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PORCINE SPERM BINDING TO OVIDUCT CELLS AND GLYCANS AS SUPPLEMENTS
TO LABORATORY SEMEN ANALYSIS

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THESIS

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ABSTRACT

Artificial insemination is a common practice within the swine industry and accurate semen evaluation is necessary to maintain reproductive efficiency. Additionally, identifying the cause of reduced fertility in sex-sorted sperm could enhance the industry. Our previous work has shown that sperm binding to oviduct cell aggregates improves fertility prediction, possibly by estimating the ability of sperm to form a reservoir in the oviduct. We have also shown that two oviduct glycan motifs, biantennary 6-sialylated *N*-acetylactosamine (bi-SiaLN) and Lewis^X trisaccharide (Le^X) bind boar spermatozoa with high affinity and specificity. Measuring binding to soluble glycans is less laborious than assessing binding to oviduct cell aggregates and more suitable for industry use. The objective of the first study was to determine if the ability to bind oviduct cells or the specific oviduct glycans that sperm bind are useful supplements to routine semen analyses. A fertility trial using semen from boars (n=30) used for homospermic matings was designed. Semen was collected and shipped overnight for laboratory analysis and for inseminations. Semen collections were analyzed from boars three times over the course of the study. Oviduct cell binding, motility, morphology and acrosome status were assessed the day the shipment was received. Binding to three soluble fluoresceinated glycans bi-SiaLN, sulfated Le^X (suLe^X), and the control lactosamine disaccharide (LacNAc) was measured the following day. The inseminations occurred at 15 farms in the greater Midwest and farrowing data from all matings from each boar were used (>50 matings/boar). Pregnancy rate (PR) and litter size (LS) were adjusted to account for different farms, number of services, number of doses inseminated, and sow parity, using the MIXED procedure in SAS 9.4. A fertility index (FI) was generated, consisting of PR x LS. This index allowed for boars to be ranked on combined PR and LS. Lastly, the GLMSELECT procedure was used to select variables having a significant impact on

PR, LS, and FI. The predictive models constructed were further analyzed using the REG procedure and accounted for 58% or more of the variation in PR, LS, and FI [PR ($p < 0.001$, $r^2 = 0.60$), LS ($p < 0.001$, $r^2 = 0.58$), and FI ($p < 0.001$, $r^2 = 0.63$)]. All three final models included traditional semen morphology evaluation traits. The model for PR also included the ability of sperm to bind oviduct cell aggregates, in agreement with our previous smaller scale study. However, binding to specific oviduct glycans was not a useful supplement to traditional semen analysis. The objective of the second study was to determine how sorting sperm by flow cytometry to separate X and Y chromosome-bearing sperm affects binding to oviduct cells and purified oviduct glycans. The sperm rich fraction from boars ($n=5$) was collected; sperm were stained with Hoechst 33342 and sorted. Sperm were separated into either X or Y chromosome-bearing cells and placed into the following treatments: 1) sperm sorted for the X chromosome, 2) sorted for the Y, 3) an equal mixture of sorted X and Y, and 4) a control of non-sorted sperm from the same collection. Samples were delivered and tested for oviduct cell binding within 12 hr of sorting. Additionally, we observed motility characteristics, acrosome status, and glycan binding to the three soluble fluoresceinated glycans previously mentioned. Results showed that the number of sperm binding to oviduct cells was reduced by more than half in the three sorted samples compared to the control. There were no differences observed between samples or individual glycans when binding of fluoresceinated soluble glycans was investigated. The percentage of sperm that were motile in the sorted samples was reduced on average by 15% from the unsorted control. All samples maintained >97% acrosome integrity after the sorting process. In conclusion, sperm binding to the complex matrix around oviductal cell aggregates was reduced after sorting but binding to purified soluble fluoresceinated glycans was not different among sperm preparations. Overall, binding to oviduct cell aggregates was correlated with

pregnancy rate and can be used in conjunction with other traditional sperm assays to predict the average pregnancy rate of an individual boar. Similarly, a reduction in fertility in sperm that are sex-sorted may be due to a reduced ability to bind to the oviduct epithelium. These results show that the interaction with oviductal epithelial cells remains impactful as a measure for potential sperm success within the female reproductive tract.

DEDICATION

This work is dedicated to my family for thinking I'm crazy while always believing in me.

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LIST OF ABBREVIATIONS

AI: artificial insemination

CASA: computer assisted sperm analysis

- AREA: size (μm^2) of all sperm heads
- ALH: mean width (μm) of head oscillation as sperm swim
- BCF: frequency (MHz) of sperm head crossing the sperm average path
- ELONG: percent of head width to head length
- LIN: departure of actual sperm track from straight line (ratio of VSL/VCL)
- STR: departure of average sperm path from straight line (ratio of VSL/VAP)
- VAP: avg. velocity of the smoothed (best fit) sperm path
- VCL: avg. velocity ($\mu\text{m}/\text{sec}$) over the actual point-to-point sperm track
- VSL: avg. velocity ($\mu\text{m}/\text{sec}$) in a straight line from the beginning to end of sperm track

DUI: deep uterine insemination

FI: fertility index

FR: farrowing rate

ORT: osmotic resistance test

HOS: hypoosmotic swelling test

ICSI: intracytoplasmic sperm injection

IVF: in vitro fertilization

PR: pregnancy rate

ROS: reactive oxygen species

TNB: total number born

UTJ: utero-tubal junction

CHAPTER 1 - INTRODUCTION

The use of fresh semen for artificial insemination (AI) in the commercial swine industry has become standard practice. It has provided many benefits, including, genetic improvement, reduced disease spread, decreased service costs and allowed for the international exchange of semen (Cole et al., 1982). Previously, accelerating genetic improvement was the primary goal of AI and has allowed for the generation of a selection system to improve different maternal and terminal traits. Currently, reducing disease spread has become the main accomplishment of AI in swine production, many farms have eliminated the introduction of new animals into the herd and rely solely on semen (Knox 2016). However, a limiting factor in swine production is semen storage. Liquid storage of boar sperm is the industry standard, and the length of long-term extenders can allow for adequate sperm storage for up to seven days if maintained at a consistent temperature (Johnson et al. 2000; Waterhouse et al. 2004; De Ambrogi et al. 2006; Pinart et al. 2015). Cryopreservation of porcine sperm is not used routinely because boar sperm succumb to more damage than their bovine counterparts (Yeste, 2016). Development of better cryopreservation protocols to produce a larger volume of viable boar sperm after thawing is currently being studied (Grossfeld et al. 2008; Bailey et al. 2008; Rodriguez-Martinez and Wallgren 2011; Okazaki and Shimada 2012; Yeste 2016).

Aside from semen storage practices, good management, starting with estrus detection is key for obtaining superior farrowing rates and litter sizes (Knox et al. 2008; Vilagran et al. 2015). After sperm are inseminated they travel through the uterus, utero-tubal junction, and bind to the lower portion of the oviduct, the isthmus, where sperm are retained in the isthmic reservoir (Hunter 1981; Suarez et al. 1991). Noncapacitated sperm with intact acrosomes are capable of

binding to form this reservoir (Fazeli et al. 1999; Gualtieri and Talevi 2000; Petrunkina et al. 2001) The carbohydrate-mediated binding within the oviduct to create the sperm reservoir is necessary because mating and ovulation are often poorly synchronized in the pig (Green et al. 2001; Suarez 2000; Wagner et al. 2002). Sugars on the oviduct have been implicated in binding sperm. This binding is based on studies using a few specific sugars as possible competitive inhibitors of sperm binding to oviduct cells, *in vitro* (Kadirvel et al., 2012; Machado et al., 2014). Only a limited number of sugars were examined in any mammal and the authentic oviduct sugar/s that bind sperm were uncertain.

A much larger number (377 glycans) were tested for their ability to bind sperm after being identified in a glass microarray (Kadirvel et al., 2012). Glycans that bound to noncapacitated porcine sperm were identified. Common among those structures were the glycan motifs, biantennary 6-sialylated *N*-acetylglucosamine (bi-SiaLN) and Lewis^X trisaccharide (Le^X). These glycan motifs were both found within the porcine oviduct (Kadirvel et al., 2012). For these studies, sulfated Lewis^X (suLe^X) was used instead of Le^X because the negative charge improved its ability to bind sperm, although it bound to the same sperm proteins as nonsulfated Le^X (Kadirvel et al., 2012).

Variation in fertility between boars impacts the swine industry (Daigneault et al., 2015; Flowers et al., 2016; Popwell and Flowers, 2004; Singleton and Shelby, 1972). Determining a way to identify and remove “poor” producing sires before they are fully in production could save time and money while improving overall production. This selection can also decrease the environmental impact of swine production by producing the same amount of animals with fewer gilts and sows. To evaluate the fertility of a semen sample, traits that are needed for a sperm to fertilize an egg are usually assessed, such as sperm motility and morphology (Gadea, 2005). But

most studies show that sperm motility and morphology alone are not adequate methods for estimating fertility (Gil et al., 2005). To accurately predict or detect fertility, assays that measure sperm function and other sperm traits seem to be necessary. Use of these assays alone or in conjunction with traditional sperm quality measures may allow for more accurate fertility prediction. **My overall goal was to design a regression model based on motility, morphology, and binding to soluble oviduct glycans to predict an individual boar's fertility.**

Using sex-sorted porcine sperm can make additional improvements in pork production efficiency. Sorting boar sperm by sex chromosomes can be done about as easily as bovine sperm, as porcine sperm have large flat heads and a 3.6% DNA difference between the X- and Y-bearing spermatozoa (Garner 2006). The restrictions associated with sex-sorted boar sperm are the large number of sperm required for an insemination dose, the difficulty in storing sorted sperm, and the physical aspects of the sorting process. It is believed that there are capacitating-like changes occurring during or after the sorting process that may inhibit the formation of the sperm reservoir (Maxwell and Johnson 1999; Spinaci et al. 2006). **Our objectives were to determine if sorting by sex chromosomes impacts the ability of sperm to bind to oviduct cells and soluble oviduct cell glycans.** These results show that the sorting process affects the ability of sperm to bind to oviduct cell aggregates. On the other hand, the sorting process did not affect that ability of sperm to bind to the soluble glycans previously discussed.

In conclusion, we identified a model for predicting the fertility of an individual boar prior to insemination. We have also identified that unknown changes are occurring on the sperm head after sorting affecting the ability to bind to oviduct cells, potentially impacting the formation of the sperm storage reservoir and reducing overall fertility.

CHAPTER 2 - LITERATURE REVIEW

The Sperm Reservoir: A Species Comparison

Sperm storage plays a key role in propagating the species. Some insects store sperm for more than a decade and some species are only able to store sperm for very brief periods. For instance, humans don't form a true sperm reservoir but a functional reservoir occurs when sperm stick to the endosalpingeal epithelium within the oviduct (Murray and Smith, 1997; Pacey et al., 1995). Human sperm do not bind to the oviductal epithelium in either ovulatory or non-ovulatory oviducts (Williams et al., 1993). However, human sperm do require interaction with oviduct cells to maintain sperm lifespan and viability (Murray and Smith, 1997). In humans, sperm are fertile for up to five days after insemination (Croxatto, 2002). In contrast, different mammalian species develop true sperm reservoirs, these reservoirs house and maintain sperm through carbohydrate mediated interactions (Hunter, 1981; Suarez et al., 1991b). The sperm reservoir formed in these mammals works well at reducing the occurrence of polyspermy and maintaining sperm viability until the oocyte is ready to be fertilized (Hunter, 1995; Hunter and Léglise, 1971).

Other species have unique sperm storage abilities. The drosophila female stores 40% of the sperm that were inseminated for up to two weeks in the seminal receptacle and a pair of spermathecae. An interesting aspect of drosophila sperm is that they swim backwards through the uterus and into the seminal receptacle. This swimming pattern is thought to occur so sperm can "back-in" to the storage organs so they have an easier time exiting and fertilizing an egg (Köttgen et al., 2011).

Birds have sperm storage tubules where sperm are housed and ready to fertilize eggs during the hen's "fertile period", which is the time between inseminations (Brillard, 1993).

Chicken hens can store sperm for 3-4 weeks from one insemination (Romanoff, 1960) and turkey hens can store sperm for 8-15 weeks (Lorenz, 1950). In contrast to mammalian sperm storage, the vagina is the selector for fertile sperm. There are two mechanisms for sperm selection in birds. The first is mechanical, only sperm with good motility can reach the egg. The second is biochemical, the vaginal mucosa interacts with the sperm selecting the best for fertilization (Brillard, 1993).

There are some species that have long sperm storage potential. A great example is the brownbanded bamboo shark, *Chiloscyllium punctatum*. In 2012, a bamboo shark at the Steinhart Aquarium in California produced a viable egg that resulted in the birth of a pup after being housed with only females for four years (Bernal et al., 2015). Sharks are known to be able to store sperm (Pratt, 1993) but four years was the longest case of successful sperm storage resulting in live offspring in sharks. Harnessing this ability could potentially help species in the wild that are threatened by habitat and overfishing. Another good example of long-term sperm storage was in the *Crotalus adamanteus*, an eastern diamond-backed rattlesnake. The female had been isolated for five years and gave birth to 19 snakelets (Booth and Schuett, 2011). Originally, in both the snake and shark cases, scientists believed the offspring were the result of virgin births, but genetic testing found DNA other than the mothers, indicating that the females had stored sperm from their last encounter with a male. Although sperm storage duration varies between species, they all have the same end goal, prolonging sperm viability.

The Formation of a Functional Sperm Reservoir

Only a small fraction of the sperm inseminated actually occupies the sperm reservoir. The current industry standard for swine is an inseminating dose of 2-4 billion sperm (Knox et al. 2008). This yields maximal pregnancy rates and litter sizes by compensating for some sperm abnormalities and inaccurate estrus detection (Flowers, 2013; Tardif et al., 1999). The primary barriers for sperm selection begin at the cervix. The majority of spermatozoa cannot overcome the obstacles presented and don't make it past the first level of selection in the cervix (Martyn et al., 2014). The second level of selection occurs within the uterus, sperm that are dead or damaged are removed through an immunological response (Knobil and Neill, 2006). Sperm then arrive in the oviduct of the female where another level of sperm selection occurs, within the sperm reservoir.

In the mammalian oviduct, there are three sections, the utero-tubal junction (UTJ), the isthmus and the ampulla. The main role of the UTJ is to prevent the entry of bacteria into the oviduct, but it also regulates sperm entry (Suarez 2008). After sperm are deposited into the vagina, they travel through the cervix and uterus to the UTJ. In some mammals like swine, semen is deposited directly into the uterus, bypassing the cervix. A study by Ardón et al. (2008), identified that the number of spermatozoa with unstable chromatin is reduced in the porcine oviduct when compared to the entire inseminating dose. This reduction suggests that sperm with damaged chromatin are unable to travel through the UTJ and bind to the oviduct, removing them from the population of sperm allowed to fertilize the oocyte. Sperm that make it through the UTJ have the opportunity to interact and bind by carbohydrate-mediated binding to the epithelium of the isthmus region of the oviduct (Hunter 1981; Suarez et al. 1991), but only if they possess the necessary requirements. Two known requirements are that sperm must have an intact acrosome

and not be capacitated, to bind and fill this reservoir (Fazeli et al. 1999; Gualtieri and Talevi 2000; Petrunkina et al. 2001). Additionally, they need to possess receptors for specific oviduct glycans, which will be discussed in depth in subsequent sections.

Sperm that are bound to the isthmus remain stored in a protective state that is characterized by the suppression of capacitation and motility, extending their lifespan (Gualtieri and Talevi, 2003; Rodríguez-Martínez et al., 2005; Töfer-Petersen et al., 2008). The mechanistic changes occurring in the oviduct to decrease sperm motility while it is stored is critical. These changes conserve energy and maintain fertility until subsequent stimulation causes sperm to release from the oviduct and fertilize the oocyte (Overstreet and Cooper, 1979; Overstreet et al., 1980). Sperm remain bound during the time between insemination and ovulation. Sperm binding to the oviduct is important for pigs because of the large variation in time between the beginning of estrus and time of ovulation (Töfer-Petersen et al., 2008). Currently, the swine industry inseminates females each day estrus is detected, ranging from 1-3 times per estrus event. The majority of females are inseminated twice, 24 hours apart, to compensate for this lack of synchronization (Knox 2016).

In addition to protecting sperm, this reservoir selects for the “best” sperm, those that are morphologically normal (Petrunkina et al., 2001), not capacitated (Lefebvre and Suarez 1996; Fazeli et al. 1999), and those that have normal DNA (Fazeli et al. 2004). Lastly, the oviductal sperm reservoir prevents polyspermy by binding and sequestering most of the sperm and releasing a smaller number at a particular time to fertilize the oocyte (Hunter and Léglise 1971; Hunter 1995). Polyspermy occurs when an oocyte is penetrated by two or more spermatozoa resulting in the death of the embryo. If sperm are surgically deposited directly into the isthmus, there is a greater incidence of polyspermy (Hunter 1973; Hunter 1976). This is most likely due to

bypassing multiple sperm selectors through the female reproductive tract, such as mucosal fluid and the UTJ, which reduce the number of sperm reaching the isthmus.

The oviduct is made up of a complex matrix of cells where sperm interact and bind. The carbohydrate-mediated binding within the oviduct to create the sperm reservoir has been studied previously in multiple species (hamster, DeMott, Lefebvre, and Suarez 1995; horse, Dobrinski et al. 1996; bull, Lefebvre, Lo, and Suarez 1997; Ignatz et al. 2001; pig, Green et al. 2001; Wagner et al. 2002; Kadirvel et al. 2012). This interaction is partially controlled by glycans present on the sperm head as well as those in the oviduct.

The Role of Complex Glycans in Sperm-Oviduct Binding

One of the major functions of glycans such as those attached to glycoproteins, glycolipids, and proteoglycans is cell adhesion. Adhesion through these glycoconjugates occurs between cells and viruses, bacteria, toxins, antibodies as well as other cells. Cells are surrounded by a complex matrix containing glycoconjugates called the glycocalyx (Brandley and Schnaar, 1986). The glycocalyx consists of monosaccharides (glucose, galactose, mannose, and fucose), the *N*-acetyl aminosugars (*N*-acetylglucosamine and *N*-acetylgalactosamine), and glucuronic and sialic acids (Brandley and Schnaar, 1986). There are three classes of glycans, N-glycans, O-glycans, and glycolipids; they are classified by their core structures (Stanley and Cummings, 2009). For N-glycans, the core of mature glycoproteins is made up of a common sequence of sugars, $\text{Man}_3\text{GlcNAc}_2\text{Asn}$ (Figure 2.1) (Stanley et al., 2009). N-glycans can have modifications to their cores. For example, they commonly have an additional fucose added by an α 1-6 linkage to the *N*-acetylglucosamine near the asparagine of the core (Figure 2.1) (Stanley et al., 2009). N-glycans can also have long branches made up of galactose added to *N*-acetylglucosamine by a β -

linkage, this is classified as a LacNAc sequence (Stanley and Cummings, 2009). These carbohydrate structures can be further differentiated by the addition of sulfates, phosphates and acetyl groups (Brandley and Schnaar, 1986).

The role of carbohydrate-mediated binding specifically in reproduction has been studied previously. DeMott and colleagues (1995), identified by blocking the terminal sialic acid in fetuin, a glycoprotein containing a complex oligosaccharide, they could inhibit sperm binding to the oviduct in hamsters. This inhibition suggests that the interaction relies on a sialic acid-like moiety. Similarly, for the horse, a glycan with terminal galactose found on oviduct cells can bind sperm (Dobrinski et al., 1996). In cattle, oviduct glycan/s with terminal fucose can bind sperm (Lefebvre, Lo, and Suarez 1997; Ignatz et al. 2001). Inhibition of sperm-oviduct binding was studied further by incubating sperm with different concentrations of carbohydrates in the pig (Green et al., 2001; Wagner et al., 2002). This variation across species in the glycoproteins within the oviduct that bind sperm requires more in-depth study to clarify whether the sperm binding glycans and putative receptors are related or whether different storage mechanisms are occurring between species.

A study completed by Kadirvel et al. 2012, identified the main carbohydrate structures that noncapacitated boar sperm bind. Using a glycan array of 377 glycans, those that noncapacitated porcine sperm bound to were identified (Figure 2.2). Common among those structures were the glycan motifs biantennary 6-sialylated *N*-acetyllactosamine on a mannose core (bi-SiaLN) and Lewis^X trisaccharide (Le^X)(Kadirvel et al., 2012). Further specificity showed that sperm preferred the linkage of sialic acid on bi-SiaLN to be at the 6- position versus being linked at the 3- position. Additionally, a branched structure is required; sperm did not bind single sialylated lactosamine trisaccharides. N-glycans with Le^X dimers, trimers and the sulfated

Le^X monomer present at the terminal end of the complex glycoprotein bound noncapacitated sperm readily. However, porcine sperm did not bind to a positional isomer, Lewis^A, indicating that sperm prefer specific sugars and not just the conformation of the glycan motif (Kadirvel et al., 2012; Machado et al., 2014). Each glycan motif had an important function because blocking each reduced the number of sperm that bound to oviduct cells by about 60% (Kadirvel et al., 2012; Machado et al., 2014). The glycans interact specifically with putative receptors on the sperm head that are also required for normal sperm-oviduct adhesion (Kadirvel et al., 2012; Machado et al., 2014).

Both bi-SiaLN and Le^X motifs bind with high affinity to the sperm head prior to capacitation and are necessary for sperm binding to the porcine oviduct (Kadirvel et al. 2012; Machado et al. 2014). Epithelial sheets collected from post-pubertal females were analyzed by nanospray tandem mass spectrometry (MS/MS). Oviductal epithelial cells contain both oligomannose and complex N-glycan structures. Multiple sperm binding motifs were identified among the complex N-glycans present on oviduct epithelial cells (Kadirvel et al., 2012). Both Le^X and bi-SiaLN were localized along the luminal epithelium of the oviduct (Kadirvel et al., 2012; Machado et al., 2014). Experiments completed previously in our lab measured the effect of occupying receptors for these multivalent glycans on oviduct cell binding using competition assays. Preincubating sperm with soluble Le^X, suLe^X, or bi-SiaLN glycans inhibited sperm binding to oviduct cell aggregates (Kadirvel et al. 2012; Machado et al. 2014). These studies concluded that when receptors on the sperm head are blocked with these specific soluble glycans they have a decreased ability to bind to oviduct cells.

Sperm interact with the oviduct by the head. Previous studies have examined the location of both suLe^X and bi-SiaLN bound to the sperm head to determine physiological importance.

(Kadirvel et al. 2012; Machado et al. 2014; Silva et al. 2014). Fluoresceinated glycans attached to a polyacrylamide chain were used in these experiments. Both suLe^X and bi-SiaLN were localized in the apical region of the sperm head in noncapacitated boar sperm. Comparatively, sperm that have undergone capacitation or are in the process of capacitating lost the affinity to bind to these glycans. Although this is great progress in the area of sperm-oviduct binding, there is still more to elucidate, as there are two parts to successful sperm storage. The first being sperm binding but the second is what causes the release of sperm from the oviduct reservoir.

Sperm Release from Oviductal Sperm Reservoir - Capacitation and the Acrosome Reaction

The end goal may be fertilizing the oocyte but it is important not to forget the process leading up to fertilization. More specifically, what are the cause/s of sperm release from the mammalian oviductal sperm reservoir? Sperm release could be due to a loss of binding sites on the oviductal epithelium and/or changes occurring on the sperm head (Suarez 2008). Among possible reasons as to why these changes occur, is the process of sperm hyperactivation.

One theory suggests that hyperactivation allows for enough force to break the interaction between the sperm head and the oviduct (Demott and Suarez, 1992). This hyperactivation may be necessary to free sperm from the oviductal reservoir but it serves additional purposes. These highly active sperm can then swim through the viscous fluid present within the oviduct (Figure 2.3) (Suarez et al. 1991; Suarez and Dai 1992; Quill et al. 2003). This mucus has been identified throughout the UTJ and the isthmus in multiple species (humans, Jansen 1980; rabbits, Jansen 1978; pigs, Suarez et al. 1991; cows, Suarez, Brockman, and Lefebvre 1997). It is also suggested that this mucus plays a role in transporting not only the sperm through the reproductive tract but

the oocyte as well, with the help of cilia (Jansen, 1978; Jansen and Bajpai, 1982).

Hyperactivation aids in sperm propulsion toward the site of fertilization. Additionally, chemotaxis has been shown to move sperm towards the cumulus oocyte complex (Zhang et al., 2015).

Different ions fluctuate through the sperm cell membrane during hyperactivation and capacitation. Sodium, bicarbonate, calcium, and potassium use different channels present on the sperm plasma membrane to move in or out of the cell. Additionally, there is a loss of cholesterol from the plasma membrane (Langlais and Roberts, 1985). Bicarbonate entry into the sperm cell activates adenylate cyclase and increases cAMP, subsequently increasing the pH and allowing the CatSper channel to open, letting calcium enter the cell (Darszon et al., 2011). This increase in intracellular calcium allows for the development of hyperactivated flagellar movement (Carlson et al., 2003; Quill et al., 2003). CatSper is a six transmembrane channel. The CatSper family consists of CatSper's 1, 2, 3, and 4. CatSper's 2, 3, and 4 differ from CatSper1 by having a smaller COOH and NH₂ termini (Darszon et al., 2011). CatSper-null mice were completely infertile, due to impairment in spermatozoa hyperactivation (Carlson et al., 2003; Quill et al., 2003). Without this hyperactivation, sperm were unable to reach and penetrate the matrix surrounding the oocyte. However, if the layers surrounding the zona pellucida were removed, CatSper-null sperm were able to penetrate the egg. Sperm required CatSper to penetrate the outer layers of the cumulus oocyte complex (Carlson et al., 2003; Quill et al., 2003; Ren et al., 2001). CatSper plays an important role for capacitation and hyperactivation in human (Tamburrino et al., 2014), equine (Loux et al., 2013), and mouse sperm (Quill et al., 2003). Additionally, a recent study of boar spermatozoa found that CatSper isn't activated by progesterone or

bicarbonate (Vicente-Carrillo et al 2016). Stimulation of spermatozoa to capacitate doesn't rely on one activator, but rather a collection of processes.

A multitude of things occur during the process of sperm capacitation. Different ions move across the sperm plasma membrane, causing different pathways to be activated. The acrosome reaction occurs but is not always simultaneous with capacitation (Vázquez et al., 1993). It is also known that capacitated sperm don't bind as readily to the oviduct as sperm that are noncapacitated (Lefebvre and Suarez 1996). These results suggest that sperm release from the oviduct is more likely due to capacitation than hyperactivation alone. Previous work in our lab has shown that soluble 6-sialylated biantennary and sulfated Le^x glycans bind to the apical ridge of the sperm head (Kadirvel et al. 2012; Machado et al. 2014). Both of these glycans showed reduced binding to sperm after capacitation had occurred.

Additionally, hormones and enzymes present within the oviduct at the time of ovulation could be promoters of capacitation and hyperactivation of sperm (Suarez et al. 1991; Thomas, Ball, and Brinsko 1994; Lefebvre et al. 1995). Progesterone begins increasing towards the time of ovulation during the estrous cycle (Hunter and Rodriguez-Martinez, 2004; Vanderhyden and Tonary, 1995; Yamashita et al., 2003). The effects of progesterone on sperm function have been studied in humans, identifying progesterone as the controller of various pathways (trypsin-like proteolytic, phospholipase A₂, and protein tyrosine kinase activity) in sperm (Calogero et al 2000). Progesterone is known to induce the acrosome reaction of bovine sperm, but doesn't have a direct impact on capacitation (Therien and Manjunath, 2003). In a study by Sagare-Patil et al. (2012), they found that low levels of progesterone have an effect on human sperm but do not cause hyperactivation or the acrosome reaction, suggesting that progesterone serves as a chemoattractant to get the sperm to the egg. In hamsters, there is an increased presence of

glycosyltransferases within the oviductal fluid at the beginning of ovulation (Tulsiani et al., 1996). Glycosyltransferases and glycosidases are families of enzymes that modify glycan chains (Rini et al., 2009). The sperm-oviduct interaction may be modified by glycosyltransferases and glycosidases, allowing for sperm release from the oviduct. Further investigation on these activities in the female reproductive tract will hopefully shed more light on the phenomenon of sperm release.

Determinants of Sperm Quality and Fertility

Introduction

Traditional seminal parameters including, motility, morphology, and sperm concentration are not sufficient to determine fertility (Gadea et al., 2004). They also cannot accurately identify subfertile males (Gadea et al., 2004). These parameters, however, are routinely used at boar studs in preparation of doses for insemination. In a review by Knox (2016), the cutoff at AI centers for sperm motility is $\geq 70\%$ and the cutoff is $< 20\%$ for morphological problems; those collections that do not meet these requirements are discarded. Boars that consistently have poor quality semen are generally culled from the herd. Semen quality is one of the top three reasons for removing a boar; the other two are for genetic improvement, or due to feet and leg problems (Knox et al. 2008). One of the main challenges in identifying an animal with superior fertility is the lack of strong correlation between *in vitro* and *in vivo* assays (Quintero-Moreno, Rigau, and Rodríguez-Gil 2004; Gil et al. 2005; Petrunkina, Waberski, Gunzel-Apel, et al. 2007; Sancho et al. 2006; Broekhuijse et al. 2012). Genetic line and boar age also influence farrowing rate. Direct boar effects explained 5.3% of the variation in farrowing rate (FR) and 5.9% of variation in total

number born (TNB). Genetic line accounted for 22 and 17% of the variation in FR and TNB, respectively. Boar age explained 0.3% of FR. Some of this variation can be explained based on animals but a large portion is not fully known. Although more in depth methodologies have been developed to assess a multitude of semen characteristics, none can accurately identify fertility status.

Capacitation Status and Fertility

Plasma membrane and acrosome integrity can be measured by various assays. These are both important for sperm survival and success within the female reproductive tract. In addition to the seminal parameters previously mentioned, detecting capacitation status by the use of Hoechst 33258 and/or chlortetracycline fluorescence could be used to improve litter size (Sutkeviciene et al. 2009; Oh et al. 2010; Kwon et al. 2015). These assays are relatively inexpensive and are not time consuming. They could be applied to routine semen analysis in combination with functional assays to identify boars that will produce larger litter sizes.

Computer Assisted Sperm Analysis on Quality and Fertility

Although sperm motility cannot identify subfertile boars alone, it is still a critical step in semen production. Sperm must be motile to complete the journey to fertilize the egg. Using a common evaluation tool, computer assisted sperm analysis (CASA), sperm motility and various motility parameters can be collected accurately and quickly (Figure 2.4). Even though this is helpful, CASA data have not predicted fertility consistently. A few studies have found significance in CASA parameters that correlate with fertility (Broekhuijse et al., 2012; Hirai et

al., 2001; Holt et al., 1997). On the other hand, some studies find no significant impact of these sperm kinetics on litter size (Didion, 2008; Kwon et al., 2015). Though the importance of this data on fertility hasn't been completely identified, CASA can be used wisely for quality control within a boar stud, to ensure proper concentrations of sperm doses (Amann and Waberski, 2014). It also allows for more accurate motility estimates, compared to subjective measurements by the human eye (Broekhuijse et al. 2011).

In a study by Broekhuijse et al. (2012), different statistical models to identify the impact of different CASA results on FR and TNB were developed. One analysis they proposed was looking at the effect of genetic line + boar + age + VCL + BCF, this model explained 9% of variation on FR. For TNB they created a model including genetic line + boar + VAP + VSL + ALH, this model accounted for 10% of the variation of TNB. In a similar study looking at a statistical model including motility and morphology traits, they found that they could accurately place 80% of the ejaculates in their respective fertility cluster (Kummer et al., 2013). These four separate clusters were based on FR and TNB. Overall, using CASA data in statistical models provided insight to explain variations of FR and TNB. Most studies have shown that, although CASA alone did not predict fertility, it may be a useful supplement to other laboratory assays.

Osmotic Swelling on Sperm Quality

An alternative for predicting fertility could be the use of an osmotic resistance test (ORT). This test also known as hypoosmotic swelling test (HOS) was originally used to detect infertility in human males (Jeyendran et al., 1984). Protocols for ORT for cattle (Revell and Mrode, 1994) and pigs (Schilling and Vengust, 1987) were developed shortly after and were

used to determine sperm samples of low quality. The osmotic resistance test is completed by incubating sperm with a solution of fructose and trisodium citrate (osmotic strength of 150 mOsm kg⁻¹) and then observing the effect of the solution on the sperm tail, in conjunction with the morphology of the acrosome (Revell and Mrode, 1994; Schilling and Vengust, 1987). This test successfully grouped males based on their results of the ORT and acrosome morphology. In bulls, this was highly correlated with nonreturn rate in females (Revell and Mrode, 1994). In boars, it was shown that the group ranked as high fertility had higher farrowing rates as well as larger litter sizes (Schilling, 1989). Like other sperm assays, this assay has seen both success and failure at predicting fertility. In a study completed by Rota et al. (2000), they identified that the ORT didn't correlate with IVF in cattle, but confirmed there is individual animal variation. Ten years later another study looked at ways to improve the assay, by using different solutions and different osmolarities. A key component of this study when samples were incubated with osmotic solutions and returned to the previous isotonic media there were significant differences (Yeste et al., 2010). In contrast, samples that were incubated with the osmotic solution and immediately followed by sperm assessment didn't have the same relationship. The use of osmotic resistance tests could help in identifying males or specific ejaculates that will potentially not perform well in the field. But more importantly, the effect of osmotic differences between seminal fluid and the fluid within the reproductive tract on sperm volume aren't well known; and to understand that role would allow for better selection for fertility (Yeung et al. 2004; Petrunkina et al. 2004; Yeung, Barfield, and Cooper 2005).

DNA Integrity and Flow Cytometry Evaluations of Sperm

It has been previously noted that sperm membrane function and DNA integrity impact fertility and embryonic development after fertilization (Silva and Gadella 2006). Observing both of these parameters can aid in the selection of superior animals based on sperm quality, resulting in the improvement of production. The DNA status of sperm is critical for successful fertilization. One way to assess DNA fragmentation is through the sperm chromatin structure assay (SCSA). Using acridine orange (AO), fluorescence of double-stranded DNA is green and single-stranded DNA red. Staining sperm with AO and evaluating the green and red ratio of those sperm with flow cytometry provides an index that is associated with fertility (Evenson et al., 1980). This assay was used to successfully predict fertility in boars that were used in heterospermic inseminations (Evenson et al., 1994). However, these results were overly simplistic, according to a larger study showed that the SCSA had a low correlation with true animal fertility because of variation in DNA fragmentation among ejaculates (Waberski et al., 2011). Nonetheless, using high-throughput multi-parameter flow cytometers to evaluate different criteria could enhance sperm quality assessment.

In addition to measuring DNA integrity, flow cytometry can be used to measure cell viability, acrosomal integrity and mitochondrial function of stained spermatozoa (Graham, Kunze, and Hammerstedt 1990). Both acrosome integrity and mitochondria are critical for sperm survival and fertilization, but viability is not a good indicator of the fertilizing ability of sperm. Although it isn't an indicator of fertility, flow cytometry can be used to identify live and dead cells, by SYBR-14 and propidium iodide, aiding in semen quality control at boar studs. Reactive oxygen species (ROS) can also be identified using flow cytometry (Guthrie and Welch, 2012). Although sperm production of ROS is necessary for normal function, too much is detrimental

(Awda et al., 2009; Barranco et al., 2016; Guthrie and Welch, 2012). Excess ROS damages the plasma membrane as well as DNA (Barranco et al., 2016; Guthrie and Welch, 2012). Flow cytometers can also provide a more accurate count of sperm concentration versus a spectrophotometer (Christensen et al., 2004). Additionally, flow cytometry can analyze multiple parameters at once but functional assays are required to identify the fertilizing capability of sperm (Gillan et al., 2005).

Sperm Binding Assays to Predict Fertility

There are a variety of functional assays to analyze spermatozoa. Some functional assays investigate the ability of sperm to bind to oocytes. *In vitro* maturation and *in vitro* fertilization can be used to compare boars based on sperm quality (Foxcroft et al., 2008; Ruiz-Sánchez et al., 2006; Xu et al., 1996; Xu et al., 1998). Fazeli and others completed a study looking at acrosome intact sperm binding to homologous zona, using a hemizona assay (Fazeli et al. 1997). This assay provides an internal control by using one oocyte cut in half to measure sperm binding to the zona pellucida (Oehninger et al., 1989). The authors expressed caution when analyzing and developing conclusions because the assay was quite different than what is occurring within the female. Lack of cumulus cells surrounding the oocyte as well as the absence of follicular fluid have an effect on sperm and should be accounted for when developing further physiological tests. Sperm penetration assays using cumulus-oocyte complexes can also rank boars by litter size (Oh et al. 2009). Measuring the number of sperm bound to the zona pellucida is an assay that can be used with other laboratory techniques. The addition of sperm-zona binding to a model aids in the prediction of fertility (Braundmeier et al. 2004; Daigneault et al. 2015). Completed work in our lab discovered a strong correlation between sperm binding to the zona and sperm

binding to oviduct cell aggregates (Daigneault et al. 2015). This correlation allows for further work utilizing oviduct cell aggregates as an indicator of potential fertility.

To study oviduct cell binding, oviductal explants or oviductal cells are collected for *in vitro* assays. Sperm bound to oviduct explants maintain motility and have normal morphology (Suarez et al. 1991; Petrunkina et al. 2001). Further development of oviduct binding assays resulted in cultured bovine oviduct cell monolayers that have similar results of prolonging sperm lifespan (Gualtieri and Talevi 2000). However, the use of three dimensional oviduct epithelial aggregates could provide a more appropriate structure for spermatozoa to bind to, versus monolayers of cells that have lost their polarity (Green et al., 2001). Additionally, incubating sperm with different antibodies or glycans could help in elucidating those factors necessary for proper binding to form the reservoir (Kadirvel et al. 2012; Machado et al. 2014). Furthermore, it is necessary to find out what causes sperm release from the oviductal reservoir, as this section of the reproductive field is still widely unknown. Oviduct cell binding assays are a useful way to measure the ability of sperm to interact and bind with oviduct cells, simulating the formation of the oviduct reservoir. Boar fertility partially depends on if sperm can bind and form this reservoir. Variation in fertility differences could be due to a lack of formation of the functional sperm reservoir (Waberski et al., 2005). The downside to using sperm-oviduct cell binding assays for sperm quality or fertility measures is that they are labor intensive. They do however, offer additional information about a characteristic required of highly fertile sperm that may be a useful supplement to basic semen analysis.

Conclusions

Although there are many assays to measure different sperm criteria, the impact of these *in vitro* assays on predicting fertility isn't consistent. This is largely due to variation between animals, ejaculates and individual sperm cells within the same ejaculate (Holt and Van Look 2004). It is also challenging to test *in vitro* assays as males in commercial semen production typically have similar fertility. This lack of accurate range can result in biased data, with strong outliers, causing significance (Mocé and Graham, 2008).

It is necessary for sperm to be motile to interact with the oviduct and to reach the site of fertilization and bind to the oocyte. It is also known that sperm with a larger number of abnormalities will result in lower litter sizes (McPherson et al., 2014). Alone none of these assays can successfully predict the outcome of fertility of an individual male. Something that is commonly suggested is the importance of using different assays simultaneously to create an appropriate predictive model (Graham 2001). Using different assays to confidently predict an individual's fertility would have a great impact on the swine industry.

On the other hand, a majority of fertility depends on the female. Artificial insemination technicians and management are key players to obtain a good level of fertility. Additionally, females may have different sperm selection criteria that can vary with age and health status (Jung et al., 2015). Petrunkina states that the major factor for a test to be able to accurately predict the fertilizing ability of sperm relies on the physiological relevance of the assay (Petrunkina et al., 2007). This is a vital dynamic of the reproductive system that isn't prevalent in *in vitro* assays. It is important to include media of suitable viscosity to match the fluid found *in vivo* when developing new assays (Hunter et al 2011). Lastly, the compensatory effect of using a high number of sperm overcomes many of the problems that may otherwise reduce fertility with

smaller doses of sperm. This compensation proves to be a challenge when completing fertility trials (Galli and Bosisio 1988; Xu et al. 1998; Gadea, Sellés, and Marco 2004; Popwell and Flowers 2004; Broekhuijse et al. 2012). Another potential problem is that only a small portion of the semen sample is analyzed in the laboratory; this sample must be representative in order to avoid misleading results. Lastly, there is usually no sperm selection with *in vitro* assays. This ignores the ability of the female reproductive tract to select sperm and remove the least fertile cells.

Sorting Sperm by Sex Chromosomes

Sorting sperm by sex chromosomes was first established in rabbits (Johnson et al., 1989). They inseminated does with Y-bearing, X-bearing, and XY-recombined sperm and saw that the number of births relatively matched their predicted values of male and female offspring. The staining procedure developed by Johnson, Flook, and Look in 1987, identified bizbenzamide (Hoescht 33342) as a suitable dye for staining DNA in spermatozoa (Johnson et al., 1987). This membrane permeable DNA-binding dye is nontoxic to spermatozoa and allows for a good split between Y-bearing and X-bearing sperm (Garner 2009). Sex-sorted semen is utilized regularly in cattle (Johnson and Welch 1999; Seidel 2007; Rath and Johnson 2008), specifically in the dairy industry, as female calves are more desirable than males. Sorting indices are an approximate value created by multiplying the area of the head (μm^2) by the DNA difference between the Y- and X-bearing spermatozoa (Figure 2.5) (Garner 2006). Bull, ram, and boar semen are highest on the sorting index because of their large flat heads and $>3.6\%$ difference in DNA. On the other hand, dog, horse and man are lower on this index because of smaller heads that aren't as flat, this makes them difficult to sort (Garner 2006). Although boar sperm are relatively easy to sort based

on meeting the DNA difference and head shape criteria, there are many factors that reduce the feasibility of sorting porcine sperm.

There is no effect of staining sperm on motility or fertility of spermatozoa (Vazquez et al., 2002). Staining with Hoescht 33342 does have a small impact on sperm viability, but sperm that are sorted have already been selected for relatively normal characteristics, so those with damage would be discarded (Spinaci et al. 2005; Garner 2009). Nevertheless, the physical process of sorting does affect the spermatozoa. Although the lasers and the electrical charge that is applied do not have a large impact on sperm viability (De Ambrogi et al. 2006), the speed through the flow cytometer seems to. Previously the routine pressure for sorting was 50 psi (Seidel et al. 1999; Schenk et al. 1999). In a study by Suh et al. (2005), they lowered the psi to 30 and found that it had less damaging effects on sperm. However, the lower pressure isn't feasible for sorting and they recommended that 40 psi be used because the effects were still less than those present at 50 psi but allowed for a greater number of cells to be sorted with less harmful outcomes.

There are two major challenges when sorting boar spermatozoa. The first being the length of time it takes to sort an inseminating dose. Flow cytometers can currently sort 20×10^6 sperm cells per hour (Suh et al., 2005). Traditional swine inseminations include 3 billion sperm twice, about 24 hours apart (Knox, 2016), which means it would take 150 hours to sort 1 traditional dose for pigs. Because cattle are generally inseminated with 15 to 20 million sperm cells and are inseminated once per estrus (Foote et al., 1997), the number of sperm needed for an AI dose is nearly 500 fold less than what is needed for swine. Despite that, progress has been made to allow the swine industry to take advantage of sex-sorted semen. Development of multiple methods for using a reduced number of sperm, such as deep uterine insemination (Martinez et al. 2002;

Vazquez et al. 2003; Grossfeld et al. 2005; Martinez et al. 2006; Bathgate et al. 2007), *in vitro* fertilization (Abeydeera et al., 1998; Rath et al., 1997; Rath et al., 1999), intracytoplasmic sperm injection (ICSI) (Probst and Rath, 2003), and surgical insemination (del Olmo et al., 2014; Fantinati et al., 2005), have allowed the production of offspring from sorted porcine sperm. Using these assisted reproductive technologies allows for litter sizes comparable to those of semen processed in the conventional method.

The second limiting factor in using sex-sorted boar semen is appropriate methods for storage. At this time it is a challenge to freeze boar spermatozoa with acceptable post-thaw rates (Gil et al., 2008; Rath and Niemann, 1997; Roca et al., 2013). The industry standard is to use cool liquid extender. Some long term extenders can help keep sperm viable for up to seven days (Johnson et al. 2000; Waterhouse et al. 2004; Marco De Ambrogi et al. 2006; Pinart et al. 2015). An additional complication is that high dilution rates of spermatozoa during the sorting process alter motility patterns and reduce sperm lifespan (Centurion et al., 2003; Maxwell and Johnson, 1999). There has been research comparing different additives to liquid extenders to improve viability. These different additives including egg yolk (del Olmo et al. 2013), spermadhesins such as PSP-I/PSP-II (Centurion et al., 2003), and antioxidants (Vallorani et al., 2010; Xia et al., 2012), have improved viability as well as fertilizing ability. It is possible to freeze sex-sorted boar sperm, but IVF is necessary to produce offspring (Bathgate et al., 2007).

One of the ways that sex sorting might damage sperm is by inducing capacitation-like changes. Modifications of the sperm membrane during sorting can alter membrane function and promote premature capacitation (Spinaci et al. 2006). Seminal plasma plays a role in inhibiting capacitation but this is removed during the sorting process (Maxwell and Johnson, 1999). This removal of seminal plasma and the high dilution rates occurring in sorted sperm may promote

capacitation-like changes. In the study completed by Spinaci et al. (2006), they found that sorting causes a relocation of heat shock protein (Hsp) 70. Heat shock protein 70 was relocated but Hsp60 and Hsp90 were not affected. This redistribution of this sperm protein suggests that there are known and unknown changes occurring on the sperm membrane that could simulate capacitation, or promote partial capacitation, reducing the lifespan of sorted sperm. Since capacitated sperm are less capable of binding to the oviduct (Fazeli et al. 1999; Gualtieri and Talevi 2000; Petrunkina et al. 2001), this may be a cause of reduced fertility. Despite these multiple challenges, using sex-sorted boar spermatozoa is desirable and could potentially become routine within the swine industry.

Figures:

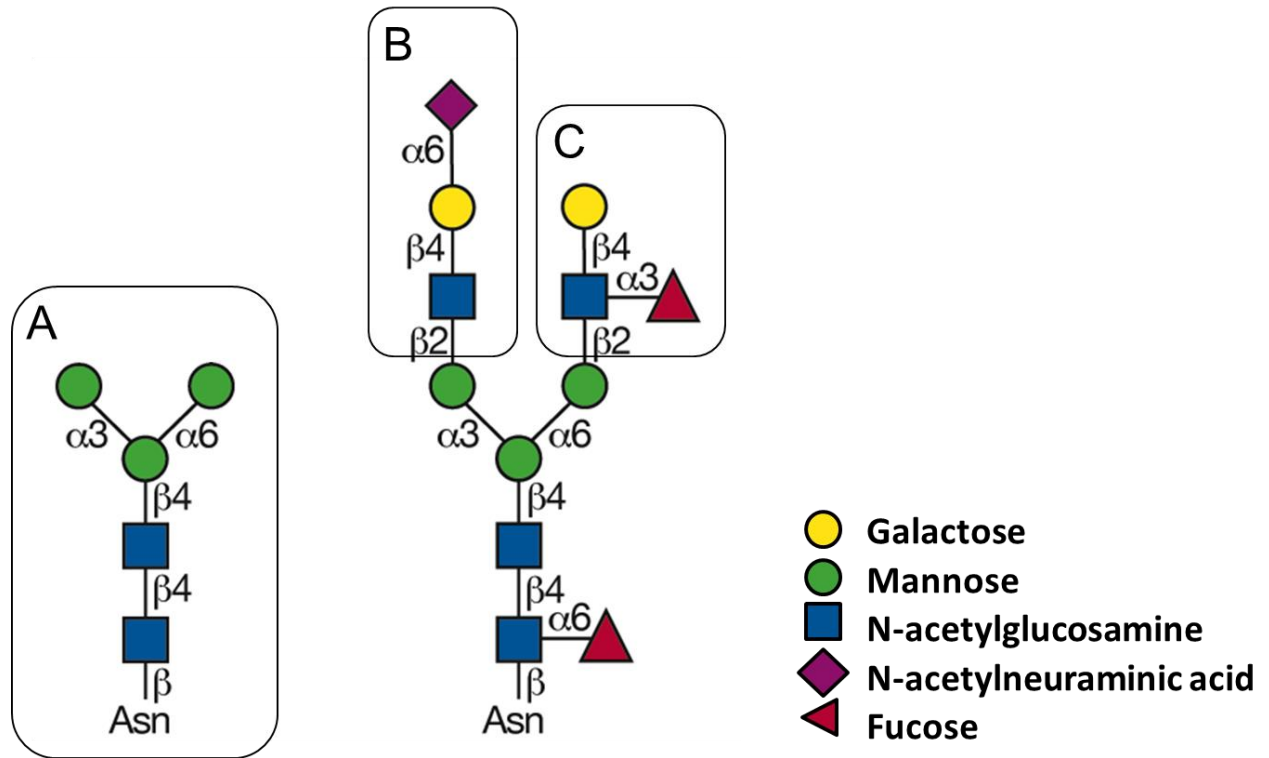


Figure 2.1: Modified from Varki et al. Essentials of Glycobiology (Chapter 8). A) The common core for all N-glycans. Common modifications to the N-glycan core B) SiaLN and C) Le^x (Varki and Lowe, 2009).

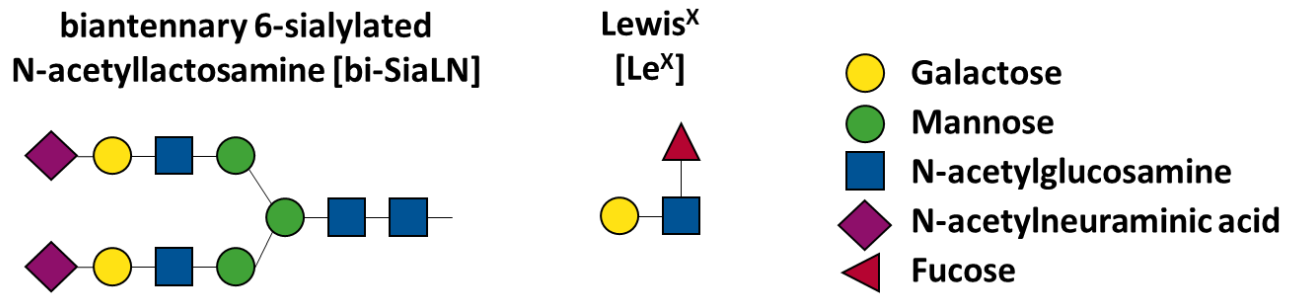


Figure 2.2: Glycans that noncapacitated boar sperm bind, biantennary 6-sialylated N-acetyllactosamine (bi-SiaLN) and Lewis^x trisaccharide (Le^x). Both were identified by Kadirvel et al. using a glycan array (Kadirvel et al., 2012).

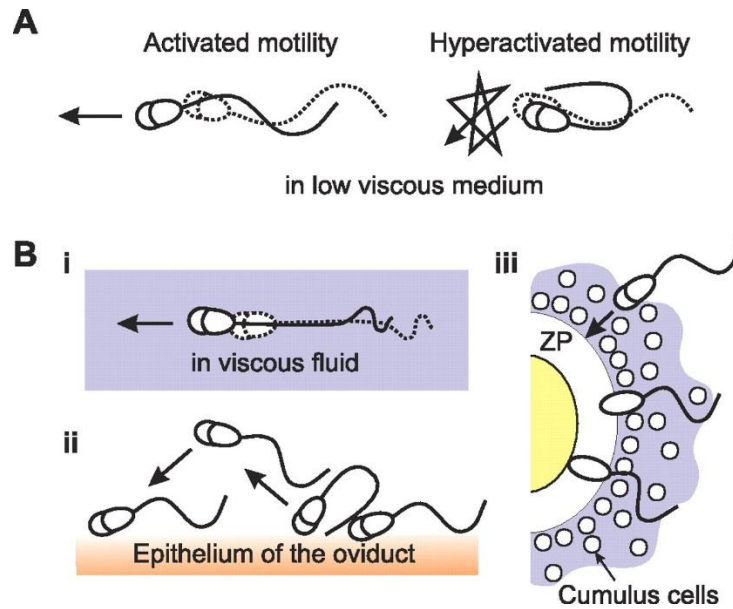


Figure 2.3: A) Visual representation of activated versus hyperactivated spermatozoa, interacting with B i) viscous oviduct fluid, Bii) oviductal epithelium, and Biii) the cumulus oocyte complex. Hyperactivated spermatozoa in viscous fluid travel in a straight path, whereas those with activated motility move forward until they reach the oviduct, where they remain in a quiescent state (Darszon et al., 2011).

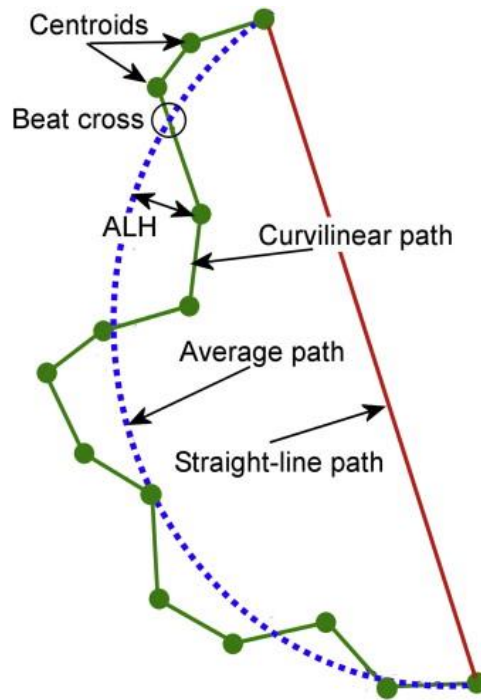


Figure 2.4: CASA Parameters (sperm movement visual) Illustration from Amann and Waberski 2014, showing how the different CASA parameters are evaluated. Centroids are given to each individual sperm for each frame analyzed. Connecting these centroids gives the sperm trajectory which is called the curvilinear path. The average time it takes for the sperm to travel along this trajectory, which is called curvilinear velocity (VCL; $\mu\text{m/s}$). The average path is computed, and the time-average velocity along the specific trajectory is termed average path velocity (VAP; $\mu\text{m/s}$). Straight-line velocity (VSL; $\mu\text{m/s}$) is the straight-line path from the beginning of the sperm trajectory to the end of the path during analysis. Following each centroid, there is a deviation from the average path, and that is called the amplitude of lateral head displacement (ALH; μm). The number of times a sperm crosses the average path is termed beat-cross frequency (BCF; number per second) (Amann and Waberski, 2014).

Dimension	Bull	Boar	Ram	Dog	Horse	Man
Length (μm)	9.1	9.0	8.1	7.0	6.5	4.6
Head sagittal section						
Width (μm)	4.7	5.0	4.0	3.5	3.4	3.2
Head Profile						
Area (μm^2)	34.5	37.5	26.6	20.9	15.2	10.8
X-Y difference %	3.8	3.6	4.2	3.9	3.9	2.8
Sorting index	131	115	112	82	59	31

Figure 2.5: Sorting indices from various species. Modified from a compilation created by Garner 2006. The sorting index is an approximation of the success sperm will have being sorted. It is calculated by multiplying the head profile area (μm^2) by X-Y DNA difference between sperm (%) (Garner 2006).

CHAPTER 3 - PORCINE SPERM BINDING TO OVIDUCT CELLS AND GLYCANS AS SUPPLEMENTS TO LABORATORY SEMEN ANALYSIS

Abstract

Artificial insemination is a common practice in swine, therefore accurate semen evaluation is necessary to maintain reproductive efficiency; however consistently accurate semen analysis has been elusive. The objective was to determine if the ability to bind oviduct cells or the oviduct glycans that bind sperm are useful supplements to routine semen analyses. Measuring binding to soluble glycans is less laborious than assessing binding to oviduct cell aggregates and more suitable for industry use. Our previous work has shown that sperm binding to oviduct cell aggregates improves fertility prediction, possibly by estimating the ability of sperm to form a reservoir in the oviduct. We have also shown that two oviduct glycan motifs, biantennary 6-sialylated *N*-acetylglucosamine (bi-SiaLN) and Lewis^X trisaccharide (Le^X) bind boar spermatozoa with high affinity and specificity. Semen from boars (n=30) used for homospermic matings was collected and shipped overnight for laboratory analysis and for inseminations to determine fertility. Semen collections were analyzed from boars three times over the course of the study. Oviduct cell binding and traditional sperm analyses including motility, morphology and acrosome status were completed the day the shipment was received. Binding to three soluble fluoresceinated glycans bi-SiaLN, sulfated Le^X (suLe^X), and the control lactosamine disaccharide (LacNAc) was measured the following day. The inseminations occurred at 15 farms (>50 matings/boar) in the greater Midwest and farrowing data from all matings from each boar were used. Pregnancy rate (PR) and litter size (LS) were adjusted to account for different farms, number of services, number of doses inseminated, and sow parity, using the MIXED procedure in SAS 9.4. A fertility index (FI) was generated, consisting of PR x LS. This index allowed for

boars to be ranked on their overall fertility. Lastly, the GLMSELECT procedure was used to select variables having a significant impact on PR, LS, and FI. The predictive models constructed were further analyzed using the REG procedure and accounted for 58% or more of the variation in PR, LS, and FI [PR ($p < 0.001$, $r^2 = 0.60$), LS ($p < 0.001$, $r^2 = 0.58$), and FI ($p < 0.001$, $r^2 = 0.63$)]. All three final models included traditional semen morphology evaluation traits. The model for PR also included the ability of sperm to bind oviduct cell aggregates, in agreement with our previous smaller scale study. However, binding to specific oviduct glycans was not a useful supplement to traditional semen analysis. Overall, binding to intact oviduct cells remains impactful as a means to predict pregnancy rate.

Introduction

Within the swine industry efficiency is key for continuous production of a high-quality protein source to feed the growing population. Isolating and removing males that will result in an overall lower production value, assessed by pregnancy rate (PR) x litter size (LS), before they are routinely collected, will subsequently improve overall reproductive efficiency. Selecting for boars with higher production values can increase the number of animals produced using the same number of females and reduce the number of nonproductive days in females. This will lower the cost per pound of pork produced and reduce the environmental impact.

One limitation in the selection of boars based on their predicted production value is that there is no single laboratory assay that can predict an individual boar's fertility. Traditional semen evaluation includes motility and morphology but these alone are not sufficient for estimating fertility (Gil et al., 2005). Using a combination of traditional and functional assays

can allow for fertility prediction with greater accuracy. Previous work has identified a correlation between sperm-oviduct binding and fertility (Waberski et al. 2005; Daigneault et al. 2015).

Formation of the sperm reservoir in the isthmic portion of the oviduct is critical for maintaining sperm viability (Gualtieri and Talevi 2003; Töfer-Petersen, Ekhlas-Hundrieser, and Tsoolova 2008; Rodríguez-Martínez et al. 2005). Sperm remain bound during the time between insemination and ovulation, which is essential for pigs because of the large variation in time between these two events (Töfer-Petersen et al., 2008). The majority of females are inseminated twice, 24 hours apart, to compensate for this lack of synchronization (Knox 2016). Insufficient sperm reservoir formation could result in higher return-to-estrus rates and reduced litter sizes.

The oviduct is made up of a complex matrix of cells where sperm interact and bind. The carbohydrate-mediated binding within the oviduct to create the sperm reservoir has been studied previously in multiple species (hamster, DeMott, Lefebvre, and Suarez 1995; horse, Dobrinski et al. 1996; bull, Lefebvre, Lo, and Suarez 1997; Ignatz et al. 2001; pig, Green et al. 2001; Wagner et al. 2002; Kadirvel et al. 2012). This interaction is partially controlled by glycans present on the oviduct as well as receptors on the sperm head. A study completed by Kadirvel et al identified the main carbohydrate structures that noncapacitated boar sperm bind. A glycan array was used, consisting of 377 glycans. The glycan array identified the glycans to which noncapacitated porcine sperm bound. Common among those structures were the glycan motifs biantennary 6-sialylated *N*-acetylglucosamine on a mannose core (bi-SiaLN) and Lewis^x trisaccharide (Le^x) (Kadirvel et al., 2012). Both bi-SiaLN and Le^x motifs bind with high affinity to the sperm head prior to capacitation and are necessary for sperm binding to the porcine oviduct (Kadirvel et al. 2012; Machado et al. 2014).

The objective of this study was to develop a model to accurately predict an individual boar's fertility based on laboratory assays, specifically the ability of sperm to bind to soluble oviduct glycans as a replacement for the more laborious sperm-oviduct binding assay. Ideally, the model would have a small number of variables to make it accurate, yet practical. Models were tested for their ability to predict PR, LS, and the product of the two, PR x LS, defined as Fertility Index (FI).

Materials and Methods

Materials

Chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) unless stated otherwise. Semen was provided by PIC (Pig Improvement Company). Reproductive tracts were provided by Rantoul Foods (Rantoul, IL, USA).

Boars and Semen Collection

Boars were all large white males selected for maternal purposes with some carcass traits, all of PIC genetics. All boars were born on the same farm. Boar age varied from 9-35 months. These boars were housed in southern Michigan, at Harmony Gene Transfer Center, a branch of Birchwood Genetics, under PIC. Boars were housed in barns kept at 61-64° F (16-18° C).

All collections were completed by the gloved hand method. Criteria for acceptable semen quality included $\geq 80\%$ motility, $\geq 70\%$ normal sperm, $< 15\%$ cytoplasmic droplets, and $< 30\%$ agglutination. After assessment, semen was extended and dispensed into doses. Doses ranged

from 3-4 billion sperm cells. These doses were cooled and packaged for shipment. They were shipped overnight to our lab as well as to the different farms for insemination. Spermatozoa from 30 different boars were collected, three separate ejaculates per each boar. Collections were from January 4th-July 11th, 2016. There was a minimum of three weeks between collections received for each boar.

Birchwood Genetics compiled various collection evaluation parameters including; boar rest interval, sperm volume/ejaculate, sperm concentration/ejaculate, total sperm/ejaculate, number of doses poured, and total sperm/dose. Overall motility and progressive motility, as well as various computer assisted sperm analysis parameters were measured. For morphology, percentages of sperm possessing normal heads, normal tails, proximal as well as distal droplets were collected.

Females and Breeding Trial

Gilts were exposed to a boar daily to stimulate puberty and estrus. Gilt boar exposure lasted approximately 10-15 min. Gilts were inseminated at the second observed estrus. Boar exposure was also used in sows to stimulate them for the duration of their insemination. Doses were administered by traditional AI. Catheters were bent and left in the female for 5-7 min after insemination to prevent backflow and allow uterine contractions to pull the remainder of the extended semen into the female. Pregnancy checks were conducted on all females starting at 3 weeks of gestation. Those that were considered open by boar exposure/heat check were considered returns. Those females that were classified as abortions were either identified by ultrasound at week 5 or visually by weeks 8-10. Some females that were classified as pregnant

were culled for unknown reasons. PIC provided the following data: number of services, number of matings, parity, and outcome. The number of services, or the number of times an individual female had an estrus. For example a service value of 1 meant it was her first estrus after weaning, a service value of 2 meant she was inseminated or skipped on her first estrus event. The number of matings is the number of doses inseminated per estrus event. Parity is the number of litters a female has had (i.e. a P3 sow has had three litters). There were five possible outcomes of each individual mating: abortion, died, farrowing (pregnant), pregnant-slaughter, or return to estrus. The farrowing data collected included: total born alive, total stillborn, and total mummified piglets. Females were all large whites of PIC Genetics with parities ranging from one to ten (P1-P10). Parity 1 females were gilts at the beginning of the trial. These animals were housed at 15 different farms all over the greater mid-west. The farms varied in size from 400-4000 females. Each boar was mated to >50 females over the course of the trial (Table 3.1).

Sperm Motility Assessment

Samples were held in NUTRIXcell+ extender (IMV Technologies, Maple Grove, MN, USA) and shipped overnight to the University of Illinois at Urbana-Champaign. Motility was assessed at time of oviduct binding assay ~36 hr after shipping. Motility analyses were conducted using Computer Assisted Sperm Analysis (Hamilton Thorne, Beverly, MA, USA). Sperm were warmed at 37° C for 15 min. Sperm were centrifuged at 2400 X g for 1 min, extender was removed and the pellet was resuspended in dmTALPC (2.1 mM CaCl₂, 3.1 mM KCl, 1.5 mM MgCl₂, 100 mM NaCl, 0.29 mM KH₂PO₄, 0.36% lactic acid, 0.6% Fraction V BSA, 1 mM pyruvic acid, 25 mM HEPES [pH 7.3], sterile filtered). Sperm suspension was kept warm and 3 µl of each sample were loaded onto a pre-warmed four chamber Leja4 slide (IMV

Technologies). A minimum of 8 fields and 450 cells were observed at 37° C and 45 frames were acquired at a rate of 60 Hz with a minimum contrast of 55, minimum cell size of five pixels, VAP cutoff of 20 µm/s, progressive minimum VAP cutoff 45 µm/s, VSL cutoff of 5 µm/s, static head size of 0.53-4.45 and magnification of 1.73. Total motility, progressive motility, VAP (average path velocity), VSL (straight-line velocity), VCL (curvilinear velocity), STR (straightness = $VSL \times 100/VAP$), LIN (linearity = $VSL \times 100/VCL$), BCF (beat cross frequency), ALH (amplitude of lateral head displacement), ELONG (elongation, percent of head width to head length), and AREA (area of sperm head) were measured. Parameters are further defined in the list of abbreviations.

Membrane Analyses of Spermatozoa

Coomassie blue staining was done to determine acrosome integrity and morphology for each sample. Spermatozoa (10 µl) from each group were fixed with 4% paraformaldehyde around 24 hr after shipping to fix cells. Cells were then centrifuged at 2000 X g for 1 min, the supernatant was discarded and the pellet was resuspended in 50 µl of 0.1 M ammonium acetate. The suspension was centrifuged again at 2000 X g for 1 min, the supernatant was discarded and the pellet was resuspended in 25 µl of ammonium acetate. The sperm suspension (10 µl) was placed onto a slide and allowed to air dry. Dried slides were stained with Coomassie Blue G-250 solution (0.22% Coomassie Blue G-250, 50% methanol, 10% glacial acetic acid, and 40% water) for 4 min. Slides were rinsed with distilled water and allowed to air dry. A drop of 1X PBS was added and topped with a cover glass. A minimum of 300 sperm/sample was evaluated using Zeiss Axio-CamHRc (Zeiss Microscopy, LLC, Thornwood, NY, USA) at 400X magnification. Spermatozoa with a stained acrosome were considered to be acrosome intact. While assessing

the acrosome status, morphology was also analyzed. The percentage of sperm with normal heads, normal tails, proximal droplets, and distal droplets were recorded. Sperm possessing multiple abnormalities were only considered once when calculating the total number of sperm.

Oviduct-Sperm Binding Assay

For each experiment, entire female reproductive tracts were collected from pre- and post-pubertal females from an abattoir. Tracts were brought back to the lab and 20-30 oviducts were removed and held in 1X PBS on ice before being processed the same day. Oviducts were transferred to a 100-mm petri dish containing 1X PBS and the ampulla was removed from the isthmus portion using a glass slide. Epithelial sheets from the isthmus were collected by gently pressing down at a 45 degree angle and squeezing the sheets out. The epithelial sheets were then transferred to a 15 ml conical tube and centrifuged at 100 X g for 30 sec. After the supernatant was removed, cells were re-suspended in 1 ml of dmTALPC. Cells were disaggregated by passage through a 1 ml pipette 10 times. Partially disaggregated cells were washed with 5 ml of dmTALPC and centrifuged at 100 X g for 1 min. The supernatant was removed and cells were re-suspended in 1 ml of dmTALPC. The cells were passed through a 23-gauge needle 10 times for further disaggregation, the volume of the cell suspension was adjusted to 9 ml with dmTALPC and cells were divided evenly into three 100-mm petri dishes. The cells were allowed to reaggregate for 1.5-2 hr at 37° C. Spherical aggregates (150-200 µm in diameter) were selected for the experiments.

About 40 spherical oviduct cell aggregates were selected and washed twice in 100 µl drops containing fresh dmTALPC. Using a Stripper Pipette (MidAtlantic Diagnostics, Inc.

Marlton, NJ, USA) with a 250 μm internal diameter tip was used to collect oviduct epithelial cell aggregates and wash them. Oviduct cell aggregates were moved to a drop containing a final volume of 50 μl with 1×10^6 sperm/ml for co-incubation at 37° C for 15 min to allow sperm to bind to the aggregates. Each group contained three 50 μl drops with 10 oviduct cell aggregates each. After incubation the sperm-bound aggregates were transferred in a volume of 3 μl onto a microscope slide. Each slide corresponded to one boar. Each droplet with 10 sperm-bound aggregates was considered an experimental unit for statistical analysis. Images were captured using a Zeiss Axioskop and AxioCam HRc digital camera (Carl Zeiss, Thornwood, NY, USA). The number of sperm bound to the periphery of each aggregate was counted and the circumference for each aggregate was calculated using ZEN AxioVision V 4.5 software (Carl Zeiss). The number of sperm bound was divided by the linear periphery of the aggregate to normalize for differences in aggregate size. Three boars were tested on an experimental day.

Fluorescent Glycan-Binding Assay

Localization of glycan-binding receptors in live spermatozoa was determined by binding between spermatozoa and three fluoresceinated glycans. Sperm from each boar were held overnight at 17° C in the original extender NUTRIXcell+. Sperm were warmed at 37° C for 15 min. Sperm were centrifuged at 2400 X g for 1 min, extender was removed and the pellet resuspended in dmTALPC. Concentration was brought to 10×10^6 sperm/ml. An aliquot of 38 μl of the sperm suspension was transferred to an eppendorf tube and 2 μl of fluorescent glycan was added (final glycan concentration of 50 $\mu\text{g/ml}$). The glycans used were biantennary 6-sialylated *N*-acetyllactosamine (bi-SiaLN), sulfated Lewis^X (suLe^X), and *N*-acetyllactosamine (LacNAc). *N*-acetyllactosamine is a disaccharide present in both motifs and was used as the control for

nonspecific binding. All glycans were coupled to a 30 kDa polyacrylamide chain modified by fluorescein addition. The sperm suspension was incubated with glycans for 30 min at 37° C. A small drop of 6 µl from the suspension was placed on a slide and covered with a coverslip. A minimum of 200 sperm was counted for each glycan, using a 63X oil immersion objective (630X magnification). Positive binding was considered when green fluorescence was detected. Each of the three glycans was tested for each of the ejaculates for every boar.

Statistical Analyses

All analyses were completed using statistical software from SAS 9.4. Farrowing data were collected by PIC. Females that had died during the trial were removed from the data set because there was no way to identify their pregnancy status. Females that were classified as abortion, farrowing (pregnant) or pregnant-slaughter (those females that were classified as pregnant but were culled before farrowing) were given an outcome value of 1, as they were pregnant for a period of time after insemination. Those females that had been marked as having returned to estrus were given an outcome value of 0 because pregnancy was not established. These two outcomes were used to determine the average pregnancy rate for each boar. Construction of the second response variable, litter size, resulted from those females that had farrowed. The raw litter size was calculated by taking the sum of total born alive, total stillborn, and total mummified piglets. Through the MIXED procedure, pregnancy rate and litter size for each boar were adjusted by farm, number of services, number of matings, and sow parity. The third response variable was a fertility index created by multiplying pregnancy rate by litter size.

After semen shipment, morphology results from four boars during one replicate were not collected. The average of the other two replicates were used to develop values for boars 8, 10,

11, and 12 to allow for complete analysis. Oviduct cell aggregates were normalized using a log transformation in SAS. The values were then back-transformed in Excel using the exponentiation function. Boars were ranked by mean binding to oviduct cell aggregates, motility, and normal morphology in the ANOVA procedure. Boars were also ranked by the three response variables PR, LS, and FI.

Using the GLMSELECT procedure, the variables that had a significant impact on PR, LS and FI were selected. This procedure created the models for PR, LS, and FI. The models were further analyzed using PROC REG with the stepwise selection function resulting in the R^2 values, partial R^2 , and p-values for each independent variable.

Results

In this study, fertility was assessed by pregnancy rate (PR), litter size (LS) and a fertility index (FI). The fertility index was developed by multiplying pregnancy rate by litter size. Boars were ranked based on each of the three response variables, PR, LS, and FI. There was consistent variation among males based on pregnancy rate and litter size. Although boars were screened by PIC using the traditional assessments of sperm motility and morphology, there were still 12 boars (Figure 3.1) that had an adjusted pregnancy rate lower than the desired pregnancy rate in females of 90% (Knox 2016). Eleven boars had average litter sizes about the industry average of 13.95 piglets/litter (PigCHAMP, 2016) and an additional 7 were above 13 piglets/litter. The remainder were below 13 piglets/litter, the lowest having an adjusted average litter size of 5 piglets (Figure 3.2). There were 7 boars with the lowest pregnancy rate and litter size, yielding the lowest group based upon fertility index (Figure 3.3).

Binding to 3-dimensional oviduct cell aggregates partially simulates the interactions occurring within the female reproductive tract, specifically with the isthmus region of the oviduct (Green et al., 2001). The 30 boars used in this study varied in the ability of their sperm to bind to oviduct cells (Figure 3.4). Binding to the oviductal epithelium also varied among ejaculate collections (Data not shown).

Because binding of sperm to oviduct cells requires a ready source of oviduct cells and is not practical for most AI laboratories, I tested whether a simpler assay that employs the glycans present among the oviductal epithelium of porcine reproductive tracts, bi-SiaLN and/or suLe^X, could predict boar fertility. Sperm were incubated with soluble fluoresceinated glycans, bi-SiaLN and suLe^X, and the percentage of sperm binding each glycan was observed, in addition to the binding patterns present on the sperm head. Lactosamine disaccharide (LacNAc) was used as a control for nonspecific binding. The three patterns present were binding to the apical ridge of the sperm head, binding to both the apical ridge and the post-acrosomal area, and lastly the post-acrosomal area only. Among the 30 boars, there were no significant differences present in the percentage of sperm that bound any of the three glycans analyzed (Table 3.2). Additionally, the percent of sperm binding to the individual patterns showed no differences (Table 3.3a-c). For further analysis, sperm binding patterns were grouped to create two new variables of total apical binding (apical ridge only + apical ridge and post-acrosomal) and total post-acrosomal binding (apical ridge and post-acrosomal + post-acrosomal only). Creating these two variables was done to determine if some boars had preferential binding to the post-acrosomal area, due to a leaky membrane. The post-acrosomal area is classified as nonspecific binding as it is not the area of the sperm head that interacts with the oviduct and glycan binding to this region is not saturable (Dutta and Miller, unpublished). However, there were no statistically significant differences in

the percentage of sperm that bound the targeted glycans (bi-SiaLN and suLe^X) for total apical binding and total post-acrosomal binding among the boars used (Table 3.4a-b). Lastly, binding to soluble fluoresceinated glycans shows no correlation with oviduct cell binding (Table 3.5). Overall, binding of soluble fluoresceinated glycans to sperm from the 30 boars was not correlated with either pregnancy rate or litter size (Table 3.6).

Sperm from samples of fresh extended boar semen were assessed based on a variety of characteristics before and after shipment. Semen was shipped to both the laboratory for assays and farms for inseminations, so motility was assessed before and after shipment. All 30 boars had $\geq 90\%$ motile cells prior to shipment (Figure 3.5). However, after overnight shipping, the average semen motility had decreased from as little as 7% to as much as 60% among the different boars. A majority of the boars showed a decrease in the percentage of motile cells of approximately 20% at the time of sperm-oviduct binding assay (Figure 3.6).

Sperm morphology is also important for successful formation of the sperm reservoir within the oviduct (Petrunkina et al., 2001; Suarez et al., 1991b). Morphology assessment was also completed before and after shipment. Morphological problems varied between boars and replicates. There was a two fold difference in the mean percentage of normal sperm across the range of values for all boars (Figure 3.6). Sperm that had no morphological defects were considered normal. Defects included proximal and distal droplets, head and tail problems, and those that were acrosome reacted.

To determine which information about each semen sample could be used to develop a simple model to predict fertility, PROC GLMSELECT was used. This procedure begins with all possible variables and selects for those that have a significant impact on the response variable individually, ensuring that the variables remain impactful on the model with the addition of

subsequent variables. Those that were significant were then ran through a regression analysis using PROC REG, and this resulted in the final models represented in Tables 3.7, 3.8, and 3.9. Table 3.7 has the estimate values in addition to the partial R-square values for PR.

Binding to oviduct cells had a significant impact on the fertility trial pregnancy rate (Table 3.7). Two CASA motility parameters were included in the final model, beat cross frequency (BCF) and curvilinear velocity (VCL). Beat cross frequency measures the number of times the sperm tail crosses a specific path, visually identifying how fast the sperm tail is beating. Curvilinear velocity was included in the final model for predicting pregnancy rate (Table 3.7). Various morphology problems were correlated with PR. The percent of head defects before shipment, and the percent of sperm with distal droplets and tail defects after shipment were useful in predicting PR (Table 3.7). Boar age negatively impacted the model for pregnancy rate as well (Table 3.7).

Those variables included in the final model to predict LS are in Table 3.8. The age of the boar had a negative impact on the model for litter size (Table 3.8). Some sperm characteristics that were included in the model to predict PR were also included in the LS model, such as the percentage of sperm with distal droplets before shipment and tail defects observed after shipment. Additionally, the percent of normal sperm after shipment had a significant impact on the litter size model (Table 3.8).

Finally, the assessments that had a significant impact on the FI, the product of PR and LS, are shown in Table 3.9. The percent of sperm with tail problems and distal droplets before shipment and the percent of normal cells were correlated with the fertility index values (Table 3.9). The CASA motility trait VCL was also included in the final model to predict FI.

Additionally, boar age remained impactful in the model to predict the fertility index value (Table 3.9).

Aside from laboratory assays, some information collected at the boar stud showed correlation with different models. Those variables with significance were the age of the boar, the total number of sperm in the ejaculate, as well as total sperm volume of the ejaculate, impacted the models. Boar age negatively impacted the model for pregnancy rate and litter size, thus impacting the model for fertility index (Tables 3.7-3.9). Some boars that were younger in age resulted in a lower fertility index value, a result of lower pregnancy rates and smaller litter sizes. Total sperm in the ejaculate impacted the model for litter size (Table 3.8). Total sperm volume of the ejaculate impacted the fertility index model (Table 3.9).

Discussion

Boar fertility plays a critical role in swine production. Predicting male fertility is challenging because males are only 50% of the equation, in addition to variation among individual ejaculates from the same male. Maintaining a high level of fertility in both males and females results in steady production. However, predicting fertility is quite complex and there is, to date, no simple assay or small group of assays that answers the question of whether a boar is going to consistently result in pregnant females producing a high number of offspring. Using a combination of different traditional semen analyses in conjunction with functional laboratory assays to identify superior or subfertile males could improve overall reproductive efficiency in swine.

Pregnancy rate and litter size cannot be determined until later in gestation and/or when the female farrows. These are both useful measures but they do not provide immediate predicting values of how a specific ejaculate will perform in the field, whereas laboratory assays can be completed in a short period of time. The sperm-oviduct binding assay is time consuming but offers some insight into how successful sperm are at interacting with intact oviduct cells. This *in vitro* assay is likely a good measure of the ability of sperm to form a reservoir *in vivo* because the oviductal cells maintain their polarity, resembling an epithelium (Thomas et al. 1997). The ability of sperm to bind to oviduct cells was included in the model to predict pregnancy rate and had the 3rd highest partial R² value (0.10). This is in agreement with previous studies looking at sperm binding to oviduct cell aggregates and the corresponding fertility of the boar (Waberski et al. 2005; Daigneault et al. 2015). Formation of the sperm reservoir to prolong sperm lifespan is important for fertilization of oocytes and pregnancy establishment (Gualtieri and Talevi, 2003; Rodríguez-Martínez et al., 2005; Töfer-Petersen et al., 2008). Samples with a large proportion of sperm that are unable to bind the oviduct appear to decrease pregnancy rates significantly.

Though the sperm-oviduct binding assay was correlated with pregnancy rate in this study, it is a time consuming assay and isn't feasible in a production setting. Binding with specific soluble glycans is a simpler assay. Incubating sperm with individual glycans can identify whether or not the specific receptors are present on the sperm head. However, in our population of boars, binding to soluble glycans showed no correlation with oviduct cell binding. Furthermore, sperm binding to glycans also had no correlation with any of the three response variables, PR, LS, or FI. Additionally, there were no statistically significant differences among boars in binding of fluoresceinated glycans. This lack of variation in glycan binding could be due to the soluble nature of the glycans. The binding affinity required to tether a highly motile cell to

an insoluble matrix is higher than to soluble glycans so the two assays have different affinity thresholds required to produce a signal, either sperm binding to oviduct cells or sperm binding to soluble fluorescent glycans.

Sperm interaction with oviduct cells requires motile sperm cells. Motility reduces on average by 5-10% after being extended and chilled for 24 hours (Knox 2006). The variability present in motility after shipment can be due to a variety of things. The semen for this trial was shipped overnight, which required a lot of handling before being used. The decrease in motility could have also impacted pregnancy rates and litter sizes in females inseminated with those particular ejaculates. Furthermore, some boar sperm perform better in different extenders (Estienne et al., 2007). The extender used in this trial was a long-term extender.

In addition to overall motility, identifying different motility parameters that had a direct effect on PR and LS, provided some insight into sperm functionality. Curvilinear velocity (VCL) is the amount of time it takes for an individual sperm cell to travel from one point to the next. Additionally, beat cross frequency (BCF) was also important in predicting pregnancy rate. BCF is the number of times the tail of an individual sperm crosses the average directional path. These parameters indicate how fast the sperm tail is beating, BCF, and how fast a sperm is traveling, VCL. These were both significant in the model for predicting pregnancy rate. Sperm are directed through the female reproductive tract by contractions and mucus (Jansen, 1978; Jansen and Bajpai, 1982), but they need to have adequate motility and a strong BCF and VCL in order to travel through the mucus in the oviduct, in order to bind to the oviduct epithelium to form the sperm reservoir (Broekhuijse et al., 2012). Similarly, sperm have a longer lifespan and are more capable of reaching the oviduct if they are actively motile. Those lacking this strong motility

have a reduced ability to travel the length of the reproductive tract to reach the area of fertilization.

Lastly, in order to form the oviduct reservoir sperm must be morphologically normal. Our study supports previous work, identifying the importance of sperm morphology on the formation of the oviduct reservoir (Suarez et al. 1991; Petrunkina et al. 2001). The two-fold variation among the boars based on the percent of normal cells after shipment again illustrates the variation among the boars used in this trial. A majority of the boars had >80% normal sperm cells after shipment. These morphological abnormalities can vary between ejaculates collected, but the standard errors for the boars present were relatively small, indicating that among the three replicates the sperm consistently had poor morphology. The high percentage of abnormal sperm could reduce the number of sperm in the reservoir, resulting in higher returns and lower litter sizes.

Examination of data from individual boars was interesting. It is known that the oviduct selects for morphologically normal spermatozoa (Petrunkina et al., 2001; Suarez et al., 1991b). Boar #25 had the lowest fertility index, which was a result of a combined lower pregnancy rate and a small litter size. Additionally, boar #25 had the lowest normal morphology and the second lowest after shipment motility, which together suggest that he should have the lowest oviduct cell binding assay results. However, that was not the case. Boar #25 ranked in the middle on his ability to bind to intact oviduct cells. This interesting result supports the hypothesis that there are other components to sperm selection that are missing during *in vitro* assays. How sperm behave within the female reproductive tract is not fully understood as we don't know the specific mechanisms controlling sperm binding and release.

Further development of more physiological assays that include a more viscous media to simulate the viscosity present in the female reproductive tract could identify males with lower fertility more accurately. Challenging sperm with a more viscous media and forcing them to travel to intact oviduct cells or immobilized glycans could provide a more physiological insight to male fertility. The relevance of a predictive assay for fertility is based on its ability to simulate what is occurring *in vivo*. Selecting assays that include aspects that match what is occurring *in vivo* should be helpful to identifying and removing subfertile males from production.

The models provided here predict the mean pregnancy rate and the mean litter size for each boar. Additionally, a fertility index was developed multiplying pregnancy rate by litter size. The fertility of these boars can be predicted with a combination of various traditional and functional assays. Motility and morphology remain strong indicators of the performance of a population of sperm and these results support their continued observation. Furthermore, the inclusion of the sperm-oviduct binding assay to predict pregnancy rate can provide more accurate fertility evaluation.

Currently, no sperm trait alone can predict boar fertility accurately; however, using a combination of traditional and functional sperm analyses can allow for more accurate prediction of both pregnancy rate and litter sizes in males. The predictive models developed in this research can aid in quality control at boar studs to monitor boar fertility. These laboratory assays can be completed before semen is used for insemination to prevent any large economic losses, due to open females and small litter sizes.

Acknowledgements

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Tables and Figures:

Frequency of Matings	
Boar	Number of Matings
1	168
2	198
3	216
4	367
5	200
6	137
7	230
8	115
9	328
10	521
11	262
12	261
13	104
14	356
15	391
16	128
17	357
18	244
19	319
20	467
21	232
22	227
23	86
24	106
25	53
26	357
27	107
28	69
29	73
30	110

Table 3.1: Frequency of the number of matings for each boar. Inseminations occurred between January-July, at 15 different farms over the greater Midwest. All boars had >50 matings.

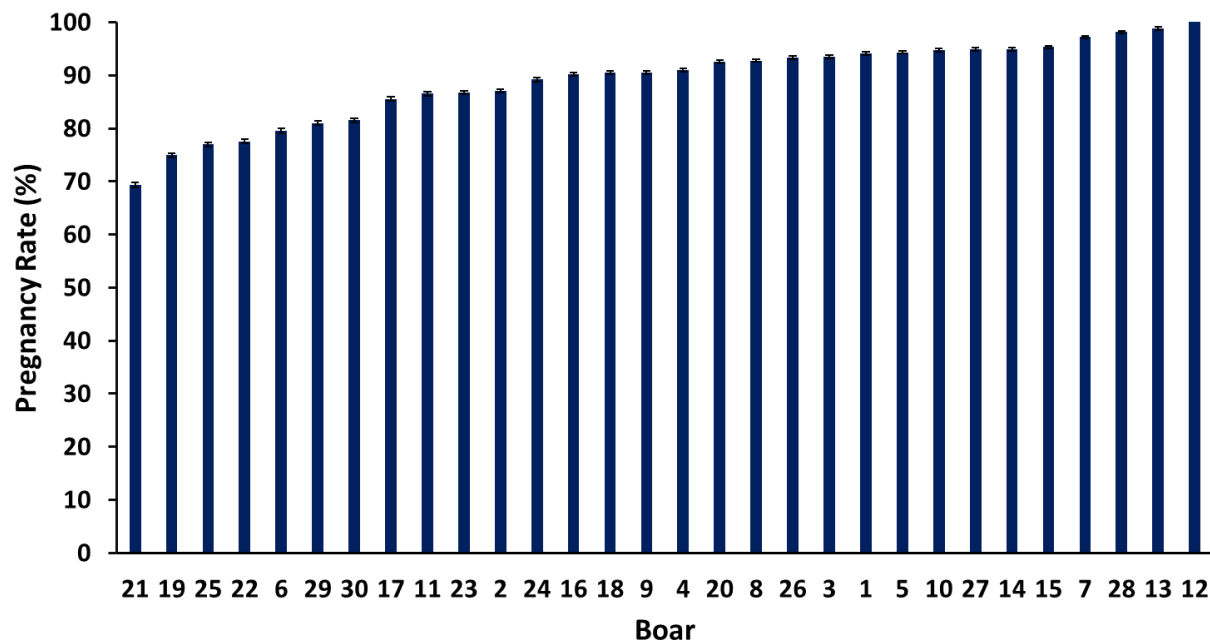


Figure 3.1: Ranking of boars based on their ability to establish pregnancy in females. Fresh semen from 30 different boars was inseminated using homospermic matings, resulting in >50 matings/boar. Pregnancy rate was adjusted by farm, parity, number of services, and number of inseminations using PROC MIXED in SAS 9.4. The mean $\pm$ the standard error is presented.

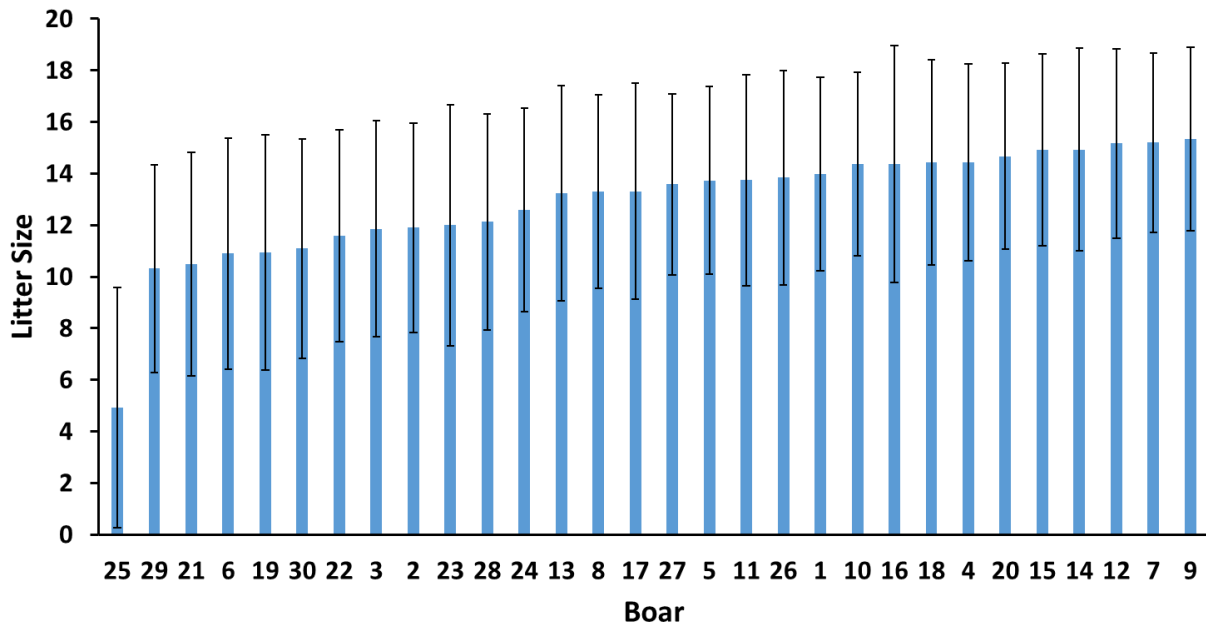


Figure 3.2: Ranking of boars based on their adjusted litter size. Fresh semen from 30 different boars was inseminated using homospermic matings, resulting in >50 matings/boar. Litter size was adjusted by farm, parity, number of services, and number of inseminations using PROC MIXED in SAS 9.4. The mean $\pm$ the standard error is presented

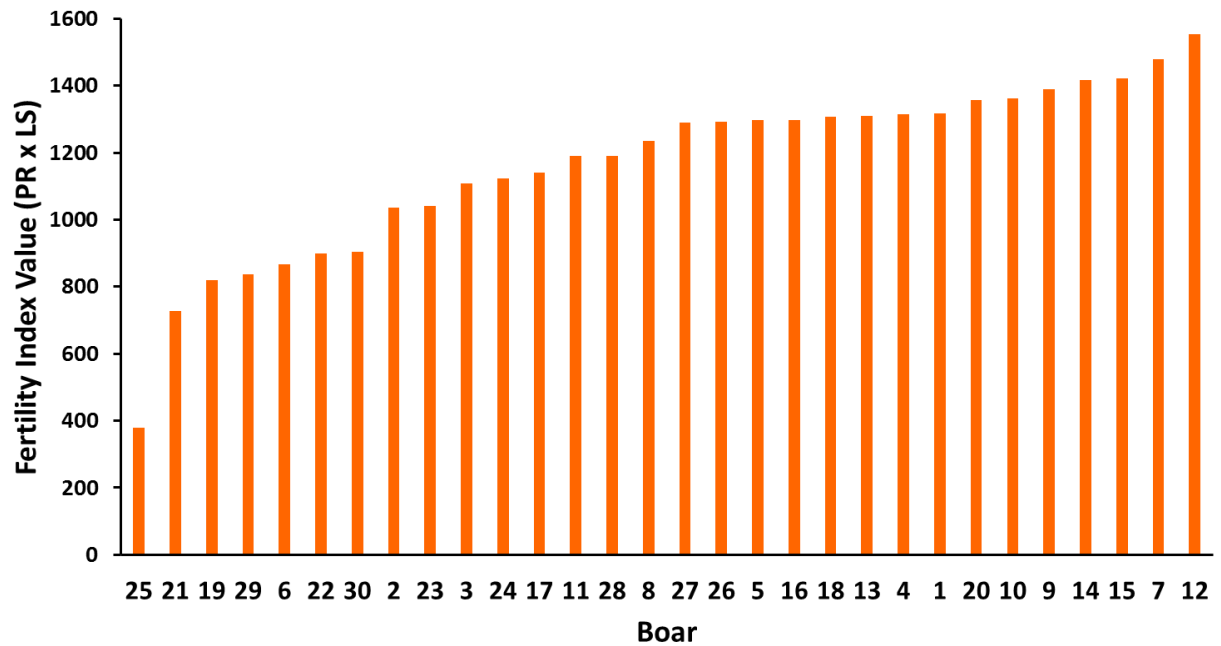


Figure 3.3: Ranking of boars based on their fertility index value. Fertility index was established by multiplying the adjusted pregnancy rate by the adjusted litter size. Both pregnancy rate and litter size were adjusted using PROC MIXED to account for farm, parity, number of services, and number of inseminations used.

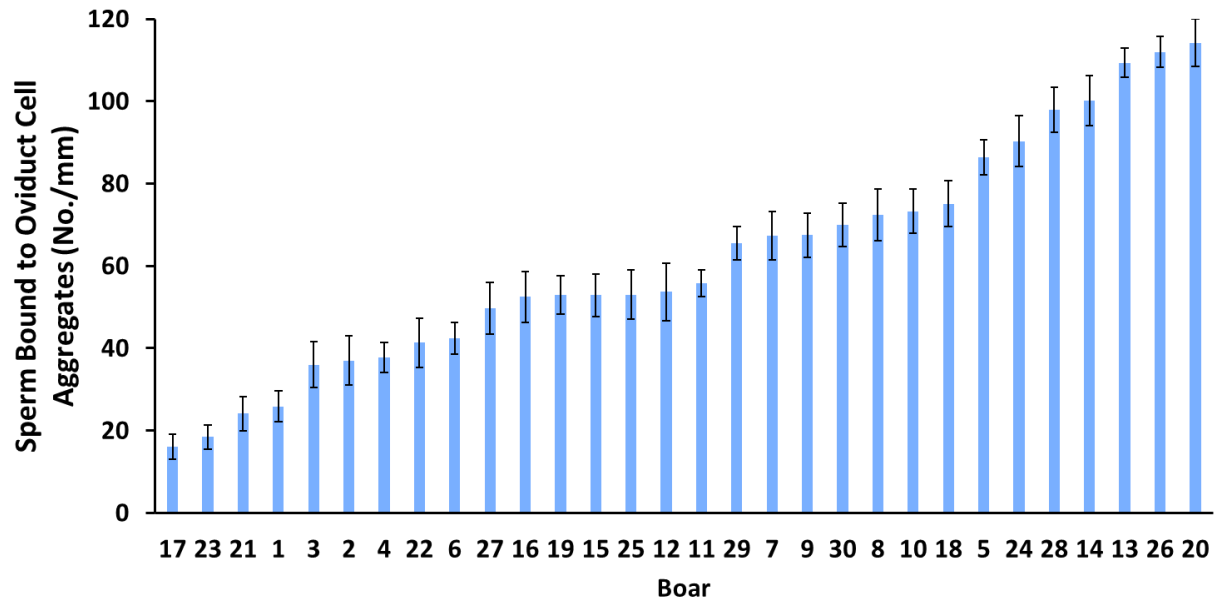


Figure 3.4: Boars ranked based on their ability to bind to oviduct cell aggregates. Sperm from 30 different boars were allowed to co-incubate with fresh oviduct cell aggregates, this assay was replicated 3 times for each boar. Sperm bound to the periphery of these aggregates (n = 30) were counted and normalized to the size of the aggregate. The mean $\pm$ the standard error is presented.

Glycan Binding (%)

Boar	bi-SiaLN	suLe ^x	LacNAc
1	98.20 ± 1.79	95.89 ± 2.75	24.68 ± 2.42
2	97.74 ± 1.82	91.81 ± 4.41	35.10 ± 11.48
3	98.56 ± 0.89	90.08 ± 2.63	36.73 ± 10.77
4	97.12 ± 1.77	94.54 ± 1.73	58.09 ± 18.50
5	97.52 ± 1.77	93.42 ± 2.53	72.01 ± 5.50
6	94.60 ± 6.71	93.07 ± 4.61	50.03 ± 3.22
7	99.54 ± 0.46	97.43 ± 2.39	55.20 ± 26.02
8	97.21 ± 1.50	92.46 ± 6.49	50.67 ± 15.86
9	99.34 ± 0.73	98.61 ± 1.59	59.79 ± 33.99
10	96.91 ± 3.04	89.58 ± 9.45	40.47 ± 11.07
11	94.20 ± 7.63	93.48 ± 4.48	39.03 ± 6.21
12	96.16 ± 4.74	87.39 ± 17.87	47.54 ± 19.75
13	96.54 ± 2.62	94.30 ± 4.40	51.48 ± 17.81
14	97.83 ± 1.55	95.65 ± 4.86	75.44 ± 18.49
15	98.13 ± 1.68	95.95 ± 2.99	48.35 ± 15.66
16	99.68 ± 0.28	96.06 ± 2.83	55.17 ± 25.00
17	99.84 ± 0.28	95.29 ± 3.90	48.92 ± 6.46
18	96.86 ± 1.91	92.55 ± 6.57	37.91 ± 16.88
19	96.42 ± 3.08	93.91 ± 2.24	52.62 ± 5.02
20	99.53 ± 0.01	95.01 ± 5.90	56.18 ± 26.77
21	96.52 ± 4.79	96.91 ± 2.69	51.46 ± 15.64
22	98.59 ± 0.81	85.26 ± 14.73	40.34 ± 17.22
23	97.48 ± 2.81	90.41 ± 12.52	39.13 ± 11.90
24	97.84 ± 3.35	96.77 ± 2.60	52.77 ± 9.37
25	98.26 ± 1.67	87.18 ± 10.03	54.73 ± 5.40
26	98.90 ± 0.96	95.54 ± 3.07	44.19 ± 21.49
27	99.22 ± 0.97	95.86 ± 3.22	40.20 ± 5.20
28	99.04 ± 0.01	97.54 ± 1.40	51.38 ± 22.72
29	97.87 ± 1.11	96.31 ± 3.84	50.86 ± 32.24
30	97.78 ± 1.06	95.90 ± 1.14	60.32 ± 22.87

Table 3.2: Percent of sperm that bound each specific glycan at any region on the sperm head. Boars (n = 30) were analyzed three separate times over the course of the study. Fluorescence detected on the sperm head was considered positive binding. This is the average total binding for each individual glycan ± the standard deviation. No significant differences were detected.

Glycan Binding (%)
bi-SiaLN

Boar	Apical Ridge	Both	Post-Acrosomal	None
1	30.54 ± 5.42	51.31 ± 5.93	16.35 ± 2.04	1.80 ± 1.79
2	34.27 ± 18.52	42.97 ± 27.13	20.50 ± 10.34	2.26 ± 1.82
3	37.52 ± 21.58	42.91 ± 23.43	18.13 ± 3.72	1.44 ± 0.89
4	27.19 ± 5.20	50.40 ± 8.16	19.53 ± 2.25	2.88 ± 1.77
5	43.69 ± 21.04	24.32 ± 13.26	29.51 ± 9.50	2.48 ± 1.77
6	28.99 ± 1.78	40.16 ± 15.43	25.45 ± 10.49	5.40 ± 6.71
7	27.01 ± 9.59	49.56 ± 17.46	22.97 ± 8.97	0.46 ± 0.46
8	42.02 ± 24.12	32.84 ± 25.03	22.35 ± 3.02	2.79 ± 1.50
9	50.64 ± 23.33	27.49 ± 15.15	21.21 ± 9.16	0.66 ± 0.73
10	28.73 ± 4.25	49.12 ± 12.61	17.71 ± 1.77	2.30 ± 3.58
11	18.34 ± 8.52	51.50 ± 27.53	24.35 ± 11.45	5.80 ± 7.63
12	22.02 ± 2.49	55.61 ± 13.89	18.52 ± 14.49	3.84 ± 4.74
13	26.44 ± 0.06	44.04 ± 3.90	26.07 ± 1.80	3.46 ± 2.62
14	37.02 ± 7.27	34.68 ± 8.87	26.13 ± 1.48	2.17 ± 1.55
15	28.70 ± 10.24	48.59 ± 7.31	20.84 ± 7.57	1.87 ± 1.68
16	42.69 ± 18.34	35.55 ± 25.26	21.44 ± 11.33	0.32 ± 0.28
17	31.80 ± 15.77	44.59 ± 17.89	23.44 ± 3.23	0.16 ± 0.28
18	15.34 ± 6.12	69.38 ± 5.24	12.30 ± 1.74	2.98 ± 2.18
19	27.45 ± 7.29	45.18 ± 19.86	23.79 ± 12.85	3.58 ± 3.09
20	47.86 ± 24.59	36.65 ± 23.56	15.02 ± 2.17	0.47 ± 0.01
21	19.15 ± 10.52	61.89 ± 24.27	15.49 ± 9.35	3.48 ± 4.79
22	32.33 ± 7.96	43.10 ± 14.72	23.16 ± 9.48	1.41 ± 0.81
23	37.93 ± 19.55	42.61 ± 29.00	16.94 ± 7.04	2.52 ± 2.81
24	40.41 ± 20.96	38.20 ± 27.03	19.23 ± 6.33	2.16 ± 3.35
25	36.60 ± 9.69	29.95 ± 11.44	31.71 ± 4.78	1.74 ± 0.96
26	24.56 ± 4.70	60.09 ± 8.34	14.25 ± 4.83	1.10 ± 0.96
27	25.16 ± 8.25	44.38 ± 29.02	29.68 ± 21.54	0.78 ± 0.97
28	30.26 ± 18.03	43.33 ± 167.76	25.46 ± 4.89	0.96 ± 0.01
29	32.04 ± 15.71	46.48 ± 25.5	19.35 ± 11.64	2.13 ± 1.11
30	29.85 ± 13.32	38.70 ± 20.81	29.23 ± 9.45	2.22 ± 1.06

Table 3.3a: Percent of sperm bound to bi-SiaLN to specific regions of the sperm head. Four patterns were detected, apical ridge only, apical ridge and post-acrosomal area, post-acrosomal only, and no fluorescence. Three ejaculates were analyzed over the course of the study, for each of the 30 boars. The mean ± the standard deviation is presented. No significant differences were detected.

Glycan Binding (%)
suLe^x

Boar	Apical Ridge	Both	Post-Acrosomal	None
1	31.12 ± 2.20	49.19 ± 7.47	15.58 ± 4.20	4.11 ± 2.75
2	43.01 ± 9.63	19.67 ± 9.78	29.13 ± 12.66	8.19 ± 4.41
3	29.69 ± 15.14	29.13 ± 21.25	31.26 ± 4.94	9.92 ± 2.63
4	23.75 ± 7.96	47.11 ± 14.70	23.68 ± 8.08	5.46 ± 1.73
5	20.03 ± 4.74	48.59 ± 15.00	24.80 ± 11.02	6.58 ± 2.53
6	32.34 ± 8.81	39.09 ± 17.38	21.63 ± 5.85	6.93 ± 4.61
7	29.50 ± 6.03	45.45 ± 10.00	22.98 ± 5.21	2.07 ± 1.79
8	36.36 ± 10.17	33.19 ± 14.66	22.91 ± 2.22	7.54 ± 6.49
9	47.32 ± 35.44	36.81 ± 30.53	14.48 ± 5.06	1.39 ± 1.59
10	32.03 ± 4.42	38.76 ± 19.42	19.93 ± 4.79	9.28 ± 10.55
11	30.31 ± 12.31	38.60 ± 21.24	24.57 ± 9.72	6.52 ± 4.48
12	28.67 ± 10.37	35.62 ± 13.46	23.10 ± 6.92	12.61 ± 17.87
13	23.44 ± 5.59	48.00 ± 16.79	22.87 ± 8.60	5.70 ± 4.40
14	46.33 ± 19.98	27.25 ± 22.75	22.07 ± 2.64	4.35 ± 4.86
15	30.78 ± 15.46	50.45 ± 21.15	14.72 ± 5.73	4.05 ± 2.99
16	29.75 ± 23.95	49.16 ± 28.28	17.14 ± 2.29	3.94 ± 2.83
17	20.64 ± 3.20	58.10 ± 6.33	16.55 ± 1.44	4.71 ± 3.90
18	19.82 ± 8.90	56.01 ± 11.38	16.58 ± 7.55	7.59 ± 6.66
19	29.76 ± 10.98	37.36 ± 15.00	26.78 ± 4.96	6.09 ± 2.24
20	35.85 ± 7.58	34.64 ± 21.09	24.53 ± 11.25	4.99 ± 5.90
21	37.13 ± 13.24	43.43 ± 17.83	16.35 ± 2.90	3.09 ± 2.69
22	42.38 ± 3.29	18.74 ± 14.13	24.13 ± 8.36	5.13 ± 2.24
23	35.22 ± 15.77	22.98 ± 25.56	32.21 ± 10.63	9.59 ± 12.52
24	31.96 ± 9.40	42.03 ± 9.32	22.78 ± 4.40	3.23 ± 2.60
25	24.68 ± 8.60	33.03 ± 19.66	29.48 ± 14.06	12.82 ± 10.03
26	33.82 ± 24.98	47.17 ± 24.91	14.56 ± 2.42	4.46 ± 3.07
27	46.70 ± 23.76	32.38 ± 23.75	16.77 ± 5.82	4.14 ± 3.22
28	16.62 ± 6.22	61.43 ± 6.8	19.48 ± 1.37	2.46 ± 1.40
29	29.52 ± 6.22	46.48 ± 9.43	20.30 ± 1.39	3.69 ± 3.84
30	23.84 ± 14.42	52.39 ± 12.94	19.68 ± 5.35	4.10 ± 1.14

Table 3.3b: Percent of sperm bound to suLe^x to specific regions of the sperm head. Four patterns were detected, apical ridge only, apical ridge and post-acrosomal area, post-acrosomal only, and no fluorescence. Three ejaculates were analyzed over the course of the study for each of the 30 boars. The mean ± the standard deviation is presented. No significant differences were detected.

Glycan Binding (%)
LacNAc

Boar	Apical Ridge	Both	Post-Acrosomal	None
1	10.08 ± 2.57	2.46 ± 2.31	12.15 ± 0.53	75.32 ± 2.42
2	14.13 ± 9.02	3.15 ± 2.43	17.65 ± 5.52	66.46 ± 13.02
3	8.47 ± 2.65	3.76 ± 0.49	24.49 ± 7.82	63.27 ± 10.77
4	20.99 ± 17.36	2.06 ± 1.99	35.04 ± 2.69	41.91 ± 18.50
5	25.58 ± 12.49	4.35 ± 4.69	42.08 ± 13.35	27.99 ± 5.50
6	16.85 ± 4.52	5.52 ± 2.90	27.67 ± 4.59	49.97 ± 3.22
7	24.02 ± 15.77	1.41 ± 0.96	30.03 ± 11.54	45.49 ± 21.30
8	19.16 ± 7.74	3.85 ± 2.03	27.66 ± 9.58	49.33 ± 15.86
9	37.22 ± 33.21	0.65 ± 0.56	32.21 ± 14.07	40.21 ± 33.99
10	21.92 ± 4.94	3.66 ± 1.65	18.49 ± 4.55	55.93 ± 9.96
11	11.36 ± 8.56	2.74 ± 2.55	24.93 ± 4.87	60.97 ± 6.21
12	15.64 ± 11.26	1.85 ± 0.93	30.05 ± 10.35	52.46 ± 19.75
13	15.22 ± 4.68	2.71 ± 1.08	33.55 ± 14.20	48.52 ± 17.81
14	25.90 ± 14.08	1.13 ± 1.01	48.40 ± 23.71	24.56 ± 18.49
15	8.60 ± 3.01	2.45 ± 2.47	37.29 ± 18.82	51.65 ± 15.66
16	25.37 ± 22.55	1.41 ± 0.86	28.38 ± 7.08	44.83 ± 25.00
17	21.66 ± 9.14	1.59 ± 1.08	25.68 ± 5.58	51.08 ± 6.46
18	9.35 ± 3.45	1.36 ± 1.32	17.78 ± 2.74	71.51 ± 0.65
19	17.31 ± 4.24	3.66 ± 1.58	31.64 ± 8.27	47.38 ± 5.02
20	25.15 ± 11.77	2.69 ± 2.66	28.35 ± 18.64	46.59 ± 23.63
21	20.07 ± 13.05	1.71 ± 2.13	29.68 ± 4.99	48.08 ± 15.13
22	11.25 ± 6.48	1.78 ± 0.42	27.31 ± 10.68	59.66 ± 17.22
23	7.79 ± 2.33	3.77 ± 1.45	27.57 ± 9.24	60.87 ± 11.90
24	19.53 ± 2.78	1.42 ± 0.41	31.81 ± 11.71	47.23 ± 9.37
25	16.32 ± 4.18	4.72 ± 3.00	34.69 ± 2.64	46.12 ± 5.67
26	23.05 ± 14.10	3.12 ± 2.12	18.01 ± 9.86	55.81 ± 21.49
27	19.27 ± 2.28	1.11 ± 0.58	19.31 ± 5.52	60.31 ± 6.06
28	20.67 ± 16.71	2.29 ± 1.18	28.42 ± 6.50	48.62 ± 22.72
29	14.12 ± 8.50	3.56 ± 2.26	33.20 ± 27.04	49.14 ± 32.24
30	20.65 ± 13.07	1.90 ± 1.84	37.48 ± 12.55	39.97 ± 22.89

Table 3.3c: Percent of sperm bound to LacNAc to specific regions of the sperm head. Four patterns were detected, apical ridge only, apical ridge and post-acrosomal area, post-acrosomal only, and no fluorescence. Three ejaculates were analyzed over the course of the study for each of the 30 boars. The mean ± the standard deviation is presented. No significant differences were detected.

Glycan Binding (%)
Total Apical Ridge

Boar	bi-SiaLN	suLe^x	LacNAc
1	81.85 ± 3.72	32.91 ± 2.46	12.53 ± 2.84
2	77.24 ± 9.14	45.27 ± 10.27	17.28 ± 6.93
3	80.43 ± 3.00	31.13 ± 14.69	12.23 ± 2.95
4	68.01 ± 8.85	22.51 ± 3.48	29.94 ± 9.79
5	69.15 ± 13.93	37.75 ± 15.38	22.36 ± 1.94
6	76.57 ± 8.63	29.97 ± 5.58	25.43 ± 14.84
7	74.86 ± 3.69	39.15 ± 11.18	23.01 ± 6.43
8	74.86 ± 3.69	39.15 ± 11.18	23.01 ± 6.43
9	78.13 ± 8.59	47.98 ± 36.13	37.86 ± 32.74
10	77.85 ± 9.02	34.33 ± 7.94	25.58 ± 6.56
11	69.84 ± 19.02	36.11 ± 17.47	14.10 ± 7.44
12	77.64 ± 11.44	32.52 ± 9.62	17.49 ± 10.42
13	70.48 ± 3.95	26.90 ± 7.30	17.93 ± 3.67
14	71.70 ± 2.62	48.50 ± 19.89	27.03 ± 13.22
15	77.29 ± 6.13	32.65 ± 15.91	11.06 ± 4.75
16	78.24 ± 11.47	30.08 ± 24.10	26.78 ± 22.76
17	76.40 ± 2.96	20.80 ± 3.48	23.24 ± 8.10
18	84.72 ± 3.86	22.80 ± 11.07	10.71 ± 2.18
19	72.63 ± 15.13	33.34 ± 13.63	20.98 ± 5.82
20	84.51 ± 2.17	36.32 ± 7.59	27.83 ± 9.14
21	81.04 ± 14.10	40.61 ± 17.93	21.78 ± 11.98
22	75.43 ± 10.29	43.79 ± 3.45	13.03 ± 6.62
23	80.55 ± 9.59	37.73 ± 14.39	11.56 ± 3.48
24	78.61 ± 8.44	34.12 ± 11.03	20.96 ± 2.42
25	66.55 ± 3.52	26.42 ± 9.49	21.05 ± 3.09
26	84.65 ± 4.92	34.91 ± 25.51	26.17 ± 12.34
27	69.54 ± 20.87	47.48 ± 23.91	20.38 ± 1.69
28	73.59 ± 4.89	17.58 ± 6.23	22.96 ± 17.12
29	78.52 ± 11.75	31.65 ± 13.23	17.68 ± 6.24
30	68.55 ± 9.79	26.06 ± 13.37	22.55 ± 11.22

Table 3.4a: Specific binding to the apical ridge of the sperm head by the three soluble fluoresceinated glycans. The three patterns on the sperm head were apical ridge only, apical ridge and post-acrosomal area, and post-acrosomal only. Patterns were re-grouped as apical ridge + apical ridge and post-acrosomal to have a total percent of sperm binding to the edge of the head which would be in contact with oviduct cells. Three ejaculates were analyzed over the course of the study for each of the 30 boars. Fluorescence detected on the sperm head was considered positive binding. The mean ± the standard deviation is presented. No significant differences were detected.

Glycan Binding (%)
Total Post-Acrosomal Binding

Boar	bi-SiaLN	suLe ^x	LacNAc
1	67.67 ± 5.81	64.77 ± 3.27	14.61 ± 2.40
2	63.47 ± 16.95	48.80 ± 11.86	20.80 ± 3.12
3	61.04 ± 21.69	60.39 ± 16.60	28.25 ± 8.17
4	53.83 ± 20.94	73.39 ± 3.98	46.43 ± 14.41
5	65.61 ± 7.21	60.73 ± 12.63	33.19 ± 5.86
6	72.52 ± 9.50	68.43 ± 4.85	31.44 ± 10.96
7	55.19 ± 25.44	56.10 ± 16.50	31.52 ± 8.15
8	55.19 ± 25.44	56.10 ± 16.50	31.52 ± 8.15
9	48.71 ± 23.99	51.29 ± 34.56	32.86 ± 13.71
10	66.84 ± 10.99	58.69 ± 14.92	22.15 ± 5.47
11	75.85 ± 16.14	63.17 ± 15.50	27.67 ± 3.84
12	74.13 ± 3.53	58.71 ± 14.63	31.89 ± 9.44
13	70.11 ± 2.58	70.86 ± 8.59	36.26 ± 13.34
14	60.81 ± 8.64	49.32 ± 20.21	49.53 ± 23.84
15	69.43 ± 10.67	65.17 ± 16.85	39.75 ± 16.39
16	56.99 ± 18.62	66.31 ± 26.45	29.80 ± 6.32
17	68.04 ± 15.64	74.66 ± 7.06	27.27 ± 6.04
18	81.68 ± 5.66	72.59 ± 7.42	19.14 ± 3.95
19	68.97 ± 7.54	64.15 ± 12.47	35.30 ± 7.09
20	51.67 ± 24.60	59.16 ± 12.94	31.04 ± 16.75
21	77.38 ± 14.99	59.78 ± 15.88	31.38 ± 2.96
22	66.27 ± 8.24	42.88 ± 17.66	29.09 ± 10.77
23	59.55 ± 22.34	55.19 ± 14.94	31.33 ± 9.69
24	57.43 ± 21.06	64.81 ± 9.99	33.24 ± 12.01
25	61.66 ± 8.13	62.51 ± 11.79	39.41 ± 3.09
26	74.34 ± 4.04	61.72 ± 22.95	21.13 ± 7.76
27	74.07 ± 7.79	49.16 ± 20.57	20.42 ± 5.45
28	68.79 ± 18.04	80.91 ± 5.47	30.71 ± 6.32
29	65.84 ± 15.10	66.79 ± 8.67	36.75 ± 25.19
30	67.94 ± 12.82	72.06 ± 3.73	39.38 ± 11.05

Table 3.4b: Specific binding to the post-acrosomal area of the sperm head by the three soluble fluoresceinated glycans. The three patterns on the sperm head were apical ridge only, apical ridge and post-acrosomal area, and post-acrosomal only. Patterns were re-grouped as apical ridge and post-acrosomal area + post-acrosomal area only to allow for grouping of sperm with potentially leaky membranes. Three ejaculates were analyzed over the course of the study for each of the 30 boars. Fluorescence detected on the sperm head was considered positive binding. The mean ± the standard deviation is presented. No significant differences were detected.

Glycan Binding vs. Oviduct Binding

Glycan	Estimate	Standard Error	<i>t</i> -Value	R ²	Adj R ²
Total Overall bi-SiaLN	2.20	1.33	1.66	0.03	0.02
Total Overall suLe ^x	1.53	0.55	2.77	0.08	0.07
Total Overall LacNAc	0.33	0.20	1.67	0.03	0.02
Total Apical Ridge bi-SiaLN	0.17	0.38	0.44	0.00	-0.01
Total Apical Ridge suLe ^x	-0.26	0.24	-1.05	0.01	0.00
Total Apical Ridge LacNAc	0.33	0.32	1.03	0.01	0.00
Total Post-Acrosomal bi-SiaLN	0.02	0.25	0.07	0.00	-0.01
Total Post-Acrosomal suLe ^x	0.43	0.23	1.85	0.04	0.03
Total Post-Acrosomal LacNAc	0.38	0.30	1.27	0.02	0.01

Table 3.5: The correlation of sperm binding soluble fluoresceinated glycans to sperm binding intact oviduct cells. The two groups of total apical ridge binding and total post-acrosomal binding regressed against oviduct cell binding, resulting in individual R² values for each glycan. Boars (n = 30) were analyzed three separate times over the course of the study.

Pregnancy Rate vs. Glycan Binding

Glycan	Estimate	Standard Error	t -Value	R ²	Adj R ²
Total Overall bi-SiaLN	0.22	0.18	1.22	0.02	0.01
Total Overall suLeX	0.07	0.08	0.88	0.01	0.00
Total Overall LacNAc	<0.01	0.03	0.16	0.00	-0.01

Litter Size vs. Glycan Binding

Glycan	Estimate	Standard Error	t -Value	R ²	Adj R ²
Total Overall bi-SiaLN	0.06	0.09	0.70	0.01	-0.01
Total Overall suLeX	0.06	0.04	1.68	0.03	0.02
Total Overall LacNAc	<0.01	0.01	0.18	0.00	-0.01

Fertility Index vs. Glycan Binding

Glycan	Estimate	Standard Error	t -Value	R ²	Adj R ²
Total Overall bi-SiaLN	7.88	9.06	0.87	0.01	0.00
Total Overall suLeX	5.89	3.84	1.54	0.03	0.02
Total Overall LacNAc	0.33	1.35	0.25	0.00	-0.01

Table 3.6: Binding to soluble fluoresceinated glycans was not correlated with pregnancy rate, litter size, or fertility index. Sperm from each boar was evaluated three times over the course of the study for each of the 30 boars. Average total glycan binding for each of the three glycans regressed against PR, LS, and FI.

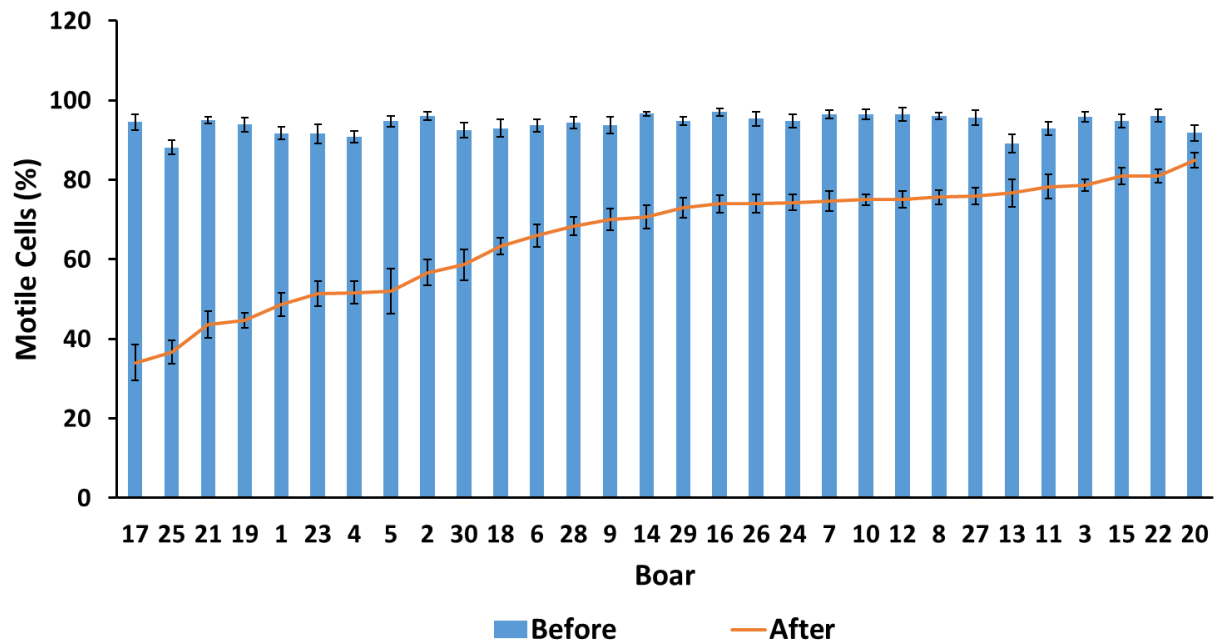


Figure 3.5: Boars ranked on the percent of motile cells after shipment. Fresh semen was analyzed three times from 30 different boars before (blue bars) and after shipment (orange line). The mean $\pm$ the standard error is presented.

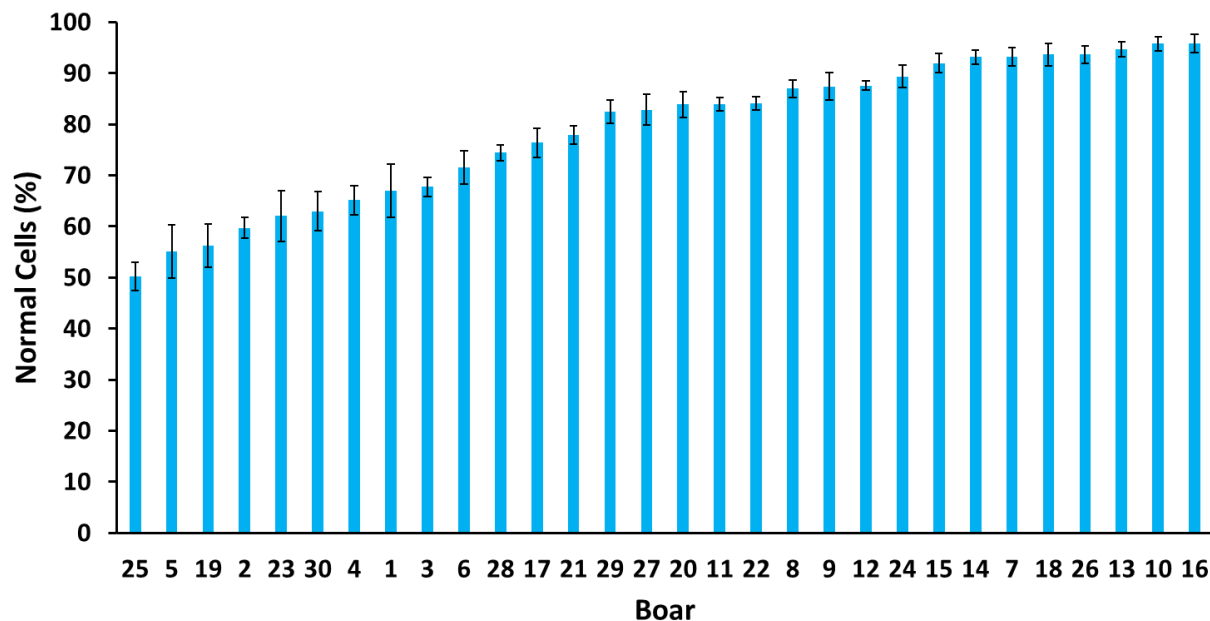


Figure 3.6: Boars ranked on the percent of normal sperm cell morphology. Fresh boar sperm from 30 boars were fixed and stained with Coomassie Blue G250 three separate times over the course of the trial. Sperm were classified as normal if they presented no defects (head/tail problems, proximal/distal droplets, and/or a reacted acrosome). The mean $\pm$ the standard deviation is presented.

Pregnancy Rate Model				
Variable	Estimate	Standard Error	t -Value	p -Value
Intercept	96.31	37.82	2.55	0.01
Boar Stud Entry Date	-0.008	<0.01	-5.68	<0.01
% Normal Tail Morphology (B)	0.47	0.17	2.79	<0.01
% Distal Droplets (B)	0.37	0.09	4.26	<0.01
CASA - VCL (A)	0.04	0.01	2.62	0.01
CASA - BCF (A)	0.50	0.14	3.49	<0.01
% Normal Head Morphology (A)	0.47	0.11	4.20	<0.01
Oviduct Binding	0.03	<0.01	3.32	<0.01

Partial Values for Pregnancy Rate Model			
Variable	Partial R ²	Model R ²	Partial p -Value
% Distal Droplets (B)	0.15	0.15	<0.01
Boar Stud Entry Date	0.15	0.30	<0.01
Oviduct Binding	0.10	0.39	<0.01
% Normal Head Morphology (A)	0.08	0.47	<0.01
% Normal Tail Morphology (B)	0.05	0.52	<0.01
CASA - BCF (A)	0.05	0.57	<0.01
CASA - VCL (A)	0.03	0.60	0.01

Pregnancy Rate Model Summary		
R ²	Adj R ²	p - Value
0.6	0.57	<0.001

Table 3.7: Multiple linear regression analysis to assess the relationship of sperm traits to adjusted pregnancy rate. Three ejaculates from 30 boars were used in homospermic matings and used for laboratory assays. Boar fertility was determined by AI. Pregnancy rate was adjusted by PROC MIXED for farm, parity, number of doses, and the number of inseminations. Variables were selected using PROC GLMSELECT. Boar and sperm traits were regressed against the adjusted mean pregnancy rate using PROC REG. Motility and morphology assessments were performed before **(B)** and after **(A)** shipments.

Litter Size Model				
Variable	Estimate	Standard Error	<i>t</i> -Value	<i>p</i> -Value
Intercept	28.08	8.38	3.35	<0.01
Boar Stud Entry Date	-0.002	<0.01	-4.16	<0.01
% Distal Droplets (B)	0.11	0.03	4.37	<0.01
[Total Sperm/Ejaculate]	0.005	<0.01	2.29	0.02
% Normal Tail Morphology (A)	0.04	0.01	2.84	<0.01
% Total Normal Morphology (A)	0.02	<0.01	3.02	<0.01

Partial Values for Litter Size Model			
Variable	Partial R²	Model R²	Partial <i>p</i> -Value
% Normal Tail Morphology (A)	0.29	0.29	<0.01
% Distal Droplets (B)	0.14	0.43	<0.01
Boar Stud Entry Date	0.08	0.50	<0.01
% Total Normal Morphology (A)	0.05	0.55	<0.01
[Total Sperm/Ejaculate]	0.03	0.58	0.02

Litter Size Model Summary		
R²	Adj R²	<i>p</i> - Value
0.58	0.55	<0.001

Table 3.8: Multiple linear regression analysis to assess the relationship of sperm traits to adjusted litter size. Three ejaculates from 30 boars were used in homospermic matings and used for laboratory assays. Boar fertility was determined by AI. Litter size was adjusted by PROC MIXED for farm, parity, number of doses, and the number of inseminations. Variables were selected using PROC GLMSELECT. Boar and sperm traits were regressed against the adjusted mean litter size using PROC REG. Motility and morphology assessments were performed before (B) and after (A) shipments.

Fertility Index Model				
Variable	Estimate	Standard Error	<i>t</i> -Value	<i>p</i> -Value
Intercept	3771.6	1795.92	2.10	0.04
Boar Stud Entry Date	-0.36	0.07	-5.38	<0.01
Sperm Volume/Ejaculate	0.51	0.23	2.19	0.03
% Normal Tail Morphology (B)	17.34	8.33	2.08	0.04
% Distal Droplets (B)	21.89	4.54	4.82	<0.01
CASA - VCL (A)	1.94	0.7	2.77	<0.01
% Normal Head Morphology (A)	5.4	1.05	5.14	<0.01

Partial Values for Fertility Index Model			
Variable	Partial R ²	Model R ²	Partial <i>p</i> -Value
% Normal Head Morphology (A)	0.28	0.28	<0.01
Boar Stud Entry Date	0.18	0.45	<0.01
% Distal Droplets (B)	0.09	0.54	<0.01
CASA - VCL (A)	0.05	0.59	<0.01
Sperm Volume/Ejaculate	0.02	0.61	0.03
% Normal Tail Morphology (B)	0.02	0.63	0.04

Fertility Index Model Summary		
R ²	Adj R ²	<i>p</i> - Value
0.63	0.61	<0.001

Table 3.9: Multiple linear regression analysis to assess the relationship of sperm traits to the fertility index. Three ejaculates from 30 boars were used in homospermic matings and used for laboratory assays. Boar fertility was determined by AI. Fertility index was developed by multiplying the adjusted pregnancy rate by the adjusted litter size. Variables were selected using PROC GLMSELECT. Boar and sperm traits were regressed against the fertility index value using PROC REG. Motility and morphology assessments were performed before **(B)** and after **(A)** shipments.

CHAPTER 4 - EFFECT OF SORTING BOAR SPERMATOZOA BY SEX CHROMOSOMES ON OVIDUCT CELL BINDING

Abstract

This study examined the hypothesis that flow sorting sperm by sex chromosomes affects oviduct cell binding which would influence formation of the sperm reservoir in the oviduct. The sperm-rich fraction from boars ($n = 5$) was collected, sperm were stained with Hoechst 33342 and sorted. Sperm were sorted based on the presence of either an X or Y chromosome and placed into the following treatments: 1) sperm selected for the Y chromosome, 2) selected for the X, 3) an equal mixture of sorted X and Y, and 4) a control of non-sorted sperm from the same collection. Samples were tested for oviduct cell binding within 12 hr of sorting. Additionally, sperm were analyzed for motility characteristics, acrosome status, and binding to the two oviduct glycan motifs that bind porcine sperm, biantennary 6-sialylated *N*-acetylactosamine on a mannose core (bi-SiaLN) and sulfated Le^x trisaccharide (su Le^x). The disaccharide found within both glycan motifs, *N*-acetylactosamine (LacNAc), was used as a control. Sperm binding to oviduct cells was reduced by more than half in the three sorted samples when compared to the control sperm that were not sorted. The percentage of sperm that were motile was also decreased significantly in each of the sorted sample groups when compared to the unsorted control. In contrast, sorting did not decrease the percentage of sperm that bound purified soluble glycans or the location on sperm to which they bound. There was also no difference in sperm acrosome status among the four groups. In summary, sorting reduced sperm binding to the complex matrix around oviductal cell aggregates but sperm binding to purified soluble oviduct glycans was not affected. The requirement for higher affinity and motility to bind glycans immobilized on

oviduct cells may explain this difference. The reduction in sperm fertility observed following sex-sorting might be explained partially by a reduced ability to bind to the oviduct epithelium.

Introduction

Sorting sperm by sex chromosomes has been practiced successfully for decades and is used regularly in dairy cattle (De Graaf et al., 2014; Johnson and Welch, 1999; Rath and Johnson, 2008; Seidel, 2007). In contrast, sex-sorting of porcine sperm is still undergoing development to make it practical for the swine industry. There is interest in using sex-sorted porcine sperm for several reasons. Increased legislation against physical castration requires alternatives such as sex biasing to reduce the percentage of males, which eliminates castration and the associated costs. It also allows for the improved production of seedstock replacement gilts. The strategic use of sorted sperm can also speed up genetic improvement and the incorporation of transgenic animals into swine industry genetics (Chang et al., 2002; De Cecco et al., 2010; Pereyra-Bonnet et al., 2008).

The sorting index, a value used to approximate the possible sorting efficiency, is defined as the product of the sperm head area (μm^2) and the DNA difference between Y- and X-bearing spermatozoa (Garner 2006). The sorting index of porcine sperm is similar to bovine sperm due to their large flat heads and a 3.6% difference in DNA, making them good candidates for sex-sorting (Garner 2006); however, many factors reduce the feasibility of sorting porcine sperm. Similar to sperm from many species, porcine sperm are damaged by staining (Spinaci et al. 2005; Vazquez et al. 2002), the physical stress of sorting (Spinaci et al. 2005; Suh, Schenk, and Seidel 2005; De Ambrogi et al. 2006) and the high dilution rates sperm undergo during sorting

(Centurion et al., 2003; Maxwell and Johnson, 1999). In addition, there are two major challenges to overcome to use sex-sorted porcine sperm. First, it takes a long time to sort an adequate number of sperm for an insemination dose. Flow cytometers currently sort 20×10^6 sperm cells per hour (Suh et al., 2005). Traditional practice for swine insemination is to inseminate approximately 3×10^9 sperm twice about 24 hr apart (Knox 2016). A common AI dose in cattle is 10 to 20×10^6 sperm and they are inseminated once per estrus (Foote et al., 1997). An AI dose of bovine sex-sorted sperm contains fewer sperm than a dose of non-sorted sperm, which makes sorting bovine sperm practical. Methods of reducing the number of sperm needed for fertility, such as deep uterine insemination (DUI) (Bathgate et al., 2007; Grossfeld et al., 2005; Martinez et al., 2002; Martinez et al., 2006; Vazquez et al., 2003), in-vitro fertilization (IVF) (Abeydeera et al., 1998; Rath et al., 1997; Rath et al., 1999), intracytoplasmic sperm injection (ICSI) (Probst and Rath, 2003), and surgical insemination (del Olmo et al., 2014; Fantinati et al., 2005) have allowed the production of offspring from sorted porcine sperm. Deep uterine insemination of as few as 50 to 70×10^6 sperm following ovulation induction, produced similar numbers of piglets as conventional AI using the same concentrations (Vazquez et al. 2003; Grossfeld et al. 2005). Furthermore, double deposition laparoscopic insemination (oviducts and uterine horns) of sows with a low dose of 3×10^6 per insemination resulted in an average litter size of 9.2 ± 0.6 piglets (del Olmo et al. 2014).

In addition to the long sorting time, the second challenge in using sex-sorted boar sperm is a lack of post-sorting storage methods. Rather than freezing, liquid storage is most often used, due to poor fertility following cryopreservation, so it is critical to have a liquid extender adequate to prolong the lifespan of the sorted spermatozoa. Additives to extender such as egg yolk (del Olmo et al. 2013), spermadhesins such as PSP-I/PSP-II (Centurion et al., 2003; García et al.,

2007), and antioxidants (Vallorani et al., 2010; Xia et al., 2012) have improved viability as well as fertilizing ability. Still, IVF was necessary to produce offspring from sex-sorted frozen-thawed sperm (Bathgate et al., 2007).

We hypothesized that one reason for lower fertility of sex-sorted porcine semen is that sorting causes capacitation-like changes to occur. Sperm that are capacitated are less able to bind the oviduct epithelium and form the sperm reservoir that supplies fertile sperm to the site of fertilization (Fazeli et al. 1999; Gualtieri and Talevi 2000; Petrunkina et al. 2001). Modifications of the sperm membrane induced by sorting could affect membrane function and promote capacitation (Spinaci et al. 2006). Membrane stability may also be affected by the removal of seminal plasma components from sperm during sorting, which otherwise inhibit capacitation and help maintain an intact acrosome (Maxwell and Johnson, 1999).

The sperm reservoir in the porcine oviduct is formed by retention of sperm by oviduct epithelial glycans containing two motifs, either a biantennary 6-sialylated *N*-acetylactosamine on a mannose core (bi-SiaLN) or a Lewis^X trisaccharide (Le^X) (Kadirvel et al. 2012; Machado et al. 2014). Both glycan motifs bind with high affinity to the head of sperm prior to capacitation and are necessary for sperm binding to the oviduct (Kadirvel et al. 2012; Machado et al. 2014).

In this study, our aim was to assess the effect of sex-sorting on the ability of sperm to bind oviduct epithelial cells and soluble oviduct glycans. The results could be used to understand the changes that occur during the sex-sorting process and perhaps devise techniques to improve the fertility of sex-sorted sperm.

Materials and Methods

Sperm Collection and Sorting

Commercial boars (n = 5) of proven fertility, housed in Wisconsin, were collected twice from July – September of 2015 for this project. The sperm rich fraction was collected using the gloved-hand technique, diluted 1:1 in a pre-warmed (36° C) proprietary media and transported to the laboratory. Semen samples were evaluated for concentration, total motility, and normal morphology characteristics. Samples were then diluted to $200 \times 10^6/\text{ml}$ with the same dilution medium and stored at 17° C overnight. Semen (1 ml) was stained with both 5 μl of Hoechst-33342 (from a stock of 5 mg/ml) and 3 μl of FD&C Yellow 6 (Sigma Aldrich, St. Louis, MO USA) from a 1:5 stock solution diluted in ddH₂O and incubated in the dark for 60 min at 34° C. Following incubation semen was filtered through a 30 μM filter into a new 5 ml flow tube. The X and Y chromosome-bearing sperm were separated using a flow sorter (Genesis MoFlo[®], Cytonome-ST, Boston, MA, USA) operated at 40 psi with a UV laser (Spectra Physics, Mountain View, CA USA). Sperm were sorted using a Tris based proprietary medium as the sheath fluid and collected in a 50 ml conical tube containing sheath fluid and 2% egg yolk. A total of 20×10^6 sperm/ml in 25 mL were collected, centrifuged at 2400 X g for 4 min and the pellets resuspended in Tris based proprietary extender at a concentration of 30×10^6 sperm/ml. Following sorting, the samples were analyzed for purity (more than 93% of sperm had the expected sex chromosome, Table 1). The motility of each population was over 85%, the viability over 90% and the percentage of morphologically normal cells was over 80% (Table 1). Sperm were cooled to 17° C and transported immediately to the laboratory at the University of Illinois.

Motility and Membrane Analyses of Spermatozoa

Post-sort motility and acrosome integrity were evaluated for each of the following treatments 1) sperm sorted for the Y chromosome (Y-bearing), 2) sorted for the X (X-bearing), 3) an equal mixture of sorted X and Y (XY-remixed), and 4) a control of non-sorted sperm from the same collection (C-not sorted). Motility analyses were conducted using Computer Assisted Sperm Analysis (Hamilton Thorne, Beverly, MA, USA). Sperm were warmed for 10 min at 38° C and 3 µl of each sample were loaded onto a pre-warmed 4-chamber Leja4 slide (IMV Technologies, Maple Grove, MN, USA). A minimum of 8 fields and 750 cells were observed at 37° C and 45 frames were acquired at a rate of 60Hz with a minimum contrast of 55, minimum cell size of five pixels, and magnification of 1.73. Samples were measured at approximately 24 hr after sorting. Parameters included total motility and progressive motility.

Coomassie Blue staining was done to determine acrosome integrity for each sample (Larson and Miller, 1999). Around 24 hr post-sort, 10 µl of spermatozoa from each group were fixed with 4% paraformaldehyde to fix cells. Cells were centrifuged at 2000 X g for 1 min, supernatant was discarded and the pellet resuspended in 50 µl of 0.1M ammonium acetate. The suspension was centrifuged again at 2000 X g for 1 min, supernatant discarded and pellet resuspended in 25 µl of ammonium acetate. The sperm suspension (10 µl) was placed onto a slide and allowed to air dry. Dried slides were stained with Coomassie Blue G-250 solution for 4 min. Slides were rinsed with distilled water and allowed to air dry. A drop of 1X PBS was added and topped with a cover glass. A minimum of 300 sperm/sample was evaluated using a Zeiss Axioskop and Axio-CamHRc (Zeiss Microscopy, LLC, Thornwood, NY, USA) at 400X magnification. Spermatozoa with a stained acrosome were considered to be acrosome intact.

Oviduct-Sperm Binding Assay

One ejaculate from each of the 5 boars was tested on an experimental day. Oviduct-sperm binding assays were completed on the same day as sorting. For each experiment, female reproductive tracts were collected from pre- and post-pubertal females at an abattoir. Tracts were brought back to the lab and 20 to 30 oviducts were removed and held in 1X PBS on ice before being processed the same day. Oviducts were transferred to a 100-mm petri dish containing 1X PBS and the ampulla portion was separated from the isthmus. Epithelial sheets from the isthmus were collected by gently pressing down a glass slide at a 45-degree angle and squeezing the sheets out. The epithelial sheets were then transferred to a 15 ml conical tube and centrifuged at 100 X g for 30 sec. After supernatant was removed, cells were re-suspended in 1 ml of capacitating dmTALPC (2.1 mM CaCl₂, 3.1 mM KCl, 1.5 mM MgCl₂, 100 mM NaCl, 0.29 mM KH₂PO₄, 0.36% lactic acid, 0.6% Fraction V BSA, 1 mM pyruvic acid, 25 mM HEPES [pH 7.3], sterile filtered). Cells were disaggregated by passage through a 1 ml pipette 10 times. Partially disaggregated cells were washed with 5 ml of dmTALPC and centrifuged at 100 X g for 1 min. Supernatant was removed and cells were re-suspended in 1 ml of dmTALPC. The cells were passed through a 23-gauge needle 10 times for further disaggregation, the volume was adjusted to 9 ml with dmTALPC and suspended cells were divided evenly into three 100-mm petri dishes. The cells were allowed to reaggregate for 1.5 to 2 hr at 39° C. Spherical aggregates (100 to 150 µm in diameter) were selected for the experiments.

About 40 spherical oviduct cell aggregates were selected and washed twice in 100 µl drops containing fresh dmTALPC. A Stripper Pipette (MidAtlantic Diagnostics, Inc. Marlton, NJ, USA) with a 250 µm internal diameter tip was used to collect oviduct epithelial cell aggregates and wash them. Oviduct cell aggregates were moved to a drop containing a final

volume of 50 μ l with 1×10^6 sperm/ml for co-incubation at 39° C for 15 min to allow sperm to bind to the aggregates. Each group contained three 50 μ l drops with 10 oviduct cell aggregates each. After incubation, the sperm-bound aggregates were transferred onto a microscope slide with a volume of 3 μ l. Each slide corresponded to one group. Each droplet with 10 sperm-bound aggregates was considered an experimental unit for statistical analysis. Images were captured using a Zeiss Axioskop and AxioCam HRc digital camera (Carl Zeiss, Thornwood, NY, USA) at 400X magnification. The number of sperm bound to the periphery of each aggregate was counted and the circumference for each aggregate was calculated using AxioVision V 4.5 software (Carl Zeiss). The number of sperm bound was divided by the linear periphery of the aggregate to normalize for differences in aggregate size.

Fluorescent Glycan Binding Assay

Localization of glycan-binding receptors in live spermatozoa was determined by binding of fluoresceinated glycans to spermatozoa. Sperm from the four groups (Y-bearing, X-bearing, XY-remixed and C-not sorted) were held overnight at 17° C. Sperm were warmed at 37° C for 10 min and the extender was removed. Sperm were centrifuged at 2400 X g for 1 min, and extender was removed and the pellet was resuspended in dmTALPC. The sperm concentration was brought to 10×10^6 sperm/ml with dmTALPC. An aliquot of 38 μ l of the sperm suspension was transferred to a microcentrifuge tube and 2 μ l of fluorescent glycan was added (final concentration of 50 μ g/ml). Glycans used were biantennary 6-sialylated *N*-acetylglucosamine (bi-SiaLN), sulfated Lewis^x (suLe^x), and *N*-acetylglucosamine (LacNAc). Multiple copies of each glycan were attached to a 30 kDa linear polyacrylamide chain providing the multivalency often observed with glycoproteins (Varki and Lowe, 2009). *N*-acetylglucosamine was used as a control

for nonspecific binding, because this disaccharide is present in both motifs. The sperm were incubated with glycans for 30 min at 39° C. A small drop of 4 µl from the suspension was placed on a slide and covered with a coverslip. A minimum of 300 sperm was counted for each glycan, at 630X magnification. Each of the three glycans was tested for all four treatment groups for each boar.

Statistical Analyses

Differences among means for oviduct binding and motility were determined by a one-way analysis of variance using SAS statistical software (v. 9.4 SAS Institute, Inc, Cary, NC, USA). The results are shown as means $\pm$ SEM and treatments were considered different when $P < 0.05$. Post-hoc analysis was conducted using Tukey's HSD for multiple comparisons when data were consistent with normality assumptions.

Results

To determine if sorting by sex chromosomes affects sperm binding to oviduct cells, sorted populations of sperm and unsorted control sperm were incubated with oviduct cell aggregates (n = 30 aggregates/treatment/boar) and binding was observed and normalized to the periphery of each aggregate. One group contained sperm sorted and selected for the Y chromosome, another sorted for the X chromosome (both greater than 93% pure), and another contained an equal mixture of X- and Y-bearing sperm. The unsorted control sperm were not processed through a flow cytometer. The normalized average of sperm bound to oviduct cell aggregates was 50.3 sperm per mm for the Y-bearing group, 53.8 sperm per mm for the X-

bearing sample, 51.7 sperm per mm in the XY remixed and 105.5 sperm per mm for the unsorted control (Figure 4.1). Y-bearing sperm, X-bearing sperm and equal mixture of X- and Y-bearing sperm showed a reduction of more than 50% in their ability to bind to oviduct cell aggregates compared to the unsorted control ($P < 0.05$). One explanation for this reduction is that the function of the sperm plasma membrane and its adhesion receptors for oviduct cells is altered by sorting (Maxwell and Johnson 1999; Spinaci et al. 2006).

Other possible explanations for the reduction in sperm binding to oviduct cells are a reduction in sperm motility or damage indicated by abnormal morphology. Sperm with decreased motility will collide less frequently with oviduct cells, reducing the opportunity to bind to cells *in vitro* or *in vivo*. Therefore, we observed sperm motility following sorting, using computer assisted sperm analysis. The percent of motile cells remained the same among the four sample groups shortly after sorting (Table 4.1). Flow cytometry removes a majority of sperm cells that are damaged and there was no difference in the percent of normal cells after sorting between the Y- and X-bearing sperm groups (Table 4.1). Viability immediately after sorting remained high in both sample groups, around 92% (Table 4.1). Motility, progressive motility, and viability were also examined, approximately 24 hr after sorting, following transport. At that time, on average, the percentage of sperm that were motile in all three of the sorted samples was reduced by 15% compared to the unsorted control ($P < 0.01$; Figure 4.2, Table 4.2). The sorted sample groups also showed a tendency towards lower progressive motility when compared to the unsorted sample, although the difference was not significant (Table 4.2). Additionally, the viability of three sorted sample groups was lower when compared to the unsorted control ($P < 0.05$). The reduction in the percentage of motile cells and a decreased viability could be a reason sorted sperm show a lower ability to bind to oviduct cell aggregates.

Damage to the sperm plasma membrane and impaired function of oviduct glycan receptors contained within the membrane is another potential cause of reduced ability to bind oviduct cells. That damage may be manifested by reduced binding to either of the two oviduct glycan motifs that bind boar sperm. To examine this possibility, we incubated spermatozoa with soluble fluoresceinated glycans and measured the percentage of sperm that bound these glycans and the location of oviduct glycan receptors. Three glycans were tested, two that bind sperm specifically, biantennary 6-sialylated *N*-acetylglucosamine (bi-SiaLN) and sulfated Lewis^X (suLe^X), and one control glycan, *N*-acetylglucosamine (LacNAc) (Figure 4.3). Sperm from the four groups did not differ in their ability to bind the three soluble glycans tested. The proportion of sperm in all four samples that bound bi-SiaLN or suLe^X averaged 82% and 83% respectively, whereas 40% of sperm bound the control, LacNAc (Figure 4.4). We also investigated whether sorting influenced the location of receptors for the three glycans. There were no differences in binding affinity to soluble fluoresceinated glycans among the sample groups (Y-bearing, X-bearing, XY-remixed, or unsorted), so the results were pooled for presentation. The glycans bound to sperm in three patterns (Pattern A: glycan binding to the apical ridge only, Pattern B: apical ridge and post-acrosomal area binding, and Pattern C: post-acrosomal area binding only) (Figure 5). Binding along the apical ridge of the sperm head is most relevant because that is where the receptors that bind to oviduct cells are located (Kadirvel et al. 2012; Machado et al. 2014; Silva et al. 2014). Patterns A and B were grouped together because both included sperm that bound the specific glycan at the apical ridge. The overall average percentages of sperm showing apical ridge binding for bi-SiaLN, suLe^X and LacNAc, were 62, 61, and 19% respectively (Figure 4.5). In contrast, the percentage of sperm that bound bi-SiaLN, suLe^X, and LacNAc in the post-acrosomal region only was 28, 29, and 18% respectively (Figure 4.5).

Because binding to soluble fluoresceinated glycans was not altered, we concluded that sperm retain the receptors for bi-SiaLN and Le^X in the same location after sex-sorting.

It has been previously determined that a sperm must have an intact acrosome to bind to the oviduct to form the sperm reservoir (Gualtieri and Talevi 2000). Sorting may damage sperm by altering the acrosome and thereby affect the function of oviduct glycan receptors. Thus, we examined the acrosome status of sperm by fixing and staining with Coomassie Blue G250 following sorting. We observed the four sample groups had all maintained >97% acrosome integrity after the sorting process (Table 4.2). From these results, we concluded that the reduction in oviduct cell binding induced by sorting was not due to premature acrosome reactions.

Discussion

When sperm are separated by sex chromosomes using a flow cytometric procedure, a reduction in fertility is observed (Vazquez et al. 2003; Grossfeld et al. 2005). The underlying cause for reduced fertility is unclear, although damage due to separation of sperm from seminal plasma may induce premature capacitation (Maxwell and Johnson, 1999). This premature capacitation may suppress fertility, in part, because capacitated sperm are less able to bind to the oviduct and form a sperm reservoir that would otherwise prolong sperm lifespan (Fazeli et al. 1999; Gualtieri and Talevi 2000; Petrunkina et al. 2001). This study tested the hypothesis that flow sorting sperm by sex chromosomes affects oviduct cell binding and binding to specific oviduct glycans (bi-SiaLN and Le^X). Separation by flow cytometry reduced binding of sperm to oviduct cells by more than half when sperm contained primarily an X or a Y chromosome or an

equal mixture of both, compared to control sperm that were not sorted. The percentage of sperm that were motile was also decreased significantly in each of the sorted sample groups but only by about 15%; not to the degree that oviduct binding was reduced. Sorting did not decrease the percentage of sperm that bound purified soluble oviduct glycans, indicating that oviduct glycan receptors were not affected by sorting.

The clear decrease in sperm binding to oviduct cells suggests that this may be a major cause of reduced sperm fertility after sorting by sex chromosomes. Although the assay measured sperm binding to oviduct cells *in vitro*, the cells were collected from fresh porcine oviducts and allowed to form spheres, retaining cell polarity and normal function (Thomas et al. 1997). Thus, the assay more closely resembles *in vivo* binding than assays using cultured oviduct cells. Furthermore, the oviduct cell aggregates retain their complex extracellular matrix that binds sperm *in vivo*.

In contrast, sperm binding to purified soluble fluorescent glycans was not affected by flow sorting. These glycans bind to and identify the location of receptors on sperm. We hypothesized that sorted sperm would have reduced ability to bind oviduct glycans but our results indicated that there was no change due to sorting. Thus, the location of oviduct glycan receptors was not influenced by flow sorting. The paradox of why sperm binding to oviduct cells was reduced but binding to oviduct glycans was not affected might be explained by considering the complexity of the oviduct cell surface and a comparison of the affinity required for binding to oviduct cells or binding to a soluble glycan. The oviduct cell matrix provides a complex profile of many different adhesion molecules that sperm can bind. These are all associated with the oviduct cells and are insoluble, requiring a higher affinity to tether a motile cell. On the other hand, the individual glycans were soluble, providing sperm the ability to bind, and not be

removed by the force generated by a motile cell. These were also individual glycans, much simpler than the matrix surrounding oviduct cells. Ignoring the contribution of other oviduct cell adhesion molecules makes the individual glycan binding results less physiological.

Unfortunately, assessing sperm binding to oviduct cells requires a ready source of oviduct cells and is a more laborious assay than measuring sperm binding of oviduct glycans. Assessing sperm binding to oviduct cells in a routine assay is not as practical as measuring sperm binding to purified soluble fluorescent oviduct glycans.

Sperm motility was reduced after sorting by about 15%, a much smaller reduction than sperm binding to oviduct epithelial cells. If motility was reduced to a greater degree, it might fully explain the reduction of sperm binding to oviduct cells because the collision frequency between sperm and oviduct cells would be correspondingly reduced. However, because there was only a relatively small reduction in the percentage of motile cells, it appears that sorting diminished adhesion to oviduct cells directly in some manner, rather than only indirectly by reducing the collision frequency between oviduct cells and sperm.

Our original hypothesis was that flow sorting might induce changes in sperm that resembled capacitation and those changes would reduce sperm binding to oviduct cells (Thomas et al. 1997; Varki and Lowe 2009; Silva et al. 2014). That hypothesis appears to be incorrect because sperm binding to purified glycans was not affected by sorting. Our previous work has shown that capacitated sperm lose their ability to bind purified soluble oviduct glycans (Kadirvel et al., 2012). Because sorted sperm still bound oviduct glycans, it appears that they had not completed capacitation. Nevertheless, it is still possible that sorting initiates only the early stages of capacitation. Sperm that have undergone complete capacitation lose their ability to bind to

these soluble glycans (Kadirvel et al., 2012; Machado et al., 2014), however, that capability was unaffected.

Another potential reason for reduced binding of sorted sperm is that the sorted sperm had lost or damaged acrosomes. Sperm must have intact acrosomes to bind oviduct cells (Vallorani et al., 2010). But, there was no effect of sorting on the frequency of sperm that had lost their acrosomes.

We expect that a reduction in the number of sperm that bind to oviduct cells *in vitro* would result in reduced fertility following artificial insemination. Sperm lifespan in the oviduct is critical to porcine fertility; sows are frequently inseminated 2-3 times at each estrus due to poor sperm lifespan (Knox 2016). Studies previously completed in our lab using cryopreserved porcine sperm, in addition to other studies, demonstrated that the ability to bind oviduct cells was related to fertility following artificial insemination (Waberski et al. 2005; Daigneault et al. 2014). In cattle, a reduction in fertility has been noted when using sex-sorted sperm (DeJarnette et al., 2011), but the cause of this reduction is not certain.

In summary, flow sorting to separate sperm into samples with either X or Y chromosomes reduced binding to the complex matrix around oviductal cell aggregates markedly but sperm binding to purified soluble oviduct glycans was not affected. The reduction in sperm fertility observed following sorting by sex chromosomes may be explained partially by a reduced ability to bind to the oviduct epithelium.

Acknowledgments

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Tables and Figures:

Sample	Y/X Purity (%)	Post-sort Motility (%)	Post-sort Normal Cells (%)	Post-sort Viability (%)
Y-bearing	94.19 ± 1.37	86.88 ± 4.39	81.80 ± 9.76	92.89 ± 3.33
X-bearing	93.17 ± 3.20	88.32 ± 5.17	81.20 ± 11.94	91.90 ± 2.80

Table 4.1: Sperm post-sorting. Purity was assessed post-sorting for both Y- and X-bearing spermatozoa by re-sorting a subpopulation of spermatozoa from each sample group. Motility, morphology and viability were also measured using computer assisted sperm analysis (n=10 replicates). Values in the table are means ± standard error of means (SEM). No differences were observed.

Sample	24 hr Post-sort Motility (%)	24 hr Prog. Motility (%)	24 hr Post-sort Viability (%)	24 hr Post-sort Intact Acrosomes (%)
Y-bearing	59.20 ± 16.22†	18.80 ± 8.73	71.56 ± 15.68‡	98.29 ± 0.74
X-bearing	67.7 ± 8.92†	20.3 ± 7.23	70.56 ± 16.79‡	97.97 ± 0.46
XY-remixed	64.9 ± 8.52†	19.5 ± 7.12	69.33 ± 15.68‡	98.17 ± 0.26
C- not sorted	83.20 ± 3.26	28.1 ± 9.65	100 ± 0.00	97.76 ± 0.65

Table 4.2: Sperm 24 hr post-sorting. Motility, progressive motility, and viability were assessed using computer assisted sperm analysis (n=10 replicates). Dagger indicates a significant difference compared to the unsorted control in the same column ($P < 0.01$). The double dagger indicates a significant difference compared to the unsorted control in the same column ($P < 0.05$). The percentage of spermatozoa with intact acrosomes was measured using Coomassie Blue staining 24 hr after sorting; sperm with staining across the apical region of the sperm head were considered acrosome intact (n=5 replicates). Values in the table are means ± SEM.

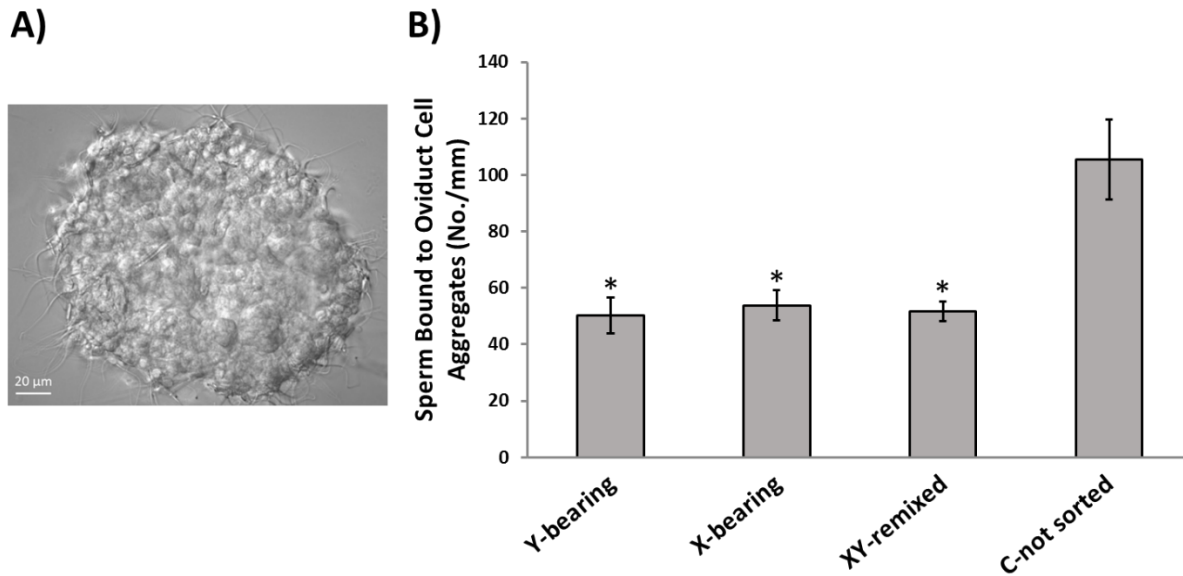


Figure 4.1: Oviduct cell binding was reduced in sorted samples. A) Example of sperm bound to an oviduct cell aggregate. **B)** Sperm were incubated with oviduct cell aggregates for 15 min ($n = 30$ aggregates/treatment/boar) and sperm bound to the perimeter were counted and normalized by the periphery of the aggregate. Asterisks indicate a significant difference compared to the control ($P < 0.05$). Four sample groups included, Y-bearing sperm, X-bearing sperm, XY an equal mixture of sorted X- and Y-bearing and C the control containing sperm that were not sorted. Means $\pm$ SEM are graphed.

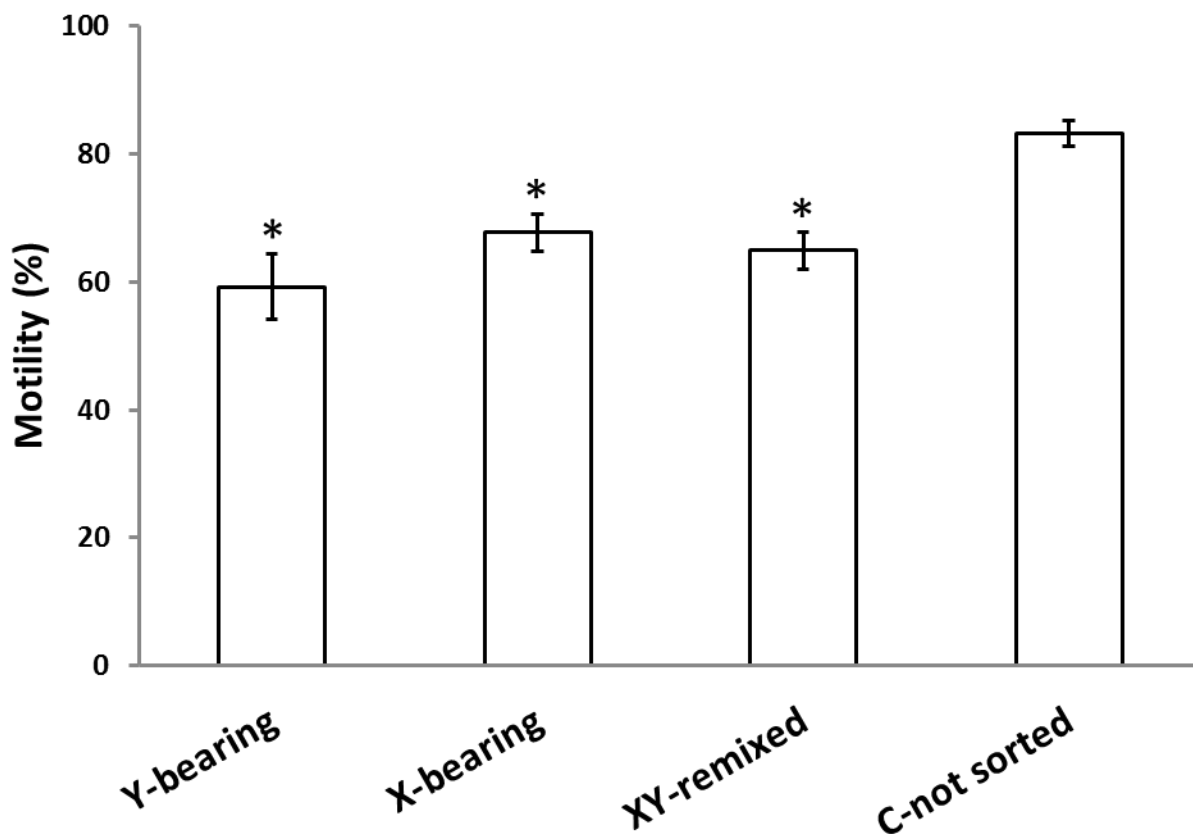


Figure 4.2: The percentage of sperm that were motile was reduced after sorting. Motility parameters were assessed using Computer Assisted Semen Analysis. Asterisks indicate a significant difference compared to the unsorted control ($P < 0.01$). Four sample groups included, Y-bearing sperm, X-bearing sperm, XY an equal mixture of sorted X- and Y-bearing and C the control containing sperm that were not sorted. Means $\pm$ SEM are graphed.

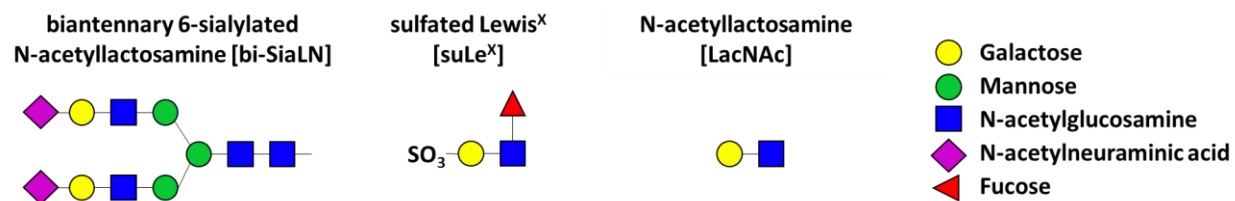


Figure 4.3: Structures of glycans used in the fluoresceinated glycan binding experiments.

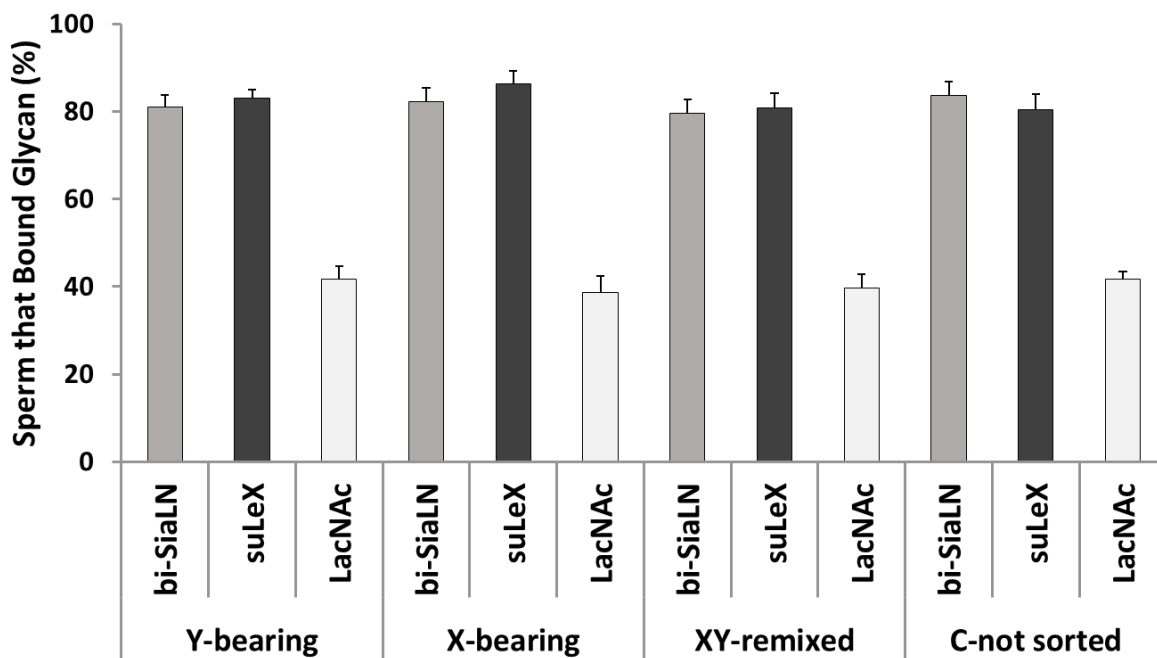


Figure 4.4: The ability of sperm to bind to soluble fluoresceinated glycans did not differ between samples. Sperm were incubated with fluoresceinated oviduct glycans and labeled sperm were counted (n = 10 replicates). The targeted glycans were biantennary 6-sialylated *N*-acetylglucosamine on a mannose core (bi-SiaLN), sulfated Lewis^X (suLe^X) and the control *N*-acetylglucosamine (LacNAC). The four sample groups included Y-bearing sperm, X-bearing sperm, XY an equal mixture of sorted X- and Y-bearing and C the control containing sperm that were not sorted. Means $\pm$ SEM are graphed.

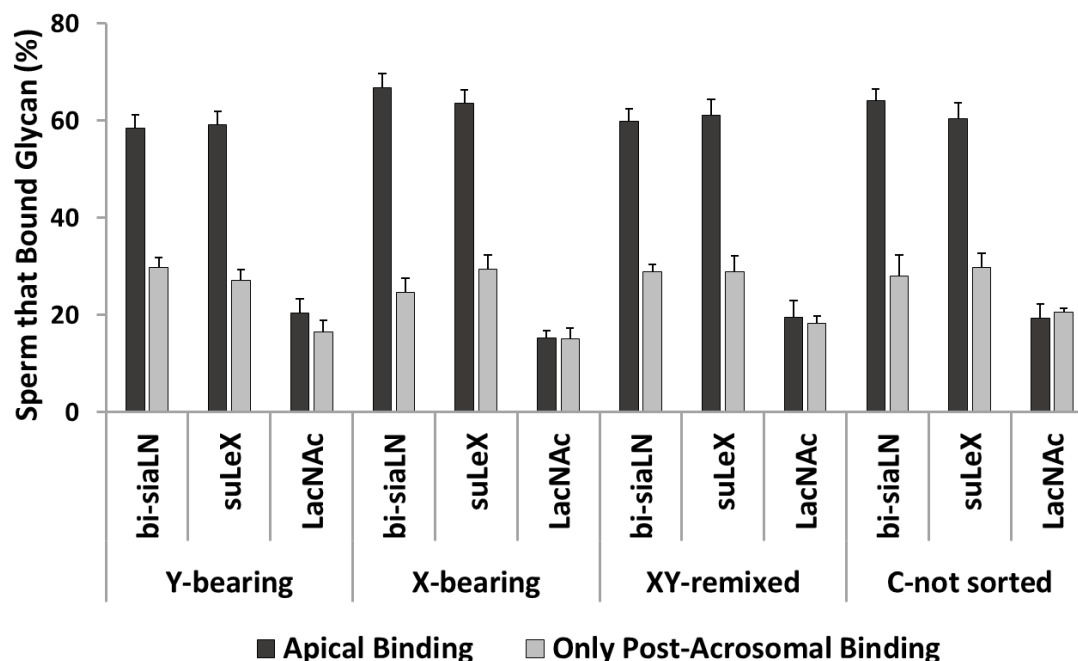


Figure 4.5: Fluorescent glycan binding patterns observed on sperm were not different between samples. Upper Panel) Pattern A: Binding to the apical ridge only, Pattern B: Binding to both apical ridge and post-acrosomal area, and Pattern C: Binding to post-acrosomal area only. Patterns A and B were combined to show all apical binding. Pattern C is considered nonspecific binding. **Lower Panel)** Sperm were incubated with glycans and the patterns present were counted (n = 5 replicates). The targeted glycans were biantennary 6-sialylated *N*-acetylactosamine on a mannose core (bi-SiaLN), sulfated Lewis^X (suLe^X), the control *N*-acetylactosamine (LacNAc). Four sample groups included, Y-bearing sperm, X-bearing sperm, XY an equal mixture of sorted X- and Y-bearing and C the control containing sperm that were not sorted. Means $\pm$ SEM are graphed.

CHAPTER 5 - OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

Summary

Currently, the swine industry uses artificial insemination for a majority of the matings occurring within the United States. Consistent use of AI requires accurate semen evaluation in order to ensure reproductive efficiency. Using both traditional and functional assays to isolate males that have poor reproductive performance and remove them from the herd could potentially save the swine industry a significant amount of money. The objectives were to determine if the ability to bind oviduct cells or the specific oviduct glycans are useful supplements to routine semen analyses and how sorting by sex chromosomes effects these interactions. Linear regression was used to isolate traits with the largest impact on each of the response variables, pregnancy rate (PR), litter size (LS), and the fertility index (FI). The predictive models constructed accounted for 58% or more of the variation in PR ($p < 0.001$, $r^2 = 0.60$), LS ($p < 0.001$, $r^2 = 0.58$), and FI ($p < 0.001$, $r^2 = 0.63$). All three final models included traditional semen morphology evaluation traits. The model for PR also included the ability of sperm to bind oviduct cell aggregates. However, binding to specific oviduct glycans was not a useful supplement to traditional semen analysis. The second study examined how sorting sperm by flow cytometry effects binding to oviduct cells and purified oviduct glycans. Sorting semen using a flow cytometer negatively impacted the ability of sperm to bind to intact oviduct cells, however, binding to soluble fluoresceinated glycans wasn't inhibited by the sorting process.

In conclusion, oviduct cell binding and the formation of the sperm reservoir are important for successful fertilization. The sperm-oviduct binding assay is correlated with pregnancy rate and can be used with other traditional sperm assays to predict the average pregnancy rate for an

individual male. Similarly, a reduction in fertility in sperm that are sex-sorted may be due to a reduced ability to bind to the oviduct epithelium. These results confirm the significance of oviductal sperm reservoir formation. This reservoir is critical for sperm to successfully reach and fertilize the oocytes within the female reproductive tract.

Future Directions

The research previously discussed isolates important criteria sperm must meet for successful fertilization, but there is still more that needs to be understood in order to accurately predict individual boar fertility and mitigate any losses in reproduction. To improve this aspect of production, it would be important to determine if you could predict how individual ejaculates will perform in the field. Further statistical analysis on the data previously collected for each ejaculate, and taking it one step further to observe each replicate individually and its corresponding matings. The number of boars being analyzed would be decreased, but a majority of the boars had at least 5 matings per replicate. This subset of data could be used to identify variation among boars in addition to variation among ejaculates from an individual boar. Further analysis could isolate ejaculates that resulted in poor oviduct binding and/or glycan binding and see how the inseminations from that ejaculate performed in the field.

Identifying boar fertility based on glycans can still be possible. The glycans used in these studies were soluble and free floating allowing sperm easy access to bind. However, if these glycans were insoluble and attached to a bead they could potentially provide results that mimic those of oviduct cell binding. Binding to something insoluble will require glycan receptors on sperm to bind with a higher affinity and possibly be an accurate and simple assay for sperm

fertility. Using a higher affinity binding assay can also be applied for sex-sorted semen. Soluble glycan binding wasn't different in the three sorted sample groups compared to the control, but an assay that required higher affinity binding to glycans may begin to demonstrate these differences that are seen in binding to intact oviduct cells. Another aspect that wasn't analyzed in these projects is that there is many components in the oviduct including multiple glycans and glycan motifs. Perhaps providing different kinds of insoluble glycans for sperm to bind on a glycobead could be a more purposeful assay. Furthermore, providing a lower concentration of glycan as to not completely saturate the receptors but allow for quantification of the amount binding and binding in their respective patterns could provide information on the binding affinity of individual sperm samples. Studying the binding affinity of sperm with these glycans could offer more understanding of what is occurring *in vivo* in cases of lower fertility.

Although binding to boar specific soluble fluoresceinated glycans wasn't able to identify subfertile males, there are further candidate glycan receptors present on the sperm head that could be used to identify fertility in males. Among those are putative glycan receptors PKDREJ, ADAM5, and lactadherin (SED1). Determining the presence and/or abundance of these receptors could aid in identifying subfertile boars. These three receptors for oviduct cell binding have previously been studied in our lab. All three of these candidate receptors are localized in the apical ridge of noncapacitated boar spermatozoa. Binding to these candidate receptors is reduced in capacitated sperm and both ADAM5 and PKDREJ binding were reduced even further when frozen-thawed sperm were analyzed. Previous work demonstrated that pre-incubating boar sperm with antibodies to PKDREJ or SED1 and then incubating with soluble fluoresceinated Le^x, resulted in lower binding to the soluble glycan. This pre-incubation shows that these receptors

are necessary to bind to these specific glycans, providing a foundation that maturation of sperm results in a modification or loss of these receptors.

Additionally, pre-incubation of sperm with both SED1 and PKDREJ antibodies had a significant impact on the ability of sperm to bind to oviduct cells. Supporting further the idea that these receptors on the sperm head are needed for adequate sperm reservoir formation. PKDREJ is a candidate receptor for both bi-SiaLN and suLe^X, and SED1 is a candidate receptor for suLe^X (Govindasamy and Miller, unpublished). Sperm reservoir formation is important to allow for sperm lifespan to be extended, especially in pigs where insemination and ovulation aren't highly synchronized. Analyzing spermatozoa from a wide population of boars for these receptors using western blot analysis and measuring the abundance of these receptors could indicate differences between boars and/or ejaculates, thus identifying boars with lower fertility.

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APPENDIX A – ADDITIONAL INFORMATION

Oviduct Binding Correlations

Variable	Estimate	Standard Error	<i>t</i> - Value	R ²	Adj R ²
Pregnancy Rate	-2.84	0.80	-3.54	0.12	0.11
Litter Size	4.83	3.15	1.53	0.03	0.015
Fertility Index	2.84	0.80	3.54	0.12	0.11
% Motile Cells (B)	-36.18	16.73	-2.16	0.05	0.04
% Motile Cells (A)	0.10	0.19	0.51	0.003	-0.008
% Total Normal Morphology (A)	1.96	0.74	2.67	0.07	0.06

Table A.1: Binding to intact oviduct cells regressed against pregnancy rate, litter size, fertility index value, motility, and normal morphology. Variables were regressed using PROC REG in SAS 9.4.

Number of Matings/Boar by Parity

Boar	Sow Parity										Total
	1	2	3	4	5	6	7	8	9	10	
1	78	39	30	19	1	1	0	0	0	0	168
2	74	54	33	21	10	4	0	0	1	1	198
3	83	62	35	20	12	1	3	0	0	0	216
4	144	76	52	74	9	4	5	2	0	1	367
5	52	55	53	28	6	1	5	0	0	0	200
6	24	40	35	21	14	1	2	0	0	0	137
7	73	53	45	30	24	4	0	1	0	0	230
8	42	24	16	17	10	3	2	1	0	0	115
9	129	91	52	46	5	4	1	0	0	0	328
10	212	117	71	79	36	5	0	1	0	0	521
11	108	70	34	32	8	4	3	1	1	1	262
12	112	59	39	38	11	1	1	0	0	0	261
13	39	22	23	13	7	0	0	0	0	0	104
14	139	92	71	50	3	1	0	0	0	0	356
15	161	109	60	31	13	8	5	2	0	2	391
16	62	34	16	12	3	0	1	0	0	0	128
17	135	86	65	45	21	4	0	0	1	0	357
18	104	58	48	22	9	3	0	0	0	0	244
19	145	57	61	36	11	3	5	1	0	0	319
20	142	108	101	78	29	6	3	0	0	0	467
21	115	45	32	27	9	2	2	0	0	0	232
22	99	42	45	19	19	1	2	0	0	0	227
23	27	22	11	15	7	2	2	0	0	0	86
24	33	31	22	13	6	0	1	0	0	0	106
25	33	8	7	4	1	0	0	0	0	0	53
26	134	109	42	44	17	8	2	1	0	0	357
27	32	18	27	16	10	2	2	0	0	0	107
28	25	15	9	17	3	0	0	0	0	0	69
29	18	23	18	10	4	0	0	0	0	0	73
30	35	30	18	16	9	1	1	0	0	0	110
Total	2609	1649	1171	893	327	74	48	10	3	5	6789

Table A.2: Number of matings/boar by sow parity.

Number of Matings/Boar by Farm

Boar	Sow Farms															Total
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
1	54	0	0	0	0	0	12	5	3	6	26	0	35	27	0	168
2	57	14	7	4	10	0	0	11	0	11	31	0	45	8	0	198
3	63	12	0	0	5	13	8	0	6	8	24	0	60	17	0	216
4	77	23	5	8	20	13	0	12	0	17	10	6	142	34	0	367
5	77	8	3	0	0	0	0	0	3	4	13	0	67	25	0	200
6	60	0	1	0	0	0	0	0	0	0	15	0	49	12	0	137
7	102	2	0	2	4	0	7	3	5	18	11	0	57	17	2	230
8	69	4	1	0	7	0	0	0	0	3	4	0	27	0	0	115
9	82	10	1	0	10	19	11	5	3	16	50	4	96	21	0	328
10	98	23	29	0	29	26	0	6	6	47	62	35	160	0	0	521
11	63	16	1	16	5	6	10	3	14	16	11	5	76	20	0	262
12	96	8	9	0	12	12	0	0	4	0	44	5	59	2	10	261
13	56	0	0	0	0	0	0	0	0	0	17	0	17	14	0	104
14	100	24	7	5	11	0	15	0	5	12	48	0	88	29	12	356
15	129	21	0	25	9	0	10	0	13	16	44	0	94	30	0	391
16	25	30	3	0	14	0	9	0	6	7	5	0	22	7	0	128
17	50	18	17	0	7	18	18	0	7	14	18	11	149	30	0	357
18	78	21	0	0	0	0	15	3	5	9	28	0	49	36	0	244
19	86	12	10	0	5	0	0	0	6	19	27	0	110	44	0	319
20	176	33	16	0	12	0	14	3	16	23	20	0	130	24	0	467
21	53	22	0	0	8	19	5	0	6	25	0	11	74	9	0	232
22	78	3	9	0	10	0	11	5	0	10	6	0	76	14	5	227
23	42	0	6	0	0	0	0	0	0	3	10	0	22	3	0	86
24	27	1	0	0	3	6	0	3	0	6	0	0	44	10	6	106
25	41	0	0	0	0	0	0	0	0	0	0	0	8	4	0	53
26	77	24	13	0	19	24	0	1	10	19	41	23	98	8	0	357
27	62	0	0	0	0	0	0	0	0	0	0	0	35	10	0	107
28	28	0	0	0	3	0	5	3	2	0	0	0	26	2	0	69
29	30	1	0	0	0	17	0	0	0	0	0	0	15	10	0	73
30	40	15	5	0	3	0	0	0	0	9	0	0	38	0	0	110
Total	2076	345	143	60	206	173	150	63	120	318	565	100	1968	467	35	6789

Table A.3: Number of matings/boar by sow farms.

Boar by Number of Outcomes

Boar	Died	Return	Abortion	Preg. Slaughter	Farrowing	Total
1	2	14	0	8	144	168
2	6	24	0	11	157	198
3	3	18	1	25	169	216
4	12	34	2	21	298	367
5	1	15	0	13	171	200
6	2	22	2	11	100	137
7	2	14	4	11	199	230
8	3	9	0	11	92	115
9	11	33	2	17	265	328
10	10	42	7	35	427	521
11	2	33	2	16	209	262
12	2	10	0	16	233	261
13	2	5	1	8	88	104
14	9	27	2	30	288	356
15	11	31	4	30	315	391
16	5	12	1	10	100	128
17	5	46	1	24	281	357
18	7	24	0	22	191	244
19	3	59	1	36	220	319
20	11	39	3	28	386	467
21	5	49	2	12	164	232
22	2	39	2	7	177	227
23	0	11	0	5	70	86
24	1	11	0	8	86	106
25	0	10	2	7	34	53
26	4	31	6	26	290	357
27	1	7	2	3	94	107
28	3	3	0	6	57	69
29	1	11	0	5	56	73
30	3	16	0	5	86	110
Total	129	699	47	467	5447	6789

Table A.4: Frequency of outcomes/boar. Outcomes of abortion, pregnant-slaughter, and farrowing were grouped, given a value of 1. Outcome of return were given a value of 0, as pregnancy was not established. These values were used to determine pregnancy rate. Those that died were removed before analysis.

Outcomes by Parity

Parity	Died	Return	Abortion	Pregnant Slaughter	Farrowing	Total
1	43	338	21	182	2025	2609
2	40	188	12	107	1302	1649
3	25	80	4	78	984	1171
4	15	63	4	62	749	893
5	5	19	5	29	269	327
6	1	4	1	3	65	74
7	0	4	0	4	40	48
8	0	2	0	1	7	10
9	0	0	0	0	3	3
10	0	1	0	1	3	5
Total	129	699	47	467	5447	6789

Table A.5: Frequency of outcomes by sow parity.

Litter Size by Sow Parity

Total Pig/ Litter	Sow Parity										Total
	1	2	3	4	5	6	7	8	9	10	
0	1	1	0	0	0	0	0	0	0	0	2
1	1	1	0	0	0	0	0	0	0	0	2
2	6	1	6	1	0	0	0	0	0	0	14
3	26	14	5	2	2	0	0	0	0	0	49
4	29	18	8	6	1	0	0	0	0	0	62
5	36	23	12	11	2	2	0	0	0	0	86
6	42	22	26	12	6	0	0	0	0	1	109
7	53	26	16	15	3	0	0	0	0	0	113
8	61	39	22	14	5	3	0	2	0	0	146
9	94	51	25	38	7	0	1	0	0	0	216
10	135	74	48	44	17	5	3	0	0	0	326
11	159	76	57	38	12	3	1	1	0	1	348
12	229	111	88	43	22	6	4	0	1	1	505
13	246	155	89	74	32	8	5	2	1	0	612
14	247	180	107	78	39	7	10	0	0	0	668
15	252	166	111	94	33	9	7	0	0	0	672
16	184	129	118	72	27	7	3	0	0	0	540
17	117	95	76	67	25	6	3	0	0	0	389
18	55	66	81	54	14	5	0	0	1	0	276
19	26	29	40	42	11	2	1	0	0	0	151
20	18	13	25	27	2	2	1	1	0	0	89
21	6	7	12	8	3	0	0	1	0	0	37
22	1	2	8	5	5	0	0	0	0	0	21
23	1	3	2	1	1	0	1	0	0	0	9
24	0	0	2	1	0	0	0	0	0	0	3
26	0	0	0	2	0	0	0	0	0	0	2
Total	2025	1302	984	749	269	65	40	7	3	3	5447

Table A.6: Frequency of total number of piglets/litter by sow parity. Total Pig/Litter was the sum of total born alive, total stillborn, and total mummified piglets.

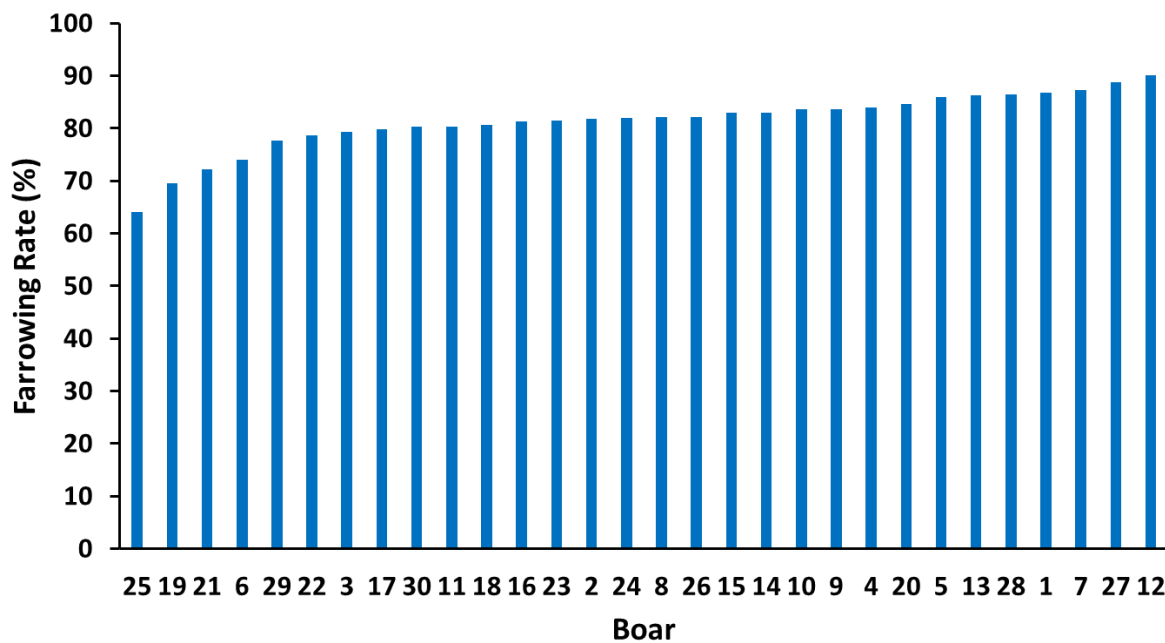


Figure A.1: Ranking of boars based on farrowing rates. Fresh semen from 30 different boars was inseminated using homospermic matings, resulting in >50 matings/boar. Farrowing rates were calculated by giving a 1 to those that farrowed and giving a 0 to those that had an outcome of abortion, pregnant-slaughter, and return. Farrowing rate was adjusted by farm, parity, number of services, and number of inseminations using PROC MIXED in SAS 9.4.

APPENDIX B - OVIDUCT BINDING ASSAY PROTOCOL

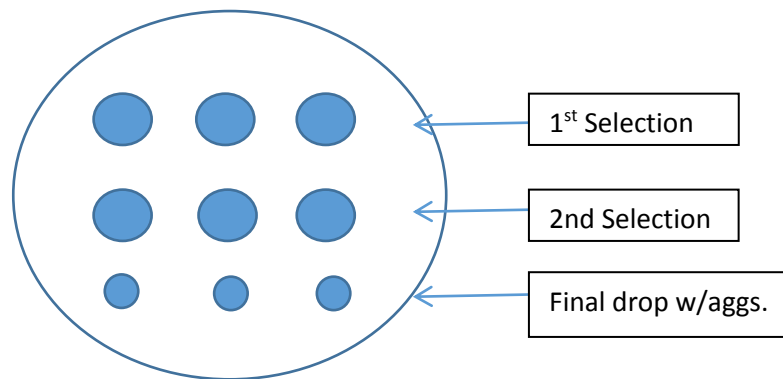
1. Collect Epithelial Cells

1. Separate isthmus from ampulla with a sharp pair of scissors (isthmus is thicker, proximal to the uterine horn and narrows proximal to the ampulla); 20 isthmus yields approximately 120 aggregates.
2. Make 0.5L of PBS (450ml H₂O and 50ml 10x PBS). Rinse isthmus' in 50 ml of PBS 3 times in a 50ml conical tube. Place conical tube with PBS into small styrofoam box on ice.
3. Add ~ 10ml of PBS at into a large petri dish (100 mm).
4. With forceps, grab 1 end of the isthmus, submerge the whole isthmus in the petri dish with PBS and remove epithelial cells by pressing down on one end of the isthmus with a glass slide held at a 45° angle and extracting cells out the other end. Repeat process until all cells are extracted. Collect cells from two isthmus' each time.
5. Gently aspirate clumps of extracted epithelial cells from the petri dish using a 200µl pipette.
6. Place aspirated cells in a 15ml centrifuge tube on ice by slowly dispensing cells as close as possible to the bottom of the tube at a 45° angle.
7. Dump the contents of the petri dish into a waste container and add 10ml of PBS and repeat process for all isthmus.
8. Allow the pellet of epithelial cells to settle for 5 min or centrifuge at speed 1 for 30 sec using a table top centrifuge.
9. Aspirate the supernatant and resuspend pellet in a total volume of 1 ml dmTALP capacitating medium.
10. Gently pipette pellet up and down ten times within the tube using a 1000µl pipette to disaggregate cells.
11. Resuspend further in dmTALP capacitating medium for a total volume of 5mL.
12. Centrifuge for 1 min at speed 1 or allow pellet to settle. Aspirate supernatant and resuspend in 1 ml of dmTALP capacitating medium.
13. Disaggregate cells very slowly using a 22 gauge needle and 1ml serological syringe 10 times.
14. Resuspend disaggregated cells in 9mL of dmTALP C in the same tube.
15. Transfer 3mL of cells and TALP mixture to 100mL petri dishes for a total of 3 dishes. Add 2 mL of TALP to each dish for a total of 5ml per dish. (Transfer equal-amounts of cells to 3 dishes for a total of 5 mL after adding TALP)
16. Incubate dishes containing cells at 39°C for 1.5-2hr at ~99% humidity for cells to reaggregate and form spherical, three dimensional structures. Observe the status of reaggregation at 1 hr to determine if sufficient aggregates exist for the experimental design.

2. Droplet Co-Incubation Preparation

Before culture time is finished, begin preparing droplets in petri dishes for aggregate co-incubation with sperm

1. In a 100ml petri dish, prepare a 3x3 droplet design per treatment
 - a. Prepare 2 rows and 3 columns of 100µl droplets of dmTALP C for selecting and washing aggregates
 - b. Prepare the 3rd row with 3, 40µl droplets. Each droplet will ultimately contain 10 aggregates used for binding sperm. The final volume of the incubation droplet will be 50 µL, including semen.
 - c. Place a small amount of mineral oil around the droplets to maintain the footprint but not to submerge the droplets



3. Remove cells from incubator and select spherical aggregates approximately ~150-200µm in size using a 3µl Drummond with a microcapillary pipette with ~275µm of internal diameter so as not to disaggregate spheres when selecting. Choose approximately 15 aggregates to add to the first wash droplet. Select 10 aggregates for a final wash placing them in the second droplet. Remove the 10 aggregates from the second droplet and place them into the third droplet to await co-incubation with sperm. You may randomize aggregate selection by selecting aggregates for the first column of every treatment from Petri Dish 1, the second column from Petri Dish 2 and the third from Petri Dish 3.

Prepare Sperm (Sperm preparation before or after aggregate selection depending upon design)

1. Warm sperm for ~10min at 39°C

2. Determine concentration of sperm using hemocytometer

-Hemocytometer Counting Instructions

- make a 1:100 dilution of sperm and water
 - Count 5 center squares in a diagonal on both sides of the hemocytometer and calculate the average from both sides (i.e.(45 sperm + 52sperm)/2 = 48.5
 - Calculate # sperm/mL: average no. sperm x DF x 0.05 = [sperm/mL x 10⁶]
- Ex: 48.5 x 100 x 0.05 x 10⁶= 242.5x10⁶ total sperm/mL

3. Add 1×10^6 sperm/mL to each droplet.
4. Add sperm to the droplet containing aggregates and bring the final volume of the droplet to 50 μ l using dmTALP C. (i.e. 2.1 μ l sperm and 7.9 μ l of dmTALP C to the original 40 μ l droplet = 50 μ l final vol.)
5. Incubate sperm and aggregates at 39°C for 15 min.
6. During aggregation time, prepare glass slides to mount 30 aggregates per slide (3 3 μ L-droplets containing 10 aggregates each per slide).
 - Clean slides with Sparkle and place a rectangular shape of petroleum jelly in preparation for a 22x40 coverslip. Draw three circles of a hydrophobic marker (PAP Pen).
 - Remove aggregates from the incubator and place 10 aggregates in each circle for a total of 30 per slide.
 - Place coverslip on top of petroleum jelly to create a seal.
 - Document binding with Axioskop software or place in 5°C overnight if necessary.
7. Use an upright scope to capture images of aggregates with bound sperm using a bright field under 200x magnification. More than one picture may be necessary to acquire all sperm. Interchange with a fluorescent field when necessary.

Alternatively: count all bound sperm and record immediately. Depending on experimental design, you may find it easier to count and record, or take pictures and count at a later time as described above.

APPENDIX C - PROTOCOL FOR LOCALIZATION OF GLYCAN RECEPTORS ON LIVE BOAR SPERM USING FLUORESCENT GLYCANS

1. Warm capacitating TALP (dmTALPC) and 1 ml of semen for 15 min at 37^o C.
2. Centrifuge sperm at 2400 X g for 1 min.
3. Remove extender and resuspend the pellet in dmTALPC.
4. Take 10 µl of semen+TALP suspension in 1.5 ml Eppendorf tube and add 990 µl of water or PBS. (The final dilution will be 100 times).
5. Count the number of sperm in 5 squares of hemocytometer.
6. Calculate concentration of sperm as follows:
 - Number of sperm in 5 squares X Dilution factor (100) X 50,000
7. Adjust concentration to 10 x 10⁶ sperm/ml.
8. Now take 38 µl of sperm suspension in a black Eppendorf tube and add 2 µl of fluorescent glycan (final concentration of glycan will be 50 µg/ml).
9. Incubate the sperm suspension for 30 min at 39^o C.
10. Place small drop of 6 µl from sperm suspension without washing over the clean slide, put coverslip and observe for fluorescence.
11. Take pictures with bright field and blue filter at 630X magnification.
12. Count at least 200 spermatozoa for each group.

APPENDIX D - COOMASSIE BLUE STAINING FOR MORPHOLOGY AND ACROSOMAL STATUS

1. Place 10ul of sperm suspension in a microfuge tube and add 25ul of 4% paraformaldehyde.
2. Incubate for 10min at room temperature to fix cells.
3. Centrifuge at 2000xg for 1 minute
4. Discard supernatant and re-suspend pellet in 50ul of 0.1M ammonium acetate.
5. Centrifuge at 2000xg for 1 minute.
6. Discard supernatant and re-suspend pellet in 25ul of 0.1M ammonium acetate.
7. Place 10ul of sperm suspension on each slide, air dry on slide warmer.
8. Dip dried slides in Coomassie Blue G-250 Solution in a Coplin jar for 2-4 min and rinse slides to remove stain. Allow slides to air dry.
9. Add a drop of PBS and top with a coverglass.
10. Take pictures of ~300 sperm and record acrosomal status.