CHARACTERIZING VAGINAL GENE EXPRESSION IN PREPUBERTAL GILTS

BY

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A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Animal Science

South Dakota State University

2023

DISSERTATION ACCEPTANCE PAGE
Shannon Dierking

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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DEDICATION

This dissertation is dedicated to my mother, Phyllis. Thank you for showing me how to kick ass and use stubbornness to my advantage. I'm in awe of how you handled the pressure of chasing your master's degree while working full-time and 'babysitting' Papa John. You taught me to pursue a career I love and take care of a family. I hope to carry myself with the same determination and poise that you had working for Ghude's all those years. Your support and encouragement have meant more than you will ever know, even if you think I don't listen.

DISSERTATION ACKNOWLEDGMENTS

The products of this dissertation are a culmination of four years of hard work, and I must acknowledge the influential people that helped get me to this point.

First, I must take a moment to thank my advisor, Dr. Crystal Levesque. She is single-handedly one of the best people I know, and I am eternally grateful that she selected me as the receiver of this gilt development trial over the last few years. I appreciate every kind word of encouragement (as there were many), as well as the occasional stern look. Her work ethic is unmatched, and if I come away with a fraction of her mentorship skills, I'll consider myself lucky. Thank you for making South Dakota a second home and creating a family within our lab group. To Drs. Jeff Clapper, Jodi Morton, Juan Carlos Pinilla, and Robert Thaler, thank you for agreeing to serve on my committee. Your insight and guidance throughout my trials is greatly appreciated. Special thanks are extended to Dr. Thomas Brandenburg, who served as graduate student representative. A sincere thank you is extended to Dr. Hossein Moradi Rekabdarkolaee for his friendship and guidance in statistics. Thank you for always listening to me drone on about "my girls" (the gilts), even if animals are one of your least favorite topics. To Drs. Rosie Nold and Eric Weaver, your mentorship means more to me than can ever be expressed with words. Thank you, for always providing me opportunities to grow in my teaching capabilities, but also for serving as role models in allowing my passion for teaching to flourish. Due to your guidance, I know a component of teaching is in my future. A special acknowledgement goes out to Ms. Kallista Roers, for allowing me to take you under my wing and show you all aspects of gilt research. I will always think fondly of your time as my "secretary" and while our schedules don't allow us that sort of quality time together anymore, please

know, I am so proud of everything you've accomplished, and I will always cheer you on. The swine industry is lucky to have you. Sincere appreciation is expressed to the Dr. Crystal Levesque lab group for all their assistance throughout these gilt trial collections. There were many late nights pulling blood samples, but your support means more than you could know. Additionally, thank you to Drs. Jinsu Hong and Jorge Perez-Palencia for their additional guidance as I went about the post-graduate job search. Thanks are extended to support staff within the animal science department, including Judy Carlson, and Aly Hinkle. Each one of you has answered my numerous questions with a smile on my face, and has always had a positive attitude, no matter what was going on that day. Veronica Shriver, I'll never forget the day my email account expired, and you set it to expire in 2027, but promised I would "finish before then." I've always enjoyed our chats, and I'll miss your tech knowledge. To my work mom, Terese VanRavensway, thank you for always being an encouraging aspect of my day. Even though you're no longer in the animal science department, I can still hear you cheering me on from the grant office. I'm going to miss knowing I can walk over and bug you whenever my heart desired.

Now, onto my friends. A perk of being in a place for four years means you establish friendships that will last you a lifetime. First, to Dr. Jodi Morton. You serve on my committee as an adjunct faculty member, but you are an even dearer friend to me. Thank you for your guidance and friendship over these last few years, and always listening to my hairbrained ideas (research or otherwise). Our similar experiences growing up have bonded us, and I look forward to our adventures in the future. To Ms. Katelyn Zeamer and Talia Everding, you are some of my favorite people to vent to. From our adventures in the barn (both good and bad), to our continued support via distance, I am so grateful

SDSU brought us together. Thank you for always listening to my vents and being the first people to pick me up when I fall. To Ms. Lily Hernandez and Ms. Taylor Andrews, even though you left me in this tundra for warmer weather, I'm so grateful when we get together, we don't skip a beat. I'm so proud of you both from afar, and I can't wait to see what you accomplish in the future. Sincere appreciation is expressed to Ms. Yemi Burden, who I created a bond with while in KY, and who has never stopped supporting me. Thank you for always having my back and allowing me the privilege of being an "aunt" to your son and daughter. I appreciate that your family always welcomes me with open arms when I visit, and I look forward to future family adventures. Within the department, I must extend utmost appreciation to my grad school "fam": Dr. Forest Francis, Ms. Becca Francis, Ms. Natalie Hoyes, Ms. Madison Korvarna, and Mr. Thiago Ribeiro, thank you for welcoming a swine person into your fold, always being down for family dinners, and tolerating my loud existence. I adore you all, and I'm grateful for the time I've had with each of you. Ms. Emily Fowler, thank you for trying to make me a more emotional person. It didn't work, but I'm glad we got to enjoy our time at the Grille, harassing Jason like it was our only job. Lastly, to Ms. Alex Kelly, Ms. Cailtyn Sullivan, and Mr. Joel Keiser. A.Kelly, thank you for being my twisted sister. You are selfless and caring, and I love that I can call you up at 4:00 on a random Wednesday for a red beer at Ray's, and you're my girl. I know the last few months of our respective programs have sent us in different directions, but please know I love and appreciate you so much. I look forward to our friendship continuing to grow throughout our adult lives and supporting each other through the next chapters. Ms. Caitlyn Sullivan, thank you for matching my sass, and allowing me to mom you. While our senses of humor can get dark

and twisty, there's no one else I would rather watch bad TV with on the couch or watch Toby attack. I cannot wait to see where your PhD takes you. You have worked so hard and deserve only good things. Lastly, to Mr. Joel Keiser. Our friendship was probably not one many people expected, given our distinct differences in personality. However, now that you are part of the "fam", I'm grateful for your no-nonsense attitude, and for always calling it like you see it. I know you will help transform Keiser Ag Bros into what you've always imagined it to be.

Additionally, I must take a moment to acknowledge SE NE, and the influential people that continue to support me, regardless of the miles between us. To Ms. Karen Teten, thank you for always being down to talk science with me, and allowing my inner nerd to come out. I appreciate that you consider me an extension of your family, and please know that your support is appreciated. To Mr. Joe Nelson, thank you for taking such good care of my person, Ashlyn. You haven't been in my circle for as long, but I always appreciate how willing you are to allow me to visit when I'm back in NE and can discuss baseball no matter what time of year. Sincere appreciation is expressed to Ashlyn (Teten) Nelson, words cannot describe how much your friendship means to me. Even though you're not really into pigs, you have continued to support me, regardless of the state or degree I have decided to pursue. Thanks for loving the Dierking boys so much.

Lastly, I must take a moment to thank the people that made me who I am today, my family. I must extend my appreciation to my older brother, Matt. You are always asking questions about my teaching experience and time in SD. Thank you for opening your home to me and the pets when I manage to sneak home for a visit, even if they are demons. To my mother, Phyllis, thank you for raising me to be so independent and

driven. You were my guide when it came to working in the swine industry, and you balanced a career you enjoyed along with raising a family. Thank you for always answering my calls, even the ones where I cried in frustration. To my father, John (or Papa John to those who know and love him), we don't agree on much, but that's because we are so similar. Thank you for covering my arm with dawn dish soap when I was in 7th grade and telling me to sleeve that gilt that was having farrowing issues. That event is ingrained in my core memory, and it is because of all the swine experiences you provided me, why I am where I am today. My work ethic and passion are a direct result of all the time spent at Dierking Farms, and for that I am forever grateful.

"The path of least resistance supports security, not growth."

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ABSTRACT

CHARACTERIZING VAGINAL GENE EXPRESSION IN PREPUBERTAL GILTS

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2023

The best-known predictor of reproductive success in gilts is the age at puberty. Early puberty (i.e., d170 – d180 of age) is associated with improved long-term reproductive performance and more full value offspring for market. The ability to predict, in the prepubertal stage, the age that a replacement gilt would be expected to achieve puberty has great reproductive and economic advantages. This work focuses on identifying biological markers that are potentially predictive of age at puberty. Study 1 identified changes in the vaginal epithelium during gilt reproductive development. Pre-pubertal gilts (n =13) were followed from d70 of age until first estrus or d213-215 of age. Blood, vaginal epithelia, and anogenital distance were collected at five timepoints during reproductive development [d70/77 (on-farm arrival), d100/110 (mid-folliculogenesis), d130 (post-folliculogenesis), d160 (start of boar exposure) and first estrus or end of trial (d214 of age)]. Total RNA was isolated from vaginal epithelia and relative gene expression of two toll-like receptors (TLR-4 and TLR-5), tacykinin precursor-3 (TAC-3), insulin-like growth factor-1 (IGF-1), and estrogen receptor-alpha (ER-α) was quantified by real time q-PCR, relative to expression of ribosomal protein lateral stalk subunit P0 (RPLP0). Expression of IGF-1 and TAC-3 were up-regulated 9- and 7-fold, respectively at d160 ($P < 0.05$). Expression of ERα tended to be upregulated 3-fold at d100 ($P = 0.08$) and expression of TLR-4 and TLR-5 was lowly detected prior to first estrus. Anogenital

distance was positively correlated to age at first estrus and negatively correlated to daily gain ($P < 0.01$; r = 0.83).

In experiment 2, pre-pubertal gilts (n = 29) were followed from d70 of age until first estrus or d215 of age. Vaginal swabs were collected at similar timepoints as described in experiment 1. By d215 of age, 19 females were classified as expressing estrus 'early' (d160 to d181), 4 were 'average' (d181- d202), and 6 were 'late' or 'anestrus' (d202 to d215). A subset of 5 gilts that expressed estrus early and 5 gilts that expressed estrus 'late' or 'anestrus' were used for vaginal transcriptome analysis using RNAseq and vaginal samples at d100, d130, and d160 of age. Data normalization used the reads per kilobase million (RPKM) method. Fold change differences were calculated across genes, within each estrus grouping (early or late). Differences in gene expression between 'early' and 'late' gilts were calculated using a Welch two-sample t-test in R (v 4.0.2). A gene selection process was used based on differential expression at each time point, between estrus groups, and across multiple time points to identify target biomarker genes. The process reduced total differentially expressed genes from > 2,000 to 6 genes of interest, with genes Lin28 homolog A (LIN28A), Anoctamin 2 (ANO2), and Lysl oxidase homolog 2 (LOXL2) more highly expressed in early estrus females. Genes of interest relative to late estrus females were Glycogen synthase 2 (GYS-2), Growth regulating estrogen receptor binding 1 (GREB-1), and Interferon alpha 16 (IFN-Alpha-16). Gene LIN28A had higher expression in early estrus gilts ($P < 0.05$). There was no significance between early and late estrus gilts for gene ANO2 but was expressed at least 10-fold greater in early estrus gilts at d130 of age. Gene LOXL2 tended to be expressed at higher levels in early estrus gilts across all time points than their late estrus counterparts ($P =$

0.06). For the late estrus gilts, GYS-2 was 35 and 15-fold greater at both d100 and d130 of age ($P = 0.04$). Gene GREB-1 was expressed more than 10-fold greater in late estrus gilts, with a tendency at d160 of age ($P = 0.09$). Lastly, IFN-Alpha-16 tended to be more expressed at d100 of age ($P = 0.06$) in late females. Overall, there are distinct differences in vaginal gene expression at a given day of age for replacement gilts that may be used to predict age at puberty. Within this dataset, 100, 130, and 160 days of age may be ideal timepoints for identification of early estrus in pre-pubertal gilts; however, a more robust dataset analysis is necessary to pinpoint the ideal day of age in reproductive development to serve as a marker for identification of early estrus.

The goal of the third chapter of this dissertation was to collect descriptive data of gilt rearing practices in the Midwest. A successful gilt development program is critical to the success of a production system because it has a direct effect on reproductive performance. While there have been reviews of literature for current industry practices employed for reproductive management (Kraeling and Webel, 2015), and performance data collected and reported by Metafarms, there is little to no data that includes both gilt management practices and decisions throughout reproductive development and a sow's first parity within a breeding herd. Therefore, the objective of this survey was to obtain an understanding of gilt development practices across the Midwest to ensure that the next phases of research have high practical application and relevance. A total of 10 respondents participated, with farms located in Nebraska, South Dakota, Iowa, and Minnesota. These farms collectively manage approximately 43,000 sows. Data was collected and analyzed for descriptive statistics within Microsoft Excel. The herd parity 3 after gilt acclimation, with a minimum parity of 2.1 and the highest parity average of a 5.

With such a young herd parity average range, it means animal removal is occurring at a young production age and warrants further investigation into reasons for removal. Based on this survey, more detailed information on gilt rearing, diet composition, and space per pig needs to be obtained.

In conclusion, vaginal gene expression appears to change in concert with circulating reproductive hormones, with significant changes in IGF-1 and TAC-3 occurring at the start of boar exposure (d160 of age). Anogenital distance was positively correlated to age at first estrus and negatively correlated to daily gain, but due to inconsistencies in measurement collection, caused by events like gilt restlessness and vulva clenching makes this unapplicable for on-farm application. There are distinct differences in vaginal transcriptome between gilts deemed 'early' and 'late' estrus. Within this dataset, there were three genes of interest that have greater expression in 'early' gilts, and three genes of interest that have greater expression in 'late' estrus gilts. Currently, d100, d130 and d160 may be ideal timepoints for identification of early estrus in pre-pubertal gilts, but a larger sample size is necessary to pinpoint the ideal deal in reproductive development. Identification of the ideal age for pubertal detection, paired with an understanding of common gilt rearing practices and diet regimes can provide swine producers with information to maximize their gilt development program and reduce the number of replacement females needed within a herd each year.

Keywords: gene, gilt, gilt-rearing, predictive, puberty detection

CHAPTER I: LITERATURE REVIEW

Sow lifetime productivity (SLP) is defined by quality piglets weaned per sow/year and longevity within the herd. A sow's reproductive success (i.e., more successful parities during their reproductive life) which results in an increase in overall herd parity and litters from older parity sows that are of higher quality (Williams et al., 2005). The introduction of high-quality, replacement gilts into the breeding herd is an often-underestimated driver of SLP. Replacement gilts are young female pigs that haven't yet produced a litter of piglets. Additionally, proper selection and management of these replacement gilts can reduce a farm's lifetime non-productive days (NPD), which is any day a sow or gilt, once part of the breeding herd, is not pregnant or nursing a litter. Therefore, the gilt is the foundation of a successful production system. Each year, nearly 3 million breeding females are removed from U.S. swine herds due to reproductive failure (Engblom et al., 2016). This equates to a 40% replacement rate. Good reproductive performance in gilts can be defined in terms of successful pregnancy at the first breeding attempt that produces a litter of high-quality piglets (defined as piglet birth weight equal to the herd average) followed by at least two additional pregnancies (Nonneman et al., 2016; Patterson and Foxcroft, 2019). Currently, one of the best predictors of SLP is age at first estrus. Gilts achieving puberty earlier tend to produce more pigs over their lifetime (Nelson et al., 1990) and have fewer days from weaning to estrus (Sterning et al., 1998). Another factor that influences SLP is nutritional management, particularly in the gilt development stage (Johnson, 2010). The growth and development of gilts is a component of their lifetime potential for reproductive

productivity (Vallet, 2015). A gilt's lifetime growth rate not only affects their ability to attain puberty, but also likely impacts their reproductive performance in later parities (Bortolozzo et al., 2009; Vallet, 2015). Due to the numerous factors that can impact SLP on a given farm, the ability to remove gilts that have a lower likelihood of reproductive success earlier in the production cycle (i.e., prior to first estrus) has the potential to not only save considerable time and effort on the part of barn production staff but also dramatically reduce overall gilt replacement costs.

1.1 Economic cost of replacement gilts

Swine production is a low-margin business, and producers have increasingly sought ways to increase efficiency in gilt development (Johnson, 2010). Combined high voluntary and involuntary culling rates of sows within the breeding herd results in the purchase of more replacement gilts. These purchases increase capital requirements for the operation and combined with an associated increase in gilt development and acclimation expense, adversely affect profitability (Stalder, 2002). Understanding components that influence gilt development costs, such as feed and non-productive days (NPD), is crucial to preventing inefficiencies in gilt pool performance (Francisco, 2003). Within a breeding herd, sow longevity is impacted by the gilt development program. Holtkamp (2019) found that while a gilt management program for a 2,400 head sow farm cost approximately $27,500 annually, it resulted in an additional $66,208 in income, which translated to a benefit: cost ratio of 2.41:1, and a return on investment of 141%. In this same report, the average parity of the herd before the implementation of a gilt management program was 3.08, and after was 3.20 (Holtkamp, 2019).

A common method to measure reproductive efficiency is through pigs weaned per sow per year (PSY) (Stalder et al., 2003, 2019; Stalder, 2009). In 2019, PSY average was 26.08 for farms in the PigCHAMP database and 26.61 for farms in MetaFarms (PigCHAMP, 2020; MetaFarms, 2020). It is closely related to the number of weaned pigs per litter, farrowing rate, NPDs, and other production factors (Bell et al., 2015). Improving PSY allows the purchase cost of gilts and feeding costs of sows to be divided among more weaned piglets, thus improving profitability of commercial swine farms (Abell, 2011). Guan and colleagues (2022) analyzed the influence of 15 different productivity measures and their impact on PSY. The four factors with the highest correlation coefficient with PSY were: mating rate 7 days after weaning (0.5058), farrowing rate (0.4427), piglets born alive per litter (0.3929), and weaned piglets per litter (0.3839). As NPD increases, sow maintenance cost increases, and farm profitability decreases (Rix and Ketchum, 2010). Increasing mating rate 7 days after weaning can improve productivity and profitability of swine farms (Guan et al., 2022) because NPD are reduced. The cost of each NPD for sows' ranges from $1.60 to $2.60 (Koketsu, 2005), this is supported by Ketchum and Rix (2012), who report that each NPD costs $ 2.25 per week per sow. One way to increase the mating rate is to lengthen the lactation period because a longer lactation length can increase the proportion of sows that display estrus within 4-6 days after weaning, which results in higher reproductive performance throughout their lifetime (Koketsu, 2017). Maximizing farrowing rate is the best way for a sow farm to limit NPD (Leman, 1992) while reducing abortions and total sow mortality are the most effective ways to improve farrowing rate (Pierozan et al., 2020).

High culling rate in commercial sow herds can impair pork operations from maximizing return on investments (Stalder et al., 2000; Pla et al., 2003). Many sows in what would be expected to be the optimal stage of productivity within their reproductive life are removed from the herd after farrowing only 1 litter, without the opportunity for an additional mating for reasons such as failure to return to estrus (Chagnon et al., 1991; Tiranti and Morrison, 2006). Approximately one-third of all removals in gilts are due to reproductive failure, where conception failure and lack of observed estrus are the major reasons (Lucia et al., 2000). In swine production, reproductive failure, which includes failure to cycle or maintain regular estrus cycles, accounts for a significant number of culls (Tummaruk, 2009; Stancic et al., 2011). By the time gilts are culled due to reproductive failure, they are substantially heavier than target market weight, and a lower price is paid. For example, based off USDA weekly market reports (ams.usda.gov), market hog prices ranged from $81 to $89 per 45 kg in August 2023, while the cull sow market price for a non-productive gilt ranged from $25 to $72 per 45 kg over the same period. Holtkamp (2019) found that implementing a specific gilt management program yielded a 10% reduction in annual gilt replacement (62% vs 52%) on a 2,400 head sow farm and resulted in an increase in 829 additional marketed pigs and 222,310 pounds of pork produced per year, which ultimately yielded a benefit-to-cost ratio of 2.41:1. This experiment accounted for genetic premiums, overhead costs, and the current low value of cull sows, which is how replacement gilts are marketed if they are above target market weight following unsuccessful breeding. Additionally, NPD is highly associated with gilt replacement rate (positively) and farrowing rate (negatively) (Leman, 1992). It's estimated that a given herd averages 35 NPD (Abell, 2011) and a decrease in NPD

increases average litters per sow per year (Xu et al., 2023). Assuming a conservative $2.00 per NPD per sow, a breeding herd with 2,400 sows can save $24,000 a year by reducing their average NPD by 5 (Abell, 2011).

1.2 Reproductive development and detecting pubertal attainment

Reproductive success of a breeding female plays a critical role in the overall productivity of pork production systems. The ability to identify early puberty and to produce a synchronous pubertal response to external stimuli (e.g., male exposure) are both dependent on the age at the start of puberty stimulation and heat detection (Patterson and Foxcroft, 2019).

Hypothalamic-pituitary-gonadal (HPG) axis and hormonal regulation of puberty. The components of the swine HPG axis are functional before puberty. The fetal anterior pituitary gland releases luteinizing hormone (LH) in response to circulating gonadotropin-releasing hormone (GnRH) at approximately d80-100 of gestation (Colenbrander et al., 1982; Bruhn and Ellendorff, 1983). After birth, serum concentrations of LH and LH pulse frequency remain high until approximately 3 months of age (Christenson and Ford, 1985). The gilt ovary displays tertiary follicles between 60 and 90 days of age (Oxender et al., 1979), when the hypothalamus and anterior pituitary become sensitive to the negative feedback of estrogen (Foxcroft, 1984; Christenson and Ford, 1985). Approximately 10 to 20 days before puberty, the pattern of LH secretion changes from high-amplitude, low-frequency pulses to high-frequency, low-amplitude pulses (Diekman, 1983; Lutz, 1984). This change in pulsatility occurs due to a reduction

in sensitivity to estrogen negative feedback and causes maturation of ovarian follicles (Berardinelli, 1984; Barb, 2010).

In sheep and rodents, the change in sensitivity of the GnRH system to the inhibitory feedback actions of gonadal steroids is deemed “gonadostat” hypothesis (Mayer et al., 2010). In females, this hypothesis stipulates that the sensitivity of the GnRH pulse generator is reduced to the negative feedback effects of estradiol-17β (E_2), also known as estrogen or oestradiol. This is the is the major female sex hormone and is involved in the regulation of the estrous female reproductive cycles (Kulin, 1969; Steele, 1974; Foster and Ryan, 1979) in the latter stages of prepubertal development. This results in reduced serum concentrations of LH until just before puberty (Lents et al., 2020). This hypothesis has been supported in female mice, where a 10-fold increase in the dose of E_2 is required to fully suppress LH secretions in adult vs. prepubertal females (Bronson, 1981).

Serum concentrations of the reproductive hormones LH, estrogen, follicle-stimulating hormone (FSH) and progesterone, can be used to identify a female in estrus, soon-to-be in estrus, or having successfully achieved estrus. In the pre-pubertal stage (d80-120), a rise in baseline concentrations of serum LH occurs (Figure 1; Evans and O’Doherty, 2001). As gilts progress toward puberty, follicles increase in size and maturity resulting in increasing concentration of serum estrogen, which stimulates a cascade of events leading to puberty (Knox, 2019). Estrogen stimulates production and subsequent release of GnRH which induces a surge of LH and FSH. The LH surge is the key event that signifies puberty attainment/estrus and ovulation (Da Silva et al., 2017b). Following ovulation, serum concentration of progesterone increases for approximately 4 d, and this

increase in circulating progesterone can be used to confirm a young female has ovulated (Evans and O'Doherty, 2001).

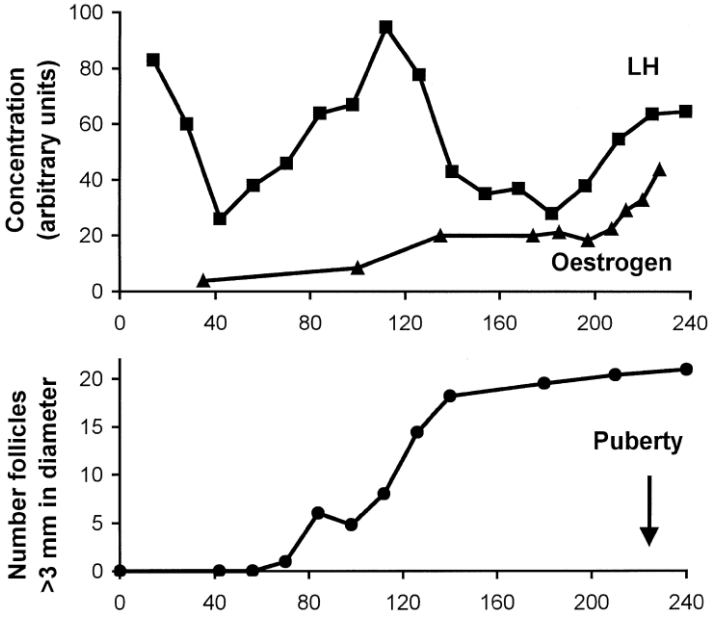


Figure 1.1. Changes in mean LH concentrations (after Pelletier et al., 1981; Diekman et al., 1983; Camous et al., 1985), estrogen concentrations (after Lutz et al., 1984; Camous et al., 1985), and number of follicles greater than 3mm in diameter with age (after Dyck and Swierstra, 1983; Grieger et al., 1986); retrieved from Evans and O'Doherty, 2001.

Detecting pubertal attainment. The best-known predictor of reproductive success in swine is age at puberty, or date of first standing estrus and is currently the only known trait that can be used to predict breeding longevity (Saski and Koketsu, 2008). Therefore, detecting pubertal attainment is critical to overall farm reproductive success. Unfortunately, it is rarely recorded if puberty occurs before boar exposure begins (Nonneman et al., 2016). Recording age at first estrus would play a key role in understanding the impacts of early pubertal attainment on overall productivity. Puberty is expected to occur between 145 and 228 days of age. While 145 days of age is reported in

the range, it is not highly relevant to commercial replacement gilts. Within commercial production, the average age of puberty is 205 days (Knox, 2009). Saski and Koketsu, (2008), as well as Patterson and Foxcroft (2019), report gilts that attained puberty at an earlier age (160-180 days of age) had increased association with overall improved lifetime reproductive performance. Current on-farm techniques to determine gilts that have achieved puberty rely heavily on human observation to identify gilts exhibiting estrus behavior. Management practices to stimulate pubertal attainment within a commercial herd commonly begin with replacement gilts being exposed to a boar for short periods each day beginning at 160 days of age to stimulate what is presumed to be the first estrus. Once the first standing estrus has been detected, gilts will be bred in the subsequent estrus (typically 21 days after first estrus has occurred). Identification of estrus in gilts by human observation can be described as a 'locked' stance, looking directly at the boar, ears erect, grunting, swollen, pink vulva, and allowing back pressure from the herdsmen. Identification of estrus in gilts by human observations alone is more challenging than older sows because they often do not exhibit distinctive estrus behavior (i.e., 'locked' stance, ears erect, grunting) (Flowers, 2020). Estrus that has occurred but not detected by behavior is deemed 'silent' or 'quiet' estrus. The detection of estrus via behavioral indices can be highly susceptible to human error and not suitable for identification of candidate gilts in the pre-pubertal phase of reproductive development (PIC, 2017). Common industry target for replacement gilts is 300 pounds at second estrus when they are bred (PIC, 2017). Gilts that are not successfully bred by the second or third estrus are typically categorized as 'reproductive failure' and culled. However, by the time she reaches her third estrus, and the decision is made to cull her, she is likely to weigh

closer to 350 pounds, much heavier than the target market weight of 280-300 pounds (PIC Road Show, 2019). As a result, by the time a replacement gilt is culled due to reproductive failure, a substantially lower price is paid for her because she is above this target market weight. Therefore, rigorous selection of gilts eligible for breeding can eliminate some of the key constraints for gilt and sow retention in the breeding herd (Patterson et al. 2016).

1.3 Predicting estrus attainment in the prepubertal phase

As previously stated, early puberty in swine is associated with increased reproductive success. This identification of females likely to attain puberty at a younger age (alternatively, at an 'old' age) has considerable economic and reproductive benefits. However, at present the ability to predict, in the prepubertal phase, which females will achieve early estrus is currently limited to intensive collection of serial blood samples for measurement of reproductive hormones (Sasaki and Koketsu, 2008).

To identify gilts in the prepubertal period that will undergo puberty, serial blood sampling is required to determine changes in serum concentration of LH over time because maturation of the ovaries is LH dependent (Evans and O'Doherty, 2001). The pulsatile release of LH means that in an individual female blood samples need to be collected every 15-30 minutes for 8 to 10 hours per day for 5-6 days to determine mean serum concentration of LH (Evans and O'Doherty, 2001). This intensive collection is severely cost prohibitive due to the labor needed to obtain the samples and the number of samples needed to characterize LH and thus attainment of puberty- which is not practical for on-farm applications.

Attempts have been made to identify a biomarker that could be used as a practical on-farm tool for prediction of age at puberty. A biomarker is a morphological or physiological indicator of a phenotypic state of an individual that can be measured accurately and reproducibly (Stribu and Tavel, 2010). They have characteristic biological properties that can be detected and measured at a development stage without being confounded by environment or management effects (Haley, 2006; Agarwal, 2008). For example, Pearce et al. (2013) reported significant follicular variation in gilts sacrificed between d90 and d100 of age, this substantiates previous observations that variation among gilts with respect to follicle size begins approximately d60 and d100 of age (Schwarz et al., 2008).

Estradiol. There has been some interest in using estradiol as an indicator of progressing reproductive development. The release of estradiol, and hence serum concentrations, are less pulsatile reducing the need for repeated blood sampling (Lents, 2019). In pre-pubertal females, circulating concentrations of serum estradiol is low, but a detectable increase was observed around 1d00 and again at d130 of age (Lutz et al., 1984; Camous et al., 1985). At around d160 of age, increased serum estradiol concentration from increasing follicle growth has positive feedback on the surge release of LH to support ovulation (Figure 1; Evans and O'Doherty, 2001). Estrogen stimulates production and GnRH and the resultant surge of LH and FSH.

Camous et al. (1985) measured urine estrone concentration in prepubertal gilts as a proxy for serum estrogen concentrations. This was used to test its suitability as a predictor of ovarian, and hence, follicular activity, because in sows, E_1, or estrone, is a weaker estrogen than E_2 (estradiol 17-β), and is excreted in the urine (Terqui, 1978).

Estrone concentration within urine increased from the prepubertal stage to puberty in three distinct periods within the 5 gilts evaluated. Age at each increase was highly variable among gilts. The range in the first rise was d68 - d110 of age, second rise from d96 - d152 of age, and third rise from d116 - d199 of age where an earlier rise in urine estrone was linked with earlier age at puberty. Serum concentration of estrogen was not determined so a direct relationship with urine estrone could not be estimated. While urine estrone concentrations appear to be related to reproductive development, it is also excreted in a pulsatile manner throughout the day, which would require collection of total urine excreted in a 24h period to determine an appropriate value for a given day, which limits its on-farm application, due to the additional labor required.

Anogenital distance. Anogenital distance (AGD) is a physical characteristic that has been associated with reproductive capabilities in females (Drickamer, 1995). In females, AGD is reported to be correlated with androgen exposure in the pre-natal environment (Vom Saal et al., 1992). In rats, rabbits, and cows it has been shown to be positively related to postnatal reproductive success (Correa et al., 2016). For women, a positive relationship exists in which each millimeter increase in AGD equated to an increase in testosterone concentration by 6 pg/mL (Gobikrushshanth et al., 2017), resulting in increased number of ovarian follicles (Mendiola et al., 2012) and greater testosterone concentrations during the early follicular phase (Mira-Escolano et al., 2014a). In an experiment involving 300 fertile women (age 33-59), a shorter AGD was associated with an increased risk for gynecological morbidity in adult women (Wainstock et al., 2019). Gynecological morbidity included breast or ovarian cancers, and vaginal adenocarcinoma (Herbst et al., 1971; Henderson et al., 1979). It appears that inconsistencies between AGD

and gynecological morbidity exist. Mendiola (2016) and Wainstock (2019) reported that a shorter AGD was associated with an increased risk for endometriosis. However, this contradicts work by several authors (Mendiola et al., 2011; Sanchez-Ferrer et al., 2017; Wu et al., 2017) who report that a longer AGD was associated with an increased risk of developing polycystic ovary syndrome (PCOS).

Sex-biased litters (> 60% either sex) or uterine position can alter AGD to either more masculine or feminine lengths, (e.g., a female between two males would have a more masculine AGD) (Seyfang, 2017). It was reported that a positive relationship between AGD and reproductive success exists where gilts born in female-biased litters had longer AGD at 16 weeks compared to gilts born in male-biased litters (Seyfang et al., 2018). The gilts from female-biased litters achieved puberty earlier and were more likely to be mated. As a result, it was suggested that AGD may be suitable as an early predictor of puberty that could rapidly be applied on swine farms (Seyfang et al., 2018). However, a direct relationship between AGD, serum concentrations of reproductive hormones and achievement of early puberty have not been reported.

Unfortunately, the correlation between AGD and reproductive success is not consistent within swine or across other species. In pigs, Drickamer et al. (1997) reported a negative correlation between AGD and reproductive success, in which a longer AGD at birth meant gilts were less likely to have reproductive success. In a rodent species, *Octodon degus*, longer AGD meant a more masculinized female, which had more robust offspring when compared to feminized females, with no differences in fertility (Correa et al., 2016). Alternatively, Gobikrushanth et al. (2017) reported that parity 1 or 2 Holsteins with shorter AGD were 2.4-3.4 % more likely to become pregnant when compared to

cows with longer AGD. While in practice, AGD could serve as an implementable tool for on-farm application, previous research highlights the lack of clarity between the direction of the relationship between AGD and reproductive success, which decreases its usefulness.

Vulva size. Vulva size by d95 - d115 of age has been positively associated with a gilt's ability to achieve puberty by 200 d of age, in which gilts with smaller vulva size in this age range are less likely to reach puberty by d200 of age (Graves et al., 2020). Recently, Rosomer et al. (2020) measured vulva size at 15 weeks in replacement gilts and hypothesized it could be used as an indicator trait for reproductive performance. Within the dataset, it was observed that gilts with a large vulva size had lower culling rates (16% vs. 26%), greater first farrowing rates (78% vs. 60%) and greater number of piglets born alive at first parity (12 vs. 11.3) compared to gilts classified as small vulva size. This indicates a relationship between vulva size and reproductive performance (Rosomer et al., 2020). Variation in vulva size within pre-pubertal gilts is associated with differences in follicular activity, specifically females with greater ovarian activity will reach puberty at a younger age and have a greater vulva size at 15 weeks (Graves et al., 2020). While vulva size shows promise in predictive capabilities for reproductive performance, estimation of genomic prediction accuracies, which calculate the difference between predicted and observed phenotypes by using cross-validation, which is crucial for on-farm application, require more investigation into determining when utilization of genomic prediction accuracies are beneficial, due to inconsistencies in results. To date, vulva size traits vary in heritability values across different gilt ages during development

(Corredor et al., 2020). In Yorkshire gilts, vulva size traits were moderate to highly heritable, and low to moderate in Landrace (Corredor et al., 2020).

Vulva width. Knauer and colleagues (2011) reported an estimated 0.57 genetic heritability for vulva width in gilts after reaching puberty. It was additionally reported that a weak but positive correlation exists between vulva width and gilt achieving first parity (r = 0.07). This is supported by Mills et al. (2020) who reported that vulva width was a differentiating factor in PSY but was only found to account for 1.5% of the variation. Vulva width tended to be greater at weaning in animals who were fertile, and those same animals achieved estrus 3 days earlier than open animals (Mills et al., 2020). This agrees with Graves (2020) that there is a positive relationship between a gilt's vulva width at weaning and the likelihood she becomes pregnant. However, this relationship is not very strong due to the variation in gilt vulva size at weaning. As such, this selection criteria cannot be used alone when selecting pre-pubertal gilts as replacements.

1.4 Advanced technologies for on-farm prediction of puberty

High throughput 'omics' are being investigated across a vast range of fields to compare distinct molecular profiles that can be used to describe phenotypic variables or characteristics (Hu et al., 2011; Xie et al., 2021).

Lipidome. Initial research by Casey et al. (2017) reported that lipid biomarkers, based on vaginal lipidomics analysis, could serve to identify replacement gilts at weaning. The vaginal lipidome is influenced heavily by the post-natal nutritional environment. Based on curve analysis [receiver operating characteristics (ROC) using area under the curve], the distinguishment between piglets that suckled colostrum and those that had been

reared solely on milk was strong (area under the curve for ROC = 0.88), which supports the use of the vaginal lipidome to distinguish early nutritional rearing environment. With respect to gilt development, Mills et al. (2021) report the vaginal lipidome, based on multiple reaction monitoring profiling, of gilts at d21 of age was linked to sow fertility over the first two parities. Relative abundance of cerotic acid, ximenic acid, nonadecanoic acid, and pentadecanoic acid were greater in infertile gilts while arachidonic acid (precursor to prostaglandins) and docosahexaenoic acid were lesser in infertile gilts. Based on curve analysis cerotic, ximenic, and nonadecanoic acid had 'fair' (area under the curve for ROC = 0.7 to 0.8) potential as biomarkers. Additionally, eicosapentaenoic acid, a precursor of prostaglandins, was greater in vaginal samples from high fertility gilts (Mills et al., 2021).

Microbiome. The relationship between a host and its microbiome has become an emerging area of study in livestock production. It is well-known that the microbiota is influenced by nutrition, temperature, and location (Kers et al., 2018). Operational taxonomic units (OTUs) are used to describe bacterial communities using amplicon sequencing of 16S rRNA gene (Gilbert et al., 2014), and are often used to infer potential functional traits since they are considered to fairly represent bacterial community members (Fernandez, 2019). The host-associated microbiota plays a role in shaping phenotypes of humans and animals (Cho, 2012). In beef cattle, the vaginal microbiota was used to distinguish between heifers that were able to establish pregnancy from those that were not (McClure, 2018). This suggests a potential avenue to identify animals with favorable reproductive performance. In sows, Sanglard (2019) investigated the relationship between the vaginal microbiota and farrowing performance. The females

were split into two farrowing performance groups (low and high) based off common performance traits, including number born alive, number born dead, number weaned, and pre-wean mortality. There was a moderate linear relationship between reproductive traits and the vaginal microbiota, with R^2 ranging from 0.19 (number born dead) to 0.46 (number of stillborns). Exploring the host microbiota and relating it to phenotypes of interest (e.g., reproductive performance) could assist the swine industry in improving animal reproductive performance. While the data described above provide insight into the vaginal lipidome and bacterial composition of breeding females, these methods do not specifically focus on periods during reproductive development in the pre-pubertal stage.

Transcriptome. The vaginal epithelium of the sow changes throughout the estrus cycle and pregnancy (Morton and Rankin, 1969; Almond and Dial, 1987). For example, pregnant sows possess a vaginal epithelial layer that is organized as 2-3 cuboidal cellular layers (Bosu, 1971; Almond and Dial, 1987), whereas non-pregnant sows display cellular layers of polygonal epithelium (3-20), epithelial ridges, mitosis, disarrangement of nuclei, cellular sloughing, and possible leukocyte infiltration (Morton and Rankin, 1969; Almond and Dial, 1987). Hympanova et al., (2019) reported that ewes experience changes in vaginal dimension and thickness of the epithelium at pubertal attainment. In mice, the estrous cycle causes a mucous layer to form during late diestrus, corresponding to the initial period of pregnancy (Sugiyama et al., 2021). Additionally, keratinization of the vaginal epithelium occurred throughout pregnancy, and was still present postpartum day 1 (Sugiyama and Machida, 2021). Similar changes could occur in swine, but differences in mice vaginal epithelium may not occur in swine, given that the vaginal epithelium of

mice is derived solely from the Mullerian duct epithelium (Kurita, 2010; Robboy et al., 2017).

Besides structural changes, evidence suggests alterations in gene expression within the vaginal epithelial are related to reproduction. Prior to pubertal development, the human vaginal epithelium is characteristically thin. At puberty onset it begins to thicken in response to estrogen stimulation while undergoing progressive cellular proliferation and growth resulting in the formation of intermediate and superficial layers of cells (Colvin, 2012). Estrogen stimulation causes glycogen concentrations in the vaginal superficial cells to increase. The metabolism of this glycogen by lactobacilli residing in the vagina results in an acidic environment (pH 3-4.5) (Brizzolara et al., 1999). This acidic environment protects against infections from various bacteria or fungi (Colvin, 2012).

Vaginal toll-like receptors. Reproductive hormones influence cellular protein function, specifically vaginal toll-like receptors (TLRs) which act to prevent infection while allowing fertilization and implantation to occur (Aflatoonian and Fazeli, 2008; Michailidis et al., 2011). These TLRs are necessary "responders" within the body and induce immune and inflammatory responses in vertebrate animals (Kannaki et al., 2011). Toll-like receptors are a family of innate immunity receptors and are meant to recognize a specific range of microbial pathogens (Clop et al., 2016). There are currently 10 TLR genes as part of the porcine genome, located at two different locations within the cell. Cell-surface TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) recognize non-nucleic acid molecules, while intracellular TLRs (TLR 3, TLR7, TLR8, and TLR9) detect nucleic acids (Clop et al., 2016). Cell-surface TLRs are highly polymorphic, which

allows the organism to broaden the types of molecules it can recognize. This is important in initiating an immune response (Clop et al., 2016). These receptors are expressed in the female genital mucosa of mice and humans, and signaling via these receptors has been reported to induce pregnancy failure (Takeuchi et al., 2015). Alteration of individual TLR expression levels occur during the estrus cycle and pregnancy (Yao et al., 2007; Takeuchi et al., 2015). Research on TLRs in the pig reproductive tract has focused on the ovary, oviduct, and endometrium (Ansari et al., 2015; Marantidis et al., 2015) with a paucity of data on simultaneous expression of TLRs in other reproductive tissues. Marantidis et al., (2015) studied all 10 TLRs in the reproductive tissue of a sexually mature sow. All 10 TLRs were expressed in these tissues, but TLRs 1-5 were found in higher abundance than TLRs 6-10 (Marantidis et al., 2015).

To date, estrogen levels have been hypothesized to be the cause of fluctuations in TLR levels. This is due, in part, to work by Yao (2007), who reported that when a mouse undergoes estrus, she is least susceptible to vaginal infection, while the most susceptibility to intravaginal infection occurs during diestrus. Work in hens confirms that TLRs may be influenced by the presence of estrogen. A comparative investigation of the expression of TLRs in the vagina of hens revealed that TLR 2-1 and TLR 4 mRNA levels were increased in mature hens compared to prepubertal hens, while TLR 2-1, 2-2 and 4 genes were downregulated in senescent hens (Michailidis et al., 2011). In addition, populations of immunocompetent cells in the vagina increased during sexual maturation then decreased in aged hens (Zheng et al., 1997). A possible factor influencing the changes in cell population is estrogen levels, which was hypothesized to also influence TLR gene expression (Michailidis et al., 2011). When treating immature hens with

stilboestrol, an estrogen analogue, immune cell populations increased within the reproductive tract (Zheng et al., 1997). This effect was not observed when hens were treated with progesterone or in controls. Rettew and colleagues (2009), discovered that estrogen has been shown to promote the expression of TLR-4. The expression of TLRs has been detected in reproductive tissues such as granulosa cells, endometrial luminal and stromal cells, trophoblasts, and cervical cells (Horne et al., 2008). Within the bovine endometrium, TLRs are critical in preventing endometrial infection. They can do this by interacting with microorganisms as they are expressed on epithelial cells of the endometrium (Davies et al., 2008). When TLR signaling is induced by molecules, many downstream targets of TLRs, including IL4, IL8, and TNF_{α} are regulated (Horne et al., 2008; Silva et al., 2010). Within the female reproductive tract, expression of many pro/anti-inflammatory molecules can be affected during formation and regression of the corpus luteum (Skarzynski and Okuda, 2010). In bovine corpus lutea, an upregulation of TLR-2 mRNA was detected in regressing corpus luteum. Localization of the TLR-2 protein was apparent in luteal cells and a prominent immunofluorescent signal that corresponds to TLR-2 was only detected in regressing CL (Atli et al., 2017). Thus, there may be a role for TLR-2 in the development, maintenance, and regression of corpus lutea.

While TLRs are influential in maintenance of pregnancy, upregulation of TLR-2 and 4 within the body may have negative effects on pregnancy or nursing ability. A common problem that occurs during parturition is mastitis. Mastitis is an inflammatory condition of the mammary gland or udder. Mastitis infections have been correlated with over expression of TLR-2 and TLR-4 in mammary glands of cattle, as well as TLR2 in swine. Previous research has shown that this correlation is even more evident with increased

severity of infection (Goldammer et al., 2004; Petzl et al., 2008; De Schepper et al., 2008; Zhu et al., 2008). Within the uterus, a common infection that may occur is endometriosis. Endometriosis is an inflammatory condition that occurs in the inner layer of the uterus. Silva et al. (2009) found significant upregulation of TLR-2 and TLR-4 mRNA in the canine endometrium. Within cattle, TLR4 mRNA expression in the endometrium was upregulated during cases of endometriosis (Davies et al., 2008).

Estrogen-receptor α. The onset of puberty is activated by an activation of the GnRH pulse generator that is dormant during prepubertal development (Wolfe, 2014). Estrogen-receptor-α (ER-α) is found in the hypothalamic arcuate nucleus (Mayer, 2010). In the proliferative phase, ER-α increases in response to estrogen and is then subsequently diminished in response to progesterone in the secretory phase (Brenner and West, 1975). Estrogen-receptor- α is predominantly expressed in the uterus, ovaries, and breasts (Jia, 2015). In women, positive feedback in the hypothalamus and pituitary are mediated by ER-α (Christian et al., 2008). However, lack of a functional ER-α in females results in a complete lack of estrogen (Smith et al., 1994; Morishima et al., 1995). Additional evidence indicates that ER-α is a necessary estrogen receptor isoform and is responsible for both negative and positive feedback actions of estrogen on GnRH/LH release (Uenoyama et al., 2021). In mice, the removal of ER-α results in polycystic ovaries without corpora lutea and the mice are reported to be infertile (Lubahn, et al., 1993). This is further supported by studies in mice and rats with knockout ER-α, where females failed to ovulate and possessed enlarged cystic follicles (Couse, 1999; Couse et al., 2003; Rumi et al., 2014).

Overexpression of ER-α can also have a negative impact. In females, two-thirds of breast cancers express an increase in ER-α, which in turn results in an increase in estrogen production and progression of tumors (Tolhurst et al., 2010). Specifically, over-expression could lead to increased cancer cell survival and the subsequent development of estrogen-independent growth, thereby contributing to resistance to endocrine therapies in breast cancer patients (Tolhurst et al., 2010).

Tachykinin-3. Another hormone that is implicated in GnRH secretion is neurokinin, which is encoded by the gene Tachykinin-3 (TAC-3) in mammals (Ojha and Nachiappan, 2019). It is widely expressed in the central nervous system but is also expressed in reproductive organs such as the ovaries (Blasco et al., 2019) and uterus (Patak et al., 2005; Canete et al., 2013). The neurokinin pathway is involved in the development of hypogonadism, suggesting that alterations in the TAC-3 gene could lead to a failure in regulating puberty (Gianetti et al., 2010). The neurokinin-positive neurons are in the hypothalamic arcuate nucleus (Goodman, 2007; Murakawa, 2016). Maeda and others (1995) reported that arcuate nucleus may regulate pulsatile GnRH secretion. In mammals, pulsatile GnRH secretion stimulates gonadotropin release from the pituitary to enhance gametogenesis and steroidogenesis (Knobil et al., 1980). Combined with this pathway described above, TAC-3 seems to regulate growth and reproduction by affecting the function of the HPG axis (Zhang, 2020).

Insulin-like-growth factor-1. Another gene of interest that has played an integral role in reproduction is insulin-like-growth factor-1 (IGF-1). Hepatic IGF-1 may be involved in maintenance of ovarian function (Adashi et al., 1994). In mammals, ovarian follicular growth consists of two distinct phases: early folliculogenesis, which involves the

maturation of the primordial follicle until the preantral stage and late folliculogenesis (Druckmann, 2002). The regulation of this initial period of folliculogenesis is dependent on IGF-1 availability (Lecomte, 1999; Quesnel, 1999). In rabbits, growth hormone action is mediated by IGF-1, considering that follicular growth and intra-ovarian concentrations of IGF-1 increased in a coordinated fashion following growth hormone administration (Yoshimura et al., 1993; Yoshimura et al., 1994). An additional experiment in mice determined that inactivation of IGF-1 results in the blocking of follicular development and growth during the preantral stage, and gonadotropin administration does not offset this inactivation (Monget, 1999). The second phase of follicular growth is gonadotropin dependent (Druckmann, 2002). It acts on luteinized human granulosa cells (Ovesen et al., 1994).

Documented changes in the vaginal epithelium throughout development and pregnancy provide insight into potential markers of reproductive efficiency in gilts. A broader screening of genes within the vaginal epithelium could show promise on specific differences between reproductively successful, or not successful, females and warrants future research in this area.

1.5 Prepubertal nutrition

Successful management of gilts is a necessary component of breeding herd management, creating the framework for the future fertility and longevity within the herd. A component of good gilt management is appropriate management of weight, physiological maturity, and a positive metabolic state at breeding (Patterson and Foxcroft, 2019). Common practice is to manage diet nutrient levels for feeding replacement gilts

like that of finisher pigs until first breeding. However, the rate of puberty attainment is sensitive to altered nutritional status during the pre-pubertal period (Beltranena et al., 1991; Prunier et al., 1993). With the increasing number of replacement females that never express normal estrus behavior (Cano, 2019), recent emphasis has shifted from feeding replacement gilts as finisher pigs to a separate entity that requires a different plane of nutrition for overall sow longevity.

Bodyweight. The previous sections of this literature review spent significant time discussing the importance of age on puberty attainment and subsequent reproductive success. An essential goal for replacement gilt nutrition is to develop females to their physiological maturity in weight, tissue composition, soundness, and reproductive performance (Faccin et al., 2022). Weight at first mating is a critical factor for greater total litter size in a gilt's lifetime production (Engblom et al., 2007; Quinn et al., 2015). Longevity and performance of gilts are impacted by their weight at puberty and a positive correlation between a heavier bodyweight and ovulation rate exists (Amer et al., 2014). However, greater body weight has also been associated with an increase in premature culling, due to higher rates of lameness (Engblom et al., 2007). Beltranena et al. (1991) described the relationship between lifetime growth rate and age at pubertal estrus using a quadratic curve. Age at first estrus decreased as growth rate increased from 0.4 - 0.53 kg/d, remained unaffected as growth rate increased from 0.53 - 0.6 kg/day, and then increased as growth rated exceeded 0.6 kg/d. One objective of gilt development is to maintain growth rates to achieve 115 - 140 kg at puberty and 135 - 160 kg at breeding (Bortolozzo et al., 2009), which would equate to >700 g/d to achieve puberty earlier and have a lower incidence of anestrus between pubertal estrus and breeding. However, recent

protocols from Denmark recommend that gilts are bred at an older ager and heavier weight (Nielsen et al., 2018). This disagrees with Williams and colleagues (2005) and Bortolozzo (2009) who state gilts are suitable to be inseminated at 135 kg of bodyweight, without any negative effects on litter performance over three parities. It has been hypothesized that growth rate and body weight of gilts are more correlated to litter size in the first parity than the total pigs born/sow (Faccin et al., 2017). Regarding a higher body weight, it is expected that higher production cost prevails when gilts are mated older and heavier due to higher maintenance requirements during their lifetime (Patterson et al., 2010).

Nutrition. One of the most manageable environmental aspects of a swine herd that has a major impact on efficiency and number of gilts that ultimately enter the breeding herd is nutrition (Klindt et al., 2001). Protein (Murray et al., 1998) and energy (Kirkwood and Aherne, 1985) intake can influence reproductive performance because growth rate and overall body composition are related to onset of puberty (Beltranena et al., 1991). It has been suggested that gilts need a minimum bodyweight and backfat level to attain puberty (Kirkwood and Aherne, 1985). Providing an appropriate growth rate is a critical aspect that will impact overall reproductive tract development. Adequate nutrition during growth is required for proper development of potential reproductive females (Klindt et al., 1999). Typically, replacement gilts are fed common grower and finisher diets which contain excess amino acids to promote maximal protein deposition (Rozeboom, 1999).

Genetic selection of highly prolific females has shifted body composition at puberty from a lighter and fatter phenotype (Beltranena et al., 1991) to a heavier, leaner phenotype (Wettere et al., 2011). Current metabolizable energy (ME, kcal/kg) for gilts

ranging from 50 - 135 kg is 3,300 kcal/kg (NRC, 2012). Restricting energy levels below growth rate requirements to slow growth during rearing can lead to delays in the expression of estrus (Miller, 2010). Moderate feed restriction (60 - 85% of ad libitum) has been hypothesized to negatively affect age at puberty in gilts (Ahern and Kirkwood, 1985). This agrees with Beltranena (1990), who reported that feed restriction delayed but did not prevent the on-set of puberty. After puberty was attained, the length of the first estrous cycle was not affected by feeding level or indirect changes in growth rate.

Previous industry practice was to restrict feed to avoid excess body condition in comparison to current practices where gilts are typically fed ad libitum during the growth phase, which may result in overfeeding and subsequently limit physiological development (Klindt et al., 1999). Developing dairy heifers that were fed for higher rates of gain had compromised performance as mature lactating cows, resulting in a decrease in mammary development, decreasing milk production (Sejrsen et al., 1982). Ad libitum feeding of pullets reduced the number and hatchability of the eggs produced (Whitehead, 1988).

Another aspect that needs to be considered is dietary energy levels during gilt development and growth, and how different energy levels can impact overall production and reproductive performance. The challenge is to ensure that body protein levels and fat mass can increase during gestation, without making gilts fat (Boyd et al., 2002; Gill, 2007). Thingnes, et al. (2014), found that gilts fed a high energy diet (13.2 - 29.0 MJ NE/d during development were younger at mating (d206 vs. d211 of age) and had more fat reserves (12.3 vs. 11.4 mm), compared to gilts fed the normal energy level.

The most common strategy to control animal growth rate is controlling daily feed intake. Implementation of this strategy in commercial swine production is not practical because replacement females are housed in groups until just prior to boar exposure, and often until after first estrus detection. Recent attempts to investigate the impact of controlling growth rate of developing gilts on attainment of puberty evaluated altered dietary lysine: energy ratio using fiber to limit intake (Calderon-Diaz et al., 2017; Lents et al., 2020). As lysine: energy ratios increased in the diets, age at puberty decreased, with average age of puberty occurring 202 d of age with a range from 166 to 222 d of age. However, it was noted rate of growth was still above that proposed for optimal reproductive development (Calderon-Diaz et al., 2017).

Amino acids. Previous studies have reported that differences in dietary essential amino acids were associated with decreased growth rate and increased body fat (Russell et al., 1983; Sorenson et al., 1993, and Main et al., 2008). These studies don't accurately portray reproductive development, as the animals were younger, bodyweight was lower, or animals did not have ad libitum access to feed. Baidoo (2001) stated that a gilt development diet should be either moderate in lysine (0.6%) with high energy (3.5 Mcal/kg) or high in lysine (1.31%) with moderate energy levels (3.2 Mcal/kg). However, he also suggested that these diets should be limit-fed to developing gilts, which given the current feeding set up and housing conditions for gilts within the swine industry, is not a highly feasible practice. In 2012, the National Pork Board surveyed the commercial swine industry to obtain diet nutrient levels that were currently being fed to developing gilts in the U.S. This survey showed that U.S. swine producers typically use higher values for standard ileal digestible (SID) lysine daily intake than those recommended by the

NRC (2012). However, commercial farms typically set dietary ME level like those recommended by NRC (2012) and the National Swine Nutrition Guide (Whitney and Masker, 2010). Following this survey, Calderon-Diaz (2015) studied different SID lysine and ME levels, in line with levels reported by the 2012 National Pork Board survey. This study was conducted over the period of reproductive development covering the period beginning at d100 of age until d260 of age. Gilts received grower diets formulated to provide 0.85 (low) or 1.02% (high) SID lysine beginning at d100 of age until 90 kg. The low-level diet falls below the NRC (2012) recommendation of 0.89% for gilts weighing 75-100 kg. The finisher diet fed to gilts contained 0.73 (low) or 0.85% (high) SID lysine for gilts weighing 100-135 kg. However, despite significant differences in lysine:ME ratio in the diets, there were no changes in growth or body composition. When looking specifically at daily lysine intake, recommendations range from 11.8 to 26.5 g/day for pigs from 46 - 136 kg (Campbell et al., 1984; Rao and McCracken, 1992; Kerr, 1993; Friesen et al., 1995, and Hahn et al., 1995). In the Calderon-Diaz (2015) study, gilts consumed an average of 26.9 g lysine/d, which is the upper range of the levels previously reported. It's likely that even the "low" lysine levels within this experiment still met the minimum lysine requirements.

Investigations into gilt nutrition have focused specifically on prepubertal nutrition and its impact on pubertal attainment. However, gaps in literature remain as far as how prepubertal nutrition can impact the proposed on-farm detectors of reproductive success. Gilt rearing could differ based on individual production systems and management decisions, and now that there are potential biomarkers proposed for on-farm use,

understanding how prepubertal nutrition may impact the transcriptome expression is a necessary step in validation of these tests across systems.

1.6 Conclusion

Reducing the number of replacement females needed within US breeding herds would benefit all stages of swine production. The overall cost of replacement gilts is substantial, and producers tend to purchase up to 40% more gilts than needed as replacements because many will never enter the breeding herd or produce a litter. If a producer purchases a replacement gilt from a genetic line, a pricing premium is placed on these females, so producers will absorb additional costs for females that guarantee they are a maternal genetic line, but not that they will successfully enter the breeding herd. In production systems that utilize their own internal multiplication and do not purchase from a genetic line, females that are culled due to reproductive failure still represent a significant economic loss due to the lower market price paid for cull females. Through the development of predictive analytics, it is expected that the quality of gilts entering a breeding herd can increase, thus reducing the number of replacement females needed for a breeding herd. Additionally, through improvement in gilt selection, other benefits would include offspring robustness, reductions in labor, and more efficient use of facility space, which would result in an overall improvement in herd reproductive efficiency. Females deemed unlikely to achieve reproductive readiness could be identified at an earlier weight and sold as a market animal which would allow for optimal value of all hogs. A reduction in days on feed would save considerable costs for the production system. Introducing

fewer, but more high-quality breeding acceptable gilts into the herd could positively impact SLP, and through proper management and selection reduce a farms' NPDs.

Therefore, the objectives of this dissertation were to 1) identify changes in the vaginal epithelium throughout gilts during reproductive development. 2) Identify distinct differences in the vaginal transcriptome between gilts deemed 'early' and 'late' estrus gilts and identify a specific day in reproductive development that may be suitable for detection of early estrus, and 3) gain insight into some common gilt rearing practices throughout farms in the Midwest and compare practices across producers.

CHAPTER II: IDENTIFICATION OF BIOMARKERS FOR REPRODUCTIVE DEVELOPMENT ASSOCIATED WITH SERUM CONCENTRATIONS OF REPRODUCTIVE HORMONES AT KEY PERIODS IN GILT REPRODUCTIVE DEVELOPMENT

Abstract

Early puberty in gilts is associated with improved long-term reproductive performance. Predicting who will achieve early puberty is limited to intensive serial blood collections for measurement of reproductive hormones. It is hypothesized that the vaginal genome is differentially altered during pubertal development and may provide insight into biomarkers of pubertal estrus. Pre-pubertal gilts (n =13) were followed from d70 of age until first estrus or d213 - 215 of age. Blood, vaginal epithelia, and anogenital distance were collected at five timepoints during reproductive development (d70/77, d100/110, d130, d160 and first estrus or end of trial). Total RNA was isolated from vaginal epithelia and relative gene expression of two toll-like receptors (TLR-4 and TLR-5), tacykinin precursor-3 (TAC-3), insulin-like growth factor-1 (IGF-1), and estrogen receptor-alpha (ER-α) was quantified by real time q-PCR, relative to expression of RPLP0. Comparison of expression of each gene relative to d70/77 was performed using the PCR package in R (version 1.2.5025) and Fisher's exact t-test for TLR-4, TLR-5 and TAC-3, and analysis of variance for ER-alpha and IGF-1. Correlation analysis examined the relationship between anogenital distance and age at first estrus. Expression of IGF-1 and TAC-3 were up-regulated 9- and 7-fold, respectively at d160 ($P < 0.05$). Expression of ERα tended to be upregulated 3-fold at d100 ($P = 0.08$) and expression of TLR-4 and TLR-5 was lowly detected prior to first estrus. Anogenital distance was positively correlated to age at first estrus and negatively correlated to daily gain ($P < 0.01$; r = 0.83).

Distinct alterations in vaginal gene expression during pubertal development support the vaginal genome as biomarker for prediction of age at pubertal estrus.

2.1 Introduction

The best-known predictor of reproductive success in gilts is age at puberty (Rosendo et al., 2007; Kuehn et al., 2009). For identification of gilts that will 'soon' undergo puberty, serial blood sampling is required to determine changes in concentration of serum luteinizing hormone (LH), because maturation of the ovaries is LH dependent (Evans and O'Doherty 2001). Within the body, LH is released in a pulsatile fashion, which requires blood sampling every 15-20 minutes for 8-10 hours per day, for 5-6 days in an individual gilt to determine mean serum concentrations of LH (Evans and O'Doherty 2001). This intensive blood sampling is not practical for on-farm use. Current on-farm techniques to determine gilts that have achieved puberty rely heavily on human observation to identify gilts exhibiting estrus behavior (Signoret, 1970). These techniques can be highly susceptible to human error and are not suitable for identification of candidate gilts in the pre-pubertal phase of reproductive development (PIC, 2017). Physical characteristics such as anogenital distance (AGD) and vulva width (Romoser, 2017; Seyfang et al., 2018; Graves et al., 2020) are proposed as a practical tool for on-farm prediction of age at puberty in the prepubertal period (PIC, 2017). In females, AGD correlates to androgen exposure in the prenatal environment and has been shown to be related to postnatal reproductive success in rats, rabbits, humans, and cows (Fouqueray et al., 2014; Correa et al., 2016; Gobikrushanth et al., 2017). Seyfang et al. (2018) proposed AGD as a suitable early predictor of puberty that could be rapidly applied on-farm, but

correlation between AGD and reproductive success is not consistent across studies. Vulva width between 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). With advancements in the field of 'omics', molecular techniques hold promise as predictive markers and the development of precision breeding technologies with on-farm application. Recent work identified associations between host vaginal lipidome and bacterial composition and gilt fertility, immune stimulation, breeding strategy, vaccination status, and pregnancy (Sanglard et al., 2020; Luque et al., 2021; Mills et al., 2021; Alves et al., 2022). However, these methods do not specifically focus on periods during reproductive development in the pre-pubertal phase as possible predictors of age at puberty. Therefore, the objective of this study was to characterize changes in the vaginal epithelium of gilts at key periods during reproductive development, in concert with changes in circulating reproductive hormones (estradiol and LH).

2.2 Materials and Methods

Animal procedures were reviewed and approved by the South Dakota State University Institutional Animal Care and Use Committee (approval number 19-006A). The original planned sample collection timeline is shown in Figure 2.1. The intent was to complete the listed collections/observations using 30 pre-pubertal gilts in a single cohort. However, due to unforeseen circumstances that occurred with the initial group of 30 gilts (deemed cohort 1) another group of 12 gilts were included (deemed cohort 2).

2.2.1 Cohort 1

D70 collection. A total of 30 pre-pubertal replacement females (PIC 1050; 22.52 ± 1.39 kg; d70 of age) from a single source were housed at South Dakota State University Swine Education and Research Facility in 2 pens (n=15/pen) with greater than 0.93m^2/pig. Gilts were provided a common diet in 5 phases (Table 2.1) with ad libitum feed intake and nutrient specifications required for growth (Table 2.1), as well as ad libitum water access with 1 nipple/cup waterer per pen.

Upon arrival, bodyweight (BW), a 3 mL blood sample [jugular venipuncture (20 g x 1.5 inches) collected in serum vacutainer (BD Vacutainer, Franklin Lakes, NJ)] and a vaginal swab was collected (Figure 2.2) using a Cervex-Brush cervical cell sampler (Fisherbrand #1437256, Fisher Scientific, Waltham, MA) placed approximately 4 cm into the vagina and then using slight pressure and 3 partial hand rotations, tissue was collected from the dorsal portion and sides to avoid the suburethral diverticulum. The cytology brush was then placed in a cryovial containing TRIzol reagent (Invitrogen, Waltham, MA), swished around for 30 seconds, and then flash frozen in liquid nitrogen. Following transport to campus, the samples were placed in a -80ºC freezer prior to RNA extraction. Blood samples were left at room temperature and allowed to clot before centrifugation at 2,500 rpm for 15 minutes at 22ºC. The serum was removed and stored in microcentrifuge tubes at -20 ºC for further analysis.

In addition, AGD (Figure 2.1) using digital calipers was measured for each gilt and was considered the distance from the top of the vulva opening to the middle of the rectum. The AGD measurements were collected while the gilts were in the weigh scale and efforts were extended to keep the females calm to reduce clenching of the vulva

during measurements. For each gilt, AGD was recorded in triplicate and measured by the same technician at each time point to maintain consistency.

D100 collection. The original intent of the collection planned for d100 was to collect serial blood samples via ear vein catheters from all 30 gilts and establish a subset of gilts with 'high' and 'low' levels of LH based on serum concentrations. As outlined in the timeline, the goal was to begin collecting blood samples at 0700 h, with collections every 30 minutes for 10 hours (0700-1600). The subsets of 'high LH' and 'low LH' gilts were expected to represent females likely to express an 'early/average' estrus or a 'late' estrus cycle, respectively. Attempts to place ear-vein catheters into gilts were unsuccessful. Due to the small size of the ear veins in the gilts we were unsuccessful in inserting the catheter through the ear vein into the jugular vein; shorter catheters were tried but were unable to collect more than 0.5 mL due to collapse of the vein during blood draw even using a small 1mL syringe. The initial plan was to insert temporary jugular catheters (Clapper, 1998). However, due to a lack of availability from manufacturers, supplies required for the protocol were delayed and only 9 gilts of the original group of 30 were selected for serial blood draws. Body weight was used as the selection criteria to attempt to choose gilts likely to express estrus at different ages where 5 of the heaviest gilts and 4 of the lightest gilts were selected from the group of 30 and assumed to represent 'early' and 'late' estrus gilts, respectively. Two gilts died within 4 h of catheterization. It is expected that one gilt had an extreme reaction to anesthesia drugs used and one died from a blood clot to the heart. For the remaining 7 catheterized gilts, serial blood samples were collected every 30 minutes for 10 hours to determine serum concentration of LH. Before the end of the collection period one gilt lost her catheter. Despite challenges with blood

collection, d100 vaginal tissue samples and AGD measures were collected as described previously on all 28 remaining in this cohort.

D130 collection. On d130 of age all gilts were again weighed, a vaginal swab collected, and AGD measurements recorded. During vaginal swab collection on one gilt, the swab tip separated from the handle and remained in the vagina. Repeated attempts were made to retrieve the tip without success, so the gilt was removed from the test and euthanized using a captive bolt gun. This gilt was one of the 6 from which serial blood samples were collected at d100. Following the d130 collection, only the 5 gilts remaining from the d100 serial blood collection were retained for the remainder of the experiment.

D133, 136, and 139 collections. A single blood draw was collected every 3 days from d133 to d139 from the 5 selected gilts and serum concentrations of progesterone were measured via radioimmunoassay (RIA) (Jolitz et al., 2015). Progesterone (P0130; Sigma Life Science, St. Louis, MO) was used to confirm that the gilts were still pre-pubertal and had not achieved an early estrus. Progesterone was the standard and radioiodinated progesterone (#07-170126; MP Biomedicals, Solon, OH) was used as the tracer. Antisera (#111.2C7.3; Enzo Life Sciences, Farmingdale, NY) was used at a dilution of 1:700,000. The intra-assay coefficient of variation was 14.7%. The sensitivity of the assay was 0.33 ng/tube. A female was considered to have ovulated based on serum concentrations of progesterone of 1 ng/mL or greater (Guthrie, 2005).

D143 and 173 collections. At d143 of age an indwelling catheter with a subcutaneous titanium port (Levesque et al., 2011) was implanted in the 3 heaviest gilts (gilt ID 0691, 0684, and 0698; 74.5 ± 8 kg) of the 5 selected (deemed 'ported' gilts). Gilts were allowed to recover from surgery for at least 7 days. Surgery for the two remaining gilts was

delayed because they were deemed excessively light BW (Gilt ID 0697: 54 kg; Gilt ID 0699: 52 kg). One of these two gilts (gilt ID 0699) was successfully catheterized on D173. By d173, the fifth gilt was still deemed too light (78 kg) and was removed from the experiment and culled.

Boar Exposure. On d160 of age, a single blood draw and a vaginal swab occurred on the 3 "heavy" ported gilts. Of the total 4 ports that were placed, patency was lost for one gilt within a week of the surgery (gilt ID 0691) and one gilt (gilt ID 0684) appeared to suffer from severe anxiety to touch following the surgery such that accessing the port was not feasible. Protocols for post-surgical daily animal care, approved by the University IACUC were followed and no indications of adverse reaction to surgical procedures (i.e., drugs used, recovery from anesthesia) was noted for this gilt so a justification for the change in behavior is unknown. Per the original collection timeline proposed in Table 2.1, daily serial blood collection was attempted in gilt ID 0698 from d160 – 174 of age. The indwelling port was accessed using sterile procedures (Appendix 1) at 0700, with a blood sample collected every 20 minutes for 4 hours. Huber needles were removed at the completion of the daily collection and reinserted the following morning. However, after completion of the first two weeks of bleeding, it became apparent that excessive scar tissue was developing in the skin surrounding the ports. The decision was made to change serial blood collection to every three days, with a 7-day rest period between every two-week bleeding interval. Therefore, serial blood collection began again with gilt IDs 0698 and 0699 on d181, 184, 187, 190, 193 of age and on d203, 206, 209, 212, and 215 of age, following a 7-day rest period. On blood collection days during boar exposure, serial samples were collected beginning one hour before boar exposure then every 20 minutes

up to 3 hours following the completion of boar exposure. Boar exposure occurred every day from 0800 - 0815 h. Two mature boars, housed separately from the gilts were alternated daily. Additionally, photos of each vulva were taken daily as another way to document estrus through an increase in size, change in color, or mucus discharge. Photos were taken daily until standing estrus or d215 of age, whichever came first. Standing estrus was detected by back pressure and human identification of estrus behavior (i.e., 'locked' stance, ears erect, grunting, swollen, pink vulva). Once standing estrus was detected a vaginal swab and AGD measurements were recorded. When standing estrus was detected, ports were accessed, and serial blood samples were collected every 2 hours for a total of 96 hours following estrus detection to characterize individual gilt LH levels and subsequent ovulation surge. A single blood sample via jugular venipuncture was then collected at d8 post-standing estrus to confirm that ovulation did occur based on serum concentrations of progesterone. Of the selected 5 gilts where standing estrus was not detected by d215, vaginal swabs and AGD measurements were recorded. Further, at d223, a single blood sample was collected via jugular venipuncture to confirm the lack of serum concentration of progesterone (deemed "anestrus"). If serum progesterone was detected that would be indicative of an undetected or 'silent' heat. Gilts that exhibited estrus, or where circulating levels of progesterone in serum was detected, were retained in the herd based on BW and herd replacement needs. The barn protocol to synchronize gilts for entrance into the breeding herd using Estrumate (Merck Animal Health, Worthington, MN) was applied. Gilts that did not exhibit standing estrus or did not have detectable serum progesterone levels after the completion of the trial were deemed 'anestrus' and shipped to a cull market.

2.2.2 Cohort 2

D77 collection. Twelve pre-pubertal replacement females (PIC 1050; 39.17 kg ± 2.72; d77 of age) arrived from a different nucleus farm than the first cohort and were offered the same diet regimen as described for cohort 1 (section 2.2.1). However, due to availability of gilts when the order was placed from the multiplier, these gilts arrived at 11 weeks of age. Therefore, the collections outlined for cohort 2 occurred on d77, 110, 130, 160 and during boar exposure. Upon arrival at d77, gilts were weighed, vaginally swabbed, and AGD measurements recorded.

D100 collection. At d100 of age, temporary jugular catheters were again attempted. However, within the first two gilts where catheter insertion was attempted, one died following the catheterization procedure. Necropsy results were inconclusive as to cause of death, and thus due to a lack of clear explanation for gilt deaths during the temporary jugular catheterizations, it was decided that serial blood collection for characterization of LH would not be continued. As with the previous cohort, LH characterization could not be used to establish 'high' and 'low' subsets, thus BW was again used.

D110 collection. On d110, a single blood draw from all remaining gilts (n = 11) was obtained for circulating hormone levels of estradiol and AGD measurements were recorded in triplicate and a vaginal swab was obtained.

The collections that occurred on d130, 133, 136, 139, and 160 of age are the same as outlined for cohort 1. For cohort 2, it was decided that the heaviest 9 gilts remaining at d130 would be retained for the remainder of the experiment and would receive indwelling titanium ports. Selected gilts received an indwelling catheter and port on d141 ± 2 of age. A single blood sample was collected from each gilt on d153 and d157 of age

to check for progesterone prior to boar exposure. Based on detectable serum progesterone concentration, it was determined that 2 gilts had achieved estrus prior to start of boar exposure at d160 of age. Boar exposure began on d160 of age and continued through d213. Collections that occurred throughout boar exposure (at standing estrus, or end of trial) were the same as those described in cohort 1.

2.3 Lab analyses

Estradiol-17β. Serum concentrations of estradiol-17β (E_2) were determined in duplicate in all blood samples by RIA (Jolitz et al., 2015). Estradiol-17β (E8875; Sigma Life Sciences, St. Louis, MO) was the standard and radioiodinated E_2 (#07138228; MP Biomedicals, Solon, OH) was the tracer. Anitsera (GDN#224 anti-estradiol-17β-6-BSA; Fort Collins, CO) was used at a dilution of 1:425,000. Sera (300 µl) was extracted with a 4 mL volume of methyl tert-butyl ether. Inhibition curves of increasing amounts of sample were parallel to standard curve. The sensitivity of the assay was 0.2 pg/tube.

Luteinizing Hormone. Serum concentrations of LH were determined in duplicate by RIA. Porcine LH (AFP3881A; National Hormone and Peptide Program, NIDDK) was used as the radioiodinated antigen and standard. Luteinizing hormone antiserum (National Hormone and Peptide Program, NIDDK) was used at a dilution of 1:200,000. Inhibition curves of increasing amounts of sample were parallel to standard curve.

Progesterone. Serum concentrations of progesterone were determined in duplicate in all blood samples by RIA (Jolitz et al., 2015). Progesterone (Sigma Life Science, St. Louis MO) was the standard and radioiodinated progesterone (MP Biomedical, Solon, OH) was used as the tracer. Antisera (#111.2C7.3; Enzo Life Sciences, Farmingdale, NY)

was used at a dilution of 1:700,000. Inhibition curves of increasing amounts of sample were parallel to standard curves.

Extraction of RNA. The cryovials were thawed on ice. When nearly thawed, samples were triturated with a needle and syringe 6 times. The sample was then placed into a 15 mL conical tube, and 600 µl of chloroform was added to each tube. Samples were shaken and spun at 4500 x g for 30 minutes at 4°C. Supernatant was then pipetted into another 15 mL conical tube, where another 200 µl of chloroform was added. After the second centrifugation, the supernatant was transferred to another 15 mL conical tube.

RNA purification. The purification of RNA from the vaginal swabs was completed using Invitrogen PureLink RNA Minikit (Fisher Scientific, catalog # 12-183-018A). A volume of 70% ethanol equivalent to supernatant volume was added to the 15 mL conical tube and vortexed. Then 700 µl of sample was pipetted into the spin cartridge with collection tube. The sample was centrifuged at 12,000 x g for 1 minute at room temperature (25°C). The flow through was discarded and the process was repeated until the entire sample was processed. A total of 700 µl of Wash Buffer 1 was added to the spin cartridge and the sample was again centrifuged at 12,000 x g for 1 minute at room temperature. The spin cartridge was placed into a new collection tube and 500 µl of Wash Buffer II [the Wash Buffer II bottle contained 60 mL of 100% ethanol added to the bottle (catalog # 12183025)] was added to each collection tube. Samples were then centrifuged at 12,000 x g for 1 minute at room temperature. The flow through was discarded and the process was repeated. To dry the spin cartridge, the sample was centrifuged at 12,000 x g for 2 minutes to dry the membrane. The collection tube was discarded, and the spin cartridge was placed into a recovery tube. A total of 30 µl of DNase/RNase free water

was added to each sample tube, and the samples were incubated for 1 minute. The samples were centrifuged at 12,000 x g for 2 minutes at room temperature, the spin cartridge was removed, and the recovery tube labelled. Concentration of RNA was determined via spectrophotometer (Nanodrop, Thermo Scientific, Washington, DE). The purity of RNA was determined by measuring the A260/A280 ratio. The ratio of all samples ranged from 1.8 to 2.0. Two micrograms of the resulting RNA were reverse transcribed into cDNA using the HighCapacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. Primers were designed using software provided by Integrated DNA Technologies (IDT, Coralville, IA), and are in Table 2.3.

Real-Time PCR. Real-time semi-quantitative PCR was used to measure the quantity of vaginal ER-α, IGF-1, TAC-3, TLR-4 and TLR-5 mRNA relative to the amount of porcine RPLP0 mRNA in each sample. Measurements of the relative quantity of the cDNA of interest was carried out using RT2 Real-Time™ SYBR Green/ROX PCR Master Mix (SuperArray Bioscience Corp., Foster City, CA). Twenty microliter reactions were measured using the Stratagene MX3005P quantitative real-time PCR instrument (Agilent Technologies, Foster City, CA). Thermal cycling conditions recommended by the manufacturer (40 cycles of 30 sec at 95ºC, 1 min at 55ºC, and 1 min at 72ºC) were used for all genes. The concentrations of forward and reverse primers used for the genes of interest and RPLP0 were 300 nM. A linear response was obtained when these concentrations of primer pairs were used with increasing amounts of cDNA. Dissociation curve analysis was performed after each real-time PCR run to confirm that a single amplicon was present. Additionally, all amplicons were electrophoresed through a 2%

agarose gel and stained with ethidium bromide to visualize that only amplicons of the appropriate size were present in each sample.

2.4 Statistical analysis

All analyses were performed in R (v 4.0.2). Anogenital distance measurements were analyzed using the Correlations package with analysis performed between AGD length at each time point between d70/77 to d215 and age at standing estrus. Relative expression is based on the expression ratio of a target gene versus a reference gene (RPLP0). The Polymerase Chain Reaction (PCR) package was used to quantify the expression of target genes (ER-α and IGF-1). Fold change differences were determined as a metric for assessing the relative alterations in gene expression between time points relative to d70/77 (genes ER-α, IGF-1, and TAC-3) and d100/110 (TLR-4 and TLR-5). This involved comparing the abundance of mRNA transcripts from d70/77 of age until standing estrus or end of trial (d215) with a fold change greater than 1 indicating upregulation and a fold change less than 1 indicating downregulation. The expression ratio results of TLR-4, TLR-5, and TAC-3 were tested for significance by Fisher's exact t-test, because these genes were not consistently found in all samples and therefore there are cells in the cross-tab. Analysis of variance was used to detect significant changes in gene expression by genes of interest (ER-α, and IGF-1) and time. Analysis of variance was used to determine significant changes for genes ER-α, and IGF-1, with main effects being time, and the response variable consisting of changes in expression. Hormone data was analyzed using descriptive statistics in Excel.

2.5 Results

Anogenital Distance. The AGD increased linearly with age ($P < 0.001$; Figure 1). There was a positive correlation ($P = 0.009$; Table 2.4) between AGD at d160 and age at first estrus and a negative correlation ($P = 0.002$; Table 2.4) with average daily gain. There was a negative correlation ($P = 0.044$; Table 2.4) between AGD at d100 and first estrus.

Estradiol-17β. A total of 9/13 females were analyzed for serum concentrations of estradiol-17β throughout boar exposure. Gilt 3152 had the highest mean serum concentration with 19.93 pg/mL ± 14.21 pg/mL (Table 2.3). She did not achieve standing estrus by d215 and did not have circulating levels of progesterone on d223. Gilt 3155 had the lowest mean serum concentration with 8.09 pg/mL ± 1.53 pg/mL (Table 2.3). She achieved puberty prior to d157 of age, and it is hypothesized that the stress related to the catheter surgery may have played a role in her early puberty. Once daily boar exposure began, gilt 3155 achieved her second standing estrus at d171.

Luteinizing Hormone. A total of 7/13 females were collected for 96 hours following behavioral signs of standing estrus, with the overarching goal of confirming that an LH surge and subsequent ovulation had occurred. For the sake of space, only collections through hour 44 were reported because the circulating levels of LH had returned to normal levels by hour 18. Of the 7 females collected, we successfully captured the LH peak for 4 (Gilt 3154, 3155, 3157, and 3162; Figure 2.5). It is suspected that the remaining 3 (Gilt 698, 3160, and 3161; Figure 2.5) had ovulated by the time serial collection began. Gilt 3154 had the highest individual LH concentration at ovulation, with 9.61 ng/mL (Figure 2.5).

Progesterone. Five of the 13 gilts had not expressed behavioral signs of estrus by the end of trial (d214 of age ± 1). In two of these gilts, serum progesterone concentrations at d160 (10.7 ng/mL) or d215 (14.9 ng/mL) indicated that ovulation had occurred despite the lack of physical estrus behaviors. Detectable serum progesterone in these 2 females indicated an undetected or 'silent' heat. Serum progesterone concentration was < 1 ng/mL in the 3 females who were deemed anestrus. The remaining females with a detected estrus were grouped into 'early' (estrus < d184 of age), 'average' (estrus d194-d195) and 'late' (estrus > d 202 of age). The early estrus group (n = 3) had serum progesterone concentration of 18.6 ± 7.5 ng/mL. The average estrus group (n = 3) had serum progesterone concentration of 23.3 ± 3.5 ng/mL. The late estrus group (n = 2) had serum progesterone concentration of 21.2 ± 4.2 ng/mL.

Gene expression. For IGF-1, there was a non-significant decrease in expression at d100 and d130. The expression of ER-α tended for lower expression at d100, with a non-significant decrease at d130. Expression of TAC-3 experienced a similar, non-significant decrease in expression at both d100 and d130. At d160, there was a 9.02-fold increase in relative IGF-1 expression ($P = 0.02$; Figure 2), a 2.57-fold tendency for an increase ($P = 0.08$; Figure 2) in ER-α expression, and a 7.88-fold increase ($P = 0.04$; Figure 2) in TAC-3 expression. Expression of TLR-4 and TLR-5 were not detected, or lowly detected, in all samples at d70, d100, d130 and d160 (n = 0/13, 3/13, 2/13, and 2/13 samples), therefore relative expression at d100/110 was used for comparison of these genes. At standing estrus or end of trial, expression of TLR-4 and TLR-5 was readily detected in 8/13 samples.

2.6 Discussion and conclusion

The objective of this study was to compare biological indicators (AGD, gene expression of the vaginal epithelium) at key points during reproductive development to circulating serum concentrations of reproductive hormones (estradiol, LH, progesterone). To date, the only known predictor of age at puberty is changes in reproductive hormones (Saski and Koketsu, 2008). Within these cohorts, AGD was utilized due to previous research associating it with reproductive ability in females and potential on-farm practicality. However, the correlation between AGD and reproductive success is not consistent across studies. Both shorter and longer AGD measurements have resulted in reproductive success (Correa et al., 2016; Gobikrushanth et al., 2017). In swine, Drickamer et al. (1997) reported a negative correlation between AGD and reproductive success, with a longer AGD at birth resulting in gilts who were less likely to be reproductively successful. Within the current study, several challenges were noted with respect to measuring AGD. In a research setting, we experienced vulva clenching, gilt restlessness, and fidgeting. While research technicians were able to allow time for the gilt to stop fidgeting, and the gilt was contained in a weigh scale which limited restlessness and movement, the added time needed for this to occur is not applicable for on-farm use. Gilts would clench during the AGD measurement, which alters the length and subsequent measurement. Fecal compaction, a common occurrence in breeding females, or expanded perineal area (Kiefer, 2021) similarly altered AGD. Additionally, like previous research (Drickamer, 1997; Correa et al., 2016; Gobikrushanth et al., 2017), the relationship between AGD and reproductive success was inconsistent. A longer length at d100 of age resulted in a smaller length at standing estrus, which would agree with Beltranena et al.,

(1991), who reported that age at first estrus decreased as growth rate increased from 0.4 - 0.53 kg/day. Alternatively, the longer length at d160 and older age at estrus from the current study agrees with other data which suggests that a faster growing gilt (i.e., larger AGD at d160) is associated with delayed puberty, specifically in females that achieved a growth rate of 0.6 kg/day (Beltranena et al., 1991). This would agree with our average gilt growth rate from on-farm arrival to d160, which was 939 g/day. If female growth rate could be managed predictably, AGD may provide more consistent measurements and predictive capabilities, particularly around d100 of age. However, given the inconsistencies reported in growth rate and AGD correlation above, as well as numerous issues that were observed during the collection period, it can be hypothesized that the potential to use AGD as a prepubertal marker of reproductive development seems limited based on this study.

In the present study, time of the preovulatory LH surge varied in relation to the onset of standing estrus, which agrees with others who reported a variation in respective LH surges (Tilton et al., 1982; Knox et al., 2003). The relationship between estrus onset and peak LH levels are consistent with the variability of other data (Parvizi, 1976; Van de Wiel, 1981). The importance of mean LH differences between gilts at puberty currently is not known (Knox et al., 2003). While not statistically significant, it appears the 3 females that achieved first estrus at an older age (Gilt 3154, 3157, and 3162; Figure 2.5) had higher LH peaks than their earlier estrus counterparts. This could be part of a compensatory response within the female reproductive tract. This is supported in human females, girls who enter puberty later progress faster to first menstruation, and those entering earlier take longer to progress to first menstruation, suggesting a compensatory

response in the female reproductive tract (Biro et al., 2001, 2006; Pantsiotou et al., 2008). According to our own data, gilt 3152 had higher mean concentrations of estradiol-17β, even though she was deemed anestrus. This disagrees with Amin (2020) who reported that regardless of age, serum estradiol-17β was higher in gilts that achieved puberty than in gilts that were anestrus. Serum progesterone followed a pattern that was indicative of a normal porcine estrous cycle. The presence of serum progesterone supports the visual identification of standing estrus and supports the hypothesis that changes in vaginal gene expression in the prepubertal phase can be correlated to reproductive development and standing estrus.

Reproductive hormones and the immune system work together within the reproductive tract. Toll-like receptors are important components of the immune system, acting to prevent infection while allowing fertilization and implantation to occur (Michailidis et al., 2011). Insulin-like growth factor-1 is important for cellular protein function within the reproductive tract and is also closely associated with rate of growth (Bosu, 1971). Estrogen-receptor-α is essential in the maturation of the female phenotype (Yao et al., 2007) and TAC-3 is critical for gonadal function (Hympanova et al., 2019). Focusing on these specific genes of interest would allow us to quantify changes in the vaginal epithelium of prepubertal gilts which could provide biological indicators of reproductive development. Research on how estrogen impacts TLR-4 production specifically in the vaginal epithelium is limited, but Yao (2007) found that estrogen upregulated TLR-4 expression in the vaginal epithelium of mice during the estrous cycle which agrees with our own data. Expressions of TLR-4 and 5 were lowly detected prior to boar exposure and readily detected at standing estrus, which would be indicative of

high estrogen level. The expression of ER-α within this study coincides with rising levels of estrogen that occur around standing estrus (Clapper, 2021). The expression of IGF-1 in this study appeared variable throughout reproductive development, but the decrease in expression at d100 corroborates with Clapper (2000) who reported that serum concentrations of IGF-I in gilts decreased from d84 - 98 of age. The decrease in expression of ER-α on d 100 coincides with the negative feedback action of estrogen that occurs during follicular development (Uenoyama et al., 2021). The increase in TAC-3 expression on d 160 may be due to the activation of the hypothalamic-pituitary-gonadal (HPG) axis. Tacykinin-receptor-3 induces the release of GnRH through kisspeptin stimulation (Fu et al., 2018; Hiney et al., 2018). It plays a key role in sexual maturation by directly stimulating kisspeptin release (Navarro, 2020), and because it is linked to the secretion of GnRH, GnRH is critical for pubertal attainment (Plant, 2015). In gilts, the HPG axis becomes activated around puberty, which leads to the onset of reproductive cycling (Calderon-Dominguez et al., 2017).

The increase in expression around the start of boar exposure (d160) for all genes suggests transcripts may be putative biomarkers that could be used in the prepuberal period to identify gilts that are likely to achieve puberty and may further be able to predict age at standing estrus for selection of gilts. This expectation requires further validation using larger datasets; these initial results support collection of additional vaginal swabs from a larger cohort of replacement gilts to strengthen predictive capability.

Table 2.1. Proposed selection timeline.

Day of age[1]	Sample type/activity	Blood analysis	Collection span
70	Blood, AGD, vaginal tissue	Estradiol, testosterone, LH	Single blood draw, AGD measure, and vaginal swab
100	Blood, AGD, vaginal tissue	LH	Serial using non-permanent jugular catheter, every 30 minutes for 10h, single AGD measure and vaginal swab
130-139	Blood, AGD, vaginal tissue	Progesterone	Single draw every 3 d, single AGD measure and vaginal swab at d130
140-145	Implantation of indwelling jugular catheter		Minimum of 7 d recovery
160-174	Start of boar exposure Blood, AGD, vaginal swab Blood	 LH Progesterone	Daily for 15 minutes Blood daily: serial sampling using indwelling catheter, every 20 min beginning 60 min prior to boar exposure until 3h after boar exposure; single AGD and vaginal swab at d160 and again at day standing estrus is observed Gilts observed in standing estrus only, single draw collected 8d after standing estrus detected
181-195	Start of boar exposure Blood, AGD Blood	 LH Progesterone	Daily for 15 minutes Blood daily, serial sampling using indwelling catheter, every 20 min beginning 60 min prior to boar exposure until 3h after boar exposure; single AGD and vaginal swab at d160 and again at day standing estrus is observed Gilts observed in standing estrus only, single draw collected 8d after standing estrus detected
202-216	If all 30 gilts have not been recorded as in standing estrus during the activities described for d160-174 and d181-195, boar exposure will be repeated one final time following the procedure described above.		

[1] Day 70 to 159 days of age expected to represent prepubertal reproductive development period; day 160 to 216 represents the periods of start of boar exposure, puberty/first standing estrus or ‘anestrus’.

Table 2.2 Composition of gilt development diets.

	Phase				
Item	5	6	7	8	Gest'n
Ingredients, %					
Corn	72.96	79.25	83.30	86.54	81.61
Soybean meal, 46.5%	23.66	17.68	13.83	10.77	14.54
L-Lysine HCl	0.4	0.4	0.38	0.36	
L- Threonine	0.14	0.13	0.12	0.11	
DL-Methionine	0.11	0.08	0.07	0.04	
L-Tryptophan	0	0.01	0.01	0.01	
Monocalcium phosphate	1.21	0.98	0.85	0.76	1.84
Limestone	1.02	0.97	0.94	0.91	1.31
Salt	0.3	0.3	0.3	0.3	0.5
Nursery Vitamin premix[2]	0.05	0.05	0.05	0.05	0.05
Trace Mineral premix[3]	0.15	0.15	0.15	0.15	0.15
Total	100.0	100.0	100.0	100.0	100.0
Calculated analysis					
ME, kcal/kg	3315	3329	3337	3342	3278
NE, kcal/kg	2418	2466	2497	2519	2446
CP, %	19.6	15.3	13.9	12.7	13.5
Ca, %	0.7	0.62	0.57	0.54	0.89
P, %	0.62	0.55	0.51	0.48	0.72
Available P, %	0.32	0.27	0.23	0.21	0.44
SID[4] amino acids, %					
Lys, %	1.08	0.94	0.84	0.75	0.55
Thr, %	0.68	0.59	0.53	0.49	0.42
Met, %	0.36	0.31	0.28	0.24	0.21
Trp, %	0.17	0.15	0.13	0.12	0.12

[1]J & R Distributing Inc. 518 Main Ave, Lake Norden, SD 57248 - USA. Minimum provided per kg of diet: Calcium 55 mg, Vitamin A 11,000 IU, Vitamin D3 1,650 IU, Vitamin E 55 IU; Vitamin B12 0.044 mg, Menadione 4.4 mg, Biotin 0.165 mg, Folic Acid 1.1 mg, Niacin 55 mg, d-Pantothenic Acid 60.5 mg, Vitamin B16 3.3 mg, Riboflavin mg, 9.9 Thiamine 3.3 mg.

[2] J & R Distributing Inc. 518 Main Ave, Lake Norden, SD 57248 - USA. Minimum provided per kg of diet: Copper 16.5 ppm, Manganese 44.1 ppm, Selenium 0.03 ppm, Zinc 165 ppm.

[3] SID = Standard ileal digestible

Table 2.3. List of primer sequences by gene and amplicon size.

Gene	Primer	Amplicon size, bp
RPLP0[1]	Forward: 5' – CTGAGTGATGTGCAGCTGATTA – 3' Reverse: 5' -CCCGTGTGTACCCATTGAT – 3'	137
ER-α[2]	Forward: 5' – GAATGTTGAAGCACAAGCGCCAGA – 3' Reverse: 5' – ACCGGGCTGTTCTTCTTAGTGTGT – 3'	91
IGF-1[3]	Forward: 5' – AGAACTGCACGGTGATCGAG – 3' Reverse: 5' – AGATGACCAGGGCGTAGTTG -3'	146
TAC-3[4]	Forward: 5' – GACTTCTTTGTGGGTCTTATGG – 3' Reverse: 5' – GCAGTTTCTACAGACGGTGG – 3'	122
TLR-4[5]	Forward: 5' – TCATCCAGGAAGGTTTCCAC – 3' Reverse: 5' – TGTCCTCCCASCTCCAGGTAG – 3'	186
TLR-5[6]	Forward: 5'- GGTCCCTGCCTCAGTATCAA – 3' Reverse: 5' – TGTTGAGAAACCAGCTGACG – 3'	187

[1]: porcine RPLP0; [2]porcine estrogen receptor-alpha; [3]porcine insulin-like growth factor 1; [4]porcine tacykinin-receptor 3; [5]porcine toll-like receptor 4; [6]porcine toll-like receptor 5.

Table 2.4. Mean and standard deviation of estradiol-17β (pg/mL) obtained during boar exposure (d160 - d215 of age) in 9 of the 13 ported females[1].

Gilt ID	Estradiol-17β Mean ± Standard Deviation (pg/mL)
698	10.17 ± 1.92
3152	19.93 ± 14.21
3155	8.09 ± 1.53
3156	11.93 ± 2.36
3157	12.21 ± 2.41
3159	12.17 ± 3.87
3160	8.71 ± 0.59
3161	15.18 ± 9.08
3162	10.21 ± 2.09

[1]Serial blood collection began on d160 of age and continued every 3 days until standing estrus or end of trial. On collection days, samples were taken every 20 min for 4 h, blood samples were pooled for estradiol-17β analysis. When behavioral standing estrus was exhibited (locked stance, erect ears, receptive to back pressure), a blood sample was collected every 2 h for 96 h and a daily pooled sample was analyzed.

Table 2.5. Significant correlations between AGD and gilt growth and puberty characteristics during reproductive development.

Trait 1	Trait 2	Correlation	*P*-Value
Age at Puberty	AGD at 160 Days	0.66 ±	0.009
Average Daily Gain	AGD at 160 Days	-0.58 ±	0.002
AGD at 100 Days	AGD at Standing Estrus	-0.41 ±	0.044

Table 2.6. Serum concentration of progesterone (ng/mL) in 13 gilts and their respective estrus allotment[1].

Estrus allotment	Sample size	Progesterone (ng/mL) + standard dev.
Silent	2	14.92 ± 4.23
Early	3	18.65 ± 7.48
Average	3	23.30 ± 3.47
Late	2	21.20 ± 4.26
Anestrus	3	*NONE DETECTED*

[1]A single blood draw was collected d8 after behavioral standing estrus or d8 after the end of trial (d215 + 8 d). Estrus allotments are representative of common industry groupings typically denoted as 'early' (d160 - 181 of age), 'average' (d181 – 202 of age) and 'late' (d202 – 215 of age) puberty.

Figure 2.1 Collection of anogenital distance of gilt.

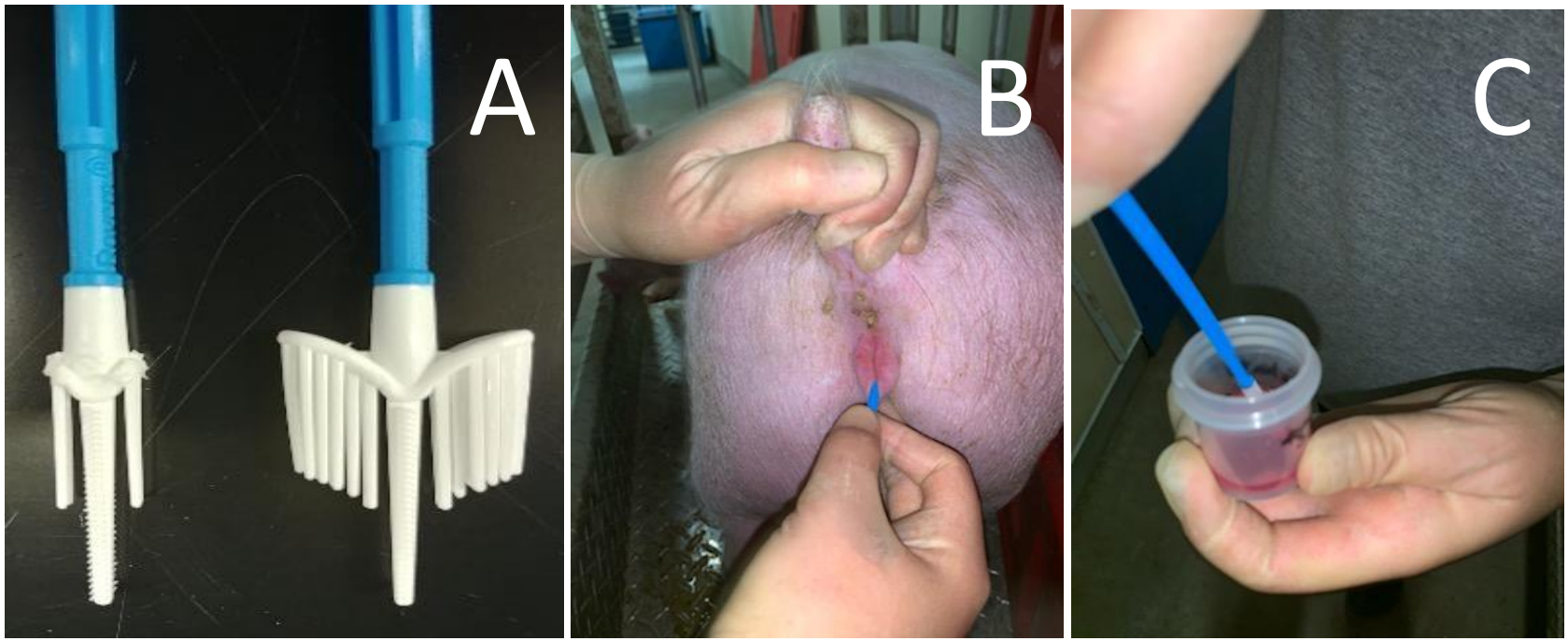


Figure 2.2. Collection of the vaginal swab. Swab brush ‘wings’ were adjusted to reflect increasing size of the vaginal area with gilt age (A). Smallest swab was applied to gilts at 70 days of age, largest swab was applied by 130 days of age. The swab was inserted approximately 4 cm into the vagina, then rotated 3 partial hand rotations (B). Swab tip placed in 3 mL Trizol reagent before flash freezing in liquid nitrogen (C).

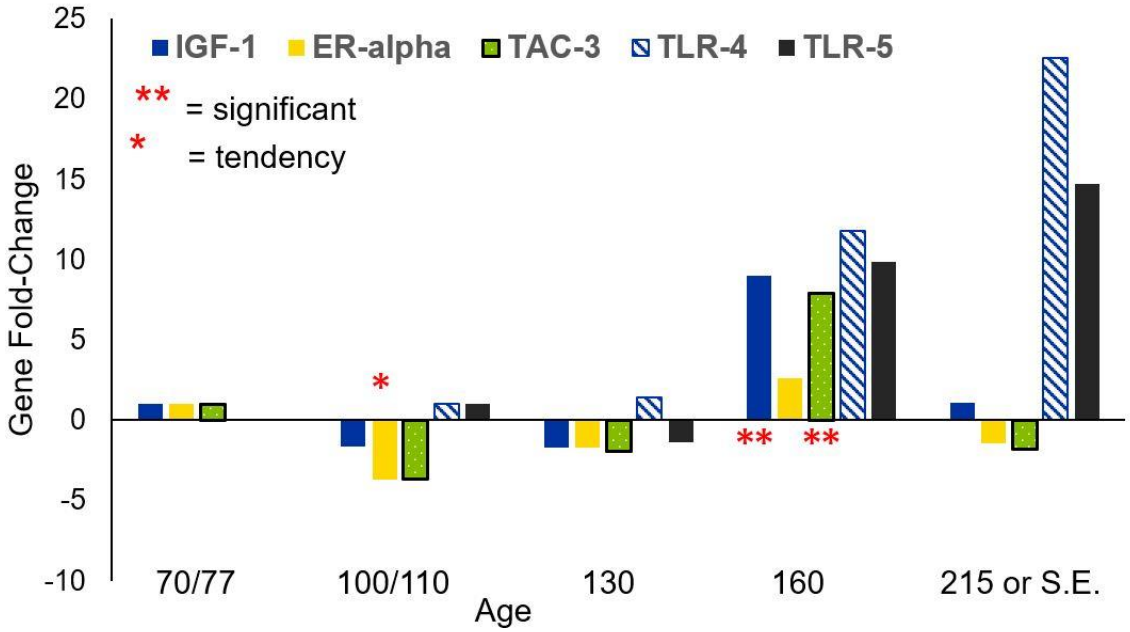


Figure 2.3. Changes in gilt vaginal gene expression during reproductive development. The d70/77 of age represents early prepubertal period, d100/110 represents mid-folliculogenesis, d130 represents post-folliculogenesis, d160 represents start of boar exposure, d215 or S.E. (standing estrus) represents end of trial. Relative expression of IGF-1, ER-alpha, and TAC-3 at each time point were compared to expression at d70/77. Due to low expression at d70/77, relative expression of TLR-4 and TLR-5 at d130, d160, and trial end were compared to expression at d100/110.

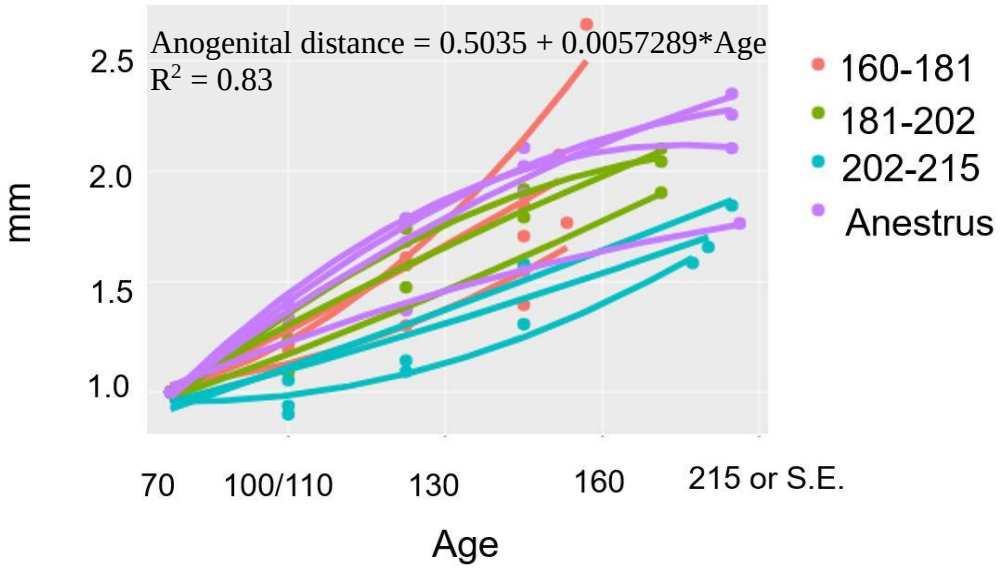


Figure 2.4. Changes in gilt anogenital distance during reproductive development. Each gilt (n = 13) was color-coded according to the age estrus was detected; estrus groupings are representative of common industry groupings typically denoted as ‘early’ (d160 - 181 of age), ‘average’ (d181 – 202 of age) and ‘late’ (d202 – 215 of age) puberty.

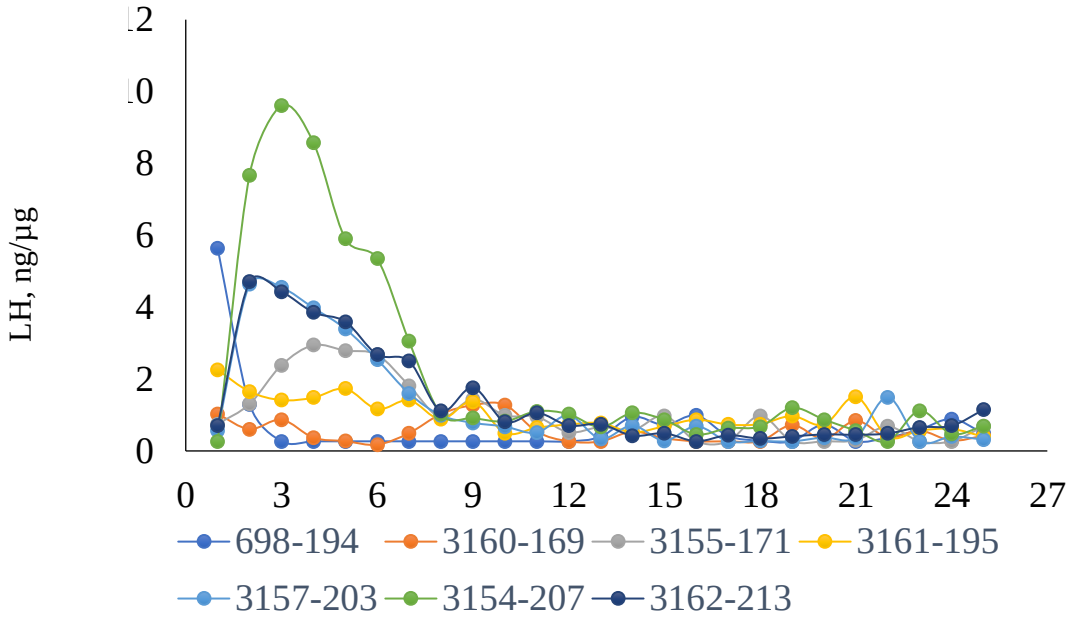


Figure 2.5. Circulating luteinizing hormone (LH) values in 7 gilts at standing estrus. Once behavioral signs of estrus were displayed, a blood sample was obtained every 2 h for 96 h. This period was utilized to confirm that the subsequent LH surge following standing estrus occurred. Each gilt number is listed, followed by their age at puberty.

CHAPTER III: DIFFERENCES IN VAGINAL TRANSCRIPTOME IN GILTS ACHIEVING PUBERTY 'EARLY' AND 'LATE' FOR PREDICTION OF AGE AT PUBERTY

Abstract

A known predictor of reproductive success in swine breeding herds is age at puberty. Early puberty has been associated with improved long-term reproductive performance. Additionally, vaginal expression of genes related to reproduction changes during reproductive development in gilts. It was hypothesized that changes in the vaginal transcriptome were correlated with age at first estrus expression and therefore likelihood of reproductive success. Pre-pubertal gilts (n = 29) were followed from d70 of age until first estrus or d215 of age. Vaginal epithelia were collected using a swabbing technique at five key timepoints during reproductive development [d70 (on farm arrival), d100 (mid-folliculogenesis), d130 (post-folliculogenesis), d160 of age (start of daily boar exposure), and first estrus or end of trial]. By d215 of age, 19 females were classified as expressing estrus 'early' (d160 to d181), 4 were 'average' (d181- d202), and 6 were 'late' or 'anestrus' (d202 to d215). A subset of 5 gilts that expressed estrus early and 5 gilts that expressed estrus 'late' or 'anestrus' were used for vaginal transcriptome analysis using RNAseq and vaginal samples at d100, d130, and d160 of age. Data normalization used the reads-per-kilobase-million (RPKM) method. Fold change differences were calculated across genes, within each estrus grouping (early or late). *P*-values were calculated using a paired t-test in R (v 4.0.2). A gene selection process was used based on differential expression at each time point, between estrus groups, and across multiple time points to identify target biomarker genes. The process reduced differentially expressed genes from > 2,000 to 6 genes of interest, 3 genes each in the early and late estrus groups. The 3

genes of interest for the 'early' were Lin-28 Homolog A (LIN28A), Anoctamin-2 (ANO2), and Lysl-oxidase homolog 2 (LOXL2). Gene LIN28A was upregulated across all timepoints ($P = 0.05$). At d130 of age, ANO2 had higher expression in early estrus gilts, although it was non-significant, and LOXL2 tended for higher expression than late estrus gilts ($P = 0.06$).

In the 'late' estrus group, the three genes of interest that remained were Glycogen-synthase-2 (GYS-2), Growth regulating estrogen receptor binding 1(GREB1) and Interferon-α-16 (IFN-α-16). At d100 of age, GYS-2 had a higher expression in late estrus gilts ($P = 0.01$). Gene GREB1 tended to have higher expression at d160 of age ($P = 0.09$) and was expressed more than 10-fold greater. Lastly, gene IFN-α-16 was at least 5-fold in expression across all timepoints ($P = 0.04$) in late estrus gilts. Distinct differences in transcripts during reproductive development occur between females achieving early or late puberty. Within this data set, d100, d130 and d160 of age may be suitable timepoints for identification of early and/or late estrus in pre-pubertal gilts.

Keywords: biomarker, early estrus, gilt, late estrus, predictive

3.1 Introduction

The research in experiment 1 determined that there are differences in gilt vaginal gene expression throughout reproductive development. Different techniques have been evaluated to identify a practical tool that could be used for on-farm prediction of age at puberty in the prepubertal period. Urine estrone concentration in the prepubertal gilts was assessed as a proxy for serum estrogen concentrations as a predictor of follicular activity

(Camous et al., 1985). Urine estrone concentrations increased in three distinct periods from the prepubertal stage to puberty, but age at each increase was highly variable and was excreted in a pulsatile manner which would require collection of total urine excretion within a 24h period.

Physical characteristics of the vulva/rectum area have also been examined. Rosomer and colleagues (2020) reported that vulva width at d105 was associated with sow productivity throughout two parities. However, only 8% of variation in age at puberty could be explained by vulva width. Vulva width at 21 d of age was predictive of whether a gilt would become pregnant, although the relationship between the two were weak and vulva width only explained 1.5% of variation in reproductive success (Mills et al., 2020). Within our own work, we examined the relationship between AGD and reproductive success. Like other studies evaluating AGD and the relationship with puberty in swine (Seyfang et al., 2018; Drickamer et al., 1997), the correlation was not consistent.

Recent advances in the field of 'omics' are being investigated across a vast range of fields to compare distinct molecular profiles that can be used to describe phenotypic variables (Hu et al., 2011; Xie et al., 2021). With respect to gilt development, Mills et al. (2021) report the vaginal lipidome, based on multiple reaction monitoring profiling, of gilts at 21 days postnatal could be linked to sow fertility over the first two parities. Relative abundance of cerotic acid, ximenic acid, nonadecanoic acid, and pentadecanoic acid were greater in infertile gilts while arachidonic acid and docosahexaenoic acid were lesser in infertile gilts (Mills et al., 2021). However, these methods do not specifically focus on periods during reproductive development. Therefore, the objective of this study was to identify key differences in vaginal transcriptome between early and late estrus

gilts. It was hypothesized that specific alternations in vaginal transcriptome could be used to predict gilts likely to achieve early or late-onset puberty.

3.2 Materials and Methods

Animal procedures were reviewed and approved by the South Dakota State University Institutional Animal Care and Use Committee (approval number 19-006A).

Cohort 3

D74 collection. Thirty pre-pubertal replacement females (PIC 1050; 31.4 kg ± 4.9; d70 of age) arrived from the same nucleus farm as cohort 1 and were offered the same diet regimen as described for cohort 1 (Chapter 2, section 2.2.1). One female died within 48 hours of on-farm arrival and the necropsy report indicated stress was the likely cause of death. The collections on d74 and 100 were the same as described for the first two cohorts.

D110 and 120 collections. Gilt BW, vaginal swab, and AGD in triplicate were collected from 29 females.

D130 collection. The collections on d130 of age were the same as described for cohorts 1 and 2 in Chapter 2 methods.

D144, 147, 150, 153, 157 collections. Unlike the first two cohorts, no females in cohort 3 received an indwelling titanium port and cephalic vein catheter. A single blood draw was collected every 3 days from d144 to 157 of age and analyzed for progesterone using RIA to confirm gilts were still pre-pubertal and had not achieved an early estrus.

D159 collection. This collection followed the same protocol as described for d160 of age collection in Chapter 2. Daily boar exposure began and continued until d213 of age. Due to an outbreak of PRRSv that occurred near the end of boar exposure the entire farm, including these gilts, was depopulated.

3.3 Lab analyses

The lab analysis for estradiol 17-β, progesterone, and RNA extraction are the same procedures as described in chapter 2.

RNA sequencing. Prior to sequencing, all amplicons were electrophoresed through a 2% agarose gel and stained with ethidium bromide to visualize that only amplicons of the appropriate size were present in each sample. A total of 30 RNA samples were sent to a commercial sequencing facility (MRDNA; Shallowater, TX). A total of 5 'early' (females who achieved puberty between d160-181 of age) females and 5 'late or anestrus' (females who achieved puberty between d202-215 of age or did not express puberty by the end of trial) females were sent for analysis: (10 samples at d100/110), (10 samples at d130), and (10 samples at d159/160) of age. The sequencing facility performed bulk sequencing, consisting of > 600 million reads, and 2 x 150 base pairs. Total counts were provided from the sequencing facility, and prior to sending the total counts, MRDNA performed data normalization utilizing the reads per kilobase million (RPKM) method. The RPKM method is made for single-end RNA-sequencing, where every read corresponds to a single fragment that was sequenced (Fundel et al., 2008; Dillies et al., 2013). Across all timepoints, 27,302 genes were expressed between 'early' and 'late' estrus gilts.

A second set of samples were sent for sequencing and included 10 females at d130 and d160 of age, 5 of which were deemed ‘early’ and 5 that were ‘late.’ and included females from cohort 3 (described earlier) and females from the lysine trial (Appendix II). The purpose of this second sequencing set was to validate the reproductive model that was created based on the 30 original samples.

3.4 Statistical Analysis

All hypothesis testing was performed in R (v 4.0.2). During the preprocessing of the data, the duplicated genes were found and removed from the data set. Anogenital length measurements were analyzed using the Correlations package. Hormone data was analyzed using descriptive statistics in Excel. To test for significant differences in RNA between early and late estrus groups, the paired t-test was employed. The null hypothesis of the Welch two-sample t-test is that there were no differences in vaginal transcriptomics between gilt puberty groups. The alternative hypothesis was that statistical differences exist in transcriptomic expression between gilt puberty groups. To develop a predictive model that can use the gene expression as input to predict late puberty as an output, a logistic model was employed. Logistic regression is a statistical method that uses probability to predict a binary outcome and classification tasks. Since the number of genes are much larger than the sample size, principal component analysis is employed to reduce the dimension of the input. This approach also reduces the effect of collinearity problems in the data. Principal component (PC) analysis involves a rotation of the axes to a set of axes that better describe the data by transforming the data into fewer dimensions (Lever, Krzywinski, and Altman, 2017). Due to removing the small PCs (removing the

PCs that had low predictive power, i.e. <5%) from the analysis, the final estimates are biased. Principal component analysis of the remaining 2,170 genes occurred (Figure 3.1 and Figure 3.2). Two principal components were produced, with one explaining 16% variance and two explaining 9% variance $p(x) = \frac{1}{1+e^{-(\beta_0 \backslash beta_1\ PC_1 + \backslash beta_2\ PC_2)}}$ with β_0 = -0.2646, β_1 = 0.2291, and β_2 = 0.1803. Using the first two PCs, a logistic regression was performed to model the probability of a gilt achieving late puberty. Because the goal of the analysis is more focused on prediction Cross-validation occurred 50 times, during which data is divided into training (80%) and testing (20%). Within this data set, 24 gilts with all genes (2,170) were used to train the logistic model using 2 principal components. No additional principal components were utilized, as adding additional ones would result in computational instability. The remaining 6 gilts (20%) were used as the test data to determine the prediction accuracy of the model. A confusion matrix was created to compare the model results to the gilts' actual estrus allotment. A confusion matrix contains information about actual and predicted classifications and is two-dimensions, with one dimension indexed by the actual class of the object (in this study, sday of age standing estrus was detected), and the other is the class that the matrix predicts (in this study, based on genes present on d100, d130 and d160, when a female should obtain puberty) (Deng et al., 2015). The results of the confusion matrix are defined within this data set as accuracy, sensitivity, and specificity (Table 3.3). Accuracy is the proportion of total number of predictions that were correct and is defined by $Accuracy = \frac{\sum_{i=1}^{n} N_{ii}}{\sum_{i=1}^{n}\sum_{j=1}^{n} N_{ij}}$.

The sensitivity (also referred to as recall), is a measure of the ability of a predictive model to select instances of a certain class from a data set and is defined by

$Sensitivity_i = \frac{N_{ii}}{\sum_{k=1}^{n} N_{ik}}$ `. The better the sensitivity (e.g., how well the confusion matrix predicts the true result) results in a score of 1.0, whereas the worst is 0.0 (Deng et al., 2015). A high sensitivity means the model is correctly identifying most of the positive results. Specificity is defined as the proportion of true negatives that are correctly identified by the model. Following the testing of the confusion matrix, the results of the testing data (n = 6) resulted in an accuracy of 0.76, a sensitivity of 0.73, and a specificity of 0.80 (Table 3.3).

The second set of RNA sequencing samples served as an additional 'check' of the predictive model created from the first 30 samples, and to identify (based on this dataset, and this predictive model), which collection day is a better predictor of standing estrus.

3.5 Results

Anogenital Distance. A strong linear relationship between AGD and age from the early prepubertal period to SE or d215 of age (R^2 = 0.85, 0.81, 0.88 in average, early, late/anestrus groups; Figure 3.4) was observed.

Estradiol-17β. Average estradiol concentrations (pg/mL) for each estrus grouping are found in Table 3.2. At d130 of age, estradiol concentrations were 21.66 ± 1.39 pg/mL. At d160 of age, mean concentrations were 14.52 ± 5.68 pg/mL. At standing estrus or end of trial, mean concentrations were 28.25 ± 8.37 pg/mL.

Progesterone. A total of 6/29 females had not expressed behavioral standing estrus by the end of trial. While two did not express behavioral estrus that was detectable to trained technicians, based off progesterone concentrations from d223 of age, two gilts achieved a

silent estrus (Table 3.3). In the early estrus group, the average concentration of progesterone was 34.24 ± 3.47 ng/mL.

RNAsequencing. Across all timepoints, 27,302 genes were expressed between 'early' and 'late' estrus gilts. The genes with significant differences are portrayed using a heat map, differentiating expression between 'early' and 'late' estrus gilts (Figure 3.4). To narrow the pool of target predictor genes, a 4-step selection process was used. 1) All genes that were differentially expressed at least 2-fold greater in early or late estrus gilts across all timepoints. The utilization of the 2-fold supports the general scientific dogma that something experiencing a minimum of a 2-fold difference might have biological or physiological importance (Kendig et al., 2010). This reduced the number of genes to 2,355. 2) All genes that were differentially expressed at least 20-fold greater at any one time point in early or late estrus gilts. The 20-fold difference supported a stronger criterion for selection, considering that the selected genes need to be heavily influenced at multiple timepoints, so a larger fold change may be reflective of that. This resulted in more than 50 genes remaining. 3) All genes at least 10-fold greater in at least 2 timepoints in early or late estrus gilts. The utilization of the 10-fold change difference still maintained the above argument for heavily influencing timepoints but allowing for more flexibility for the genes that are still important, but the encoded protein was generally expressed at lower levels. After this selection step, there were 11 genes of interest remaining. 4) A minimum of 3 females expressing known genes at both d100 and d130 of age. Of the 11 genes that remained in selection step 3, a total of 5 genes were unknown according to searches on NCBI Blast. That left 6 remaining known genes (Table 3.1), which were deemed suitable target biomarkers for greater investigation. The

six genes that remained were split into genes of interest for ‘early’ and ‘late’ estrus gilts. The 3 genes of interest for the ‘early’ were Lin-28 Homolog A (LIN28A), Anoctamin-2 (ANO2), and Lysl-oxidase homolog 2 (LOXL2). Gene LIN28A was upregulated across all timepoints ($P = 0.05$). Gene ANO2 had higher expression in early estrus gilts at d130 of age, though this was not significant. Lastly, gene LOXL2 had a higher expression at d130 of age in early estrus gilts compared to late estrus gilts ($P = 0.06$).

In the ‘late’ estrus group, the three genes of interest were Glycogen-synthase-2 (GYS-2), Growth regulating estrogen receptor binding 1(GREB1) and Interferon-α-16 (IFN-α-16). Gene GYS-2 had a higher expression in ‘late’ estrus than in ‘early’ estrus ($P = 0.01$) and was 35-fold greater at d100 of age. Gene GREB1 tended to have higher expression at d160 of age ($P = 0.09$) and was expressed more than 10-fold greater. Lastly, gene IFN-α-16 was at least 5-fold in expression across all timepoints ($P =0.04$).

The second set of samples for RNAsequencing initially had 25,897 differentially expressed genes. Using at least a 2-fold greater expression in early or later at d160 of age, 3545 and 1896 genes, respectively were identified. Within the genes differentially expressed in early vs late estrus females, 8 were expressed at least 80-fold greater and 7 of these were expressed at least 2-fold greater at d100 and/or d130 of age in the first set of 30 samples (Table 3.5). Within the late vs early comparison, one gene, Dimethylarginine dimethylamino hydrolase-1(DDAH1) was expressed 28-fold greater in late estrus females at d160 of age and was expressed 1.6 and 1.9-fold greater at d100 and d130 of age, respectively in the first cohort of 30 females. Of the 6 genes of interest listed as ‘target genes’ from the first 30 samples, LIN28A, GYS-2 and IFN-α-16 were also identified in the second sample set. The LIN28A was expressed 39-fold greater in early

estrus females at d160 of age, GYS-2 was expressed 2-fold greater in late estrus females at d160 of age, and IFN-α-16 was expressed 8-fold greater in early estrus females at d160 of age.

Predictive model. By using the first two principal components, 25% of the variation in the input data has been explained. The total accuracy, sensitivity, and specificity for the full data (30 samples) were determined. Total accuracy was reported at 0.8, sensitivity at 0.8, and specificity at 0.8. From the second group of sequencing samples, it was determined that collections on d130 of age predict the correct estrus grouping 30% of the time, but the collection on d160 of age predicts the correct estrus grouping 75% of the time. When comparing these two days within reproductive development, the d160 appears to show promise as the point in time to successfully predict females that will achieve early puberty.

3.6 Discussion and conclusion

The studies herein determined there are distinct differences in transcript abundance at d100, 130, and 160 of age between gilts achieving 'early' or 'late' puberty. Considering the potential target genes from the first sample set, GYS2 is commonly associated with glycogen storage disease, caused by genetic defects in glycogen synthase (Kamenets et al., 2020). It is known to influence polycystic ovary syndrome in human females (Hwang, 2012) which is in line with the greater expression in late vs early estrus females in this work. Type 1 interferons (IFN) are members of an antiviral cytokine family (Webb, 2018). The activation of IFN- α -16 is caused by an increase in TLR-7 gene expression. In adolescent females, IFN- α -16, increases right before puberty (Webb and Butler,

2018). The potential predictive role of IFN- α -16 is less clear where expression was greater in late estrus females in the first sample set and greater in early estrus females in the second sample set. The greater expression in early females is more in line with available data in humans. The overexpression of LIN28A has been shown to alter pubertal timing in female mice (Corre et al., 2017), but this disagrees with our research, as females that had higher levels of LIN28A were categorized as 'early' puberty. Genes ANO2 and LOXL are both associated with uterine function, however ANO2 is specifically involved with uterine contractions and is commonly found in uterine smooth muscle (Bernstein, 2014), but most research is tied to pregnancy maintenance, so the connection to pubertal gilts is unclear. The loss of LOXL2 promotes uterine hypertrophy (Lu et al., 2022). The expression of LOXL2 in late estrus gilts could be tied to maintenance of reproductive organ growth as the female continues to grow. Lastly, GREB-1 had higher expression in 'late' compared to 'early' females, and in humans, GREB1 is required for progesterone-driven human endometrial stromal cell decidualization (Cheng et al., 2018) and overexpression has been associated with estrogen-driven ovarian cancers.

Within this dataset, there was significance in transcriptomes at all three timepoints, with multiple genes of interest still significant. From an applicability on farm, identifying a single time point to take vaginal swabs, instead of multiple days is necessary for ease of use. Moving forward, increasing the sample size to be included within this predictive model is critical to confirm what day of development the vaginal swab should occur for early estrus detection.

Based on the predictive model, the model needs more samples to confirm that d160 is the ideal collection day for determination of estrus attainment. Use of other variable selection models, such as lasso or elastic nets could also positively influence the accuracy of the prediction model. These methods capture the possible underlying sparsity in the data and reduce the number of parameters that need to be estimated, which often leads to an increase in prediction accuracy.

This experiment determined that there are indeed distinct differences in the vaginal transcriptome throughout reproductive development. Currently, the d160 predictive model appears to show the most promise for detecting early estrus in prepubertal gilts. Events that occur throughout reproductive development could play an influential role in estrus attainment.

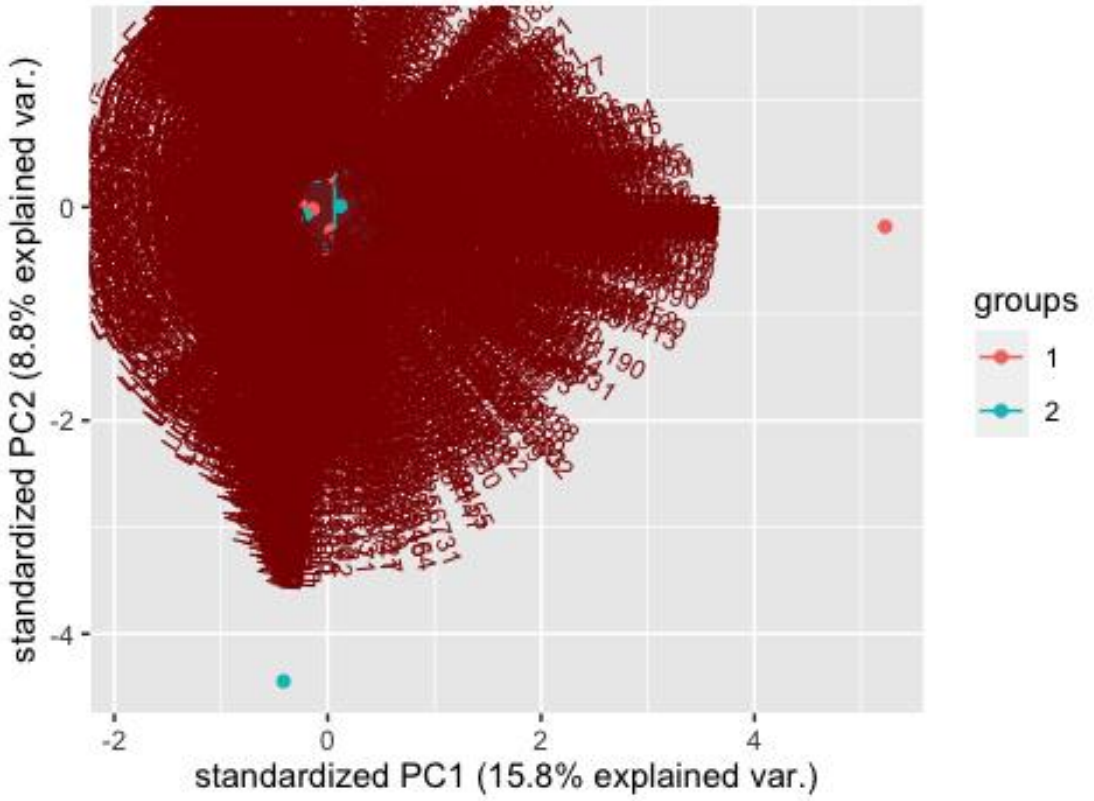


Figure 3.1. Principal component analysis number one. This analysis explained 16% of variation within the transcriptome data. Gilts were grouped by 1 (early estrus) or 2 (late or anestrus).

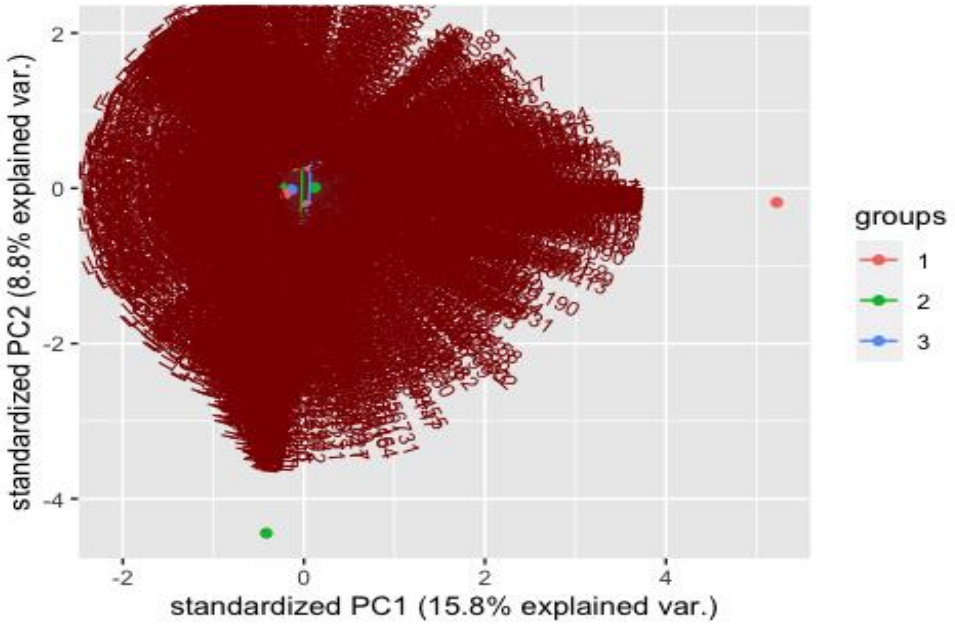


Figure 3.2. Principal component analysis number two. This analysis explained 9% of variation within the transcriptome data, analyzing d100 (1), d130 (2) and d160 (3) of age.

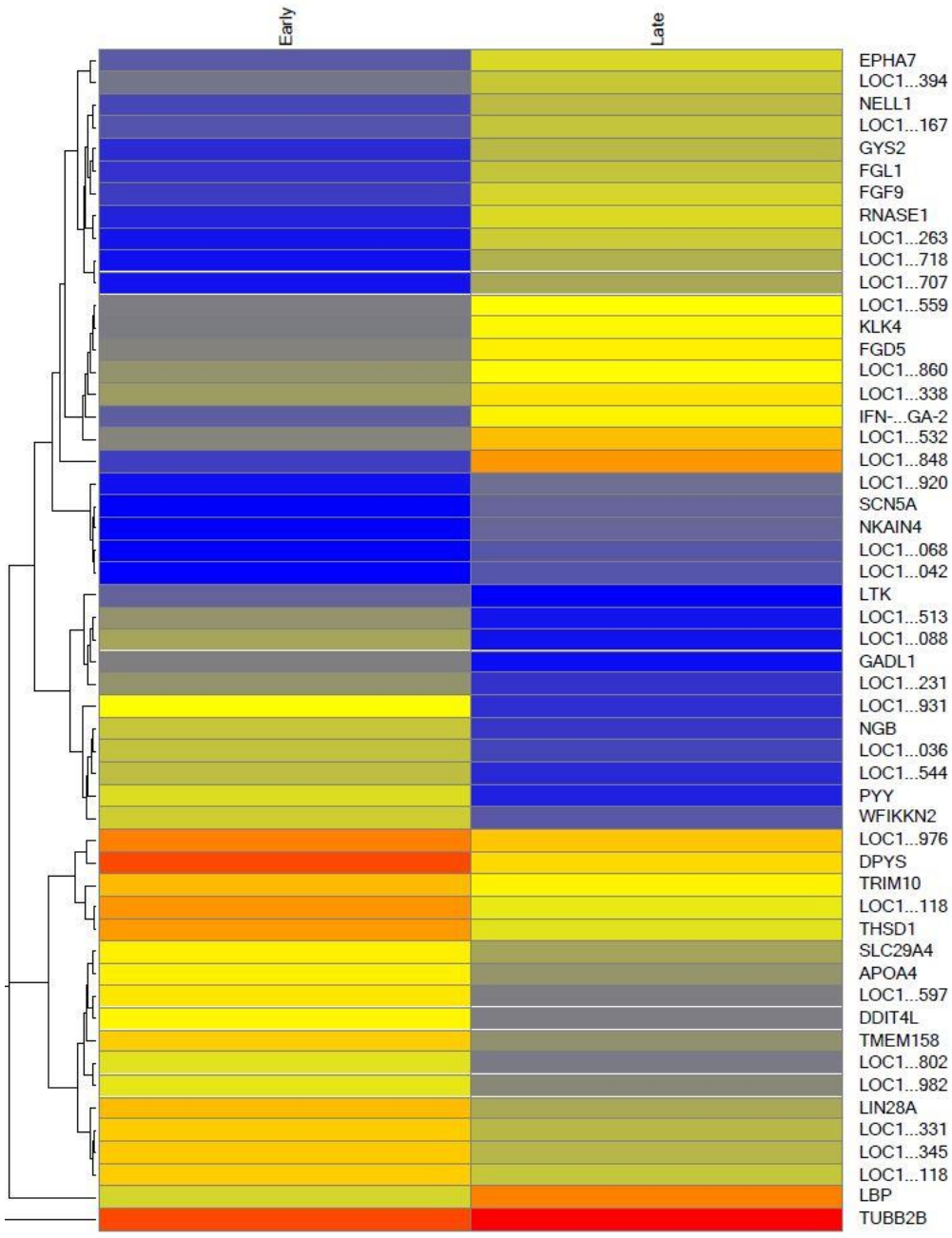


Figure 3.3. Heat map portraying the differences in gene expression between 'early' and 'late' estrus gilts across all time points. The blue color denotes down-regulation, and yellow denotes up-regulation.

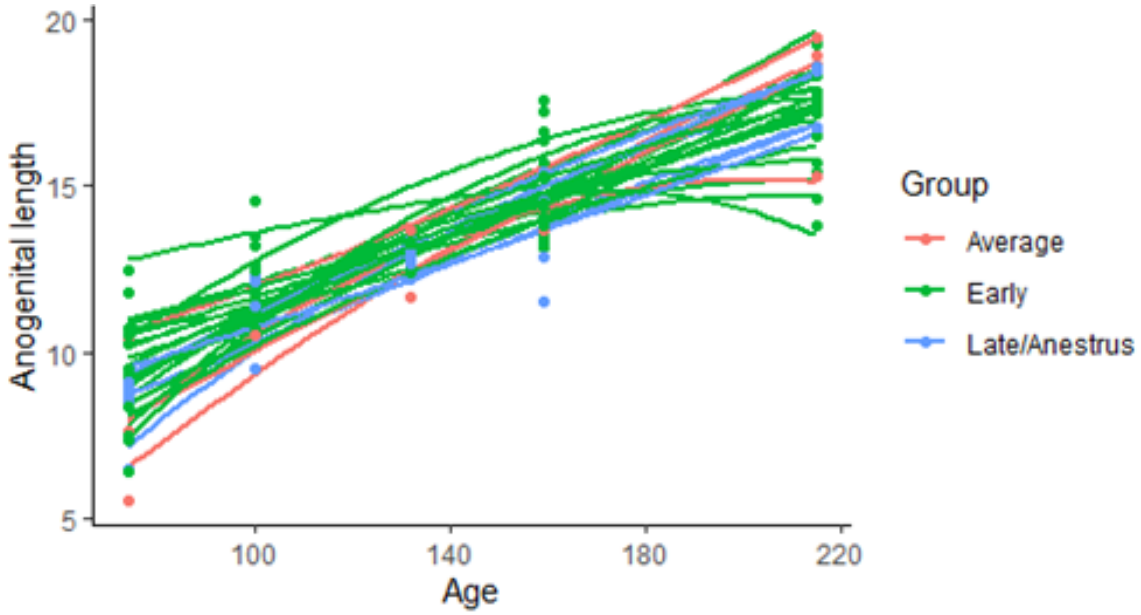


Figure 3.4. Change in anogenital distance in gilts throughout reproductive development. Each gilt (n =29) was color-coded according to the age first standing estrus was detected; estrus groupings are representative of common industry groupings typically denoted as ‘early’ (160 - 181 of age), ‘average’ (181 – 202 of age), and ‘late’ (202 – 215 of age).

Table 3.1. Serum concentration of estradiol-17β at 3 timepoints during reproductive development.

Collection timepoint	Estradiol-17β mean ± standard deviation (pg/mL)
d130 of age	21.66 ± 1.39
d160 of age	14.52 ± 5.68
Standing estrus or end of trial (d215 of age)	28.25 ± 8.37

Table 3.2. Serum concentrations of progesterone from 29 gilts and their respective estrus allotment[1].

Estrus allotment	Sample size	Average progesterone concentration (ng/mL) + standard dev.
Silent, late	2	14.92 ± 4.23
Early	19	34.77 ± 7.48
Average	4	34.24 ± 3.47
Anestrus	4	*NONE DETECTED*

[1]A single blood draw was collected d8 after behavioral standing estrus, or d8 after the end of trial (d215 + 8 d).

Table 3.3. Known genes of interest and function following gene ‘cut’ process.

Gene of interest	Function
Lin-28 Homolog A	Tissue repair
Anoctamin 2	Uterine contractions
Lysl oxidase homolog 2	Uterine function
Glycogen synthase-2	Glycogen synthesis
Growth regulating estrogen receptor binding 1	Endometrial stromal cell decidualization
Interferon-α-16	Cytokine activity

Table 3.4. Top 7 genes differentially expressed in greater relative proportion in early estrus females at d160 of age identified in both sample sets.

Gene of interest	20 sample set (fold change)	30 sample set	
		Fold change	D of age
Testis-expressed 15	82	2	d100 of age
Chromosome 19 open reading frame 84	91	< 2	d100 and d130 of age
Kallikrein related peptidase 10	97	4.5	d130 of age
Keratin	160	2	d100 and d130 of age
Involucrin	186	3	d100 of age
		2	d130 of age
Olfactomedin 4	282	6.3	d100 of age
Kallikrein related peptidase 11	332	9	d100 of age

CHAPTER IV: A SURVEY OF GILT REARING PRACTICES IN THE MIDWEST

Abstract

A successful gilt development program is critical to the success of a production system because it has a direct effect on reproductive performance. While there have been reviews of literature for current industry practices employed for reproductive management (Kraeling and Webel, 2015), and performance data collected and reported by Metafarms, there are little to no data that investigates both gilt management practices and decisions throughout reproductive development and her first parity within a breeding herd. Therefore, the objective of this survey was to obtain an understanding of gilt development practices across the Midwest to ensure that the next phases of research have high practical application and relevance. A total of 10 respondents participated, with farms located in Nebraska, South Dakota, Iowa, and Minnesota. These farms collectively manage approximately 43,000 sows. The questions ranged from gilt on-farm arrival to first parturition litter information. Data were collected and analyzed for descriptive statistics within Microsoft Excel. Some of the least varying responses included the gilt selection process, in which 10 respondents reported that gilt body size (weight) was the most important metric for selection, followed by foot/leg structure. The herd parity range for the sow farms was 2.9 - 3.1 after gilt acclimation, which indicates an overall young herd, which means sow removal is occurring at a young age. Based on this survey, more detailed information on gilt rearing and diet composition needs to be obtained.

Keywords: gilt rearing, management, gilt development

4.1 Introduction

Within a breeding herd, gilts are often the largest age group of females, accounting for nearly 24% of a sow farm animal inventory (Koketsu, 2005; Tummaruk et al., 2009). A successful gilt development program is critical to a production system because it has a direct effect on reproductive performance (Kansas State gilt development fact sheet, 2023). Good reproductive performance in gilts is defined as a successful pregnancy at the first breeding attempt that produces a litter of high-quality pigs followed by at least two additional pregnancies (Nonneman et al., 2016; Patterson and Foxcroft, 2019). In swine herds, the highest level of culling occurs after the first and second parities, with 34% resulting from reproductive failures (Hadas et al., 2015). As a result, gilt reproductive performance impacts overall herd reproductive performance and success (Kaneko and Koketsu, 2011). Understanding and refining management practices during gilt development could improve various aspects of gilt reproductive performance and stimulate changes at the herd level that could be beneficial to both reproductive development of the gilt and the producer. Understanding variation within rearing practices across a population of farms can aid in identifying when practices need improvement. Implementing effective gilt pool management can allow producers to meet targets for body condition and reproductive maturity (age at first estrus), and ultimately reduce annual replacement rates (Patterson and Foxcroft, 2005). However, there are no reports using commercial herd data to study associations between management practices and gilt reproductive performance in the same model. The objective of this survey was to obtain descriptive data regarding gilt development management practices and facilities, identify practices perceived by producers to be more useful from a management perspective, and obtain insight into where future research around management practices

might focus to assist swine producers. The survey included quantitative measurements, including pig space, estrus detection and induction protocol, and diet concentration throughout reproductive development.

4.2 Materials and Methods

Institutional Animal Care and Use Committee approval was not required for this study because no animals were used. The Institutional Review Board at South Dakota State University granted approval to conduct this survey (IRB – 002772 – EXM).

A questionnaire was developed to ensure that all the same questions were asked of participants. Initial testing of the survey was performed by a gilt development unit (GDU) manager for Christensen Farms (Sleepy Eye, MN). A point of emphasis during the review process was to reduce the number of open-ended questions and to reduce the amount of time a participant would need to complete the survey. Prospective participants were obtained from contact with the state's respective pork producers councils in South Dakota (SD), Iowa (IA), and Nebraska (NE). Additional contacts in Indiana (IN) were invited using contact information provided by industry professionals. Candidate producers were identified as owning a sow herd with more than 10 sows and purchasing their replacement females from a genetics company. Prospective participants were first contacted by email or telephone, and if willing to participate, were polled via the electronic questionnaire by use of QuestionPro (Appendix AI). Participants were asked to answer questions about gilt rearing and types of facilities used with specific questions for gestation housing (GH) or open-pen housing (OP). They were also asked questions about their estrus induction protocol, gilt development and gestation diets, and farrowing protocols for gilts during their first parity. Ten farms completed the entire survey with 2,

1, 3, 2, and 2, from MN, IA, SD, NE, and IN, respectively. According to the survey metrics, 70 different participants viewed the survey, 34 participants responded partially, and 23 drop-outs occurred with an average of 10 minutes per submission. Response data from the survey was entered into a Microsoft Excel (Microsoft) spreadsheet for summarization.

4.3 Results and Discussion

The overall objective of this survey was to determine current gilt development management practices across sow farms in the Midwest. There are limitations to this survey that may affect the applicability of these results. This survey only represents a portion of sows in the US (~43,000), and hence, may not be representative of the entire swine producing sector in these states. In addition, a particular short-coming of this survey is insufficient data regarding diet lysine and net energy concentrations for each herd. Two specific questions were asked, one encompassing gilt development diet composition and one encompassing gestation diet composition. Respondents were asked to provide predicted diet net energy/phase (Mcal/kg), and if different diets/phases existed, to provide information for those as well. There were no responses to this question, although one participant did specify that their diet recommendations are to PIC specifications. Depending on the weight range for prepubertal gilts, PIC recommendations range from 2,998 Kcal ME/kg to 3,131 Kcal ME/kg. Obtaining true dietary components, specifically in the gilt development period, would allow producers to compare across farms, and provide the opportunity to fine-tune management practices to help raise the best replacement gilts possible.

General gilt housing information

The initial survey questions asked basic barn information, including ventilation type, type of facility, as well as a feeder and waterer summary while gilts were housed in the GDU. The questions regarding ventilation and type of facility were removed from analysis, given the wide variety of answers provided by respondents. The most common water source was a well (n = 8), with two farms (SD-1 and SD-3) reporting rural water usage. The farms polled had an average of 2 waterers per pen in the GDU, with NE-1 reporting 4 waterers per pen and MN-2 reporting 1 per pen, but unfortunately respondents did not provide space/pig. In terms of feeder type, most were dry self-feeders (n = 5), 4 were wet-dry feeders, and one handfeeding. In the future, these types of questions could be removed from the survey, if the survey is only distributed electronically. The purpose of these types of questions were to obtain an understanding of gilt feeder space, but when asked directly, respondents either a) did not submit a response, or b) simply provided feeder space estimates per pig but did not provide how it differs across growth. If this survey is performed on-farm, these questions would provide insight into feed and feeder management, pan score, and feeder space per pig could be calculated. In addition, a 500-g feed sample could be collected from multiple feeders. These samples could be pooled together and mixed, and a representative subsample could be analyzed for proximate analysis and amino acid concentration. This method of diet sample collection on farm was introduced by Jones et al. (2010), where a single composite analysis was collected to account for analysis cost and avoided using a probe to reduce biosecurity risk. The laboratory results could be compared to the diet formulations provided by consulting nutritionists, and there could be a comparison between expected and actual nutrient (eg.

lysine). Comparing expected vs. actual values would provide an indication of feed mixing adequacy (Zeamer et al., 2021).

Gilt development

Producers were surveyed about their gilt development unit, and were asked about two different timepoints, on-farm arrival and 'current' (age at survey completion) (Table 5.1). Within these responses, IA-1 had the highest number of gilts delivered at a single time with 750, however their frequency of gilt delivery was not reported. Moving forward, obtaining information like number of rotations/calendar year within the GDU would allow us to calculate true gilt numbers for each producer, and could provide better understanding of the number of gilts that are purchased for an individual farm. This type of information would also help tie in average gilt age and weight at time of survey, based on the number of gilt cohorts currently in their respective GDUs. These types of question would provide insight into the most common phases of development that gilts are being fed. If this information can be connected with specific diet information for the individual farm, then it could be compared across systems and means and standard deviations of dietary components could be provided. Moving forward, direct contact with the nutritionists who formulate the diets for the individual farms may prove to be more fruitful, as the nutritionists would have a better idea of common diet formulations and the age at which the phases change. The producers may be more concerned with proper gilt management within the barn and day-to-day operations and therefore not as involved in the nutrition formulation for the gilts.

Gilt selection criteria

Following reproductive failure, failed structural soundness exams are the second highest reason for sow removal from a breeding herd (Penn State Extension, 2022). Feet and leg soundness are paramount to gilt selection and play a key role in overall gilt success. Therefore, a portion of this survey focused on gilt selection criteria (Table 5.2). Due to the type of answer selection process (select one, order of importance), it is hypothesized that these questions were quicker to answer and resulted in quicker response times. For example, when asked about assessing gilts at breeding, and ranking importance of selection, there were a total of 38 responses (which included producers that started but did not finish the survey), with the response marked as 'most important' consisting of body size (Table 5.2, n =10), and both foot and leg structure and teat number ranking second in importance for gilt selection prior to breeding (Table 5.2, n = 9). Two respondents listed "other" as their selection criteria but did not provide any further information. In this survey, body size referred to weight at selection. It is recommended that gilts be bred at a target weight of 135 - 150 kg (297 - 330 lbs) (Williams, 2005; Kim, 2016), with most recent PIC recommendations suggesting between 136 - 159 kg (300 - 350 lbs) (PIC, 2021). It has been recommended that gilts are bred at this weight range to protect against the detrimental effects of lean tissue loss during first lactation in subsequent reproductive performance (Patterson and Foxcroft, 2019). Gilts that weighed less than 135 kg at first breeding had fewer total pigs born over three parities than their heavier counterparts (Williams, 2005). While it is beneficial for a gilt to reach a minimum weight at first breeding, avoiding excessive weight gain is important. Ad libitum feed intake for current fast-growing genotypes is a concern

because of potential lameness and leg problems (Farmer, 2018). Ad libitum feeding after 10 weeks (approximately 77 d of age) has been shown to increase the risk of osteochondrosis by 20% for each 100 g increase in ADG (de Koning et al., 2013). It is hypothesized that heavier gilts at first service tended to have more nutritional maintenance demands over their productive life (Bortolozzo, 2009). When targeting gilt nutrition, it's important to aim for a lifetime growth rate >700 g/day. Females with a lower lifetime growth rate (<700 g/day) had fewer total pigs born (Patterson and Foxcroft, 2019). While an understanding of gilt weight at first estrus or first breeding is a crucial step in meeting target gilt breeding weights, these records are not readily available across the industry (Khanji, 2019). While most producers can estimate gilt weight by visual appraisal, nearly all farms do not possess a scale to confirm visual weight assessments. An on-farm method that could provide weight estimates would be utilization of a weight tape (PIC, 2022). This method utilizes a basic allometric growth curve which takes advantage of the high correlation between heart girth circumference and body weight (Khanji, 2019).

An interesting response was that respondents listed foot and leg structure as a second priority compared to body size. Given the reports that lameness (i.e. foot and leg issues) is a top reason for culling and that the greatest proportion of sows culled due to lameness are young (< parity 3; Grandjot, 2007), the fact that it is a second priority compared to body size leads to more questions. Why is body size more of a selection priority than foot and leg structure? We hypothesized that producers expect a larger body size at farm arrival could lead to earlier puberty and subsequently producing litters sooner, which would be beneficial to overall productivity. Another hypothesis is it a labor issue, where a

visual of the body size is easier than identifying all feet and legs of several hundred replacement females. Moving forward, if providing this questionnaire on farm, asking specific follow-up questions to gain an understanding of why body size is a stronger criterion for selection over foot and leg structure. Purchased gilts should be screened for reproductive traits (ex: vulva size, teat number) as well as feet and leg structure on arrival (National Hog Farmer, 2010). As a follow-up, asking specific questions of the producers regarding soundness components that they require could help explain why that producer may have foot and leg structure issues, if any once those gilts enter the breeding herd. There are some very useful online resources that provide excellent visuals to aid producers in the selection process for foot and leg structure. An ideal replacement should have good cushion and flexion in her joints, toes that are big and evenly sized, and large feet (Pork Checkoff, 2010). Gilts that are unsound display one or more of the following symptoms: splay footed, pigeon-toed, buck-kneed, post-legged, sickle-hocked, straight pasterns, uneven toes, and goose stepping (Stalder, 2010). Of these structural issues described above, several have been shown to impact overall sow longevity (Serenius et al., 2004, 2006, and 2007). Gilts with buck-kneed front legs leave the herd earlier than their sound counterparts (Image 5.1.) and females with front legs like this should be removed without further consideration. Moving forward with this survey, an on-farm application would provide the opportunity to identify gilts that either meet the soundness criteria, or do not. It is also the hypothesis of this author that most producers do not actually count teat numbers on their own replacement gilts, given that most genetic companies provide a teat number guarantee.

Another structural aspect to consider when selecting replacement females is the position of the rear leg pastern. It is important to select replacement gilts that do not possess rear legs of a gilt that would be deemed sickle-hocked (Image 5.2). Sows housed in gestation stalls that possess rear leg structure like sickle-hocked appear to sit on their butt like a dog and can increase the risk of the animal becoming splay-legged (Stalder et al., 2010). Teat number had the same priority in selection as foot and leg structure (n =9), and vulva size was the criteria selected most often as 'least important' with 8.

Boar exposure, heat detection and breeding

Farms SD-2 and IA-2 completed questions related to the sections noted above only, thus were removed from all remaining sections. Kraeling and Webel (2015) reported that after the first recorded estrus, gilts should be acclimated to stalls or breeding and gestation housing at least 16 d prior to breeding. This appears to be a commonly accepted practice within the respondents polled, as farms that utilize stall housing reported that stall-breaking began on average 25-26 weeks of age, with NE-1 reporting the youngest training (stall-breaking) occurring at 18 weeks (Table 5.3). The recording of pubertal estrus and number of estrous cycles for a gilt is more important than chronological age when determining the time of mating in gilts (Foxcroft and Patterson, 2010). Interestingly, within this subset, there was a range in the age at which boar exposure began. Within respondents of this data set, the average age range that boar exposure began was 25-26 weeks of age (n = 5), or approximately 175-182 days of age. This is an older age than what has been reported previously (Patterson and Foxcroft, 2019), that stated that male exposure of the replacement gilt should begin at 160 d of age. Gilts that

achieve puberty between 160-180 d of age are associated with improved reproductive performance (Sasaki and Koketsu, 2008). But, if daily boar exposure isn't beginning until approximately 180 d of age, there is a strong possibility that these females are being identified as reaching puberty at 190 d of age or older, when they may have achieved puberty earlier. This means that there could be a high number of gilts that are bred at 3rd estrus instead of 2nd, which would accrue higher feed costs maintaining these females prior to breeding.

Once boar exposure began, 4 of the 10 farms reported that it occurred daily for 10-15 minutes, with the goal of allowing individual females contact with the boar for 1-2 minute before moving to the next pen or stall area. It appeared that MN-1 had the longest time for exposure, consisting of 22 h per day of alley exposure to the gilts when trying to achieve pubertal attainment, and SD-3 reporting the longest period between exposure, occurring 3 days per week for 2 h per day.

Once puberty had been recorded, 6 of the 10 farms reported that they utilize heat-no-service (HNS). Utilization of HNS within a herd resulted in an increase of lifetime total born and gilts inseminated after the first HNS produced 1.57 more pigs per lifetime than those inseminated at pubertal attainment (Malanda, 2020). With support for utilization of HNS across the commercial industry, asking additional questions of the 2 respondents that do not utilize HNS could provide information on that farm's specific animal flow and breed targets. This information could then clarify why these farms do not utilize HNS.

If females have not had a recorded cycle, only 3 of the 8 respondents utilize PG-600 injection. The other respondents (n = 4) simply culled these females for reproductive

failure. However, 2 of the 3 farms that reported PG-600 usage, culled females after 1 additional failed cycle if intervention did not help.

Breeding and gestation information and housing

One of the most staggering results from this survey was the reported average parity across farms of 2.9 - 3.1, but two farms (MN-1: 2.5, MN-2, 2.3) reported lower parity average. It has been proposed that 52% of sows in a herd should be in parities 3 - 6 (Stalder et al., 2000; Sporke, 2007). Additionally, good reproductive performance in gilts can be defined in terms of successful pregnancy at the first breeding attempt that produces a litter of high-quality piglets (defined as piglet birth weight equal to the herd average) followed by at least two additional pregnancies (Nonneman et al., 2016; Patterson and Foxcroft, 2019). Given the average parity range reported in this dataset, it can be hypothesized that many of these females being introduced to the herd are not achieving the 3+ litters necessary to economically "break even" for the producer (Engblom, 2016). A follow-up survey question could potentially ask for the parity distribution, instead of parity average, so that it could provide a better overview of the true parity distribution within a farm.

At breeding, all farms reported a minimum of 2 doses with 5 farms reporting up to 3 doses/cycle (Table 5.4). A minimum of 2 semen doses is beneficial for pregnancy attainment, given that in swine, estrus can last between 45 - 65 h (Soede, 1997). This means typically ovulation can occur 24 - 48 h from the onset of estrus, and data suggests that all follicles ovulate over a 1 – 3-h period (Soede, 1992; Flowers, 1993; Soede, 1997).

The farms that contain open pen housing (n = 4), reported placing gilts in pens following the last insemination dose, where they stayed for the remainder of gestation until loading into farrowing rooms (Table 5.4). On average, farrowing rooms are loaded between 110 - 114 d of gestation, with NE-1 reporting that rooms are loaded later than d114 of gestation (Table 5.4). It is hypothesized that the time frame in which sows are loaded into rooms is dependent on animal flow, as well as labor availability for cleaning and disinfecting farrowing rooms between groups.

Farrowing information

During parturition, if a gilt is experiencing farrowing difficulties, the most common method of intervention was sleeving every 20 - 30 min (n = 6; Figure 5.1). However, 4 of the farms (MN-1, NE-1, NE-2, and SD-3) also reported oxytocin injections as another method of intervention. The only farm that reported Ca/P administration was SD-1. The MN-1 farm reported the highest total born and liveborn with 15.7 and 14.2, respectively. Unfortunately, 4 of the respondents only listed one value for total born and liveborn, so we are unable to make observations for their average gilt litter information. Only 3 farms (30%) count stillborn and mummies (IN-1, IN-2, and MN-1), so that data remains inconclusive. If farms are simply not recording the stillborn information, it could be considered a missed opportunity in identifying new disease outbreaks on farm or mitigating potential feed mycotoxin issues that may arise. Moving forward, accessing specific litter information could be beneficial, as that information could be compared to management practices during farrowing (e.g., sleeving interval, oxytocin injection, etc.), and provide more information on successful management practices.

A problem that can occur during parturition is savaging. Savaging is aggressive behavior by newly farrowed sows or gilts towards their own offspring (Chen et al., 2008). In this dataset, a savaging sow was defined as a deliberate attack on one or more piglets that resulted in piglet death by biting (Chen, 2008). A total of three options commonly used to help alleviate gilt savaging was described, which included pulling piglets off, acepromazine injection, and underline rubbing (Table 5.5). Of the respondents, only 2 reported all 3 avenues to alleviate savaging (NE-2 and MN-1). The most common response was acepromazine injection (n = 4; Table 5.5), and 3 reported pulling piglets off and underline rubbing (Table 5.5). The SD-1 farm reported that alcoholic beer administration worked better than acepromazine injection within his own herd, and while a unique response, it shows the individuality of management practices across farms. When asked if the gilt is culled for savaging, 5 of the 8 responded "no." This is an understandable response since savaging is a generalized behavior that may not occur in subsequent parities (Chen, 2007).

The last portion of this survey investigated gilt retainment within a herd following her first lactation. Respondents were asked to provide an estimate for how many animals farrow as a P1, and farrow as a P2. According to IN-1 and IN-2, 75 - 76% return as a P2 within their herds. As for SD-1, NE-1, and NE-2, 80% return as a P2. The highest percentage of gilts returning as a P2 was reported by MN-2, who responded that 85 - 90% return, on average. The largest response for culling a gilt after her first parity was farrowing difficulty (n = 7), followed by structure (n = 5). This is one of the questions that other respondents (who dropped out of the survey) chose to complete, which is why the total number of respondents is higher than what has been reported so far. In future

surveys, asking for specific feedback beyond simply “farrowing difficulty” may provide more insight into management practices, specifically as far as what is considered farrowing difficulty within those farms.

4.4 Conclusion

This survey was able to obtain information that is not readily available across the swine industry. It also highlighted the different management decisions that can be made across systems. Moving forward, specifically focusing the survey on direct areas of gilt rearing may improve overall feedback from producers. Additionally, allowing producers that utilize internal multiplication to participate may also improve response numbers. On-site visits may allow us to obtain diet sample information that could be reported across different weight ranges or receiving feed nutrient targets from the relevant nutritionist who has access to diet formulations could also be beneficial.

This survey is the first of its kind in providing general management practices for gilt rearing. Though the number of respondents who completed the survey was small (n = 10), they accounted for approximately 43,000 sows within the industry and provides a strong starting point for future data collection to better understand management practices throughout gilt rearing and acclimation into the breeding herd.

In conclusion, this survey still provided insight into gilt rearing practices, regardless of the low number of respondents. Moving forward, providing questions that are easier to answer, such as multi-select, may help improve overall completion. Additionally, asking if a follow-up call could occur would allow the surveyor to ask additional questions for clarity. One of the biggest problems within these responses was the lack of specification

by producers. Another point to consider is opening the survey up to producers who utilize internal multiplication. There are several group chats on WhatsApp that a researcher could get connected too, with producers from South Dakota, North Dakota, Wyoming, and Canada. The producers within these chats seem very interested in learning, and there is open discussion within the chats regarding common production measures, such as farrowing targets, litter weights, etc. Lastly, utilizing state pork producer expos could drum up additional interactions and potential respondents. This way, producers being asked the questions could put a face to the name of who is reaching out, and if the opportunity for a prize was offered, it may also increase participation.

Table 4.1. Gilt delivery information.

Barn[1]	Gilts in GDU (on-farm arrival)	Weight of gilts, lbs. (on-farm arrival)[2]	Avg age of gilts, wks. (on-farm arrival)	Avg weight of pigs at survey, lbs.[2]	Avg age of gilts at survey, wks.
IN-1	720	40, 80	8, 11	40-265	8-24
IN-2	420	40, 90	9, 12	40-265	9-26
MN-1	190	15	3	80-300	12-27
SD-1	60	Not reported	24	Not reported	Not reported
SD-2	1200	14	3	Not reported	Not reported
NE-1	400	18	3	135	3-18
NE-2	2000	35	2	200	12-28
SD-3	480	160	14	205	Not reported
IA-1	750	200	21	Not reported	Not reported
MN-2	2000	50	13	50-290	12-26

[1]Barn name was provided as state abbreviation in which the barn is located, followed by the number of respondents from that state.
[2]Pig BW was provided by the producer as a visual estimate.

Table 4.2. Gilt selection criteria prior to breeding herd implementation.

Order of importance[1]	Selection criteria[1]	Number of respondents
1	Body size	10
2	Feet and leg structure	9
3	Teat number	9
4	Vulva size	8
5	Other[2]	2

[1]Order of importance was based on the average number of respondents that placed each criterion by "priority" during the selection process.
[2]Respondents who answered "other" in their selection criteria were asked to provide what criteria they consider, but no responses were provided, so it is unknown what other criteria are being considered in their selection process.

Table 4.3 Stall-breaking, heat detection, and breeding protocols.

Barn[1,2]	Age (wks.) at stall-breaking	Age (wks.) at boar exposure start	Boar exposure occurrence, time allotted	HNS[3], yes or no	Gilt cycle at first breeding	Pharmaceutical intervention if not cycling[4]	Culled for failed conception (number of cycles)
IN-1	26	25	Daily, 10 minutes	Yes	2-3	PG-600	1
IN-2	NA	25	Daily, 10 minutes	Yes	2-3	PG-600	1
MN-1	26	21	22 hours/day	Yes	2	PG-600	2
SD-1	NA	Not reported	Daily, 15 minutes	No	1	No	3
NE-1	18	18	Daily	Yes	2-3	No	2
NE-2	28	26	Daily	Yes	2	Not reported	1
SD-3	26	26	3 days/wk, 2 hours	No	2	No	2
MN-2	25-27	25-26	Daily, 15 minutes	Yes	2	No	2

[1]Barn name was provided as state abbreviation in which the barn is located, followed by the number of respondents from that state.
[2]Farms named SD-2 and IA-2 were removed from this table, as no answers were provided for this portion of the survey.
[3]HNS = heat-no-service
[4]Respondents who provided PG-600 as an intervention method also emphasized that it is used sparingly if the females have not cycled naturally.

Table 4.4. Breeding and gestation information.

Barn[1]	Barn type	Total number of females	Average parity	Number of gilts entering herd/year	Number of inseminations	Farrowing rooms loaded, d of gestation
IN-1	Open pen	6400	2.9-3.1	4300	2-3	110-114
IN-2	Stall	5600	2.9-3.1	3300	2-3	110-114
MN-1	Stall	3745	2.5	1800	2-3	110-114
SD-1	Open pen	75-80	4.5-5.0	60-65	2-3	110-114
NE-1	Open pen	5500	4.6	825	2	114 or later
NE-2	Stall	7300	3.0	120/wk	2-3	110-114
SD-3	Stall	3000	3.4	1500	2	110-114
MN-2	Open pen	5000	2.3	2600	2	110-114

[1]Barn name was provided as state abbreviation in which the barn is located, followed by the number of respondents from that state.

Image 4.1. Front leg view utilized when selecting replacement gilts. (Photo retrieved from Replacement Gilt Evaluation Pocket Guide, Pork Checkoff, 2010).

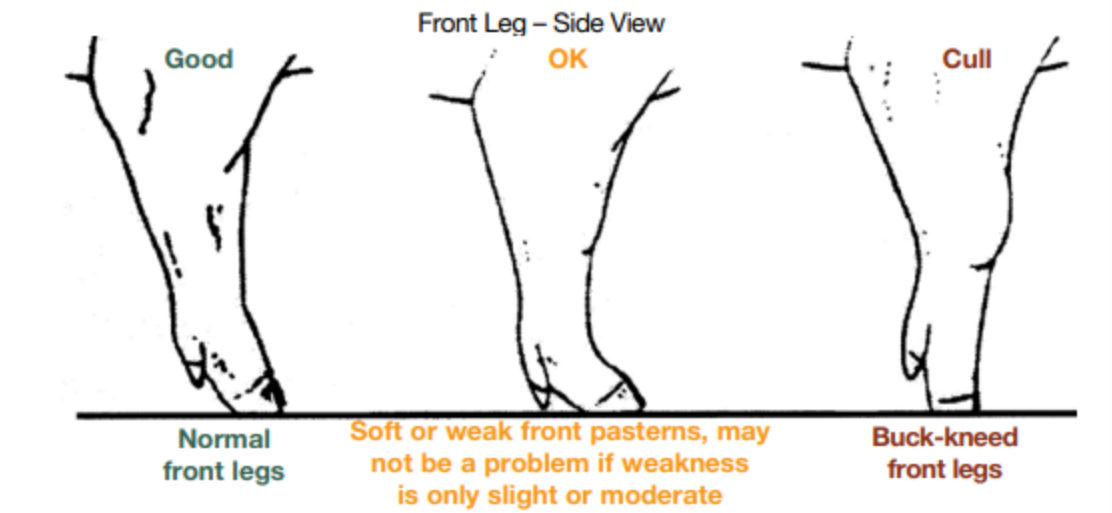


Image 4.2. Rear leg, side view utilized when selecting for replacement gilts. (Photo retrieved from Replacement Gilt Evaluation Pocket Guide, Pork Checkoff, 2010).

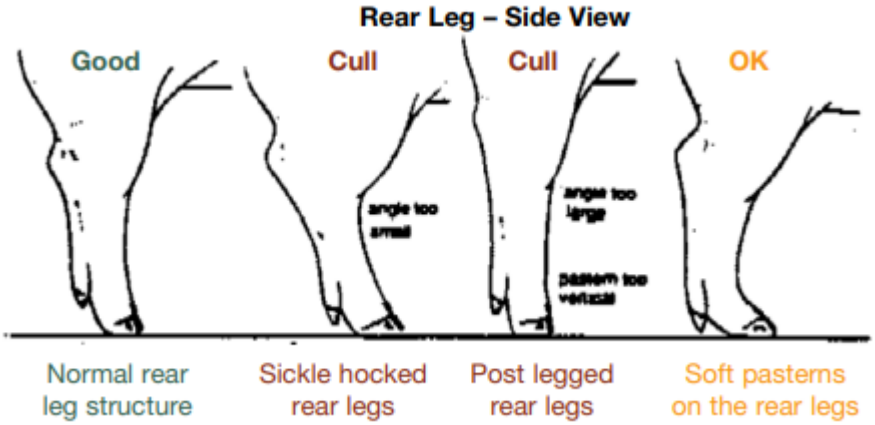
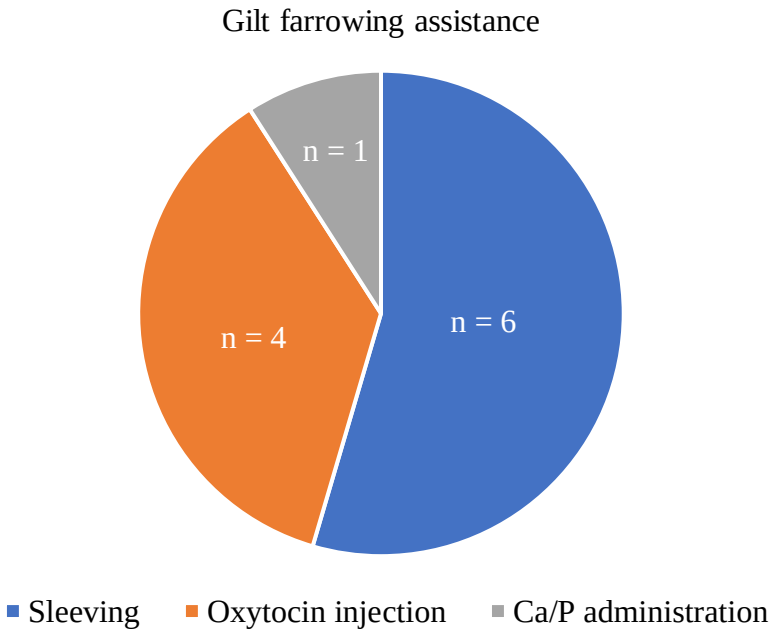


Figure 4.1. Gilt farrowing assistance method, and number of respondents.



CHAPTER V: CONCLUSION

The primary focus of this project was to identify genes in the vaginal transcriptome during prepubertal stage of reproductive development that have potential to predict age at puberty. The assessment of this was accomplished in 2 stages: confirmation that changes in the vaginal epithelium do occur in the prepubertal stage of reproductive development, and that these changes work in concert with circulating reproductive hormones. In addition to changes in the vaginal epithelium, AGD was also collected as an additional means to determine if it were an applicable prepubertal test to predict age at puberty.

The use of AGD as a potential predictive on-farm tool was inconsistent within these cohorts. Collection of AGD was a time-consuming endeavor because of gilt restlessness and fecal compaction. For on-farm applications, these variables could increase measurement variation reducing accuracy and predictive potential. The time required to accurately measure the distance while managing gilt movement makes it unappealing for on-farm application.

Changes in the vaginal epithelium do occur throughout reproductive development, with the most noticeable changes occurring at d160 (start of boar exposure). The predictive model shows promise at d160 of age. While this is considered the start of boar exposure for most farms, based on the producer survey data, farms are not actually breeding their gilts until third estrus. If that is the case, the ability to cull the gilts at d160 of age provides the opportunity for feed cost savings for the producer, and the female can be sold at the regular market weight, as she would be expected to still be within the packer-accepted range.

APPENDICES

Appendix I. Producer Survey Questions

The questions listed with this appendix were asked to producers either electronically or on the phone. Please see Chapter 4 for the full discussion of this producer survey.

There are two sections to this survey. Section 1: will cover common aspects of the gilt development unit (GDU), or any space therein that may house pre-pubertal gilts before they are transferred to the breeding herd. Section 2: will cover management techniques once gilts are part of the breeding herd and then moved into the farrowing rooms.

Section 1: Gilt Development Unit (GDU); The questions asked below covers reproductive development for the current farm, prior to implementation into the breeding herd.

Animal Summary:	
Total number of gilts in GDU (initial arrival)	
Average body weight of gilts in GDU (initial arrival)	
Average age of gilts in GDU (initial arrival)	
Average body weight of gilts in GDU (current)	
Age range of gilts in GDU (current)	
Total number of pens in GDU	
Average square feet/pig (current)	
Genetics company/line	

Feeder & Waterer Summary:	
Type of waterers	
Water source (rural or well)	
Number of waterers/pen	
Feeder type (dry, wet/dry)	
Inches of feeder space/pig (current)	

Feed (Diet) Summary:	
Number of diet phases (from on-farm arrival to movement to breeding herd)	
Predicted lysine content/phase (please list each phase and estimated lysine content)	
Diet NE/phase (Mcal/kg) (please list each phase and estimated lysine content)	

Section 1: Gilt Development Unit (GDU); The questions asked below covers reproductive development for the current farm, prior to implementation into the breeding herd.

Animal Summary:	
Total number of gilts in GDU (initial arrival)	
Average body weight of gilts in GDU (initial arrival)	
Average age of gilts in GDU (initial arrival)	
Average body weight of gilts in GDU (current)	
Age range of gilts in GDU (current)	
Total number of pens in GDU	
Average square feet/pig (current)	
Genetics company/line	

Feed (Diet) Summary:	
Number of diet phases (from on-farm arrival to movement to breeding herd)	
Predicted lysine content/phase (please list each phase and estimated lysine content)	
Diet NE/phase (Mcal/kg) (please list each phase and estimated lysine content)	

1. How frequently does this current farm receive gilts?

2. What is the health status/requirements of the source farm from which you are receiving your gilts?

3. What type of selection process does this farm utilize?
   - ☐ Single
   - ☐ Double

4. At farm arrival, what problems (conformational, illness, etc.) would cause your farm to not keep her in the GDU (humane euthanasia or culling)?
   - ☐ Defects, animal welfare issue
   - ☐ Conformation
   - ☐ Other (if so, please explain)

When assessing the gilts at breeding herd selection, rank the following common characteristics in order of importance.

Foot/leg structure	
Body size	
Vulva size	
Teat number	
Other (please describe)	

5. Within this farm, what is the temperature set point within the GDU when the gilts arrive?
   If weans, then what? :
   If feeders, then what?:

6. What is the current farm's isolation length for new gilts?

7. What is the testing protocol for these gilts?
   - ☐ Pre isolation
   - ☐ Post isolation
   - ☐ Both

8. What diseases are tested for (check all that apply)?
   - ☐ PRRSv
   - ☐ Myco
   - ☐ Swine influenza
   - ☐ Seneca
   - ☐ Other, please specify.

9. At what age does “stall breaking” occur?

10. If utilizing pens, what is the stocking density or space/gilt?
    Pre-induction:
    During heat induction:

11. At what age does boar exposure begin?

12. How often does boar exposure occur (ex: daily, every other day), and how long does each occurrence approximately last (ex: 10 minutes, 20 minutes).

13. What is the approximate age of the boars that are used for exposure?

14. How many pens of gilts is a single boar used for each day during exposure? (ex: 4 pens, 10-15 minutes each). Or, if gilts are brought into the herd late and placed directly into breeding stalls, what is your boar to gilt ratio?

15. Do you utilize HNS (heat no service)?

16. At what cycle will the gilt be bred (ex: second, third, etc.)?

17. At what age will gilts be moved from the GDU to the gestation area?

18. Pharmaceutical intervention taken if not cycling? If so, what?

19. What is the acclimation to herd health status protocol? (Ex: feedback, cull sows, etc.) ☐
    Feedback
    ☐ Vaccination (commercial)
    ☐ Vaccination (autogenous)
    ☐ Live animal introduction

Now that I know more about your gilt development prior to breeding, I would like to know more about their transition into the farrowing herd.

Section 2: Breeding Herd/Farrowing

Animal Summary:	
Toal herd size of facility	
Average parity of sows in the breeding herd	
How many gilts enter the herd a year?	
Barn type: stall vs open pen	
Gestation Feed (Diet) Summary:	
Predicted lysine content/phase	
Diet NE/phase (Mcal/kg)	

PEN HOUSING QUESTIONS ONLY:

1. If utilizing pen housing, at what day of pregnancy do females enter pens?

2. If pen housing, how are females fed in pens? (Ex: ESF, floor feeding, stanchion-short or long, lock-in stalls, Gestal combo).

HEAT DETECTION/BREEDING:

1. During heat checking, how many boars are utilized?

2. How many cycles is a gilt allowed before she is culled for failed conception?
   ☐ 1
   ☐ 2
   ☐ 3 or more cycles (if more than 3, please explain)

3. Does the farm utilize standard or post-cervical AI?

4. How many times will a sow/gilt be AI'd in each cycle?

5. On what day (on average) are farrowing rooms loaded?
   - ☐ Prior to d 110 of gestation
   - ☐ D 110- 114 of gestation
   - ☐ Later than D 114

6. What are the intervention protocols during farrowing for gilts?
   - ☐ Sleeving every 20-30 minutes
   - ☐ Oxytocin injections
   - ☐ Ca/P administration
   - ☐ Other (please specify):
   - ☐ All of the above

7. Average litter size of first parity gilts?
   Total born:
   Born alive:
   Stillborns:
   Mummies (if counted):

8. If a gilt savages during farrowing, is there any intervention performed during farrowing (ex: pulling pigs off).
   - ☐ Pull pigs off
   - ☐ Acepromazine injection
   - ☐ Udderline rubbing
   - ☐ All of the above
   - ☐ Other (please specify):

9. If a gilt savages during farrowing, are they culled after that lactation?

10. How many animals that farrow as a P1 farrow as a P2?

11. What are the reasons a gilt might be culled after her first parity?

- ☐ Health
- ☐ Structure
- ☐ Farrowing performance
- ☐ Farrowing difficulty
- ☐ Other, please specify:

Appendix II. Dietary lysine level on age of first estrus and vaginal transcriptome changes in pre-pubertal crossbred market gilts

Research objective: Determine the impact, if any, varying levels of g/kg gain lysine has on gilt pre-pubertal development and vaginal gene expression.

IACUC number: 2108-046A

Diets:

1. Proposed optimum: 21-22 g/lysine per kg/gain (Calderon-Diaz (2017); Lents et al., 2020).
2. Baidoo (2001): two phase diets; Phase 1: (100-120 days of age); 21.15 g/day SID; Phase 2: (120-160 days of age); 18.9 g/day SID.
3. 160 days of age to standing estrus or end of trial (215 days of age), all gilts will receive PIC dietary recommendations for replacement gilts).

Planned experimental collections:

A. Body Composition
    a. Backfat thickness (d100, 120, 140, 160, 180, 200, S.E. or end of trial)
    b. Leptin sample (d100, 120, 140, 160, 180, 200, S.E. or end of trial)
    c. Weight (d70, 100, 120, 140, 160, 180, 200, S.E. or end of trial)
B. Reproductive performance
    a. Vaginal epithelium samples (d70, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, S.E. or end of trial).
    b. Anogenital distance samples, in triplicate (d70, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, S.E. or end of trial).
    c. Measure concentrations of progesterone 8 days after standing estrus.

The collections outlined above were supposed to occur during this trial. However, on December 1, 2021, the SDSU onsite broke with PRRSv 1-4-3. To garner some data from this group of females, it was decided that the trial would proceed as planned, but collections would be reduced.

Trial d0-40

At trial start, a total of 97 gilts (41 kg ± 7 kg; 4 or 5 pigs/pen; 0.99 or 0.79 m^2 per pig) were utilized in a 50-day feeding trial. Pens were allocated to one of two dietary

treatments, with a total of 10 replicates per treatment. All pens contained one 2-space dry feeder and one cup waterer for ad libitum access to feed and water. Data collection began December 2021 and concluded April 2022. The barn was mechanically ventilated; at the beginning of each group, the temperature setpoint was 19.4°C and then decreased 0.06°C daily until reaching 14.4°C. High, low, and average room temperatures were recorded between 0600 and 0800 h daily.

Throughout the trial, feed disappearance was monitored for each pen. Gilts on the control (CON) regime received a standard corn-soybean meal diet that contained 2, 475 Mcal/kg, and 0.77% Lysine (20 g/day lysine target) in phase 7, and 2,475 Mcal/kg, and 0.63% (16.5 g/day lysine target) Lysine in phase 8. Gilts on the lysine (LYS) regime also received a standard corn-soybean meal diet, with the LYS diet containing 2,475 Mcal/kg in phases 7 and 8, and targeted g/lysine day of 16.5 g/day, respectively. Diet formulation can be found in Figure AII.1. Feed disappearance and body weight (BW) was measured every 10 days to determine average daily gain, daily feed intake, and gain-to-feed ratio. A vaginal swab was collected on these days, with the swab being placed into a plastic bag and flash-frozen in liquid nitrogen. No TRIZOL was used during the collection procedure.

In January, it was decided that the site would depopulate and repopulate following deep cleaning and sanitation. At that time, it was decided that females younger than a certain age within the trial were removed from test, given that they would not be close to d160 of age at the start of boar exposure.

Trial d 40-50

To condense the gilt age within a tighter window, on trial d40 (d140 of age), a total of 11 pens were removed from the trial. Of the 9 remaining pens, females of both light and heavy ADG were selected for boar exposure. A total of 20 females were selected, with 10 females per treatment, and 5 light (ADG of 760) and 5 heavy (ADG of 822) were selected. Females were moved into empty finishing pens within the facility and transitioned to a gestation diet.

Boar exposure

Boar exposure began at d160 of age, with two boars being used each day. One boar would interact with 5 females at a time for approximately 7 minutes, then moved to the next set of 5 for an additional 7 minutes. This way, 20 females could be heat-checked at a time. Once standing estrus was detected, a vaginal swab, BW, and single blood sample were obtained. An additional blood sample was obtained d8 after standing estrus for progesterone concentration.

Samples analysis

When extracting the RNA from the vaginal swabs, there was RNA degradation in many samples. Under normal conditions, RNA concentration should contain a minimum of 75 µg/mL, with an A260/280 ratio greater than 1.80. Unfortunately, within the samples from this trial that extraction occurred, most samples averaged a 260/280 ratio less than 1.75, and many had concentrations less than 50 µg/mL, particularly those that were taken during reproductive development (e.g., d103, d130). In addition, due to the degradation

of many of the samples, complete collection profiles (meaning vaginal swabs across all collection days of the trial), were unachievable. Estrus groupings are shown in table AII.2.

RNA sequencing

A total of 6 gilts from this trial were sent for RNAsequencing (MRDNA, Shallowater, TX). The initial intent was to send 10 samples for sequencing but given the small sample size of females that underwent boar exposure (n =20), there were not enough 'early' or 'late' estrus gilts from this trial. Additionally, once samples were received, two samples (314-d160, 342-d160) did not contain enough workable material and had to be removed from analysis. These samples were entered into the predictive model in Chapter 3 to serve as an additional 'check.' The overall goal was to use the predictive model to determine which day of collection (d130 or d160) is a better predictor of when a female will achieve puberty. Within this analysis, the d160 predictive model was 75% accurate, when comparing what the model predicted and the actual estrus grouping the female was assigned.

While this trial was unable to be fulfilled to the intent it was planned, interesting discoveries still arose. Moving forward, providing more data to this predictive model is critical to overall predictive accuracy. Unfortunately, identifying a better method of vaginal epithelial cell collection without the use of TRIZOL is going to be necessary to avoid RNA degradation in the future. The biggest reason that TRIZOL was not used in this study was due to cost associated with the number of samples that were initially collected (97 pigs x 4 timepoints). The goal moving forward is to find the minimum

amount of TRIZOL needed to successfully protect the RNA, while maintaining an acceptable A260/280 ratio as well.

Figure AII.1. Composition of gilt finisher diets.

	Phase[1]				
Item	CON-7	LYS-1	CON-8	LYS-8	Gest'n[1]
Ingredients, %					
Corn	80.22	80.06	80.39	80.39	81.61
Soybean meal, 46.5%	13.30	13.3	13.3	13.3	14.54
Soybean hulls	3.80	3.8	4.35	4.35	
L-Lysine HCl	0.33	0.4	0.15	0.15	
L- Threonine	0.09	0.12	0.03	0.03	
DL-Methionine	0.03	0.06	0.00	0.00	
L-Tryptophan	0.02	0.03	0.00	0.00	
L-Valine	0.00	0.02	0.00	0.00	
Monocalcium phosphate	0.77	0.77	0.50	0.50	1.84
Limestone	0.94	0.94	0.78	0.78	1.31
Salt	0.3	0.3	0.3	0.3	0.5
Nursery Vitamin premix[2]	0.05	0.05	0.05	0.05	0.05
Trace Mineral premix[3]	0.15	0.15	0.15	0.15	0.15
Total	100.0	100.0	100.0	100.0	100.0
Calculated analysis					
ME, kcal/kg	3350	3350	3350	3350	3278
NE, kcal/kg	2475	2475	2475	2475	2446
CP, %	13.9	13.9	12.7	12.7	13.5
Ca, %	0.57	0.57	0.46	0.46	0.89
P, %	0.47	0.47	0.42	0.42	0.72
Available P, %	0.26	0.26	0.21	0.21	0.44
SID[4] amino acids, %					
Lys, %	0.88	0.93	0.74	0.74	0.55
Thr, %	0.58	0.60	0.52	0.52	0.42
Met, %	0.27	0.30	0.24	0.24	0.21
Trp, %	0.16	0.17	0.14	0.14	0.12

[1]Gestation feed intake varied across gilts, from 4-6 pounds at the beginning of boar exposure, due to extreme differences in size.

[2]J & R Distributing Inc. 518 Main Ave, Lake Norden, SD 57248 - USA. Minimum provided per kg of diet: Calcium 55 mg, Vitamin A 11,000 IU, Vitamin D3 1,650 IU, Vitamin E 55 IU; Vitamin B12 0.044 mg, Menadione 4.4 mg, Biotin 0.165 mg, Folic Acid 1.1 mg, Niacin 55 mg, d-Pantothenic Acid 60.5 mg, Vitamin B16 3.3 mg, Riboflavin mg, 9.9 Thiamine 3.3 mg.

[3] J & R Distributing Inc. 518 Main Ave, Lake Norden, SD 57248 - USA. Minimum provided per kg of diet: Copper 16.5 ppm, Manganese 44.1 ppm, Selenium 0.03 ppm, Zinc 165 ppm.

[4] SID = Standard ileal digestible

Figure AII.2. Final estrus grouping and age at standing estrus. Females were selected by light or heavy ADG (g/day), as well as birth date, to ensure that their age was as close to d160 as possible.

D50 ADG (g/day)	Date of S.E.	Estrus Allotment	Age at S.E.
640	3/12/2022	Early	D182
730	Unknown	Silent	
760	3/25/2022	Average	D195
800	Did not stand	Anestrus	
880	3/29/2022	Average	D199
1070	3/15/2022	Average	D185
1090	3/13/2022	Average	D183
1130	Did not stand	Anestrus	
1150	4/2/2022	Late	D203
1200	3/15/2022	Average	D185
710	2/25/2022	Early	D166
780	Did not stand	Anestrus	
850	3/18/2022	Average	D188
880	Did not stand	Late/anestrus	D203
890	3/18/2022	Average	D188
1060	Unknown	Silent	
1070	2/24/2022	Early	D165
1110	Unknown	Silent	
1160	2/24/2022	Early	D165
1230	3/29/2022	Average	D199

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