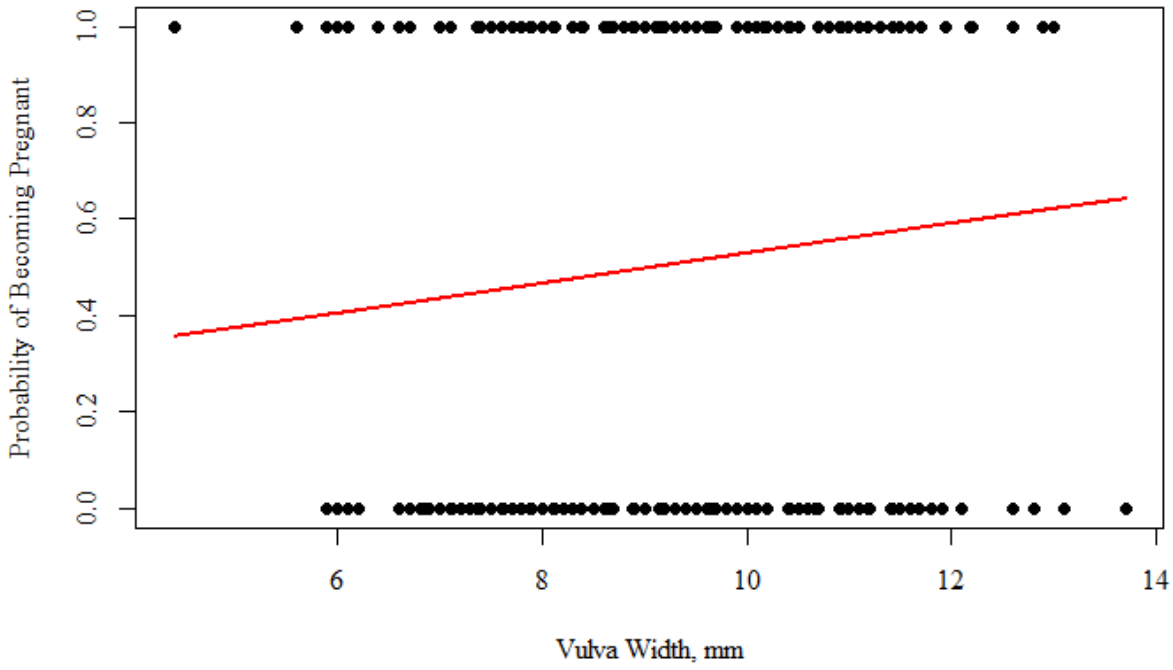

Figure 3.4 Vulva width as a predictor of gilt fertility at 21 ± 4 days of age.

Binomial regression analysis was used to determine if vulva width at 21 ± 4 days of age could be a predictor of a gilt fertility. A Nagelkerke r^2 was calculated to determine if there was a relationship between vulva width at 21± 4 days of age and gilt fertility.

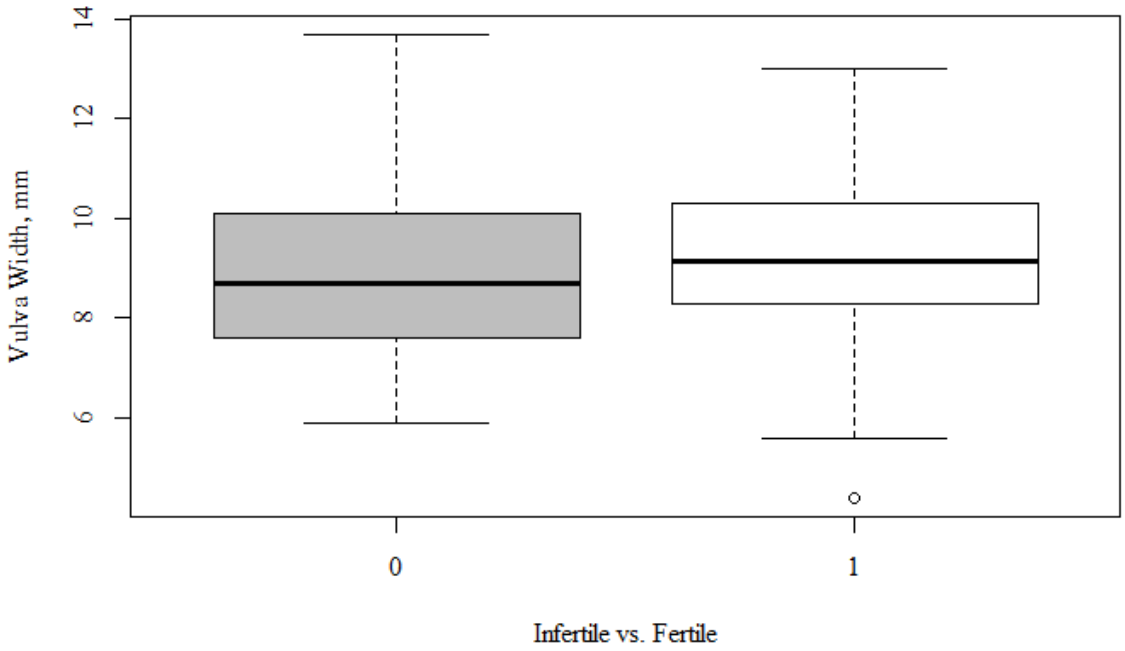

Figure 3.5 Distribution of vulva width between infertile (0) and fertile gilts (1).

Depiction of the distribution of vulva width at 21 ± 4 days of age in both infertile and fertile gilts.

3.5 Discussion

Achieving an adequate return on investment in replacement gilts is a challenge that the swine industry currently faces. The leading cause of removals from sow herd is related to poor reproductive performance. This is due in part to the lack of the ability of the producer to identify reproductively sound gilts early in life (MetaFarms, 2020; PigChamp, 2020). Because the perinatal environment is associated with long-term fertility, we aimed to determine whether several easily measured morphological and developmental markers could be related to fertility outcomes. We found that among all traits assessed, only vulva width at weaning was weakly related to long-term fertility of gilts.

In this study, consistent with modern swine farm management, we found the combination of genetics, cross-fostering, weaning schedule, and selection of gilts into the gilt development unit (GDU) resulted in the similar birth weights, nursing litter sizes, weaning weights, average daily gain from birth to weaning, number of days nursed, and birth litter size across all fertility phenotype groups. The very light birthweight piglets were not selected into the on-site nursery which could explain the high degree of similarity for animals that made it into the GDU who had an average birth weight of 1.72 kg. However, it is interesting to note that animals culled between weaning and 25 weeks of age were born into a larger litter, had a smaller body size at both birth and weaning, and a lower ADG between birth and weaning. All of these traits are indicators that animals culled between weaning and 25 weeks likely to consumed less colostrum, thus negatively affecting the overall developmental trajectory of the animal (de Passille and Rushen, 1989; Milligan et al., 2002; Le Dividich et al., 2005; Devillers et al., 2011; Quesnel et al., 2012b; Morton et al., 2019).

During the first two weeks of life, the gilt's reproductive tract undergoes morphologic and molecular changes across the uterus and cervix (Bartol et al., 2006a) and into the vagina (Harlow et al., 2019d). The amount of colostrum consumed in the first days postpartum is related to long-term fertility (Vallet et al., 2015a), and affects reproductive development centrally and peripherally (George et al., 2019). Morphologic and molecular changes in the gilt's upper and lower reproductive tract are affected by colostrum intake (Bagnell et al., 2009; Bartol et al., 2009; Harlow et al., 2019a), with several studies linking relaxin in milk to postnatal reproductive tract development in swine (Bagnell et al., 2005; Frankshun et al., 2012).

The vulva is an external extension of the female reproductive tract, and previous studies have shown a relationship between sow reproductive efficiency to vulva width at 95 days of age

(Graves et al., 2020) likely correlated to pubertal increases in estrogen. Our findings revealed that there is a positive relationship between vulva width at weaning and the probability of a gilt becoming pregnant. Fertile gilts also had numerically heavier weaning weights than the infertile gilts which suggests they may have had a subtle advantage in the perinatal environment. The association between colostrum intake and reproductive tract development before weaning, suggests that vulva size at weaning may be an indicator of colostrum consumption and therefore reproductive status later in life. Due to the physical similarities at 21 days of age, it is difficult to discern which animals will become the most prolific gilts on the farm. Future research will thus likely need to investigate the potential in evaluating the molecular environment of the gilt's reproductive tract at 21 days of age to determine if biomarkers can be used to differentiate between phenotypes.

3.6 Conclusion

Identifying gilts with the greatest reproductive potential at 21 days of age rather than at 175 days of age would be incredibly valuable to the swine industry. Our data indicated that there is a positive relationship between vulva width and probability of a gilt becoming pregnant, however, this relationship was not very strong due to the variation in vulva size at weaning and thus cannot be used alone when making gilt selection decisions. Vulva width may be more beneficial when used in conjunction with current selection strategies.

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CHAPTER 4. BIOMARKERS PREDICTIVE OF LONG-TERM FERTILITY FOUND IN VAGINAL LIPIDOME OF GILTS AT WEANING.

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4.1 Abstract

A marker indicative of fertility potential of replacement gilts early in development would decrease culling rates in the sow herd, improve sow herd reproductive efficiency, and reduce production costs. The objective of this study was to determine if vaginal lipid profiles at 21 d postnatal (PN) could predict sow reproductive performance. Vaginal swabs of the anterior vagina were taken at 21 ± 4 d PN from gilts born on a commercial sow production facility for lipidomic analysis. Animals were followed prospectively for two years and assigned to reproductive performance categories based on observation of estrus or piglets weaned per sow per year (PSY) across two farrowings. Lipids were extracted from cellular material collected with swabs taken from high fertility (HF; n=28; ≥26 PSY) and infertile (IF; n=34; no estrus, no pregnancy) animals and multiple reaction monitoring (MRM) profiling was used for lipidome analysis. Relative abundance of arachidonic acid (ARA, C20:4) and docosahexaenoic acid (DHA, C22:6) were lower (P<0.05) in IF gilts than HF gilts, whereas abundance of the free fatty acids cerotic (C26:0), ximenic (C26:1), and nonadecanoic (C19:0) acids were greater (P<0.05) in IF gilts. Additionally, eicosapentaenoic acid (C20:5) a precursor of prostaglandins was higher (P<0.05) in IF gilts. The perspective of having a panel of lipids captured with vaginal swabs at weaning that can predict reproductive efficiency of gilts shows promise and warrants future research in this area.

4.2 Introduction

Sow's reproductive efficiency has a major effect on economic profitability in the swine industry. A common method to measure reproductive efficiency is number of piglets weaned per sow per year (PSY) (Stalder et al., 2003; Stalder, 2009; Stalder et al., 2019). In 2018, the average PSY for US sow herds was 25.3 (PigCHAMP, 2019). Another way to measure farm reproductive efficiency is sow replacement rate. Despite high yielding sows, the annual removal rates of animals from herds averaged 45% across the US in 2018, with culling primarily due to poor reproductive performance. The high culling rate of sows from herds is due, in part, to the lack of the ability of the producer to identify reproductively sound gilts early in life.

To maximize economic returns and efficiency for the farm there is a need to identify animals with the greatest reproductive potential as early in their lifetime as possible. An efficient gilt management system has three selection timepoints: at weaning, gilts selected to receive boar exposure, and from this group, gilts identified as exhibiting estrus (Patterson et al., 2010). Minimization of differences in size and growth rates of animals at 21 days of age due to perinatal management practices like cross fostering to equalize piglet access to sow nipples, and thus milk during lactation, make the initial step of replacement gilt selection at weaning challenging. Currently, the most predictive parameter of sow reproductive efficiency occurs at the second stage and is average age at first estrus. However the second stage is more than 200 days into the production timeline, with studies finding 26% of gilts failing to show estrus from boar exposure alone (Patterson et al., 2010). To increase the number of available breeding females, P.G. 600 is often administered to gilts that do not show estrus following boar exposure to induce puberty. During the same experiment, Patterson et al. (2010) observed 20% of gilts that received P.G. 600 following boar exposure did not respond, which suggests that a subpopulation of gilts are truly

infertile due to unknown reasons. Therefore, there is a need to identify a biomarker indicative of lifetime sow fertility early in life.

A biomarker is a morphological or physiological indicator of a phenotypic state of an individual that can be measured accurately and reproducibly (Strimbu and Tavel, 2010). Studies aimed at identifying markers of fertility in swine found that vulva width at 105 days was associated with sow productivity through two parities (Romoser et al., 2020). Whereas vulva width at 115 days of age was positively associated with the ability to achieve puberty by 200 days of age, however, only eight percent of the variation in age at puberty could be explained by vulva width alone. In an attempt to establish earlier markers predictive of gilt fertility, we evaluated whether phenotypic traits, such as birth weight, weaning weight, vulva length, vulva width, birth and nursing litter size were related to reproductive performance later in life (Mills et al., 2020b). Although, vulva width at 21 days of age was predictive of whether or not a gilt would become pregnant later in life, this relationship was weak and vulva width only explained 1.5% of the variation in reproductive success (Mills et al., 2020b). Serum immunocrit ratio at 24 h postnatal is used as an indicator of colostrum intake, and since perinatal nutrition is linked to reproductive tract development (Bartol et al., 1993b; Bartol et al., 2006b; Bagnell et al., 2009; Bartol et al., 2009; Frankshun et al., 2012; Bagnell and Bartol, 2019), Vallet et al. (2015) investigated its relationship with fertility outcomes in gilts. Immunocrit ratio at 24 h had a 41% predictive ability for age at first estrus with higher immunocrit levels being associated with an earlier age at puberty (Vallet et al., 2015a). However, immunocrit has not been established as a biomarker of fertility. This may be because of labor involved in blood sampling piglets and the variability in values across studies (Peters et al., 2016).

Most biomarkers used for diagnosis and prognosis are genes, proteins, and metabolites. The advent of high throughput omics tools for studying genome, transcriptome, proteome and metabolome has enabled the generation of large amount of data for global comparison of changes in molecular profiles that underlie phenotypes (Hu et al., 2011). These tools also provide opportunities for rapid screening of samples for biomarker discovery and have proved successful in identifying molecular markers diagnostic and prognostic of multiple human diseases, including cancers. Metabolites and lipids in tissues are reflective of pathways and processes within the system. Emergent technologies in mass spectrometry, specifically the utilization of multiple reaction monitoring (MRM) profiling, has enabled rapid and efficient identification of biomarkers of healthy or normal individuals from disease states (Xie et al., 2021). In our previous exploratory studies, we found that lipids captured with vaginal swabs taken from two-week old piglets and measured using MRM profiling distinguished the vaginal lipidome between gilts fed colostrum versus milk replacer (Casey et al., 2018a). In particular, vaginal swabs taken on d 14 PN revealed that a glycerolipid containing arachidonic acid (C20:4) was a potential biomarker for colostrum intake (Casey et al., 2018a). In a follow-up study utilizing MRM profiling, we found the vaginal lipidome on d 2 PN of gilts fed milk replacer reflected the lipids present in milk replacer, thus indicated that nutrition in the perinatal environment influences the composition of the reproductive tract and could reflect the gilt's reproductive success later in life (Harlow et al., 2019a). Therefore, the objective of this study was to determine whether there were lipid biomarkers in cellular material captured with vaginal swabs taken at weaning predictive of lifetime sow productivity. Specifically, the MRM profiling method was used to profile lipids collected with vaginal swabs at 21 days postnatal (PN) in gilts that were later identified as being infertile (IF) or highly fertile (HF) sows.

4.3 Materials and Methods

4.3.1 Animals

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at Purdue University prior to beginning the study. Animals used were a subset of a larger previously described study (Mills et al., 2020b). Briefly, gilts (n=2146) born on a commercial farm in central Indiana were enrolled in a longitudinal observational study (Figure 1). Initial individual weights were recorded between d 0 and 2 postnatal and are referred to hereafter as birth weights. Piglets were weighed and processed on postnatal d 2-3 (ear tag, 200 mg iron, tail docking, antibiotic administration) as per routine management on the farm. Cross-fostering between litters of similar aged piglets occurred after piglets were given an ear tag (litter size standardized to 14 ± 1 piglets). Data on farrowing date, birth litter size, size of gilt's litter at her weaning, birth weight, and weaning weight were entered into MetaFarms (MetaFarms, Inc., Burnsville, MN) database for each gilt.

Gilts (n=1084) were weaned at 21 ± 4 d, selected as potential replacement sows, and moved to the farm's onsite nursery (Figure 1). Gilts that were not selected as replacement females were transferred to an offsite wean to finish facility for market pig production. Immediately following selection into the onsite nursery, swabs of the anterior vagina were taken for lipidomic analysis. Gilt's vulva was sprayed with ethanol and wiped clean with gauze before the swab was taken. A human pap smear brush (Rovers® EndoCervex-Brush®, Oss, Netherlands) was placed into the vagina as far as possible and then rotated clockwise to get a representative scraping of the anterior vagina. Swabs were taken in duplicate and then placed in 15 mL polypropylene conical tubes (Corning™ Falcon™, Corning, NY) and immediately placed on ice, and transferred to the Purdue laboratory and stored at -80ºC until analysis.

Gilts (n=400) approaching 25 weeks of age were selected to move from the on-site nursery into the gilt development unit (GDU) to receive daily, full-contact boar exposure to induce puberty. Following observance of a second estrus, gilts were moved to a gestation crate to be bred using artificial insemination (AI) on their third estrus. Gilts failing to show estrus by 28 weeks of age were administered a full dose of P.G. 600 (Intervet America, Inc., Millsboro, DE) and bred as soon as they came into estrus. Gilts that never showed estrus were removed from the selection pool. Breeding date, age of first estrus, and induction of estrus with P.G. 600 were entered into the Metafarms database.

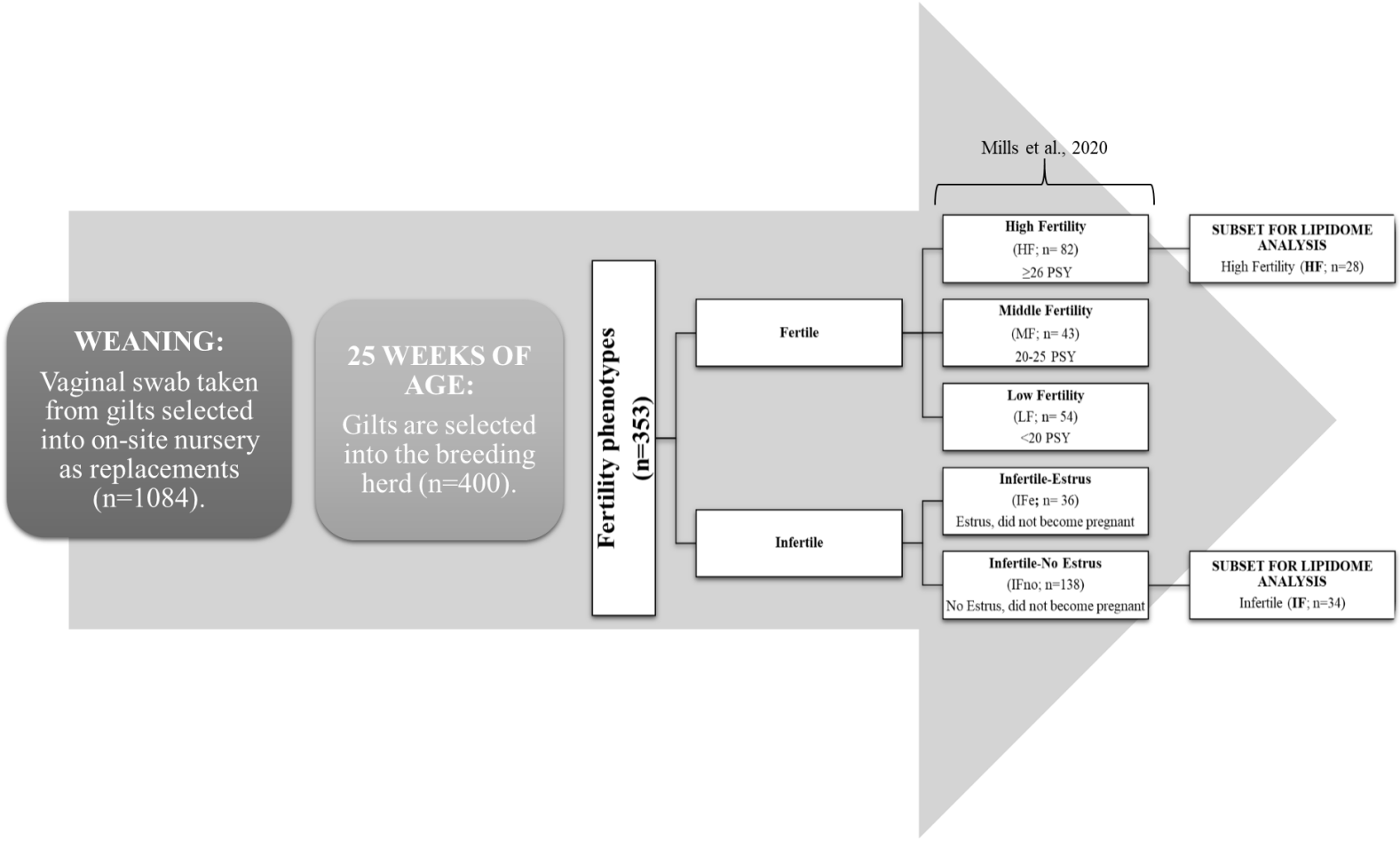

Figure 4.1 Longitudinal study design and fertility phenotype classification

Figure depicts the timeline of the study highlighting the three stages of replacement sow selection and characterization of fertility phenotypes. Fertility phenotypes were characterized based on pigs per sow per year (PSY) and whether gilts exhibited signs of estrus. A subset of animals from the most extreme fertility phenotypes previously described in Mills et al., 2020 were used for lipidomic analysis. Gilts classified as infertile for lipidome analysis never exhibited estrus and were never bred. Fertile gilts selected for lipidome analysis were from the high fertility group where gilts had at least 26 PSY.

4.3.2 Categorization of Fertility Groups

Performance data from breeding herd animals (n=400) were extracted from Metafarms reports which included date of birth, dates of estrus detection, herd removal (cull) date, reason for removal, farrowing date and litter information (total born, number born alive, preweaning mortality). Gilts that were culled from the breeding herd for non-reproductive reasons were removed from data set. Gilts that remained (n=353) were divided into fertile and infertile groups (Figure 1). Fertile animals were classified into one of three subclasses: sows with at least 26 PSY were defined as High Fertility (HF; n=82), 20-25 PSY were classified as Middle Fertility (MF; n=43), and less than 20 PSY were classified as Low Fertility (LF; n=54). PSY was chosen as it encompasses multiple facets of fertility and prolificacy, such as ovulation rate, wean to estrus interval, farrowing rate, and embryo survival rate (Vinsky et al., 2006; Soede et al., 2011; Koketsu et al., 2017). Infertile animals were divided into two subclasses based on whether they exhibited estrus following routine boar exposure or P.G. 600 administration. Infertile-Estrus (IFe; n= 36) were gilts that showed signs of estrus but did not become pregnant and Infertile-No Estrus (IFno; n=138) were gilts that never exhibited signs of estrus. A subset of the most fertile (HF; n=28) and infertile animals that did not exhibit estrus (IF; n=34) were randomly selected for lipidome analysis. Because all gilts within the study were reared under the same environmental conditions, detectable differences in the vaginal lipidome are likely subtle. Therefore, to identify a potential biomarker of fertility, the vaginal lipidome of animals from extremes of the fertility spectrum identified by Mills et al. (2020b) was analyzed.

4.3.3 Statistical Analysis of Phenotypic Traits

Analysis of phenotypic traits such as birth litter size, nursing litter size, average daily gain from birth to weaning, birth weight, weaning weight, and vulva width by fertility category was performed using the GLM procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC).

4.3.4 Lipidomic Extraction and Multiple Reaction Monitoring (MRM) Profiling

Lipids were extracted from swabs using the Bligh and Dyer method (Bligh and Dyer, 1959). Samples were thawed at room temperature before the start of the extraction. Swabs were rinsed with 500 µl deionized water and vortexed to remove cellular material in the same 15 ml polypropylene conical tubes. Two hundred µl of sample was transferred to a new 1.7 ml tube (Axygen®, Corning, New York). Four hundred fifty µl of methanol prepared with 1mM butylated hydroxytoluene and 250 µl of chloroform was added to the solution and vortexed. Samples were then incubated for 15 min at 4º C. An additional 250 µl of both deionized water and chloroform were added to the tube and centrifuged 10 min at 3000 rcf at 4º C. The solution was separated into three phases consisting of the polar, protein, and organic (lipid) phase. The lipid phase was removed, placed into 1.7 ml microcentrifuge tubes, and dried in a vacuum concentrator for 8 h. Dried pellets were resuspended 200 µl of acetonitrile, methanol, and ammonium acetate 3:6.65:0.35 (v/v/v). Further 10X dilution of sample in solvent was used for direct injection.

MRM profiling was done using a two-part process beginning with a discovery phase followed by a screening phase. The discovery phase was used to determine which lipids were detectable in the samples. For this, ten µl from each sample was pooled by phenotype into a 1.7 ml tube and then dried by nitrogen flow for 8 h. Dried lipid extracts were diluted in 200 µl of acetonitrile/methanol/ammonium acetate 300mM 3:6.65:0.35 (v/v/v). Eight µl from each pooled

sample were injected into the microautosampler (G1377A) in a QQQ6410 triple quadrupole mass spectrometer (Agilent Technologies, San Jose, CA) equipped with an ESI ion source. A solvent solution containing acetonitrile with 1% formic acid at 10 µl/min was pumped between injections (CapPump G1376A, Agilent Technologies, San Jose, CA). Pure methanol was injected between samples to remove any remaining lipids from the previous injection.

During the discovery phase, pooled samples were screened for the chemical classes of acylcarnitine (AC), cholesteryl ester (CE), ceramide, free fatty acids (FFA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylserine (PS), and triacylglycerol (TAG) lipids using MRM profiling methods previously reported (de Lima et al., 2018; Dipali et al., 2019; O'Neil et al., 2020; Suarez-Trujillo et al., 2020). Additionally, lipids from previous research that had been found by MRM profiling to discriminate between gilts fed different postnatal diets were also screened (Harlow et al., 2019b). Our studies described in Harlow et al., 2019, identified 146 lipids that discriminated between gilts that suckled colostrum versus bottle fed milk replacer the first 48 h postnatal, and are referred to as Method 1 (Harlow et al., 2019a). Lipids in Method 1 were primarily TAG, PE, PC, and PG. A second set of 197 lipids distinguished between gilts fed a lard based fat supplementation and unsupplemented animals, and are referred to as Method 2 (Harlow et al., 2019a). Lipids in Method 2 were primarily glycerolipids (Harlow et al., 2019a). Processing the initial chemical class data was completed using MSConvert20 which converted each set of profiling method data into mzML format. Signal intensity for ions present in the samples was obtained using an inhouse script. Ions with values >30% in at least one of the samples compared to a blank within each profiling method were selected for the screening phase.

Due to the large number of MRMs to be screened, the samples were interrogated using four lists of MRMs, which we refer to as methods:

1. Phosphatidylcholine lipids (PC)
2. Free fatty acids (FFA)
3. Lipid classes defined by Harlow et al., as Method 1
4. Lipid classes defined by Harlow et al., as Method 2

4.3.5 MRM Profiling Data Analysis in MetaboAnalyst

For data analysis, the relative ion intensity of a given MRM in each sample was calculated by class of lipid or method employed in each screen, following removal of MRM ion pairs with intensities less than 1.3-fold of the blank sample. Relative ion intensity of MRMs was calculated by dividing intensity by the sum of intensities of all lipids within a sample by screening method. Tentative lipid class attributions based on associated functional group and biological information were assigned to unidentified MRM ion pairs to increase potential biomarker discovery using Lipid Maps (http://www.lipidmaps.org/). Relative intensity of MRM ion pairs were uploaded into MetaboAnalyst 4.0 (Pang et al., 2020) and data were normalized using autoscaling. Student t-test analysis was used to identify MRMs that distinguished between fertility phenotypes, using an alpha of 0.05 of nominal *P*-value to identify differentially distributed lipids. Biomarker analysis was completed on differentiating lipids using classical univariate receiver operating characteristic (ROC) curve analysis with area-under-the-curve (AUC) value used to determine a lipid's potential as a biomarker. The following AUC scale was used to evaluate lipids as potential biomarkers: excellent = 0.9-1.0; good=0.8-0.9; fair=0.7-0.8; poor=0.6-0.7; fail=0.5-0.6 (Xia et al., 2013).

4.4 Results

There were no differences between highly fertile (HF) and infertile (IF) gilts in their birth and weaning weights, nor their birth or suckling litter size (Table 4.1). Moreover, there was no difference in vulva size at weaning, which is consistent with our previous findings of the entire population (Mills et al., 2020b).

Table 4.1 Birth and weaning weight, litter size and vulva width at weaning of high fertility (HF) and infertile (IF) gilts.

On-farm Characteristic	HF (n=28)	IF (n=34)	SE	*p*-value
birth litter size, piglets	13.04	12.82	0.49	0.74
nursing litter size, piglets	13.54	13.41	0.14	0.53
average daily gain[1], kg/d	0.16	0.16	0.01	0.96
birth weight, kg	1.77	1.67	0.06	0.20
weaning weight, kg	5.29	5.12	0.29	0.65
vulva width, mm	9.00	8.81	0.29	0.65

[1]Average daily gain from birth to weaning

The MRM profiling discovery phase identified a total of 269 MRMs related to distinct molecules in pooled samples from HF and IF vaginal swabs. Over 20% of MRMs related to lipids were categorized as phosphatidylcholines (PC). Free fatty acids (FFA) represented approximately 13% of the lipids profiled, and triacylglycerols (TAG) made up approximately 12% of the lipids found. The remaining were acylcarnitines (AC), phosphatidylethanolamines (PE), cholesteryl esters (CE), sphingomyelins (SM), phosphatidylserines (PS), ceramides (Cer),

phosphatidylinositol (PI), and phosphatidylglycerols (PG) in that order (Figure 4.2). Thus the majority (57%) of the vaginal lipidome was comprised of cell membrane lipids, which are represented by the PC, PE, SM, PS, Cer, PI and PG classes.

MRMs identified in high quantities during the discovery phase along with MRMs previously identified as most discriminating based on perinatal nutrition (Method 1 and 2; Harlow et a., 2019) were profiled in individual samples during the screening phase. Tentative attributions were assigned to differentially abundant lipids within each screening method (Table 4.2). Lipids related to the FFA class were the most distinguishing between phenotypes (Figure 4.3). Scores plots show that approximately one-third of the IF gilts separated from the group. When data were analyzed using a FDR-cut off of 0.05 no lipids were found to distinguish between groups. However, using a nominal p-value cut-off of 0.05, six of the of the 36 FFA lipids were found different between HF and IF gilts (Table 4.2). Arachidonic acid (ARA) and docosahexaenoic acid (DHA) were higher (P<0.05) in HF than IF gilts, whereas cerotic acid C(26:0), ximenic acid C(26:1), nonadecanoic acid (C:19:0), and pentadecanoic acid (C15:0) were lower (P<0.05) in HF than IF gilts. Of the 181 lipids screened by Method 1, 12 were differentially abundant (P<0.05) between HF and IF gilts. HF had a greater amount of MRMs attributed as PC(32:1) and SM(d18:0/18:0). Infertile animals had a greater amount of MRMs related to PC(40:6), PCo(38:0), cholesteryl ester 18:0, docosadienoylcarnitine, PC(40:7), PS(32:2), PC(40:6), Lyso-PG(20:5), PSo(18:0), and PC(40:8). Only one of the 102 MRMs in Method 2 was different (P<0.05) between HF and IF animals and was the unidentified MRM of 554.79 -> 282.192, which was higher (P< 0.05) in HF compared to IF animals. Based off the precursor ion we attributed this MRM to be a ceramide.

ROC curve analysis was performed to determine the potential of lipids differentially abundant between HF and IF as biomarkers of fertility (Table 4.2; Figure 4.4). Cerotic acid (C26:0), ximenic (C26:1), and nonadecanoic acid (C19:0) had AUC between 0.7-0.8, indicating fair potential as biomarkers. All other distinguishing lipids had an AUC of 0.6-0.7, indicating poor potential as biomarkers of fertility status (Table 4.2).

Table 4.2 Relative difference between HF and IF in abundance of lipids extracted from vaginal swabs taken at 21 days of age.

Method	Tentative Attribution/MRM ID	Lipid Class	Common Name	HF-IF FC[1] (log2)	*p*-value	AUC[2]
FFA	C(26:0)	free fatty acid	Cerotic Acid	-0.30	<0.01	0.74
	C(26:1)	free fatty acid	Ximenic Acid	-0.43	0.01	0.71
	C(19:0)	free fatty acid	Nonadecanoic acid	-0.16	0.01	0.73
	C(20:4)	free fatty acid	Arachidonic Acid	0.28	0.03	0.66
	C(15:1)	free fatty acid	Pentadecenoic acid	-0.25	0.04	0.64
	C(22:6)	free fatty acid	Docosahexaenoic Acid	0.41	0.05	0.60
M1	PC(40:6)	Glycerophosphocoline	Phosphatidylcholine 40:6	-0.17	0.01	0.68
	PCo(38:0)	Glycerophosphocoline	Phosphatidylcholine O-38:0	-0.23	0.02	0.64
	CE(18:0)/ST(16:0)	Cholesterol ester/Sitosteryl ester	CE(18:0)/ST(16:0)	-0.30	0.02	0.65
	PC(32:1)	Glycerophosphocoline	Phosphatidylcholine (32:1)	0.16	0.03	0.65
	PC(40:7)	Glycerophosphocoline	Phosphatidylcholine (40:7)	-0.17	0.03	0.64
	PS(32:2)	Glycerophosphoserine	PS(32:2)	-0.31	0.03	0.63
	PCo(40:6)	Glycerophosphocoline	Phosphatidylcholine O-40:6	-0.16	0.04	0.65
	LPG(20:5)	Glycerophosphoglycerol	Lyso PG 20:5	-0.30	0.04	0.63
	PSo(18:0)	Glycerophosphoserine	PS O-18:0	-0.31	0.05	0.62
	PC(40:8)	Glycerophosphocoline	Phosphatidylcholine 40:8	-0.14	0.05	0.62
	SM(d18:0/18:0)	Sphingomyelin	Sphingomyelin d18:0/18:0	0.14	0.05	0.62
M2	554.79 -> 282.192	Not Identified	n/a	0.19	0.02	0.66

[1]Fold change

[2]AUC=area under the curve from receiver operator characteristic (ROC) curve analysis to evaluate potential as a biomarker to distinguish animals based on fertility group.

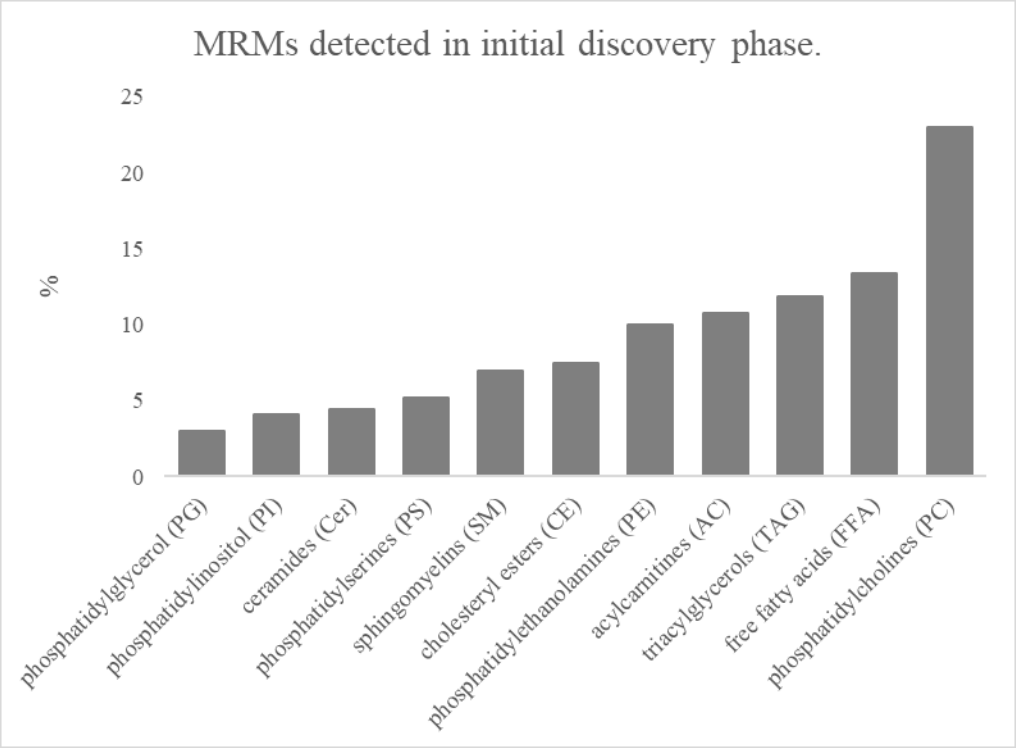

Figure 4.2 Number of MRMs related to the lipid classes screened

MRMs presenting at least 1.3x fold-change in at least one of the samples interrogated in the initial discovery phase.

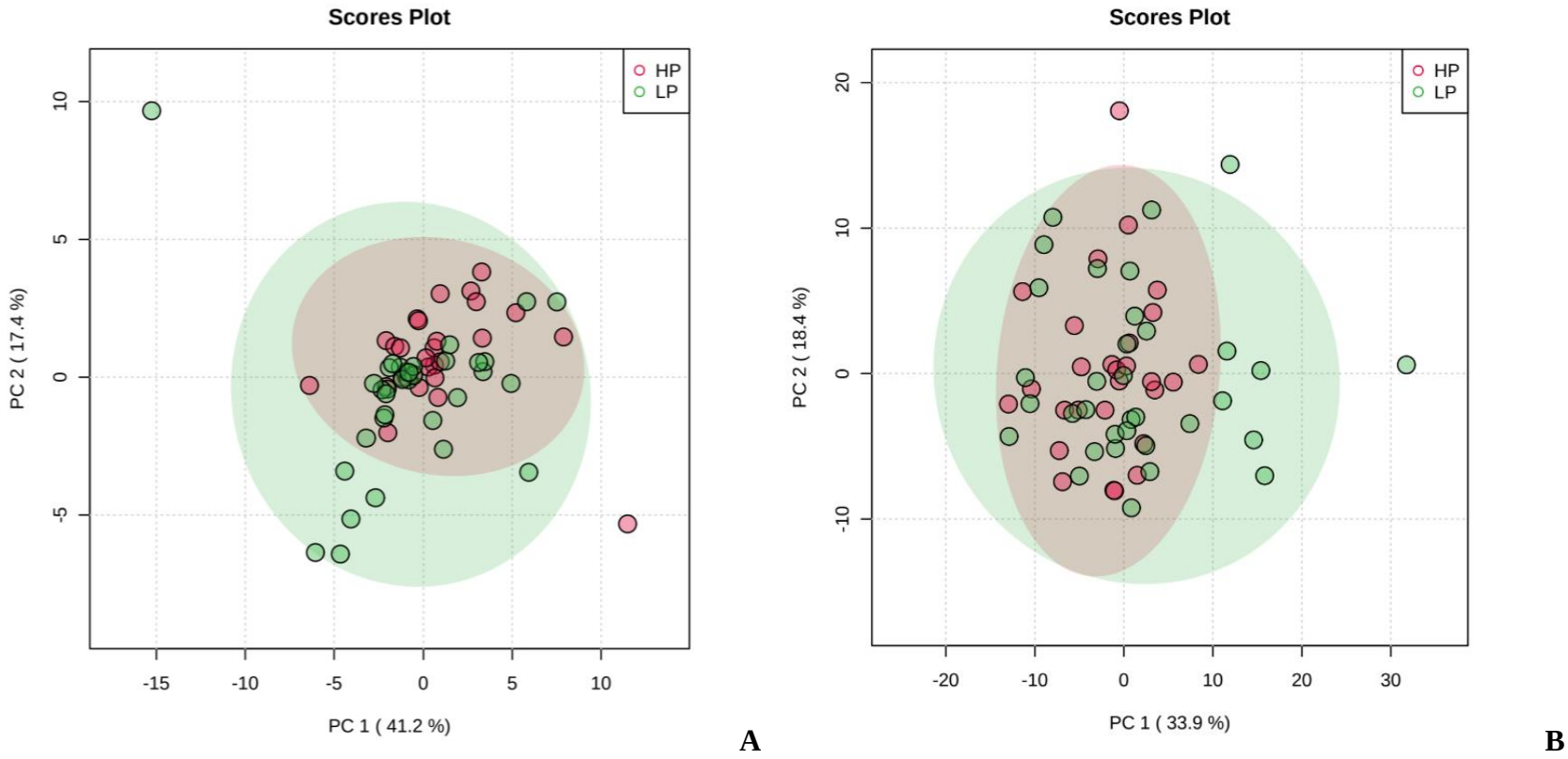

Figure 4.3 PCA Scores plots of screening methods that displayed the greatest separation between phenotypes.

(A) Scores plot for FFA method. (B) Scores plot for Method 1. Red circles represent the high fertility (HF) phenotype. Green circles represent the infertile (IF) phenotype. The same individual IF animals clustered together away from the rest of the population for both FFA method and Method 1 based on their vaginal lipidome at 21 days of age.

Correlation analysis of distinguishing lipids revealed that ARA had an inverse relationship ($P<0.05$) to cerotic acid (r=-0.57), nonadecanoic acid (r=-0.35), and ximenic acid (r=0.50). Approximately one-third of IF animals exhibited a lipidome signature of low ARA coupled with high levels of cerotic, nonadecanoic, and ximenic acid. Ximenic acid, cerotic acid, and nonadecanoic acid had a positive relationship to each other, with ximenic and cerotic acid having a very strong relationship to each other (r^2=0.94). IF animals with high levels of cerotic acid also had high levels of Lyso PG (20:5) (r^2=0.41). DHA had a relatively weak relationship when compared to all other lipids in the analysis ($-0.15 \leq r^2 \geq 0.2$). The phenotype driving the correlation between cerotic acid, ximenic acid, and ARA was the IF group (Figure 4.4).

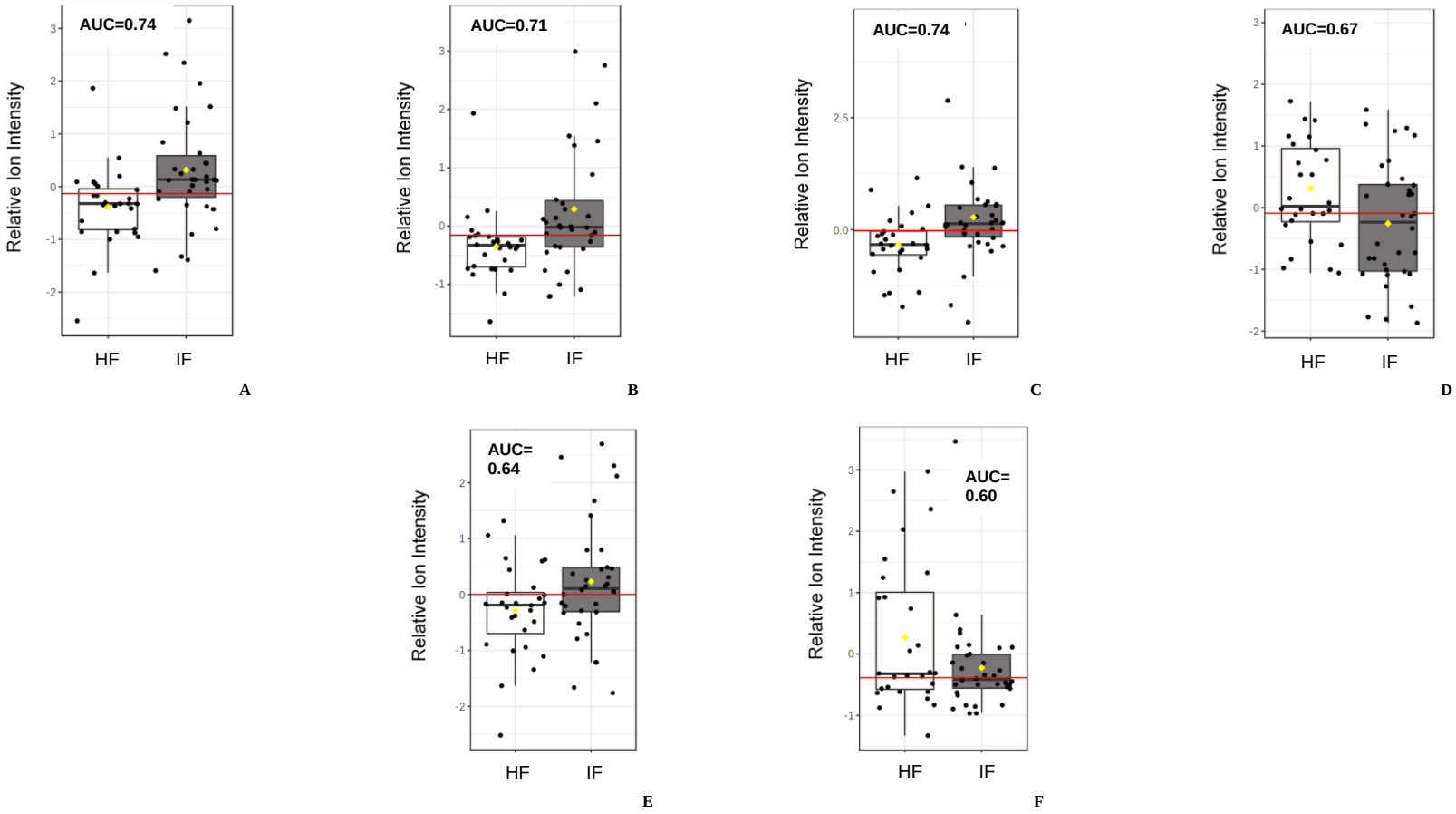

Figure 4.4 ROC curve analysis and distribution of differentially abundant FFAs.

(A) Area-under-the-curve (AUC) value and distribution of high fertility (HF; white box) versus infertile animals (IF; gray box) for C(26:0); Cerotic Acid. (B) C(26:1); Ximenic Acid (C) C(19:0); Nonadecanoic Acid (D) C(20:4); Arachidonic Acid (E) C(15:1); Pentadecenoic Acid and (F) C(22:6); Docosahexaenoic Acid. The following AUC scale was used to evaluate lipids as potential biomarkers: excellent = 0.9-1.0; good=0.8-0.9; fair=0.7-0.8; poor=0.6-0.7; fail=0.5-0.6. The yellow notches within the distribution plots represent the 95% confidence interval around the median for each group. If the notches do not line up, the medians are likely different. The red line represents the median. The y-axis for box plots represent the relative ion intensity of lipids within each sample.

4.5 Discussion

Our overall aim was to determine whether lipids captured from vaginal swabs taken at weaning could be used to predict fertility outcomes in swine. Approximately one-third of the IF animals clustered together and away from the population in principal component analysis (PCA) of FFA and lipids in Method 1. Further analysis found that vaginal lipid profiles of approximately one-third of the infertile animals at weaning were high in cerotic, ximenic, nonadecanoic, and eicosapentaenoic acids and low in arachidonic and docosahexaenoic acids. These animals were also driving the associations between arachidonic acid and cerotic acid, ximenic acid, and the Lyso PG lipids (Figure 4.5). Thus, data indicate that while all infertile animals did not show distinct lipidomes, likely reflecting multiple factors that affect fertility, approximately one-third showed lipidome profiles at weaning that distinguished them from highly fertile animals.

Arachidonic (ARA) and docosahexaenoic (DHA) acid levels in vaginal swabs were higher in gilts that were high fertility at maturity. ARA and DHA are classified as polyunsaturated fatty acids (PUFAs) and are known to be positively related to female reproductive success (Nehra et al., 2012; Smit et al., 2013; Elis et al., 2016; Sinedino et al., 2017; Maillard et al., 2018). Linoleic acid (LA) is the precursor to ARA and alpha linolenic acid (ALA) is the precursor of DHA. Neither LA nor ALA can be synthesized by the body and must be consumed in the diet (Whelan and Fritsche, 2013). DHA and ARA can also be derived from the diet and transferred through colostrum and milk to the tissues of piglets (Arbuckle and Innis, 1993; Lin et al., 2015; Roszkos et al., 2020). ARA levels are high in milk (Davis et al., 1994). In gilts fed colostrum versus milk replacer, gilts that received colostrum had a greater amount of ARA in the vaginal lipidome on PN d 14 signifying ARA as a marker of colostrum intake (Casey et al., 2018a). The level of colostrum

intake in the first 24 h postnatal was associated with long-term fertility in swine (Vallet et al., 2015a). Therefore, these data together establish support for a connection between level of colostrum intake, level of ARA in vaginal lipidome and long-term fertility in swine.

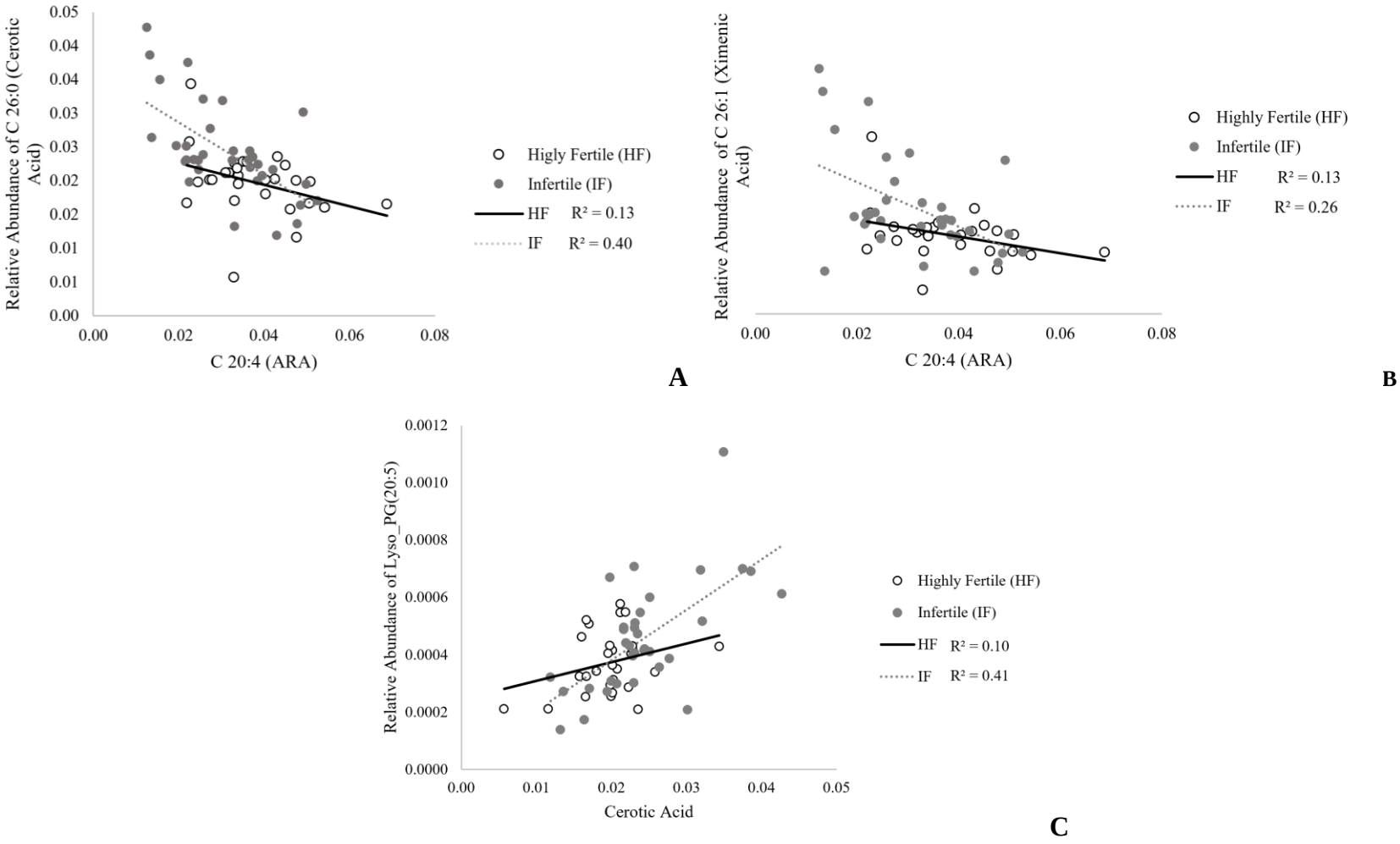

Figure 4.5 Correlation analysis of distinguishing free fatty acids and glycerophosphoglycerols within IF and HF animals

(A) Correlation analysis of the relative abundance of C(26:0) Cerotic Acid and Arachadonic acid (ARA). (B) Correlation analysis of the relative abundance of C(26:1) Ximenic Acid and ARA. (C) Correlation analysis of the relative abundance of Lyso_PG (20:5) and Cerotic acid.

Level of perinatal exposure to DHA has been linked to tissue levels. DHA levels within plasma, liver phospholipids, and brain plasma membrane of two week old pigs was found related to levels in milk fed to them as neonates (Arbuckle and Innis, 1993). Moreover, rats and humans fed a diet high in DHA had increased reproductive success with greater oocyte quality at an advanced maternal age (Nehra et al., 2012). Holstein cows consuming DHA postpartum had an increased number of large follicles (Elis et al., 2016), increased pregnancy rate at first breeding, and a greater resumption of cyclicity following calving (Sinedino et al., 2017). DHA treatment of bovine granulosa cells in culture increased proliferation and progesterone secretion (Maillard et al., 2018). Feeding DHA to gestating gilts and sows increased litter growth and larger corpus lutea size on the ovaries when they cycled after litters were weaned (Smit et al., 2013). Similarly, sows supplemented with DHA during lactation appeared to have improved quality of ovarian follicles and increased survivability of embryos (Roszkos et al., 2020). DHA has inhibitory effects on prostaglandin-H-synthase which may be beneficial for the preparation of endometrium to maintain pregnancy (Bilby et al., 2006). Thus, the relationship between DHA and fertility is well documented, and now studies need to be designed to determine if increasing exposure of gilts to DHA during the perinatal period affects their long-term fertility.

ROC curve analysis indicated that nonadecanoic acid had the highest AUC value of all distinguishing lipids. Nonadecanoic acid is a 19-carbon saturated fatty acid that has ties to reduced fertility. Men with asthenozoospermia had higher levels of nonadecanoic acid within their seminal plasma than their healthy counterparts (Tang et al., 2017). This group found high levels of C19:0 to be a potential biomarker of asthenozoospermia related infertility in men (Tang et al., 2017). However, little is known about the relationship between elevated nonadecanoic acid and impaired female fertility. Pentadecenoic acid (C15:1) was also found to be higher in IF gilts. Odd-chain

fatty acids must be broken down via alpha-oxidation within the peroxisome (Jenkins et al., 2015). Therefore, a buildup of odd chain fatty acids in IF gilts may be indicative of a peroxisomal issue.

ROC curve analysis indicated that cerotic and ximenic acid were fair biomarkers of infertility and were higher in vaginal swabs of IF gilts at weaning. Cerotic acid, is a saturated very long-chain fatty acid (VLCFA) consisting of 26 carbons (Leon et al., 1965; Kim et al., 2019b). VLCFAs with C22 and C24 are found ubiquitously throughout the body, whereas VLCFAs with 26 carbons or more are sub-classified as ultralong-chain FAs (ULCFAs) and are found in the skin, retina, testis, and brain (Sassa and Kihara, 2014). The outermost layer of the epidermis is comprised of saturated and monounsaturated UCLFAs which are essential in creating a barrier against water loss and invasion of pathogens (Proksch et al., 2008). Similar to the epidermis, the lumen of anterior region of the vagina is composed of stratified epithelium. Therefore, the presence of ULCFAs captured on vaginal swabs is to be expected. However a higher proportion of them, as evident in the IF group, may be indicative of alterations in lipid metabolism that affects health and fertility potential (Whigham et al., 2013). Fatty acids longer than 22 carbons are exclusively broken down in the peroxisome (Steinberg et al., 2006). Impairment of VLCFA degradation pathways is associated with several diseases related to impaired peroxisomal function and biogenesis (Sassa and Kihara, 2014). Cerotic acid build-up is found in individuals with adrenoleukodystrophy (ALD). ALD is an X-linked recessive condition characterized by buildup of VLCFA in tissues due to a mutation in the *ABCD1* gene (Lu et al., 1997; Moser et al., 2007). ABCD1 functions to transport VLCFA into peroxisomes. In addition to the development of cognitive impairment, males with ALD are infertile. Accumulation of saturated fatty acids within Sertoli cells is hypothesized to be a cause of infertility in men with ALD (Powers and Schaumburg, 1981; Moser et al., 2007) .

The eicosanoid Lyso-PG (20:5) was higher in IF animals and positively related to cerotic acid levels in vaginal swabs. The fatty acid associated with Lyso-PG (20:5) is a PUFA that is converted to prostaglandins (Rodriguez-Estrada et al., 2014). Infertile gilts also had a higher abundance of certain phosphatidylcholines, namely PC(40:6), PC(40:7), PCo(40:6), and PC(40:8), which are highly unsaturated and compatible with IF animals having a buildup of eicosanoids in vaginal tissue at weaning. In a fertility trial, cows who were less receptive to pregnancy had a greater abundance of PC(40:6) and PC(40:7) in their uterine lipidome (Belaz et al., 2016). Perigonadal tissue from reproductively old mice also had a higher abundance of PC(40:6) and PC(40:7) (Dipali et al., 2019). Another disease associated with the presence of elevated VLCFAs is Zellweger syndrome. Individuals with this condition lack intact peroxisomes and cannot properly metabolize prostaglandins (Diczfalusy et al., 1991).

Across the entire larger study, 39% of the animals were categorized as infertile, no estrus (138 out of 353) on this commercial operation that produces its own replacement gilts (Mills et al., 2020). However, infertile gilts from this operation and study were not followed to slaughter to harvest ovaries to verify infertility status of these gilts. If the current study's findings are reflective of true sensitivity and specificity for infertility identification, this test-analysis could bring down the rate of infertile animals in this commercial herd to 26%, by getting rid of the 13% that showed this lipidome profile at weaning. That said, it is also important to note that rates of reported infertility in gilts vary across studies and management systems.

For example, in a controlled study conducted by Patterson et al. (2010) where all animals received proper boar exposure and accurate documentation of estrus, 20% of gilts who did not cycle following boar exposure did not respond to puberty induction with P.G. 600. Whereas, an observational study of a commercial operation in Southwest China, reported 56% of gilts were

culled due to failure to show estrus at greater than 9 months of age, but were never given P.G. 600 (Wang et al., 2019b). The differences observed between the commercial operation and the highly controlled studies could be attributed to variation in management practices prior to and around the time of puberty or in data reporting. Therefore, more research is needed to validate this technique's ability to identify true infertility. Although the true predictive ability of MRM profiles of vaginal lipidome at weaning still needs to be determined to validate biomarkers of infertility, the economic impact and production efficiency that may be gained for the producer supports continued research in this area. Identification of infertile animals at an early age would reduce the overall number of resources used to raise replacement gilts that provide minimal economic return. Gilts unable to achieve puberty are sold to cull markets for meat consumption. Cull markets are utilized by producers when an animal is too large or heavy for conventional meat packing plants. However, selling gilts to a cull market does not cover the economic loss associated with raising gilts to puberty or premiums paid for genetics, thus creating large cost differential for the producer likely accounting for millions of dollars lost each year.

4.6 Conclusions

Through the use of untargeted lipidomic analysis techniques, potential biomarkers predictive of sow fertility have been identified in the vaginal lipidome of gilts at 21 days of age. Low ARA combined with higher abundance of very long chain fatty acids and phospholipids with prostaglandins in vaginal swabs taken at weaning was predictive of infertility in approximately one-third of IF animals in this study. ROC curve analysis supports further exploration of cerotic acid (C26:0), ximenic acid (C26:1) and nonadecanoic acid (C19:0) as potential biomarkers of reproductive efficiency, as well as studies aimed at understanding the mechanistic relationship of

a greater proportion of long chain fatty acids and higher proportion of PUFA in vaginal swabs of three-week old piglets that in the long-term are infertile. Application of these findings has the potential to improve reproductive efficiency of swine farms, and in this way reduce economic losses for the producer.

4.7 Acknowledgments

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CHAPTER 5. FUTURE DIRECTIONS

The aim of the work presented herein was conducted to identify biomarkers predictive of fertility in boars and gilts using omics tools for discovery. As stated throughout this dissertation, there is no single test for fertility prediction. Reproductive efficiency in both sows and boars is an important economic trait for producers. However, emphasis of genetic selection is placed on progeny performance traits such as feed efficiency, growth rate, and carcass characteristics, which are often negatively related to fertility (Oh et al., 2006b; Safranski, 2008). Subfertility negatively impacts farrowing rate and litter size and subsequently results in increased sow culling rates related to reproductive failure (Ruiz-Sánchez et al., 2006; Roongsitthichai et al., 2013; Roca et al., 2015). Therefore, in order to increase farm efficiency and reduce culling rates, there is a need to identify subfertile and infertile animals prior to their entry into the breeding herd.

5.1 Boars

Shotgun proteomic analysis of seminal plasma samples from the boar's first ejaculate support that differences in protein profiles may prospectively reflect differences in boar fertility phenotype (Mills et al., 2020a). Only one protein, inositol-1-monophosphatase (IMPA1)—the enzyme responsible for releasing free inositol into the body, was lower in abundance when compared to high performance boars across the three subfertile phenotypes (Mills et al., 2020a). Interestingly, boars within the low total born and low farrowing rate phenotype had signs of blood microparticles within their seminal plasma. We also identified several proteins higher in abundance in high fertility boars previously associated with higher fertility PSP-1, MAN2B2, SOD1, PRDX6, and GPX6. However, the common denominator distinguishing these boars is that

the seminal plasma of subfertile boars has a reduced ability to protect sperm cells from reactive oxygen species. Sperm cells are susceptible to oxidative damage from the female reproductive tract and oxidative stress has been associated with pregnancy failure and embryonic loss (Sanocka et al., 1997; Aitken et al., 2003). The presence of high levels of antioxidant species such as superoxide dismutase, glutathione peroxidase, and catalase in seminal plasma function to protect sperm from oxidative stress during transit through reproductive tracts (Garrido et al., 2004; Bromfield, 2014).

Taken together, the abundance of a panel of seminal plasma proteins, rather than a single protein, should be screened to predict reproductive performance of boars. Among the potential candidates to screen for subfertility would include higher abundance of proteins categorized as blood components and inflammation markers of subfertility, and lower abundance of antioxidant levels and IMPA1, or simply inositol levels. To move forward and translate findings into practice at the commercial boar study level, a panel of proteins needs to be validated for sensitivity and specificity of fertility/subfertility prediction before implementation. Experimentation should begin by screening seminal plasma samples for specific proteins associated with subfertility found in our preliminary data. Those proteins include inositol-1-monophosphatase (IMPA1), hemoglobin (HBB and HBA), alpha-2-macroglobulin (A2M), macrophage migration inhibitory factor (MIF), superoxide dismutase (SOD1), peroxiredoxin 4 (PRDX4), peroxiredoxin 6 (PRDX6), and glutathione peroxidase 6 (GPX6). Following detection and validation, we must aim to create a statistical model that is able to predict fertility based on protein abundance in seminal plasma to be utilized for selection decisions at the level of the boar stud. However, it is important to note that to create an accurate model for fertility prediction requires a large sample size i.e. thousands of animals to achieve the appropriate effect size.

Furthermore, exploration into the reason as to why blood microparticles are present in subfertile boars and whether their presence changes over time would provide valuable information on whether there are ways to manage boars to prevent subfertility. Hemoglobin subunit beta was also identified in the seminal plasma proteome of adult boars by González-Cadavid et al. (2014b) using two-dimensional SDS-PAGE. However, abundance was not related to differences in sperm quality parameters. The presence of blood microparticles in the seminal plasma proteome of subfertile boars may suggest a disruption of the blood-testis barrier which functions to protect developing sperm cells from being attacked by the immune system. Trials evaluating how levels of blood microparticles change over time and in the presence of different stressors should be performed to help understand what might be causing reduction in fertility.

5.2 Gilts

In chapter 3 of this dissertation, on-farm indicators which included perinatal data and vulva size at 21 days of age were evaluated. A weak relationship was found between vulva width at weaning and probability that a gilt would become pregnant (Mills et al., 2020b). Vulva width explained only 1.2% of the variation evident in reproductive performance, thus vulva width at weaning is not sensitive enough to select for breeding herd replacement gilts. It is also important to note, findings from this study demonstrate there was no difference in birth or weaning weight, average daily gain or nursing litter size of gilts that became highly fertile production sows versus those found infertile. The commercial operations are challenged with replacement gilt selection, as differences are not readily apparent until animals are 6 months of age.

Following the results from Mills et al. (2020b), it was apparent that a more sensitive test was necessary to pick up any potential differences or biomarkers predictive of fertility between

gilts at 21 days of age. Based on previous work out of our own group, it was hypothesized that lipids captured from vaginal swabs taken at weaning could be used to predict fertility outcomes in swine (Casey et al., 2018b). In chapter 4, relative abundance of multiple MRMs related to lipids was different in profiles of biological material captured on swabs of gilts at 21 d of age that were later found to be infertile and high fertility sows (Mills et al., 2021a). Vaginal lipid profiles of approximately one-third of the infertile animals at weaning were found to be high in cerotic, ximenic, nonadecanoic, and eicosapentaenoic acids and low in arachidonic and docosahexaenoic acids. Lipidome profiles are reflective of what *has happened* in a system and vaginal tissue is composed of stratified epithelium. The stratification of the cells and movement from basal to apical layers reflects a history of exposures. Our studies of the vagina showed that the layers of stratification increased in parallel with alterations in abundance of proteins as the tissue developed over the first two weeks postnatal (Harlow et al., 2019c), and thus samples taken at two and three weeks postnatal likely reflect biochemical products related to nutritional environment in the perinatal period. Beta-oxidation of VLCFA and break-down of odd chain fatty acids and eicosanoid derivatives occur in peroxisomes (Lodhi and Semenkovich, 2014). Infertile animals at weaning had a greater abundance of VLCFA, eicosanoids and odd chain fatty acids and low DHA and ARA in cellular material captured with vaginal swabs. We hypothesize that the low levels of ARA and DHA were due to lower levels of colostrum intake, and the build-up of VLCFA, odd-chain fatty acids and PUFA reflected a factor in infertile animal's perinatal environment that affected peroxisome function or biogenesis (Farr et al., 2016).

Arachidonic (ARA) and docosahexaenoic (DHA) acid levels in vaginal swabs were higher in gilts that were high fertility at maturity. ARA and DHA are polyunsaturated fatty acids (PUFAs) that have been associated female reproductive success (Nehra et al., 2012; Smit et al., 2012; Elis

et al., 2016; Sinedino et al., 2017; Maillard et al., 2018). Both precursors of ARA and DHA, linoleic acid and alpha linoleic acid, respectively, must be consumed in the diet as they are essential fatty acids (Whelan and Fritsche, 2013). Colostrum also provides a source for ARA and DHA and can be transferred through colostrum to the tissues of piglets (Arbuckle and Innis, 1993; Lin et al., 2015; Roszkos et al., 2020). Low colostrum intake has been associated with poorer fertility outcomes (Vallet et al., 2015b). It has also been observed that gilts reared in smaller litter sizes have less competition for colostrum and milk than those reared in larger litters (Flowers, 2009c). Therefore, low levels of ARA and DHA may be an indicator of low colostrum intake in IF gilts.

Components of milk, specifically milk fats, stimulate the activation of PPARs (Parodi, 2016; Shi et al., 2019). Therefore, there is the potential that peroxisomal deficiency or dysfunction resulted in the build of cerotic (C26:0), nonadecanoic (C19:0), Lyso-PG(20:5), in about one-third of the IF gilts, due to IF gilts likely consuming less milk than HF gilts. VLCFAs with 26 carbons or more are sub-classified as ultralong-chain FAs (ULCFAs) and are found in the skin, retina, testis, and brain (Sassa and Kihara, 2014). However, elevated levels of VLCFAs may be indicative of alterations in lipid metabolism which could affect health and fertility potential (Whigham et al., 2013). The peroxisome is exclusively responsible for the degradation of fatty acids longer than 22 carbons (Steinberg et al., 2006) and the impairment of VLCFA degradation pathways is associated with several diseases related to impaired peroxisomal function and biogenesis such as adrenoleukodystrophy (Lu et al., 1997; Moser et al., 2007; Sassa and Kihara, 2014). Another disease associated with the presence of elevated VLCFAs related to peroxisome dysfunction is Zellweger syndrome (Diczfalusy et al., 1991).

Lipid accumulation, or lipotoxicity, is associated with mitochondrial and peroxisomal dysfunction, endoplasmic reticulum stress, and negatively impacts fertility (Minge et al., 2008;

Jungheim et al., 2010; Wu et al., 2010; Luzzo et al., 2012). The build-up of ULCFA and eicosanoids in tissue of IF animals suggest that peroxisomal dysfunction may be at the root of the problem. Peroxisomes are required for the first steps of beta-oxidation of ULCFA and degradation of eicosanoids (Poulos et al., 1992; Sassa and Kihara, 2014; Dennis and Norris, 2015). Individuals with Zellweger syndrome have mutations in genes that encode for peroxin (PEX) proteins. Peroxins are required for peroxisome biogenesis (Distel et al., 1996), and peroxisome proliferator activator receptors (PPARs) activate genes related to fat oxidation and proliferation of peroxisomes in the liver through induction of PEX proteins (Lodhi and Semenkovich, 2014).

To explore this as a potential mechanism, we queried data from Rahman et al., where they evaluated the effects of exposure to colostrum versus milk replacer on the uterine transcriptome (Rahman et al., 2016). Briefly, for their study, gilts were either allowed to nurse from sows *ad libitum* or were bottle fed a commercial milk replacer for 48 hours after birth. Animals were euthanized at 48 h postnatal, uterine tissues removed and RNA was isolated for transcriptome analysis using RNA-seq (Rahman et al., 2016). Transcriptome data were searched for peroxisomal related genes, and we found that peroxisomal biogenesis factor 5 like (PEX5L), acyl-coenzyme A 3 (ACOX3), peroxisome proliferator activated receptor alpha (PPARα), and arachidonate 15-lipoxygenase (ALOX15) were numerically, but not significantly, higher in gilts that nursed sow's milk versus replacer-fed gilts. PEX5L is a paralog to PEX5 which is responsible for the assembly and function of peroxisomes (Stelzer et al., 2016b). Mice with knockout for PEX5 have an accumulation of VLCFAs due to the inability to transport VLCFAs into the peroxisome and a disruption of the PEX5 gene is common in Zellweger Syndrome patients (Baumgart et al., 2001). Another gene associated with Zellweger syndrome is ACOX3. ACOX3 functions in the regulation of peroxisomal lipid metabolism and is considered the rate-limiting step in the degradation of

methyl branched fatty acids (Hunt et al., 2012). PPARα is transcription factor that regulates genes involved in lipid degradation and inflammation, where PPARα is primarily responsible for the degradation of inflammatory fatty acid residues (Chinetti et al., 2000). Lastly, ALOX15 acts on a number of PUFAs to synthesize lipid mediators such as eicosanoids and also regulates inflammation (Stelzer et al., 2016b). These data show that piglets exposed to colostrum had greater levels of expression of peroxisome related genes. Therefore, future trials should aim to validate colostrum's influence on the lipidome signature observed in IF gilts. Additionally, future work evaluating PEX5, ACOX3, ALOX15, and PPARα expression and their relationship to colostrum intake should also be conducted to potentially identify the mechanism through which colostrum is influencing infertility in gilts.

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